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THERAPEUTIC EFFECTS OF *Bokhi* FROM CAMELS ON UTERINE LEIOMYOMA

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ABSTRACT

Bokhi is a transparent, water-soluble, sticky and odorous liquid containing sex steroids, that is secreted by male camels from their occipital or poll glands during the mating season. We investigated whether *Bokhi* has positive therapeutic effects on the treatment of uterine leiomyoma (ULM). ULM model rats were established by multipoint subcutaneous injections of a combination of diethylstilbesterol and progesterone for 11 successive weeks. Rats were then treated by oral administration of *Bokhi* for a further 7 successive weeks. Following the 11 weeks of injections the levels of serum estradiol (E_2), progesterone (Pro), follicle-stimulating hormone (FSH), tumour necrosis factor (TNF-α) and nitric oxide synthase (NOS) in ULM rats were significantly higher than the non-ULM control rats ($P < 0.05$). This demonstrated that development of the ULM model was successful. After 7 weeks of oral treatment with *Bokhi* there was no significant difference in the levels of E_2, Pro, FSH, TNF-α, iterleukin-2 (IL-2) and NOS between the ULM rats fed high doses of *Bokhi* (HDB) and the non-ULM control rats ($P > 0.05$). This demonstrates that high doses of *Bokhi* from camels could improve ULM and that there should be further research on *Bokhi* and its potential therapeutic uses.

Key words: *Bokhi*, camel, occipital gland secretion, sex steroid, uterine leiomyoma

Uterine leiomyoma (ULM) is the most common benign tumour in the human female reproductive organs (Gambadauro *et al,* 2012) and the incidence rate is as high as 70% (Shen *et al,* 2009). There is no effective long-term medical therapy and surgery remains the mainstay of treatment for these patients (Islam *et al,* 2013). Therefore, studies to improve treatments for ULM have attracted a lot of attention in recent years.

Bokhi is the Mongolian name for the material that is secreted by male camels from the occipital or poll gland which is located on the neck midline behind the ear and is composed mainly of sweat glands and sebaceous glands. During the rutting season the occipital gland is activated; its morphological structure changes and its function is enhanced. In non-rutting season the occipital gland becomes atrophied and is completely degraded in castrated camels. *Bokhi* is a transparent, water-soluble, sticky and odourous liquid. The main components of *Bokhi* are sex steroids including sex pheromones that induce female camels into oestrus (Guo *et al,* 2013). Few studies have investigated the pharmacological role of *Bokhi*.

The main treatments for ULM are surgical or drugs. However, there are no specific drugs for the treatment of ULM and most of them have side effects. Therefore, it is of great importance to find an effective drug for the treatment of ULM. Traditionally *Bokhi* has been taken as an infusion in Mongolia for the treatment of ULM and kidney-yang-deficiency syndrome but there have been no studies to quantify any positive effects. This study evaluated the potential therapeutic effects of *Bokhi* from camels on ULM in rats.

Materials and Methods

Preparation of Bokhi

At the peak of the male camel rutting season, *Bokhi* samples were collected from the neck of mature, domesticated, bactrian camels from Alashan in Inner Mongolia. The samples were collected in sterile screw bottles and frozen for transportation to the laboratory. Each sample was placed in 200 ml of distilled water and soaked for 24 h. The resulting '*Bokhi* solution' was filtered through filter paper and the filtrate freeze-dried to produce a black solid powder.

Chemicals

Diethylstilbestrol injections were procured from Tianjin Jinyao Amino Acid Co., Ltd. (Tianjin,

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China). Progesterone injections were procured from Zhejiang Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). Sodium chloride (control) injections (0.9 %) were procured from Jilin Kelun Connell Pharmaceutical Company (Jilin, China). Sildenafil citrate was procured from Pfizer Pharmaceuticals Limited (Liaoning, China). Serum E_2, Pro, FSH, TNF-α, IL-2 and NOS reagent kits were purchased from Nanjing Institute of Biological Engineering (Jiangsu, China).

