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Ameliorative effects of a Chinese herbal formula, fufang meidengmu on uterine leiomyoma in a mice model

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Abstract: Fufang Meidengmu (FFMDM) is an ethnic herbal medicine form Yunnan province of China, which is often used for the treatment of uterine leiomyoma (UL). Combined Gancao (*Glycyrrhiza uralensis* Fisch.) and Haizao (*Sargassum pallidum* (Turn.) C.Ag) in FFMDM represent an herbal pair in the so-called "eighteen antagonistic medicaments" according to traditional Chinese medicine. In this study, we explored the prevention and treatment effects of FFMDM component compatibility on UL in mice. Female Kunming mice were injected for different periods of time with different concentrations of estradiol benzoate (EB) to investigate a feasible method to establish a mice model of UL. Treatment with 0.3mg/kg EB for 15 days was found to be the optimal condition for UL mice models. We then investigate the role of Gancao and Haizao in FFMDM, and explored the underlying mechanism of action of UL mice. Our findings suggested that Gancao and Haizao exerted the favorable effects. In addition, FFMDM is effective in the treatment of UL, and its mechanism was associated with the estrogen (ER) and progesterone receptors (PR).

Keywords: Estrogen, fufang meidengmu, inflammation, nos activity, progesterone, uterine leiomyoma.

INTRODUCTION

Uterine leiomyoma (UL) is benign tumors from smooth muscle cells and it is common in women of reproductive years at 30-50 years old, the prevalence rate is 20%-40%, but the incidence rate is 70%-80% at age 50 (Giuliani *et al.*, 2020; Machado-Lopez *et al.*, 2021). UL have become the main cause of hysterectomy and infertility, which seriously affect women's health and quality of life (Herve *et al.*, 2018). At present, UL are recognized as a hormone-dependent tumor, E_2, P and their receptors can play an important role in its occurrence and development (Reis *et al.*, 2016). Currently, the drugs used to treat UL are mainly inhibiting steroid hormones, such as antiprogestins, aromatase inhibitors, but these can only reduce the size of a tumor and the tumor will continue to grow after drug withdrawal (Feng *et al.*, 2021). Although hysterectomy is a relatively thorough method, it is not suitable for the patients with fertility requirements and the high costs associated with surgical treatment poses a heavy economic burden (Harrington *et al.*, 2020). Therefore, it has become a vital task for clinical and scientific researchers to develop a safe and effective new clinical drug for the treatment of UL by modern scientific means.

FFMDM is a preparation of Dai and Lahu indigenous medicine selected by the Pu'er National Institute of Ethnic Traditional Medicine in the process of excavating and sorting out ethnic folk medicine, it has been used in folk for more than 500 years. FFMDM has the effects of clearing heat and detoxicating, resolving hard lumps, relieving swelling and pain, it has been reported that FFMDM can improve the hyperplasia of mammary glands and regulate the hormone level *in vivo* (Feng Deqiang, 2006; Yu Chenghao, 2012). In addition, FFMDM is also found that it has good effects on UL in clinical treatment (ya, 2013), but the pharmacological research has not been reported. What's more, Gancao (*Glycyrrhiza uralensis* Fisch.) and Haizao (*Sargassum pallidum* (Turn.) C.Ag) in FFMDM represent an herbal pair in the so-called "eighteen antagonistic medicaments" according to the Chinese medicine. However, until now, the compatibility mechanisms remain unknown.

Therefore, in this study, we took FFMDM intervention while making a UL model, from organ coefficients(ovary, uterus), histopathology, sex hormone levels (E2, P and their receptors) and inflammation factors to explore the prevention and treatment effects of FFMDM on UL.

MATERIALS AND METHODS

Preparation of FFMDM

FFMDM was composed of Luxiancao (*Balanophora involucrata Hook.* f.) 10g, Zijinteng (*Wisteria sinensis* Sweet) 10g, Meidengmu (*Maytenus hookeri* Loes.) 13g, Baihuasheshecao (*Hedyotis diffusa* Willd.) 10g, Chonglou (*Paridis polyphylla* Smith var.) 3g, Haizao (*Sargassum pallidum* (Turn.) C.Ag.) 10g and Gancao (*Glycyrrhiza uralensis* Fisch.) 3g. All the above drugs were purchased from Pu'er National Institute of Ethnic Traditional Medicine. According to the conversion of doses by human and animal, the dosages of FFMDM selected in this experiment were 4.21g/kg, 12.64g/kg and 25.45g/kg, respectively. The dosage of 12.64g/kg was selected for FFMDM group, FFMDMa group and FFMDMb group, those dosages used in the experiment were safe. The extract of FFMDM was prepared by the following

