

Original Article

Effect of puerarin on vaginal connective tissue in patients with pelvic organ prolapse

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Received October 26, 2015; Accepted June 28, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Aims: The objective was to determine the impact of puerarin on the vaginal connective tissue of pelvic organ prolapse (POP) patients. Methods: We performed a prospective evaluation of patients who presented with POP between June 2010 and November 2012. Fifty-two women were enrolled and divided into a puerarin treatment group and control group. Patients in the puerarin group were treated with 0.429 mg/day puerarin for 30 days. The control group received placebo for 30 days. After treatment, all of patients accepted pelvic organ prolapse repair and biopsies of prolapsed tissue were obtained. Morphologic changes in the two groups were evaluated by light microscopy. The expression of elastin, collagen, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP) and TGF- β was evaluated in all patients using immunohistochemistry. Result: Patients treated with puerarin had significantly less prolapse symptoms than control patients. Moreover, the expression of elastin, collagen, NPY, VIP and TGF- β was significantly increased in the puerarin group. Conclusion: Puerarin is a phytoestrogen that has therapeutic effects in patients with POP.

Keywords: Puerarin, connective tissue, pelvic organ prolapse

Introduction

Pelvic organ prolapse (POP) is caused by the descent of pelvic organs into the vagina, which is a result of failure of the vagina and its supportive connective tissues to provide primary support to the pelvic organs. POP is a global health care problem, and while this condition is not fatal, it can significantly interfere with the quality of life. Although the cause of POP is multifactorial, connective tissue metabolism plays an important role in its pathogenesis [1, 2].

Estrogen deficiency is a known risk factor for POP. Estrogen replacement therapy traditionally has been used to improve the structural integrity of the pelvic tissue, with favorable effects on urinary incontinence. Moreover, it has been suggested that estrogen restores collagen metabolism to a premenopausal state [3]. In a double-blind, placebo-controlled trial of postmenopausal women with urinary stress incontinence, Jackson found that estradiol therapy resulted in both new synthesis and new degradation of collagen. The major problem with the current estrogens used in Menopausal Hormone Therapy (MHT) is that they are not tis-

sue selective [4]. In addition, estrogens can exert effects on multiple organs, and can play an important role in breast tumor development.

Puerarin is an isoflavonoid isolated from the root of the plant *Pueraria lobata*. It is endemic to Thailand and has been prescribed in China for the treatment of cardiovascular disease. In addition, puerarin has also been reported to exert estrogen-like and antioxidant effects. Therefore, puerarin acts as a phytoestrogen and is a possible therapeutic agent in neurodegenerative diseases [5].

We found that POP patients treated with puerarin showed normalization of POP. We therefore investigated the mechanisms underlying the bioprotective effect of puerarin on the connective tissues of POP patients.

Materials and methods

Preparation of puerarin

Puerarin was obtained from the root of *P. lobata* (Willd.) (Marco Pharmaceutical Co. Ltd., Thai-

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Table 1. Baseline Characteristics and Clinic feature between two groups (mean \pm SD)

	Puerarin group n = 30	Control group n = 22	P
Age (years)	71.4 \pm 8.90	67.50 \pm 7.46	0.10
Menopausal Period (years)	20.0 \pm 12.8	16.05 \pm 8.42	0.089
BMI	23.98 \pm 2.66	22.83 \pm 2.35	0.15
Times of pregnancy	4.67 \pm 1.95	3.67 \pm 1.46	0.076
Parity	3.27 \pm 1.78	2.62 \pm 1.36	0.070
POP-Q stage (%)			
I	0	0	-
II	4 (13.3%)	4 (18.2%)	0.12
III	19 (63.3%)	12 (54.5%)	0.068
IV	7 (23.3%)	6 (27.3%)	0.09

Table 2. POP-Q stage before and after puerarin treatment

Symptom improvement	Puerarin group	Control group
Positive (%)	57.29 \pm 6.43	0
Strongly positive (%)	34.67 \pm 5.65	0

land) and was tested by the Shanghai Center for Disease Control and Prevention.

Study subjects

The study was approved by the Institutional Review Board of Renji Hospital, Shanghai Jiao Tong University School of Medicine. Informed consent was obtained from 52 women with POP and/or stress-induced urinary incontinence (SUI) undergoing transvaginal repair surgery at the Department of Obstetrics and Gynecology of Renji Hospital between June 2010 and November 2012. Patients at different POP stages (II-IV) were divided blindly into two treatment groups, control and puerarin. Patients in the puerarin group were orally administered 0.429 mg of puerarin per day for 30 days. And the control patients were orally administered placebo of same look. At the 31th day, Plasma estradiol-17 β (E2) levels were determined using an enzyme-linked immunosorbent assay (ELISA).