Animals and treatment groups

Forty-eight healthy adult female Spague Dawley (SD) rats (weighing 200-210g, specific pathogen free) were obtained from Vital River Laboratory Animal Technology Company Limited (Beijing, China) and allowed to acclimate to the animal facility for 1 week before starting the experiment. Animals were maintained in controlled environment (room temperature of 21–23°C, relative humidity of 45–65%) in a 12h light/dark cycle with free access to food and water. All protocols were approved by the animal care and use committee at Inner Mongolia Agricultural University.

Treatment administration

Rats were randomly allocated to 4 groups as follows: control, ULM model, HDB (High Dose *Bokhi*) and LDB (Low Dose *Bokhi*), 12 rats per group. Establishment of the ULM rat model followed the recommended methods from published work (Jia *et al,* 2012). Except for the control group, the Model, HDB and LDB groups were all injected with diethylstilbestrol (0.2 mg/kg body weight) once a day and progesterone (5 mg/kg body weight) 3 times a week for 11 consecutive weeks. Rats in the control group received only multipoint subcutaneous injections of 0.5 ml/kg body weight of medical physiological saline as a control for the injection process. To determine whether the ULM model had established, blood was taken from all rats, centrifuged (3,000 g centrifugation for 10 min) and the serum isolated. The levels of E_2, Pro, FSH, TNF-α and NOS in the serum from each rat was determined using commercial assay kits according to the manufacturer's instructions. From the 12^{th} week the control and ULM model groups were given 10 ml/kg body weight normal saline by intragastric administration every day. In contrast, the HDB group was given *Bokhi* 50 mg/kg body weight daily and the LDB group was given *Bokhi* 10 mg/kg body weight daily for 7 weeks. All rats were weighed every other day and at the same time, their activity, hair gloss and shedding were observed and recorded. After the final treatment blood samples collected as described previously and all rats were sacrificed.

Statistical analysis

Data, unless otherwise indicated, was expressed as mean ± standard deviation (SD). SPSS 17.0 software was used for all statistical analysis. GraphPad Prism 7 software was used to produce all Figs. R language software was used for principal components analysis using the ggplot 2 package for data visualisation. Probability levels of <0.05 were considered significant.

Results and Discussion

Observations on the general state and condition of rats

Before implementation of the model, there were no obvious differences between the groups. Within 2 weeks of beginning model implementation rats in the ULM groups (Model, HDB and LDB) were showing signs of hair loss. With increasing time the number of rats with hair loss increased in these groups, as did the area of skin with hair loss. Rats in the ULM groups also appeared apathetic, lethargic and prone to arching behaviour. These phenomena were not observed in control group.

Following intragastric administration of *Bokhi* hair loss gradually stopped and began to grow again. The hair gradually regained luster and the apathy and lethargy decreased. With prolongation of treatment time differences between the general state of the *Bokhi* groups and the control group gradually decreased. However, there was no sign of improvement in the general state of the rats in the model group. It was apparent that treatment with *Bokhi* had a positive effect on alleviating the symptoms of ULM.

Evaluating successful establishment of the ULM model in rats

In order to determine whether the ULM model was established successfully in ULM model groups, blood samples were taken during the 11-week model establishment period and the levels of serum hormones determined (Fig 1). The levels of E_2, Pro, FSH, TNF-α and NOS in the ULM model groups (Model, HDB and LDB) were not significantly different to each other ($P > 0.05$). However, the levels of E_2, Pro, FSH, TNF-α and NOS in the ULM model groups were all significantly different to the levels in the control group ($P < 0.05$). This showed that the ULM model was established successfully.

