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Original Article

Effects of pueraria root (*pueraria radix*) on the content of collagen and elastin in pelvic floor dysfunction patients

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Abstract: This study was performed to investigate the effects of *Pueraria radix*, the root of a legume *Pueraria lobata* (Wild.), on the content of collagen and elastin and discuss its role in treating patients with pelvic floor dysfunction (PFD). This prospective, multi-center, and randomized study was performed on postmenopausal women diagnosed with PFD planning to undergo vaginal hysterectomy. Included patients were randomly assigned into pueraria-treated group or control group. Then randomization was stratified by the Pelvic Organ Prolapse (POP) stage [early POP stage (I, II) *vs* advanced POP stage (III, IV)]. The primary outcome was the morphology and content of collagen and elastin, which was detected by hematoxylin-eosin (HE) and Weigert's staining, or immunohistochemistry. The intraoperative outcome, postoperative complication and pathologic changes of endometrium and serum estradiol level were also recorded. Totally 60 patients were included, with 30 women in each group. For each group, there were 15 patients with PFD at early POP stage and 15 patients at advanced POP stage. Preoperative administration of pueraria tablet could improve the morphology and significantly increase the content of type I/III collagen and elastin ($P < 0.001$). Additionally, patients in pueraria-treated group had significantly shorter duration of operation ($P = 0.001$), less blood Loss during operation ($P < 0.001$). Furthermore, pueraria tablet treatment is safe and well-tolerated, with comparable adverse effects in the control group. In conclusions, preoperative administration of *Pueraria radix* could remarkably increase the content of collagen and elastin, which might be beneficial for postmenopausal women with PFD.

Keywords: Puerariae, pelvic floor dysfunction, extracellular matrix, collagen, elastin

Introduction

Pelvic floor dysfunction (PFD) is a complex and often debilitating group of conditions which include urinary incontinence, voiding dysfunction, pelvic organ prolapse (POP), and anal incontinence [1]. It is a common problem in women and strongly linked to childbirth and aging [2]. However, the pathology of PFD was still unclear, which limited the development of clinical treatment for PFD. Recent studies have found that collagens and elastin, as the major supporting components of pelvic floor extracellular matrices (ECMs), may play important roles in maintaining the strength and tenacity of the pelvic connective tissues [3, 4]. Especially in postmenopausal women with low estrogen levels or abnormal metabolism, collagen deficiency and elastin degradation are closely related to the development of PFD [3].

Pueraria root (*Pueraria radix*) widely known as Ge-gen (Chinese name) is the root of a legume *Pueraria lobata* (Wild.) Ohwi, and it is also a kind of traditional Chinese medicine [5, 6]. For clinical use, *Pueraria radix* is often made into powder or further pressed into tablet, and applied widely for treating fever, diarrhea, emesis, cardiac dysfunctions, liver injury, weight loss, and toxicosis [7]. *Pueraria radix* contains variable amounts of phytoestrogens such as puerarin, daidzin, genistin and genistein, which have been indicated to elicit estrogenic activity [8, 9]. Previous study has shown that vaginal estrogen application preoperatively could increase the synthesis of mature collagen, decrease the activity of degradative enzyme, and then improve the maintenance of connective tissue integrity of the pelvic floor in postmenopausal women with prolapse who planned surgical repair of POP [10]. However, widespread

use of estrogen brings considerable worries about many side effects caused by this treatment [11]. Thus, plant estrogen-like compounds have attracted a wide range of attention as potential alternatives to estrogen therapy for management of menopausal symptoms with few side effects. Thus, we speculated that *Pueraria radix* administration might be helpful for the treatment of patients with PFD.

In the present study, we investigated the effect of preoperative administration of pueraria tablet in patients with PFD planning to undergo vaginal hysterectomy through detecting the content of collagen and elastin, following by the intraoperative outcome, postoperative complications as well as the pathologic changes of endometrium and serum estradiol level after surgery. We hoped our study would provide basis for the further investigation of treatment of PFD by *Pueraria radix*.