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procedures: Chonglou (*Rhizoma Paridis* Smith var), Luxiancao (*Balanophora involucrata* Hook. f.) and Gancao (*Glycyrrhiza uralensis* Fisch.) were made into coarse powder, 8 times, 5 times and 3 times of volume of 95% ethanol were added and extracted for three times. Then, Baihuasheshecao (*Hedyotis diffusa* Willd.), Zijinteng (*Wisteria sinensis* Sweet), Meidengmu (*Maytenus hookeri* Loes.), Haizao (*Sargassum pallidum* (Turn.) C.Ag.) and the residues of Chonglou (*Rhizoma Paridis Smith* var), Luxiancao (*Balanophora involucrata* Hook. f.) and Gancao (*Glycyrrhiza uralensis* Fisch.) after alcohol extraction were extracted three times with 8 times, 6 times and 4 times of distilled water and then the filtrate of three times was collected and added to the previous alcohol extraction. The total extraction was concentrated and stored in the refrigerator at -4℃. FFMDMa was obtained by removing Gancao (*Glycyrrhiza uralensis Fisch.*) from FFMDM, FFMDMb was obtained by removing Haizao (*Sargassum pallidum* (Turn.) C.Ag) from FFMDM, FFMDMa and FFMDMb were extracted in the same way as FFMDM. The extraction was restored to room temperature before use.

Animals

Kunming female mice, SPF grade, 18-22g, 4-6 weeks old, were purchased from experimental animal research institute of Chinese Academy of Medical Sciences (Certificate of quality No.: scxk (Beijing) 2014-0004). The mice were fed at the laboratory temperature of 22℃-26℃ and humidity of 45%-65%, free access to water and food and the ventilation was well. The experiment was approved by the animal ethics committee of Yunnan University of Chinese Medicine (R-062020S081).

Construction of a UL model in mice and treatment

This experiment was divided into three parts, all of which were grouped according to the random number table method. In the first part, 96 mice, 24 in each group, were divided into control group, model 1 group (0.3mg/kg EB), model 2 group (0.6mg/kg EB) and model 3 group (0.9mg/kg EB), 8 mice for 15, 30 and 45 days respectively. Control group was intramuscularly injected with NS (normal saline), model groups were intramuscularly injected with EB, once a day for 15, 30 and 45 consecutive days. In the second part, 50 mice were divided into five groups, the normal control group, the EB model group, FFMDM group, FFMDMa group, FFMDMb group, 10 in each group. Control group and model group were given NS and treatment groups were intragastrically administrated with the extract of FFMDM, FFMDMa and FFMDMb, once each day for 15 consecutive days while modeling. In the third part, 50 mice were divided into five groups, the normal control group, the EB model group, FFMDM1 group, FFMDM2 group and FFMDM3 group, 10 in each group. Control group and model group were given NS and treatment groups were intragastrically administrated with the extract of FFMDM1 (25.45g/kg), FFMDM2 (12.64g/kg) and FFMDM3 (4.21g/kg), once a day for 15 consecutive days while modeling. The change of body weight and the growth of mice were recorded every other day.

Uterine histopathologic examination

24 hours after the last intragastric administration, blood was taken from the eyeballs of mice, then mice were killed and the uterus was taken out. The shape of the uterus was photographed and the wet weight of the uterus was weighed. Uterine coefficient=wet weight of the uterus/ weight of the mice after 12 hours of fasting×1000. Then 0.4cm uterine tissue was taken and embedded from the same part above the uterine dividing horn, then it was hematoxylin-eosin (HE) stained and observed under the optic microscope (NIKON YS100, Japan); at the same time, the thickness of uterine smooth muscle was measured.

Detection of serum E_2, P, TNF-α and IL-2

The levels of E_2, P, TNF-α and IL-2 in serum were detected by enzyme-linked immunosorbent assay, the activity of NOS was detected by NOS test kit. The detection steps were followed strictly according to the instructions.

Immunohistochemical staining

Uterine tissue from the same part of mice uterus above the uterine dividing horn was taken and fixed with 10% formaldehyde, after uterine tissue was dewaxed, hydrated and repaired antigen. Then, uterine tissue was incubated with 3% H_2O_2 for 10 minutes. Next, the ER, PR and second antibodies were added and incubated for 1h at 18℃-25℃. Then, the uterine tissue was washed with phosphate buffer for 10 min and redyed and observed under optical microscope (NIKON YS100, Japan), the area of positive cells was measured by 600μm×800μm rectangular box in image analysis system.

STATISTICAL ANALYSIS

The experimental results were expressed as mean±SE (standard error) and analyzed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA) statistical software. One-way analysis of variance (ANOVA) was used for the data conforming normal distribution and uniforming the variance, Nonparametric test was used for the data disobeying normal distribution. $P<0.05$ suggested that it was statistically significant.