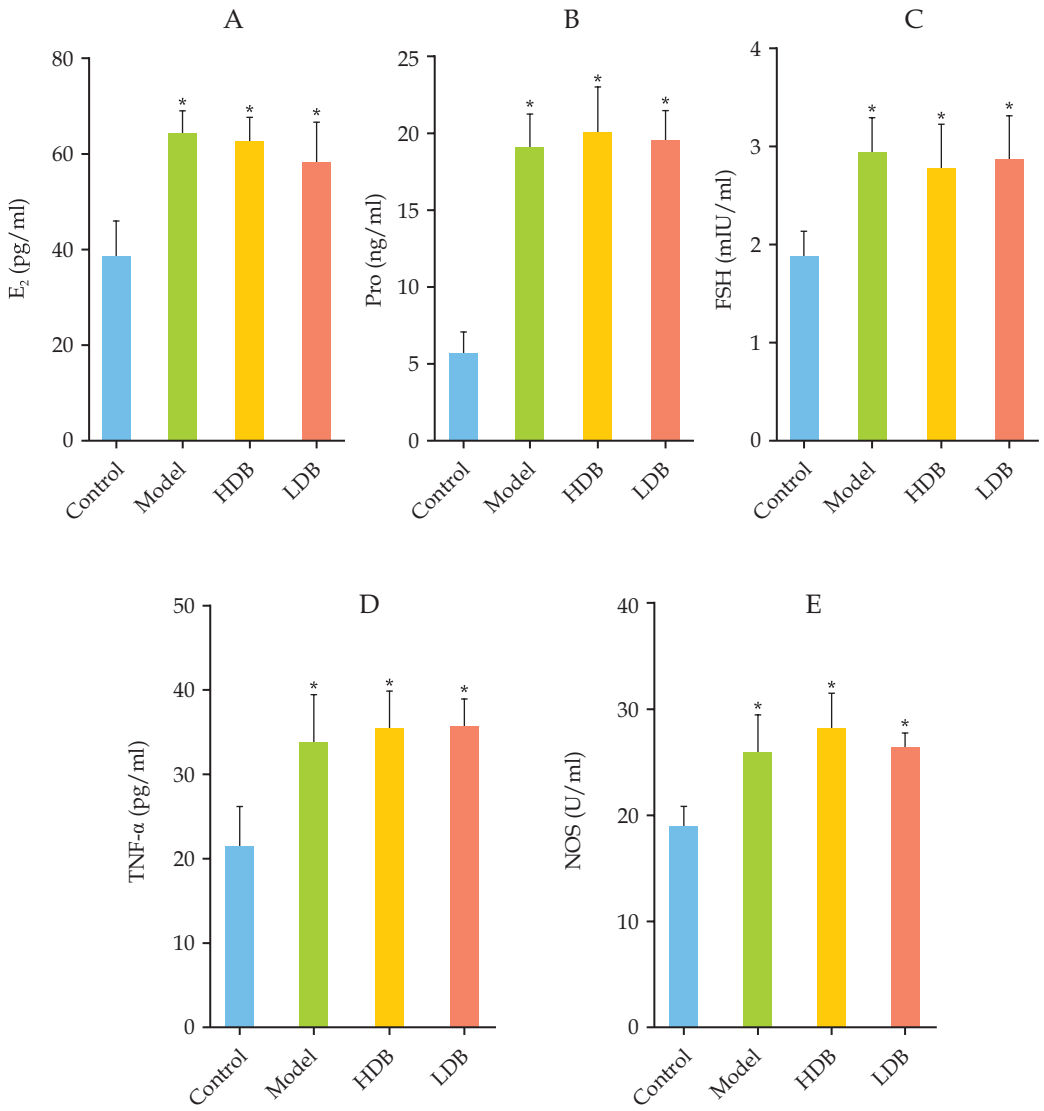

Fig 1. Levels of serum hormones E_2 (A), Pro (B), FSH (C), TNF-α (D) and NOS (E) during the establishment of the ULM model. *$P < 0.05$ compared with the Normal group.

Effects of Bokhi therapy on uterus coefficients in ULM model rats

The uterus coefficients of rats in all the *Bokhi* treatment groups were significantly different to the uterus coefficients in the model group ($P < 0.05$, Fig 2), but were not significantly different to the control group ($P > 0.05$). Following establishment of the ULM model, treatment with *Bokhi* restored uterus coefficients to the levels of the control group.