Materials and methods

Design and patients

Postmenopausal women with clinical diagnosis of PFD and prepared to undergo vaginal hysterectomy were recruited from Shanghai Punan Hospital, Shanghai Seventh People's Hospital or Renji Hospital of Shanghai Jiaotong University School of Medicine from December, 2009 to November, 2012 for this prospective, multicenter, double-blind, and randomized study. Ethical approvals for human subjects were obtained from the research ethics committees of all the three Hospitals; informed consent was obtained from each patient. To enroll, women could have no exogenous estrogens treatment in the prior three months. In addition, patients with functional ovarian tumor were not included.

Sample estimation

Since few studies were performed to explore the efficacy of pueraria tablet in patients with PFD, the sample size was evaluated following our preliminary experiment. Sample size was to provide 80% power to detect a 1.5-fold content of type I collagen detected by immunohistochemistry in patients with PFD orally administered of pueraria tablet (0.33 g/tablet, one tablet per day) compared to PFD patients without pueraria tablet treatment with a 2-sided 5% significance level. Assuming a 10% early termination rate, a minimum of 30 patients per group (total 60 patients) were to be enrolled in each group.

Randomization and masking

A computer-generated random allocations sequence (SPSS version 19.0, SPSS, Chicago, IL, USA) prepared by an individual who is independent of the study team was used to allocate participants to either pueraria-treated group (treated with pueraria tablet) or placebo group at a ratio of 1:1. Randomization was stratified by the Pelvic Organ Prolapse (POP) stage [early POP stage (I, II) *vs* advanced POP stage (III, IV)], which was defined according to the standardized Pelvic Organ Prolapse Quantification (POP-Q) staging system [12]. In addition, patient, study staff and investigators were masked to treatment assignment throughout the primary analysis period. The experimental designers of this study were unmasked to treatment assignment.

Procedures

Patients in the pueraria-treated group were administered of oral pueraria tablet (0.33 g/tablet, including 0.425 mg isoflavones). Patients in the placebo group were given placebo tablet, which were designed to taste, smell and look similar to the pueraria tablet. Patients in both group were required to take one tablet per day until the vaginal hysterectomy. Treatment lasted 60 days. Patients were not allowed to take any concomitant medications associated with the treatment of PFD during the trial. The vaginal hysterectomy performed on patients from both groups were executed by the same surgical team in Renji Hospital.

Outcomes

The primary outcome of the present study was the type I/III collagens and elastin morphology and content in uterosacral ligament tissues, which was evaluated by immunohistochemistry. The second outcomes included intraoperative outcome (duration of operation, blood loss), postoperative complications (stump inflammation, stump granulation and stump bleeding), pathologic changes of endometrium and serum estradiol level postoperatively.

Table 1. Basic characteristics of the patients

	Patients at early POP stage (n = 30)			Patients at advanced POP stage (n = 30)		
	Pueraria-treated group (n = 15)	Control group (n = 15)	*P*-value	Pueraria-treated group (n = 15)	Control group (n = 15)	*P*-value
Age (years)	71.39 ± 5.56	70.94 ± 6.85	0.8448	72.11 ± 4.32	71.86 ± 5.61	0.8922
Post-menopause years (years)	19.75 ± 6.88	21.42 ± 4.59	0.4408	20.21 ± 5.43	20.92 ± 5.03	0.7131
BMI	24.26 ± 1.89	23.06 ± 1.72	0.0797	23.95 ± 1.64	23.68 ± 1.39	0.6305
Times of gravidity (times)	3.48 ± 1.62	3.08 ± 1.62	0.5045	3.81 ± 1.49	3.74 ± 1.55	0.9006
Times of parturition (times)	2.12 ± 0.71	2.09 ± 0.58	0.9001	2.31 ± 0.54	2.14 ± 0.42	0.3441

BMI: Body Mass Index; POP: pelvic organ prolapsed. Independent samples t-test was used for comparing the differences between pueraria-treated and control group in age, post-menopause years, BMI, times of gravidity and times of parturition.