RESULTS

Different EB concentrations and treatment times have varied effects on morphology of the uterus

As shown in fig. 1A, the shape of uterus in control groups were long and thin, light red, smooth and uniform, without edema and nodule. However, in the model groups

the shape of uterus were dim in color, deformed with irregular nodule protuberances, short and thick and the diameter of the uterine horn became larger with edema. The uterine coefficient and thickness of uterine smooth muscle in model groups were increased as compared with control group, especially the model group with 0.3mg/kg EB for 15 consecutive days was significant (p<0.01) (fig. 1). The pathology result revealed that the control group mice had normal uterine tissue morphology, the endometrial thickness was thin, the muscle fiber had not proliferation and hypertrophy and were arranged orderly, most of them were long strips and some were oblate. However, the endometrium of the model groups were focal hyperplasia, the arrangement of muscle fiber was disordered and irregular (fig. 2).

Effects of the extract of FFMDM, FFMDMa and FFMDMb on uterus morphology in mice

The uterine coefficient of the model group increased evidently than that of control group. However, the uterine coefficient in the FFMDM, FFMDMa and FFMDMb groups decreased and the improvement was more significant in the FFMDM and FFMDMa groups than that of model group (p<0.01) (fig. 3A). The shape of uterus in the control group was long and thin, Y-shaped, smooth and uniform, without edema and nodule. The shape of uterus in the model group was deformed with irregular nodule protuberances, short and thick. In the FFMDM, FFMDMa and FFMDMb groups, the shape of uterus were light red with slightly nodular and coarser; especially the improvement in the FFMDM group was more dramatical than that of the model group (fig. 3B). Under optic microscope (HE 4×10 and 40×10): the myofibers of uterus in the control group were arranged regularly and without hyperplasia, the nucleus staining was fuzzy. The uterine muscle fiber in the model group was thickened, the boundary was unclear, the nucleus was increased irregularly and oblate, some of them had mitotic phase, the gland was expanded seriously and a large number of inflammatory cells could be seen. In the FFMDM, FFMDMa and FFMDMb groups, the muscular layer evidently thinned, the uterine muscle fiber was relatively orderly, endometrial hyperplasia was weak than that of model group and the nuclei were mostly oval. However, the improvement of uterus in FFMDMa and FFMDMb groups was weak as compared with the FFMDM group (fig.3C).

Effects of the extract of FFMDM, FFMDMa and FFMDMb on the contents of E_2 and P in UL mice

As shown in fig. 4, the contents of E_2 (fig. 4A) and P (fig.4B) were decreased in the FFMDM, FFMDMa and FFMDMb groups as compared with the model group, especially the decrease of FFMDM group was significant (p<0.01).

Effects of FFMDM on uterus morphology

The uterine coefficient (fig. 5A) in the model group increased remarkbly as compared with the normal control group (p<0.01), the uterine coefficient in the treatment groups decreased than that of the EB model group (p<0.05) and the decrease in the FFMDM1 group was more obvious (p<0.01). The shape of uterus in the control group was thin, smooth and even in texture, without swelling and nodule. However, the shape of uterus in the model group was deformed with irregular nodule protuberances and some of uterine tissue was edematous, which were improved in the treatment groups (fig. 5B). Under optic microscope, the endometrial layer in the control group had no hyperplasia and the uterine muscle fiber was well-arranged. The thickness of the uterine smooth muscle in the model group was increased and the muscle fiber were hypertrophied and disordered. The endometrial thickness and the muscular layer were obviously reduced in the treatment groups, the uterine muscle fiber were more tidy than the model group (fig. 5C).

Effects of FFMDM on contents of E_2, P, TNF-α, IL-2 and NOS in serum of UL mice

The level of E_2, P, TNF-α and NOS in the model group were dramatically higher and the content of IL-2 were evidently lower than the control group (p<0.01). Nevertheless, the contents of E_2, P, TNF-α and NOS in treatment groups were lower and the content of IL-2 were higher than the model group (p<0.05) and there were dose-dependent (fig. 6).

Effect of FFMDM on expression of ER and PR

In the control group, the cells in the muscular layer of mice uterus were dyed brown, evenly distributed and few in number. While in the model group, both ER and PR positive cells in the model group were increased evidently and stained, distributed intensive. FFMDM treatment significantly reduces ER and PR levels compared to the model (fig. 7).

DISCUSSION

The etiology and pathogenesis of UL are still unknown. It is crucial significance to study the pathogenesis of UL by establishing a stable and reliable animal model. At present, people mainly use exogenous hormones to induce the establishment of UL animal models, including single estrogen induction and combined induction of estrogen and progesterone(Lin *et al.*, 2019; McWilliams *et al.*, 2017). Prior of UL animal model establishment, different concentrations of estrogen and different time points were examined to choose the optimum conditions. The results revealed that the endometrial thickness was thin, the muscle fiber had not proliferation and hypertrophy and were arranged orderly in the control group.