Pre- and posttreatment POP staging

Before treatment, all patients underwent physical examination in the lithotomy position. The pelvic organ prolapse quantification system (POP-Q) was used to stage prolapse. The methods and definitions conformed to the standards

recommended by the International Continence Society [6]. A second examination was performed 30 days later, just before surgery. No change in symptoms was marked as (-), and symptom improvement by one stage, two stages or more was marked as (+) and strong (+) respectively.

Morphological examinations

Full-thickness samples (100-300 mg) were obtained from the upper one-third of the anterior vaginal wall during the transvaginal repair surgical procedure. Tissue samples were rinsed in saline and fixed in 10% paraformaldehyde. Tissue was dehydrated, embedded in paraffin, and serially sectioned (3 μ m). Five random sections from each tissue sample were stained with hematoxylin & eosin for routine light microscopic examination.

Immunohistochemical analysis of elastin, collagen, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and TGF- β

All biopsies were performed by one surgeon, and the immunohistochemical evaluation was performed by an experienced histopathologist who was blinded to the clinical data. Light microscopy examination was performed under 400 \times magnification. Cells that stained with a buffy color were considered positive. No staining reaction was marked as (-), and weak, moderate and strong staining reaction was marked as (+), (++) and (+++), respectively. Ten high-power fields were randomly assessed for each preparation.

Paraffin sections were deparaffinized with xylene, treated with a graded series of ethyl alcohol, rehydrated, and rinsed with phosphate-buffered saline (PBS) three times. Trypsinization was performed for 10 min with 0.1% trypsin to restore the antigen. Sections were incubated at room temperature for 10 min with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity and then rinsed three times with PBS. The sections were then placed in normal goat serum and incubated at 4°C overnight with one of the following primary antibodies diluted in PBS: (1) rabbit polyclonal antibody against elastin; (2) rabbit polyclonal antibody against collagen; (3) rabbit polyclonal antibody

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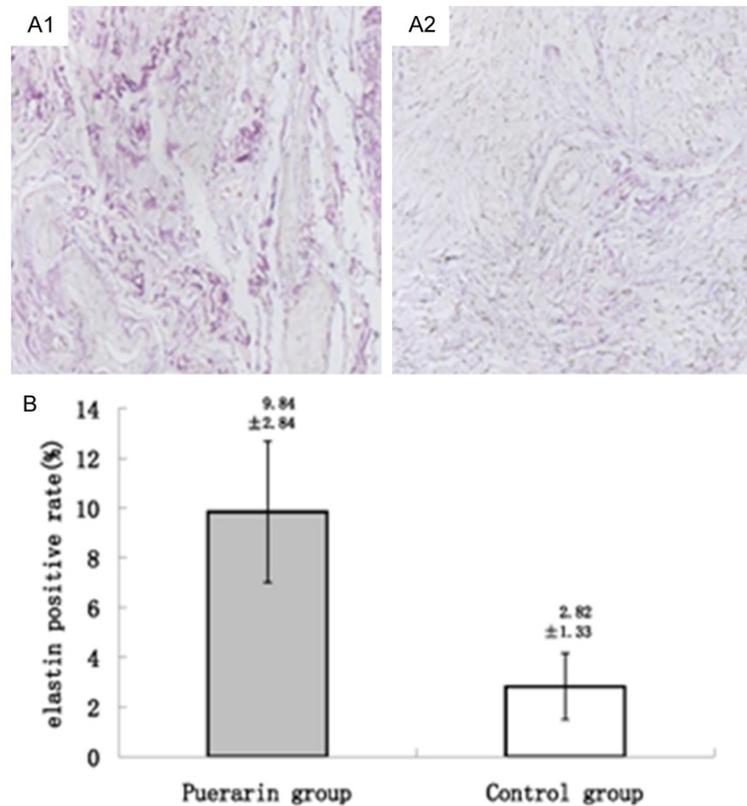


Figure 1. Elastin expression in vaginal connective tissue after treatment. A, C elastin fiber distribution by immunohistochemical staining; A. Puerarin group; B. Control group. C. The change of elastin positive rate after Puerarin treated.