Histological analysis

At the time of surgery, about 100 mg uterosacral ligaments were obtained from all patients and immediately fixed in buffered paraformaldehyde (10%) for 24 h. After rinsing, samples were immersed in paraffin at 58°C overnight and then processed continually for 4 h after replacing the paraffin. Then paraffin-embedded tissue samples were cut into 4-μm sections for further analysis.

A part of sections were stained with hematoxylin-eosin (HE) and Weigert's elastin stain after deparaffinization and rehydration [13]. All these stained sections were observed by light microscopy (BZ-9000; Keyence Co., Osaka, Japan) and the images were acquired using a digital camera (C-5060, Olympus, Tokyo, Japan).

For immunostainings, the other part of sections were incubated with 0.01 M citrate buffer (pH = 6) and received microwave treatment at 100°C for 15 min for antigen retrieval after deparaffinization and rehydration. Afterwards, sections were incubated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity and 5% normal goat serum for 30 min to block nonspecific binding at room temperature. Afterwards, the sections were respectively incubated with in rabbit-anti-human type I collagen polyclonal antibody (1:100, Boster Biology, WuHan, China), rabbit-anti-human type III collagen polyclonal antibody (1:150, Boster Biology, WuHan, China) and rabbit anti-human elastin antibody (ZSGB Biology, Beijing, China) at 4°C overnight, followed by incubated with secondary antibody (Envision + HRP Rabbit, DAKO Cytomation, Carpinteria, CA) at room temperature for 30 min. Finally 3,3-diaminobenzidine was used for the color development and hematoxylin was used for counterstaining. Negative controls were obtained by substituting the primary antibody with PBS. Images were obtained using a Zeiss Axioplan 2 microscope at a 400× magnification (Carl Zeiss, Germany) and analyzed using the Kontron KS400 version 3.0 image-processing software (Carl Zeiss, Germany). On each slide, 3 views were randomly selected and 5 pictures were acquired for each view. The gray levels from 10 to 120 were considered positive expression. According to the gray levels, the area ratio of positive cells and overall view was used for the semi-quantitative analysis of proteins expression. The evaluation of positive expression were conducted by two independent researchers who were blind to the experiment grouping design without duplication. Inter-examiner agreement was assessed using weighted coefficient Kappa. A Kappa score higher than 0.80 indicated a high inter-observer agreement.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) software. Data were presented as mean ± standard deviation (S.D.). Independent samples *t*-test was used for the comparison between groups. Chi-square test was used for comparison of percentage. $P < 0.05$ was considered statistically significant.

Results

Patients

A total of 60 patients were eligible for our study, with 30 women in each group. Women in each group was stratified based on POP stage. For each group, there were 15 patients with PFD at early POP stage and 15 patients at advanced POP stage. The basic characteristics of patients in each group were presented in **Table 1**. There

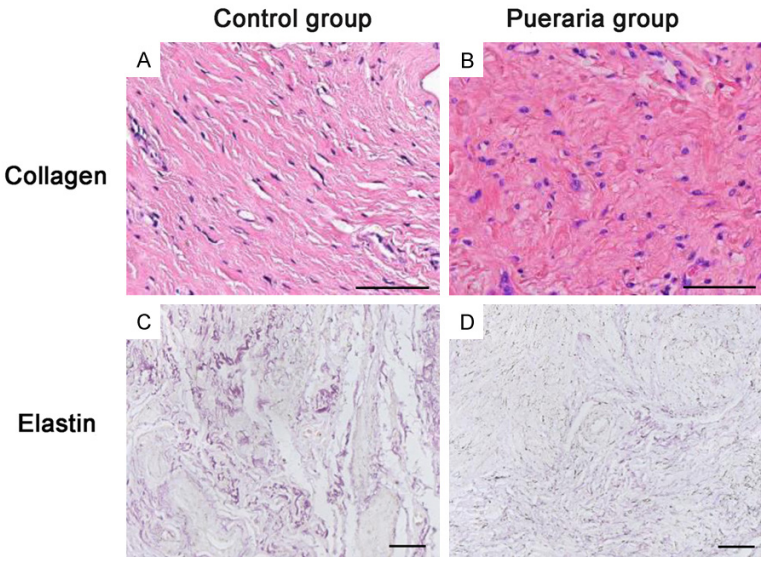

Figure 1. Morphology of collagen and elastin fibers. A: Hematoxylineosin (HE) staining in control group; B: HE staining in puerariae-treated group; C: Weigert's staining in control group; D: Weigert's staining in puerariae-treated group; all the bars were 50 μm.