Effects of Bokhi therapy on hormones in ULM model rats

Levels of the hormone, E_2, in the HDB and LDB rats were significantly different to rats in the model group ($P < 0.01$, Fig 3 A), but were not significantly different to the control group ($P > 0.05$).

There were no significant differences in Pro between rats in the HDB group and rats in the control group ($P > 0.05$, Fig 3 B). However, there were significant differences in Pro between rats in the LDB group and rats in the control group ($P < 0.01$). Compared with the model group, there were significant difference in Pro between rats in the Model group and rats in both the HDB group and the LDB group ($P < 0.01$).

There was no significant difference in levels of FSH between rats in the HDB group and rats in the control group ($P > 0.05$, Fig 3 C). However, levels of FSH were significantly different between rats in the LDB group and rats in the control group ($P < 0.01$). Levels of FSH in rats from the HDB and LDB groups

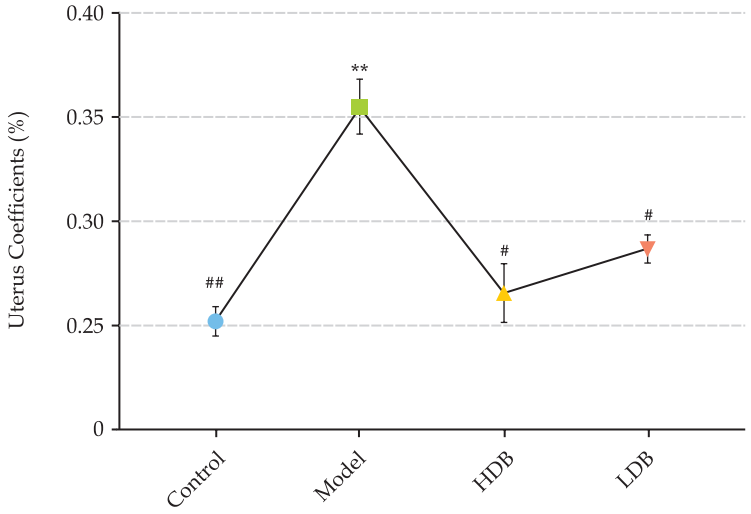

Fig 2. Effects of *Bokhi* on the level of uterus coefficients in ULM model rats compared with control rats and rats receiving *Bokhi* therapy. $^{**}P < 0.01$ compared with the control group; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the model group.

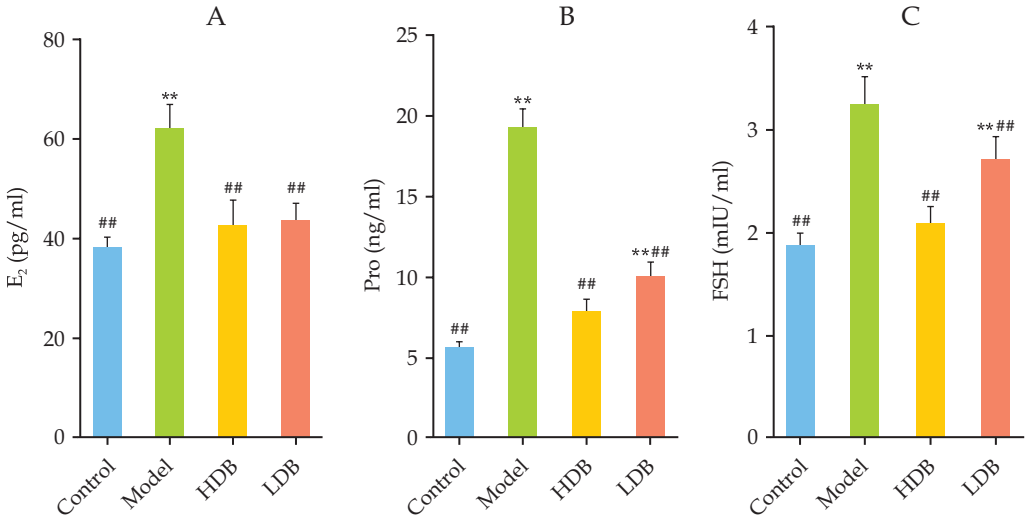

Fig 3. Effects of *Bokhi* on the level of serum hormones E_2 (A), Pro (B), FSH (C) in ULM model rats compared with normal rats and rats receiving *Bokhi* therapy. $^{**}P < 0.01$ compared with the control group; $^{\#\#}P < 0.01$ compared with the model group.

were significantly different to levels of FSH in the model group ($P < 0.01$).