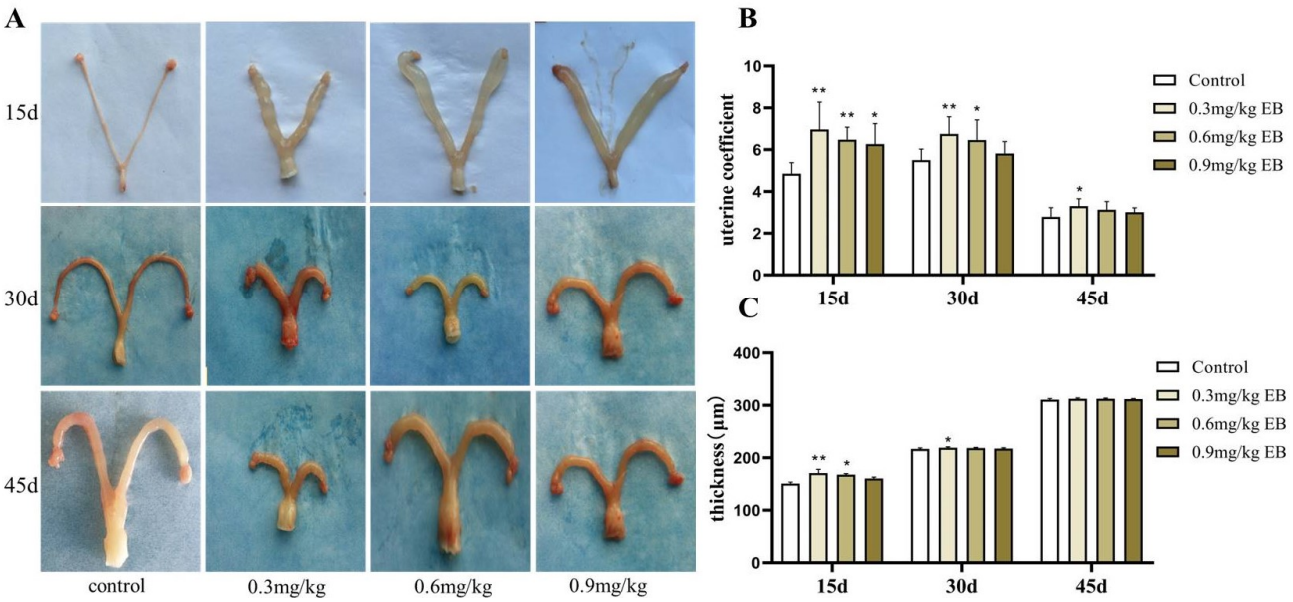

Fig. 1: Different EB concentrations and treatment times have varied effects on morphology of the uterus. (A) Uterine appearance. (B) Uterine coefficient. (C) Thickness of the smooth muscle. Compared with the control group, $^{*}p<0.05$, $^{**}p<0.01$.