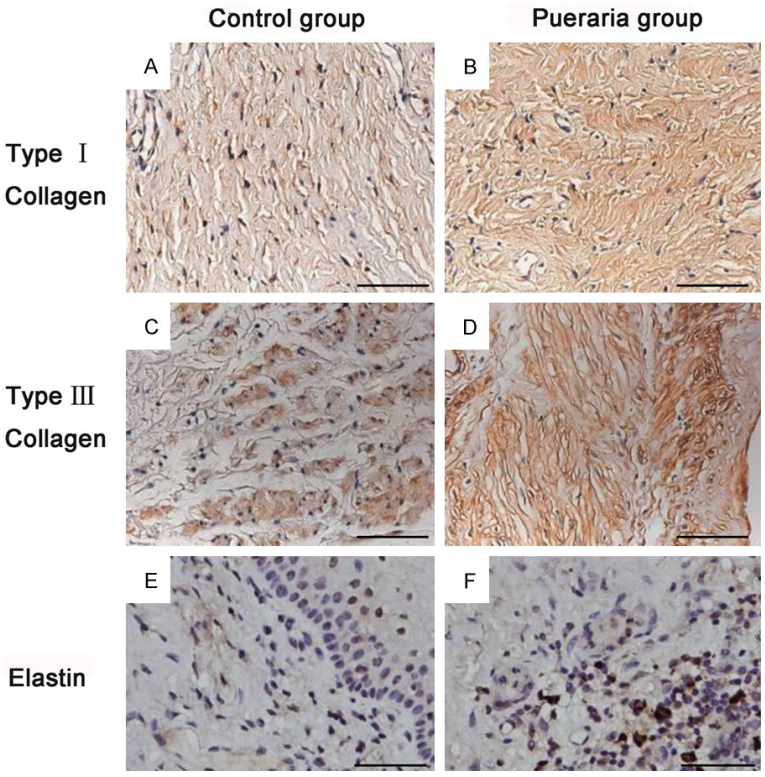

Figure 2. Effects of puerariae on the expression levels of Type I/III collagen and elastin analyzed by immunohistochemistry staining. A: Type I collagen immunohistochemical staining in control group; B: Type I collagen immunohistochemical staining in puerariae-treated group; C: Type III collagen immunohistochemical staining in control group; D: Type III collagen immunohistochemical staining in puerariae-treated group; E: Elastin immunohistochemical staining in control group; F: Elastin immunohistochemical staining in puerariae-treated group; all the bars were 50 μm.

was no significant difference in terms of age, post-menopause years, body mass index (BMI), times of gravidity and times of parturition between puerariae-treated and control group among patients with PFD at early POP stage or advanced POP stage ($P > 0.05$).

Primary outcome

The morphology and content of type I/III collagens and elastin in uterosacral ligament tissues were considered to be the primary outcome in our study. Kappa score for estimating the inter-examiner reliability for evaluating the positive expression of these proteins was of 0.897.

First, we compared the morphology of collagen and elastin between pueraria-treated and control group after HE and Weigert's elastin staining. As presented in **Figure 1A** and **1B**, the collagen fibers were stained pink. Compared to the control group, deeper pink appearance, larger amount of collagen fibers and more uniform and intensive distribution of collagen were observed in the pueraria-treated group. Meanwhile, the elastin fibers were stained purple by Weigert's staining and displayed in **Figure 1C** and **1D**. The elastin fibers with larger amount and more uniform distribution were found in the pueraria-treated group compared to the control. These results indicated that collagen and elastin fibers morphology could be improved in patients after pueraria tablet treatment.