against VIP; (4) rabbit polyclonal antibody against NPY; (5) rabbit polyclonal antibody against TGF- β (All antibodies were obtained from Ebioscience). After rinsing three times with PBS, the bound antibody was visualized using the peroxidase-avidin-biotin complex method, which involved incubation with a biotinylated goat anti-rabbit secondary antibody (Boshide Biotech, Wuhan, China) at a dilution of 1:300 vol/vol. DAB (100 μ l) (Maixin Biotech, Fuzhou, China) was used as the chromogen after the sections were incubated with horseradish peroxidase-labeled streptomycin at room temperature for 30 min. The sections were rinsed with distilled water, counterstained with hematoxylin, dehydrated with a graded series of ethyl alcohol, lucidificated with xylene, mounted in neutral gum, and examined by light microscopy. In the control experiments, the primary antibody was replaced with PBS. Rodent brain was used as negative and positive control samples.

Statistical analysis

For univariate analysis, Student's *t*-tests were used for categorical variables. All analyses were performed using the SPSS software. Comparisons were considered significant for *p* values < 0.05.

Results

The 52 patients' demographic characteristics are shown in (Table 1). Patients from the two treatment groups were of similar age, parity, BMI and menopausal status. There was no significant difference in serum E2 in the puerarin group before and after treatment (before: 50.92 ± 37.79 , after: 37.01 ± 21.07 , $P > 0.05$), and patients in this group showed significant improvement in symptoms. The control group showed no change in symptoms. After treated with Puerarin, the POP patients had symptom improved significantly. And patients in control group had

their symptom unchanged (Table 2). Otherwise, the endometrial thickening are found in 2 patients of puerarin group, which is not found in control group.

Vaginal specimens from the puerarin group had a much more disordered and loose elastin fiber distribution than those from the control group. In keeping with this, immunohistochemical staining demonstrated greater elastin expression in the puerarin group (9.84%) than in the control group (2.82%) (Figure 1).

Vaginal specimens from the puerarin group had a much more disordered and loose collagen fiber distribution than those from the controls (Figure 2). The immunohistochemical results were in agreement, as they showed increased expression of collagen I and III in the specimens of patients treated with puerarin. In particular, collagen I expression increased to a greater extent compared to collagen III expression in the puerarin-treated group compared to the

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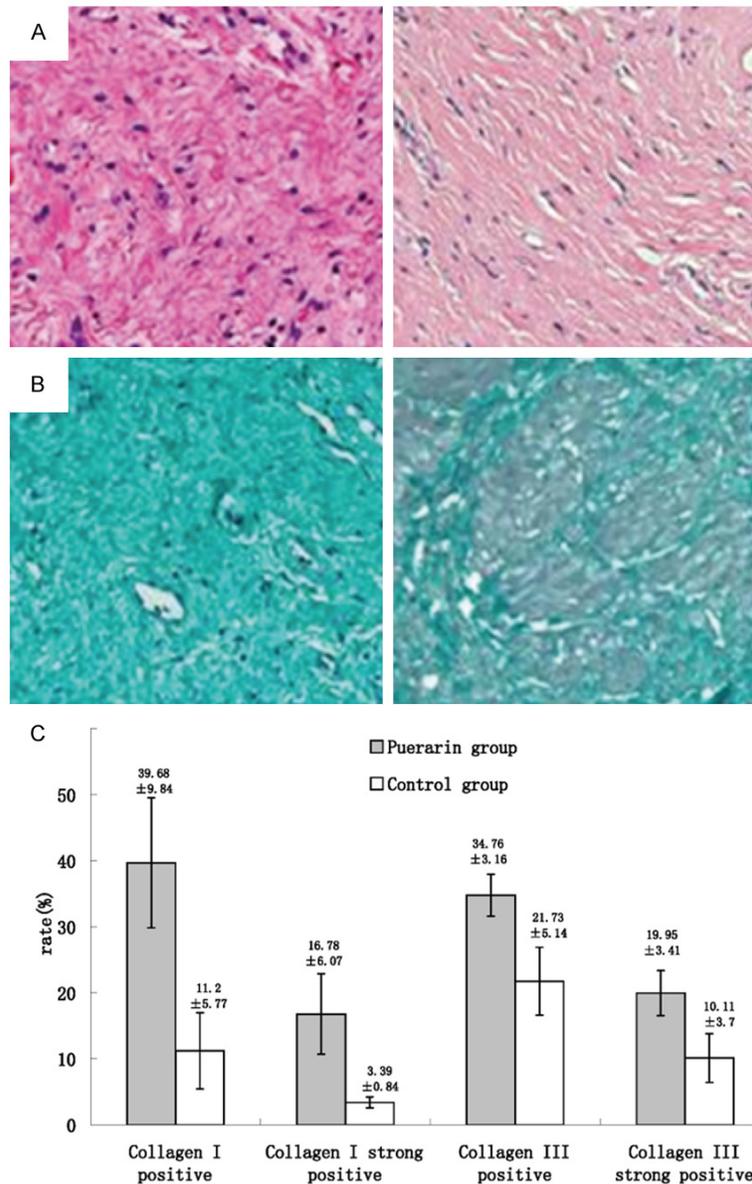