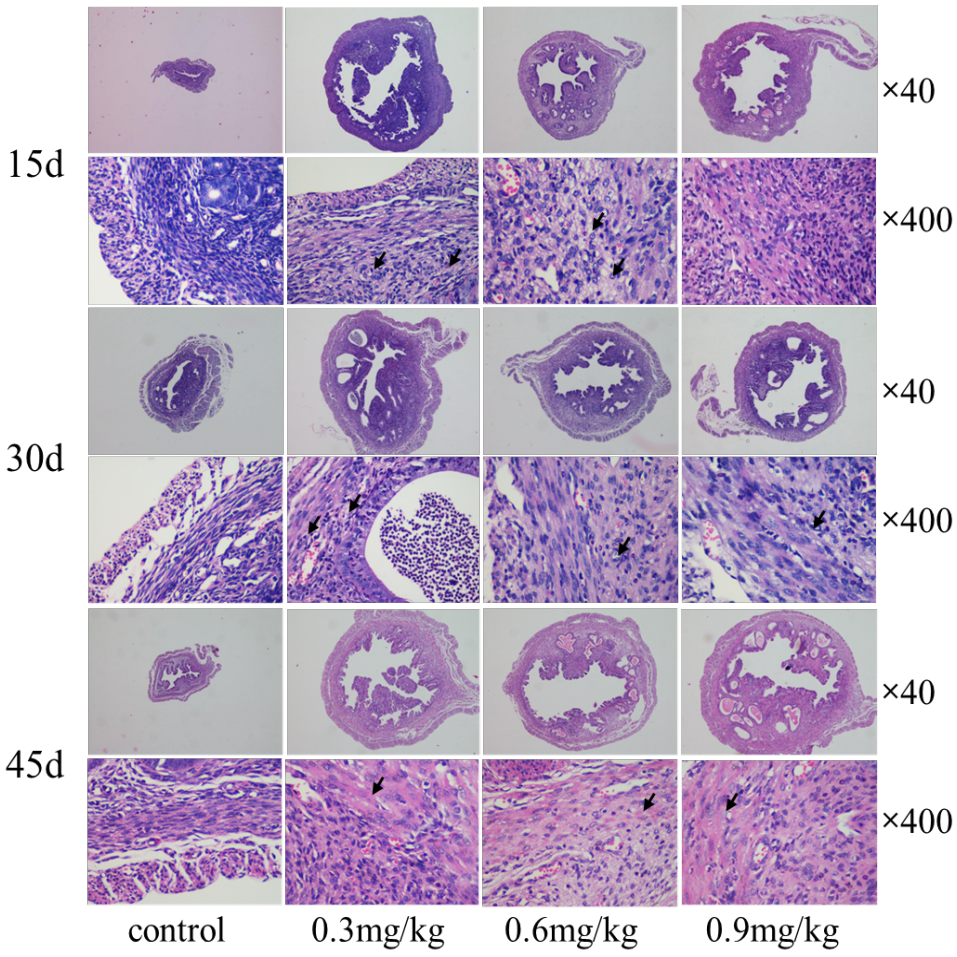

Fig. 2: Pathological changes of the uterus in different groups. (HE:4×10 and 40×10).

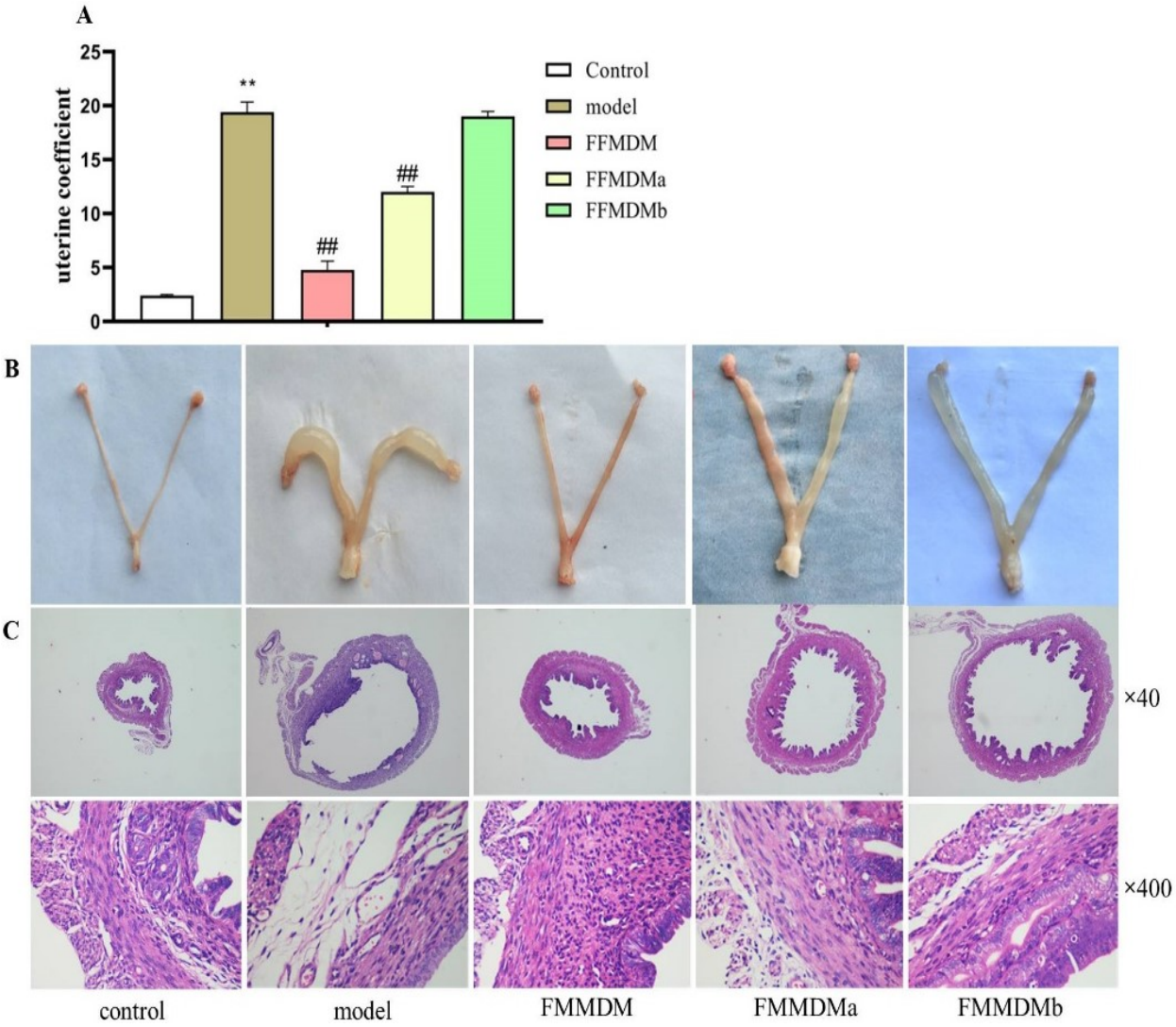

Fig. 3: Effects of the FFMDM, FFMDMa and FFMDMb on uterus and ovary coefficients in UL mice. (A) Uterine coefficient. (B) Uterine appearance. (C) Uterine tissue sections stained with HE. $^{**}p<0.01$ in comparison with control group; $^{\#\#}p<0.01$ in comparison with model group. FFMDM: Fu-fang-mei-deng-mu; FFMDMa: FFMDM remove Gancao (*Glycyrrhiza uralensis Fisch.)*; FFMDMb: FFMDM remove Haizao (*Sargassum pallidum(Turn.) C.Ag*).