We also tested the content of type I/III collagens and elastin in both control and pueraria-

Table 2. Post-operational clinical outcomes of the patients

	Pueraria-treated group	Control group	*P*-value
Duration of operation (min)	44 ± 13	57 ± 16	0.001
Blood Loss during operation (ml)	100 ± 20	130 ± 15	< 0.0001
Complications (%)			
Stump granulation	2/30 (6.67%)	4/30 (13.33%)	0.389
Stump bleeding	0/30 (0%)	2/30 (6.67%)	0.150
Stump inflammation	2/30 (6.67%)	8/30 (26.67%)	0.038

Independent samples t-test was used for comparing the differences between pueraria-treated and control group in duration of operation and blood loss during operation. Chi-square test was used for comparing the incidence of complications in pueraria-treated and control group.

treated group. Following the results in **Figure 2**, there were more positive cells of type I, III collagens and elastin expression in pueraria-treated group than the control. After statistical analysis, administration of oral pueraria tablet could significantly increase the expression of both collagens and elastin in uterosacral ligament tissues in patients with PFD at either early POP stage or advanced POP stage ($P < 0.001$) when compared to the patients in the control group. Our results indicated that preoperative treatment of pueraria tablet would increases the content of collagen and elastin in the uterosacral ligament tissues of patients.

Secondary outcomes

Intraoperative outcome: The intraoperative outcomes including duration of operation and blood loss were shown in **Table 3**. Patients in the pueraria-treated group had significantly shorter duration of operation ($P = 0.001$) and less blood loss during operation ($P < 0.001$), indicating an improvement of intraoperative outcome after preoperative administration of pueraria tablet.

Postoperative complications: As presented in **Table 2**, there was obviously lower incidence of stump inflammation in patients orally administered with pueraria tablet preoperatively than those without ($P = 0.038$), while there were no significant difference of incidence of stump granulation ($P = 0.389$) and stump bleeding ($P = 0.150$) between patients from two groups. These results suggested that preoperative treatment of pueraria tablet was well-tolerated in women with PFD.

Pathologic changes of endometrium and serum estradiol level: Atrophic endometrium was found in patients both from pueraria-treated group and control group. Additionally, there were no significant difference of serum estradiol level between patients with PFD from both groups ($P = 0.465$). However, there was a decreased tend compared the pueraria-treated group with control group (**Table 4**).

Discussion

PFD is a common problem in women that seriously influence the life quality of them. Menopause is considered a risk factor for the development of the PFD, and the decline in sex steroid hormones of pelvic supportive tissues may be the physiological basis for the increased risk [14]. Although the precise mechanism of these hormones on these supportive tissues in the pelvic floor remains poorly understood, it was speculated that the supplementation of estrogen would be helpful for the treatment of patients with PFD [10]. However, many side effects of estrogen treatment limit the widespread use of this therapy. *Pueraria lobate* (Wild.) Ohwi which is an isoflavone/phytoestrogen-rich tuberous herb grows in many parts of the world. The root of *Pueraria lobata* named as *Pueraria radix* is a traditional medicine and used for treating many diseases [15]. This herb contains puerarin, daidzin, genistin and genistein and other phytoestrogens and all of these compounds elicit estrogenic activity [8]. We speculated that *Pueraria radix* might be also helpful for the treatment of patients with PFD.

As described previously, the pelvic organs support is provided by both levator ani muscles and the vagina connective tissue, which opposes the increasing downward forces from intra-abdominal pressure and gravity [16]. For the vagina connective tissues, there are variable amounts of collagen, smooth muscle, elastin and nonfibrillar matrix [17]. Previous biochemical studies indicated that women with pelvic organ prolapse has a lower collagen content of vaginal connective tissues and the decrease of the collagen content may be induced by menopause [18, 19]. Additionally, vaginal estrogen

Table 3. Comparison of the expression of extracellular matrix proteins between different groups