After 7 weeks of treatment with *Bokhi*, these results showed that levels of hormones in the model group were seriously unbalanced, while in the *Bokhi* treatment groups there was a regulatory effect on E_2, Pro and FSH. The effects of HDB were the best as hormones levels returned to normal levels.

Effects of Bokhi therapy on TNF-α in ULM model rats

After 7 weeks of treatment with *Bokhi*, the level of serum TNF-α in rats from the HDB group was not significantly different to the level of serum TNF-α in rats from the control group ($P > 0.05$, Fig 4). However, the level of serum TNF-α in rats from the LDB group were significantly different to the level of serum TNF-α in rats from the control group ($P < 0.05$). Level of serum TNF-α in rats from the model group were significantly different to the level of serum TNF-α in rats from both the *Bokhi* therapy groups ($P < 0.01$). Thus, *Bokhi* effectively reduces the level of serum TNF-α in ULM rats.

Effects of Bokhi therapy on IL-2 in ULM model rats

There were no significant differences in the level of IL-2 in rats from the HDB and LDB groups compared with the control group ($P > 0.05$, Fig 5). However, there were significant differences in the

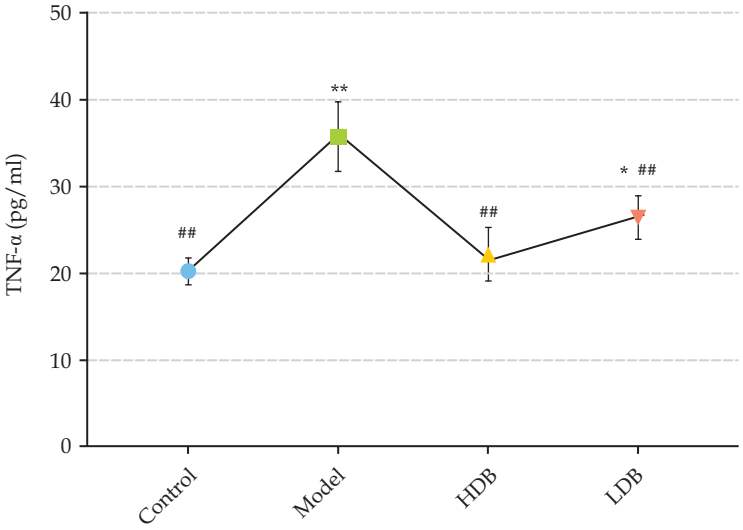

Fig 4. Effects of *Bokhi* on the level of TNF-α in ULM model rats compared with control rats and rats receiving *Bokhi* therapy. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the control group; $\#\#P < 0.01$ compared with the model group.