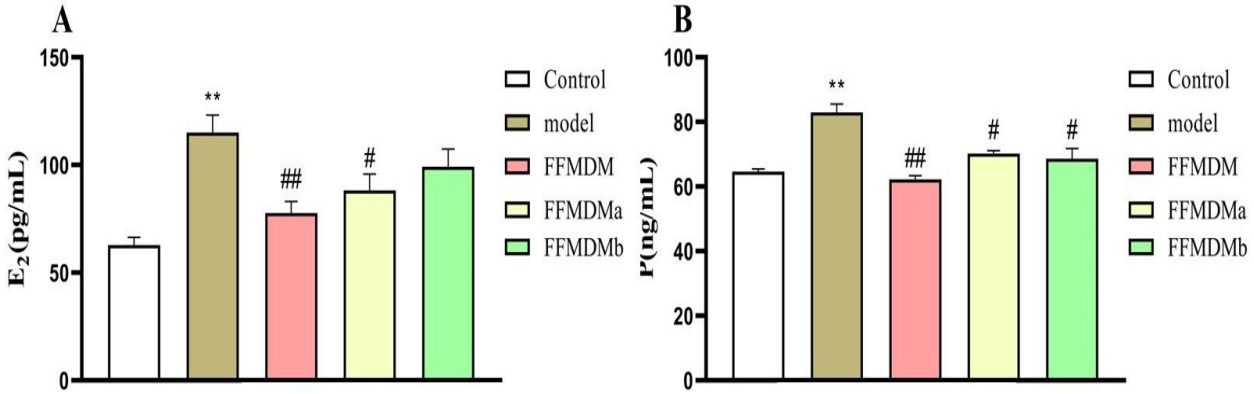

Fig. 4: Contents of E2 (A) and P (B) in UL mice. $^{**}p<0.01$ in comparison with control group; $^{\#}p<0.01$, $^{\#\#}p<0.01$ in comparison with model group. FFMDM: Fu-fang-mei-deng-mu; FFMDMa: FFMDM remove Gancao(*Glycyrrhiza uralensis Fisch.)*; FFMDMb: FFMDM remove Haizao(*Sargassum pallidum(Turn.) C.Ag*).

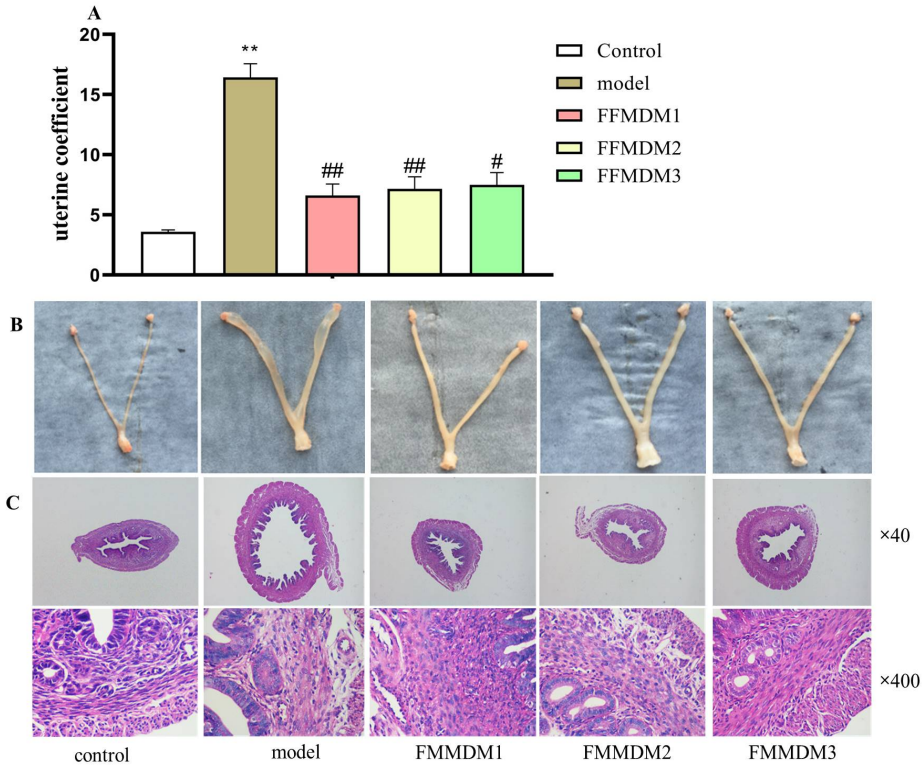

Fig. 5: Effects of the FFMDM on uterus and ovary coefficients in UL mice. (A) Uterine coefficient. (B) Uterine appearance. (C) Uterine tissue sections stained with H-E. $^{**}p<0.01$ in comparison with control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ in comparison with model group. FFMDM1: 25.45g/kg; FFMDM2: 12.64g/kg; FFMDM3: 4.21g/kg.