Proteins	Mild POP (n = 30)			Severe POP (n = 30)		
	Pueraria-treated group (n = 15)	Control group (n = 15)	*P*-value	Pueraria-treated group (n = 15)	Control group (n = 15)	*P*-value
Type I collagen (%)	39.68 ± 9.84	11.20 ± 5.77	< 0.0001	32.74 ± 8.06	8.96 ± 3.34	< 0.0001
Type III collagen (%)	34.76 ± 3.16	21.73 ± 5.14	< 0.0001	29.75 ± 3.01	17.89 ± 3.82	< 0.0001
Elastin (%)	11.16 ± 3.60	1.23 ± 0.47	< 0.0001	6.33 ± 2.34	0.69 ± 0.30	< 0.0001

POP: pelvic organ prolapsed. Independent samples t-test was used for comparing the proteins expression levels of pueraria-treated and control group.

Table 4. Postoperative pathology and serum estradiol levels in the two groups

	Pueraria-treated group	Control group	*P*-value
Endometrial evaluation	Atrophic	Atrophic	
Estradiol (pmol/L)	43.08 ± 20.21	46.72 ± 18.06	0.4649

Independent samples t-test was used for comparison of estradiol levels in pueraria-treated and control group.

application preoperatively could increase the synthesis of mature collage and decrease degradative enzyme activity, then improved the maintenance of connective tissue integrity of the pelvic floor in postmenopausal women with prolapse [10]. It was speculated that similar effects of phytoestrogen-rich herbs *Pueraria radix* on collagen content could be obtained, meanwhile with few side effects. *Pueraria radix* is always made into powder or further pressed into tablet in clinic. In the present study, preoperative administration of pueraria tablet (containing 0.425 mg isoflavones) could improve the morphology of collagen and elastin, and increase the collagen type I and III content as well as elastin content, all of which could improve the maintenance of strength and tenacity of the pelvic connective tissues in patients with PFD.

The main bioactive component of *Pueraria radix* is puerarin, which is also commonly regarded as phytoestrogen and has shown the estrogenic activity in certain animal models [20]. It was reported that a short term injection of puerarin could up-regulate the number of uterine glands in immature ovariectomized rats, and a long term injection of puerarin obviously increased the percentage of keratinocytes in mature rats, indicating the estrogenic activity of that puerarin [21]. In addition, it was also reported that puerarin could enhance the expression of type I collagen in baboon osteoblasts [22], and inhibit the deposition of collagen I and collagen III in chronic hypoxia hypercapnia rats [23]. Moreover, puerarin also can help rehydrate the skin and strengthen collagen and elastin [24]. Therefore, we inferred that positive effects of preoperative administration of pueraria tablet on collagen and elastin content might be attributed to the function of puerarin.

In addition, we also investigated the effects of preoperative administration of puerariae tablet on intraoperative outcome and postoperative complication. Our results suggested preoperative administration of pueraria tablet is a safe and well-tolerated therapy for patients with PFD. The pathologic changes of endometrium and serum estradiol level after vaginal hysterectomy were also detected. We found that the serum estradiol level was similar between the pueraria-treated and control group, and there was a decreased tend compared the pueraria-treated group with control group. This result was consistent with the study of Trisomboon *et al* [25], which reported that serum estradiol levels were unchanged, but tended to decrease in aged menopausal cynomolgus monkeys treated with pueraria mirifica. Meanwhile, no different pathological changes were found between both groups.

However, there were several limitation in our study. Firstly, follow-up data to further investigate the beneficial effects of preoperative administration in patients with PFD has not afforded. Secondly, we did not measure the thickness of the vaginal wall in patients due to the limitation of technique. Thirdly, the sample size in our study is relatively small. Therefore, the beneficial effects of preoperative adminis-

tration of *Pueraria radix* should be interpreted cautiously and further studies with larger sample size and more clinical parameters are still needed.

In conclusion, preoperative administration of *Pueraria radix* could improve the morphology and significantly increase the content of collagen type I, III and elastin in women with PFD. Meanwhile, this therapy is safe and well-tolerated, and even beneficial for the intraoperative outcome. Thus, *Pueraria radix* might be helpful for treating patients with PFD, while further study with larger sample size is still needed to validate the results of this study.

Disclosure of conflict of interest

None.

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