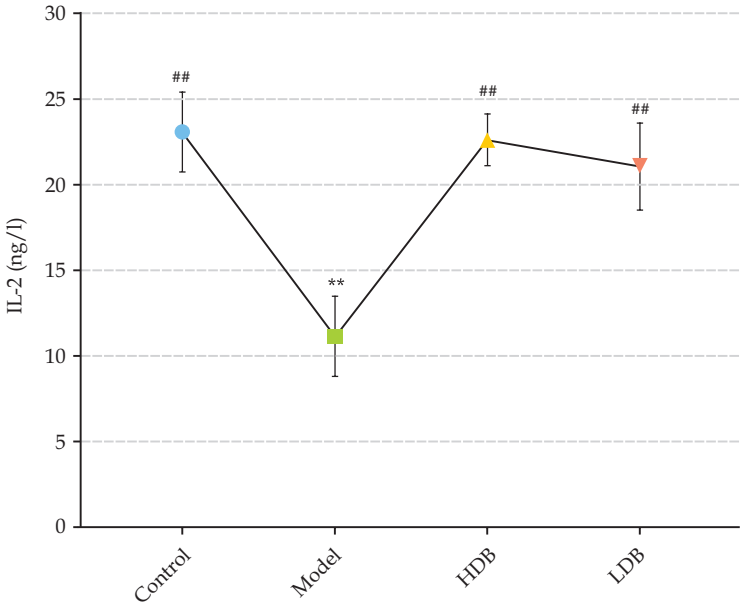

Fig 5. Effects of *Bokhi* on the level of IL-2 in ULM model rats compared with control rats and rats receiving *Bokhi* therapy. $^{**}P < 0.01$ compared with the control group; $\#\#P < 0.01$ compared with the model group.

level of IL-2 in rats from the HDB and LDB groups compared with rats from the model group ($P < 0.01$).

Effects of Bokhi therapy on NOS in ULM model rats

Levels of NOS in rats from the HDB and LDB groups were not significantly different compared with levels in rats from the control group ($P > 0.05$, Fig 6). However, levels of NOS in rats from the HDB and LDB groups were all significantly different to the levels in rats from the model group ($P < 0.01$).

Principal Component Analysis (PCA)

The data for levels of E_2, Pro, FSH, TNF-α, IL-2 and NOS after therapy were used in PCA (Fig 7). The model group and the control group were very distant from each other showing that the biochemical functions of the model group had been pathologically changed. It also indicated that the establishment of the ULM model rats had been successful. The HDB group was the closest to the control group and also distant from both the Model and LDB groups. Therefore, the treatment effect achieved by high doses of *Bokhi* on ULM rats was the most beneficial.

The experimental results of therapeutic effects of camel *Bokhi* on ULM model rats showed that camel *Bokhi* had a significant regulatory effect on the levels of E_2, Pro, FSH, TNF-α, IL-2 and NOS.

Although, gonadotropins (Plewka *et al,* 2014), adipokines (Wakabayashi *et al,* 2011) and ovarian peptides (Islam *et al,* 2014) have been postulated to have some influence on fibroid onset and growth, oestradiol and progesterone are the strongest candidates for these roles (Moravek *et al,* 2015). In the pathogenesis of ULM, the effects of oestradiol and progesterone are interrelated and involve the mediation of receptors, transcription factors, kinase proteins, growth factors and numerous autocrine and paracrine factors (Ono *et al,* 2012). The blockade of pituitary gonadotropin release with gonadotropin-releasing hormone (GnRH) analogs or antagonists is an effective strategy to control fibroid symptoms and arrest their growth (Islam *et al,* 2013; Engel *et al,* 2007). Although, the inhibitory effect of these peptides might be related to their direct action on GnRH receptors in the uterus (Malik *et al,* 2016; Balkwill, 2009) or via downregulation of gonadotropin levels reducing the direct stimulus of gonadotropins on luteinising hormone (LH) and follicle-stimulating hormone (FSH) receptors within the leiomyomas (Plewka *et al,* 2014), the most probable explanation for the effectiveness of this therapy is ovarian blockade and the consequent decrease in circulating estradiol and progesterone levels. Therapeutic effects of camel *Bokhi* might be due