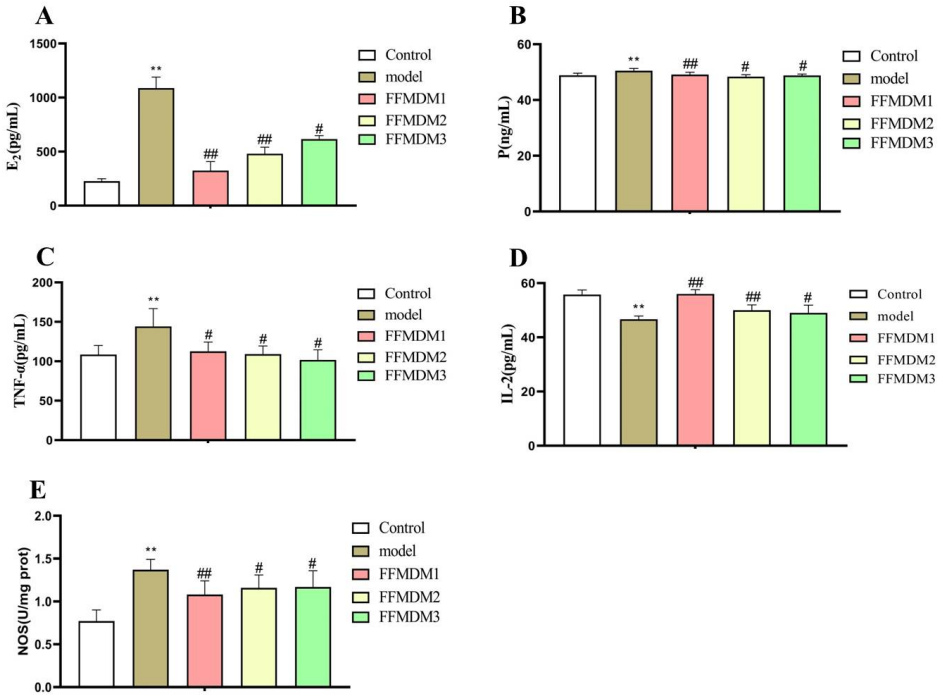

Fig. 6: Effects of FFMDM on contents of E_2 (A), P (B), TNF-α (C), IL-2 (D) and NOS (E) in serum of UL mice. $^{**}p<0.01$ in comparison with control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ in comparison with model group. FFMDM1: 25.45g/kg; FFMDM2: 12.64g/kg; FFMDM3: 4.21g/kg.

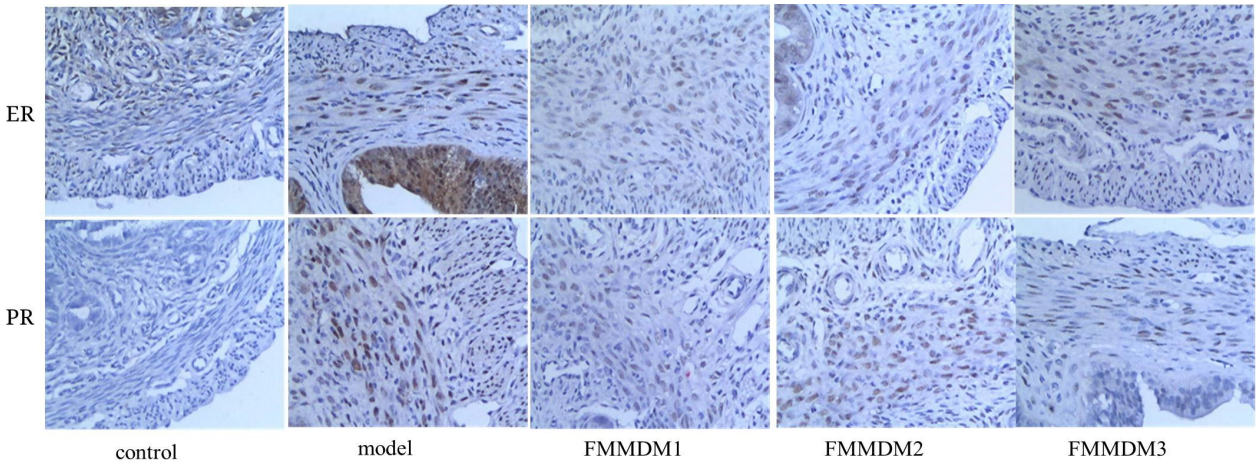

Fig. 7: Effect of FFMDM on expression of ER and PR, the original magnification was200×. FFMDM1: 25.45g/kg; FFMDM2: 12.64g/kg; FFMDM3: 4.21g/kg.