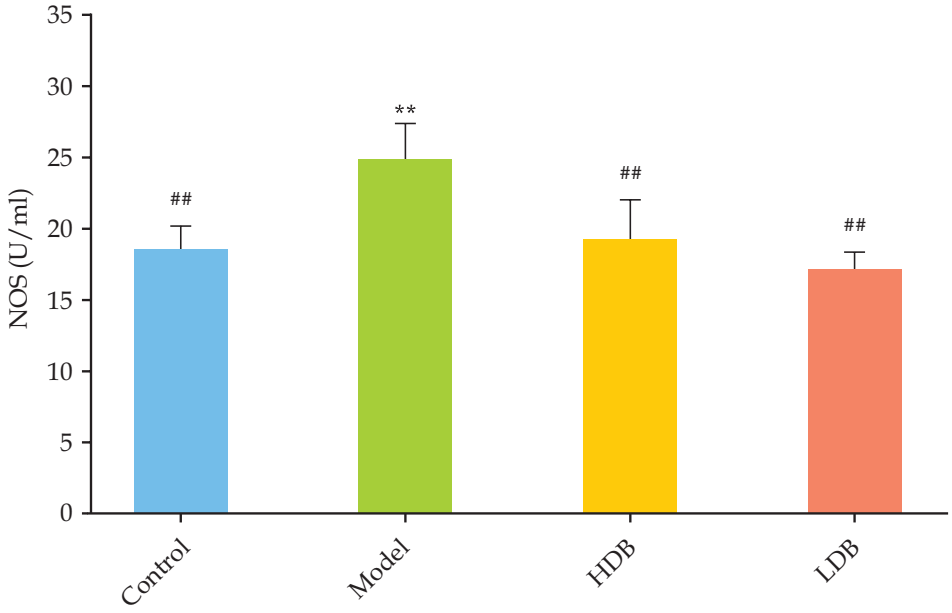

Fig 6. Effects of *Bokhi* on the level of NOS in ULM model rats compared with control rats and rats receiving *Bokhi* therapy. $^{**}P < 0.01$ compared with the control group; $^{\#\#}P < 0.01$ compared with the model group.