However, the endometrium of the model groups were focal hyperplasia, the arrangement of muscle fiber was disordered and irregular, some lymphocytes and eosinophils diffused; meanwhile, the uterine coefficient and the thickness of uterine smooth muscle were increased than that of control group, indicating the modeling was successful and we found that the model group was more stable by intramuscularly injecting with 0.3mg/kg EB for 15 consecutive days than the other model groups.

Uterine leiomyomas can be classified into the category of "zhengjia" in traditional Chinese medicine and its main pathogenesis is considered to be qi stagnation and blood stasis. The treatment is mainly to promote blood circulation and remove blood stasise. The FFMDM is a preparation of Dai and Lahu indigenous medicine collected and sorted out from the folk and it has been clinically proved that it can treat UL. Nevertheless, because there are Gancao (*Glycyrrhiza uralensis* Fisch.) and Haizao (*Sargassum pallidum* (Turn.) C.Ag) in the FFMDM, which violate the principle of eighteen incompatible medicament, this experiment designed FFMDM, FFMDMa and FFMDMb groups to probe into the actions in animal model of UL. The results showed that FFMDMa and FFMDMb were not as effective as FFMDM in improving UL.

The occurrence of UL is closely related to steroid hormones and estrogen is generally considered as the initial factor to promote the growth of UL, it can affect the permeability and promote angiogenesis, especially in the proliferative stage, the content of estrogen in UL tissue is obviously increase as compared with normal myometrium tissue (Afrin *et al.*, 2021). In luteal phase, the mitosis of myoma cells is significant due to progesterone. Some studies have shown that the ER, PR, mRNA of ER and PR content in the local tissue of uterine myoma are higher than those in the normal tissue around the uterus (Liao *et al.*, 2019; Yang *et al.*, 2016). ER and PR can also recognize and combine with E_2, P and their analogues respectively and play certain effect (Amazu *et al.*, 2020). The results showed that FFMDM decreased the contents of E_2 and P and the expression of ER and PR and showed a certain dose-dependent.

It is reported that the occurrence of UL is related to the low immune function in vivo, which may be related to the disorder of myoma cell proliferation and apoptosis(Liu *et al.*, 2019). TNF-α is expressed in immune cells, but it is also found in smooth muscle cells as a response to tissue injury or upon immune response. When TNF-α is abnormally increased, it will cause immune disorder and vascular dysfunction of patients, make myoma cells escape from host immune surveillance and grow continuously and damage blood vessels (Ciebiera *et al.*, 2018). IL-2 has dual immune regulation on immune cells. In clinical, IL-2 has been used as a candidate drug for immunotherapy of tumor cell growth to stimulate the activation of T cells and the expression of related antigens (Demir *et al.*, 2019; Hsieh *et al.*, 2007). Large dose of IL-2 can promote the activation and proliferation of NK cells (Wang *et al.*, 2013). The results of this experiment revealed that the FFMDM can decrease the content of TNF-α and increase the content of IL-2, we think FFMDM can enhance the immune function and inhibit the inflammatory reaction.

NOS is the only limiting factor of NO synthesis *in vivo*. However, NO participates various physiological processes of blood vessels and it is an important information molecule of organism, meanwhile, it also participates in the regulation of female reproductive process(Thomsen *et al.*, 1994). When NO an increased excessively, vascular endothelium-dependent contraction is impaired (Tang *et al.*, 2009; Wrona *et al.*, 2022).

Especially, during menstruation, the uterus cannot contract to stop bleeding, resulting in excessive menstruation in patients with UL. The expression of NOS is higher than that of normal myometrium in UL, which makes myoma cells stay in capillaries due to mechanical resistance and promotes the growth of myoma cells(Chen Fanglan, 2002). The increase of NOS in serum indicates that there is tissue hyperplasia in vivo. In this experiment, the activity of NOS in the model group was higher than that of control group, but in the treatment groups it was lower than the model group, indicating that FFMDM can play an anti-fibrosis role by regulating the activity of NOS.

CONCLUSION

All in all, FFMDM has an obvious inhibitory effect on UL, it is suggested that FFMDM not only targets one location but also has multiple targets. Our study has proved that FFMDM has positive prevention and treatment effects on UL, which is achieved by reducing uterine volume, changing uterine structure, regulating hormone levels and inhibiting inflammation. Our work provides a new basis for the prevention and treatment of UL for FFMDM.

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