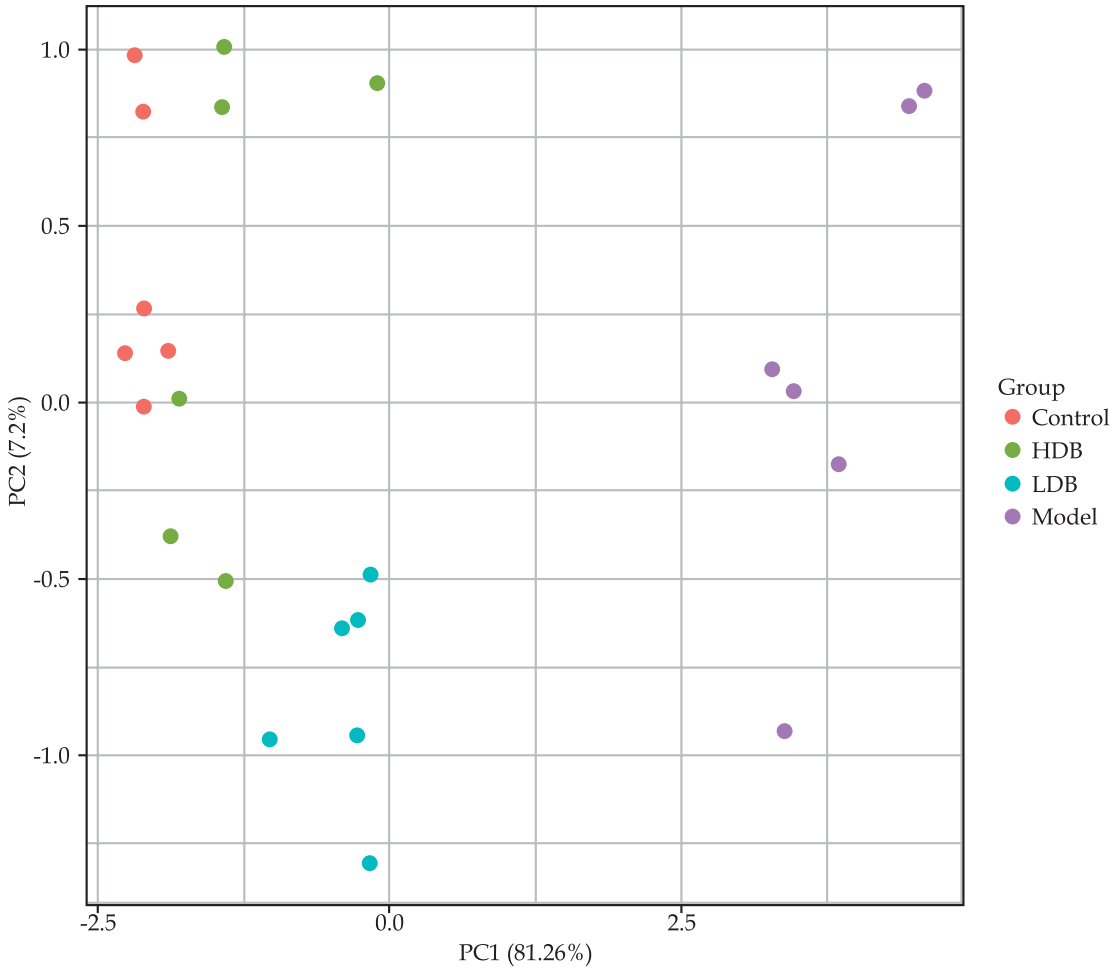

Fig 7. Effects of *Bokhi* on PCA in ULM model rats compared with control rats and rats receiving drug therapy.

to the inhibition of FSH secretion resulting in reduced levels of E_2 and Pro.

TNF-α is a pleiotropic cytokine involved in inflammation, immunity, migration, cellular homeostasis and tumor progression (Balkwill, 2009). The expression of TNF-α in ULM was higher than that in the adjacent myometrium (Kurachi *et al,* 2001). The research (Wang *et al,* 2015) reported that TNF-α upregulates matrix metalloproteinase-2 (MMP-2) expression and stimulates cell migration through activation of the extracellular signal regulated kinase (ERK) signaling pathway in leiomyoma smooth muscle cells (SMCs), but not in normal myometrial SMCs. *Bokhi* can significantly reduce the level of TNF-α and thus reduce cell migration.

IL-2 can not only promote the proliferation and differentiation of effector T cells, but also plays a key role in maintaining the stability of regulatory T cells (Treg) (Malek, 2008; Boyman and Sprent, 2012). IL-2 is also an important signal to maintain viability; if proliferation of T cells leads to the removal of IL-2 then this results in cell apoptosis (Chihara, 1998). Our experimental results showed that after treatment with *Bokhi* levels of IL-2 in ULM rats were improved, thus increasing the immunity of ULM rats.

Nitric oxide (NO) is a potent vasodilator produced by nitric oxide synthase (NOS) (Sengoku *et al,* 2001). NOS has been reported to be expressed most prominently in the uterus (Telfer *et al,* 1995; Telfer *et al,* 1997; Tseng *et al,* 1996).

Research has reported that the degree of expression of NOS was higher in the uterus of women who had ULM than in women without ULM (Oh *et al,* 2013). Therefore, by decreasing the level of NOS, *Bokhi* also reduces ULM.

The main components of *Bokhi* are sex steroids. The action of sex steroids in the myometrium are locally mediated by numerous growth factors, cytokines and chemokines. Disruption of autocrine/ paracrine signaling is central to inducing healthy myometrium transformation into the leiomyoma phenotype (Ciarmela *et al,* 2011). A complete understanding of the actions of sex steroids on ULM may provide new perspectives for disease treatment with minimal interference in the systemic and physiological functions of these hormones. *Bokhi*, might act as an oestrogen receptor antagonist and thereby decrease the concentrations of growth factors within the ULM (Palomba *et al,* 2005).

Most of the drugs used in the treatment of ULM are based on animal studies. The differences in ULM mechanisms between humans and animal models are probably due to a complex interaction of different factors. One example is that the differences between rodent and primate endometrial growth (Kurita *et al,* 2005) and between oestrous and menstrual cycles, which determine obvious differences in the endometrial impact of leiomyomas in rats and humans (Bulun, 2013; Hirshfeld-Cytron *et al,* 2011). Therefore, the therapeutic effect of camel *Bokhi* on ULM needs further clinical research in humans.

Acknowledgements

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