Figure 2. Collagen expression in vaginal connective tissue. A, B. Light microscopic images of collagen fiber morphology in both groups. A: H&E staining. Left: Puerarin group; Right: control group. B: Masson staining. Left: Puerarin group; Right: control group. C. Collagen I and III expression in vaginal connective tissue.

controls (I/III positive ratio: 1.14 ± 0.28 vs. 0.52 ± 0.25). The expression of NPY and VIP was significantly increased after puerarin treatment compared to control treatment ($P < 0.05$) (Figure 3). Similarly, the expression of TGF- β was also significantly increased after puerarin treatment ($P < 0.05$) (Figure 4).

Discussion

Puerarin (4',7-dihydroxy-8- β -D-glucosylisoflavone) is a C-glycoside compound that is found

in abundance in the root of *P. lobata*. Several isoflavonoids are known to weakly bind to estrogen receptors (ERs) due to their biphenolic structure. Isoflavonoids can mimic or modulate the actions of endogenous estrogens by acting as partial ER agonists or antagonists. The compounds found in extracts obtained from the *Pueraria* root may constitute a new class of tissue-selective estrogens. They can reverse weight gain, fat accumulation and metabolic syndrome in postmenopausal women [7].

At least 17 phytoestrogens, mainly isoflavones, have been isolated using high-performance liquid chromatography. Both *in vitro* and *in vivo* studies have been conducted to evaluate their estrogenic activity in reproductive organs, bones, cardiovascular diseases and other climacteric-related symptoms [8]. These studies show that phytoestrogens may interact with ERs that mediate endocrine homeostasis, causing damage to reproductive health [9, 10]. Although phytoestrogenic activities are weaker than endogenous estrogen activities, the consumption of phytoestrogens may have clinically significant consequences.

All patients in this study were postmenopausal women. Menopause is associated with a profound drop in estrogen levels in women. This estrogen deficiency initiates early menopausal symptoms including hot flashes, mood swings and vaginal dryness, and contributes to long-term conditions such as osteoporosis, cardiovascular disease, and urogenital atrophy [4]. In our study, although the serum E2 concentration did not increase after puerarin treatment, most patients showed improved or normalized POP-Q stage. This supports the phytoestrogenic effect of puerarin.

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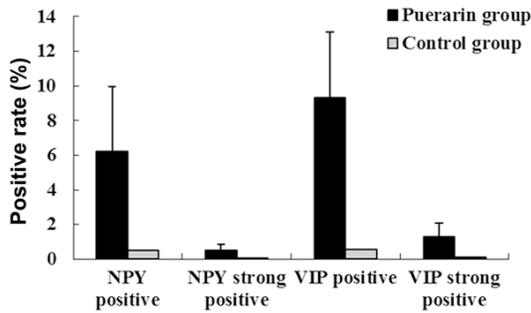


Figure 3. NPY and VIP expression in vaginal connective tissue of both groups.

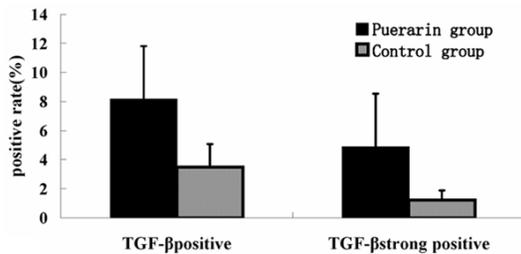


Figure 4. TGF- β expression in vaginal connective tissue of both groups.

The vaginal wall is composed of four layers: a superficial layer of stratified squamous epithelium, a subepithelial dense connective tissue layer composed primarily of collagen and elastin, a layer of smooth muscle referred to as the muscularis, and an adventitia composed of loose connective tissue. The connective tissue underlying the vagina contains relatively few cells: beside fat cells and mast cells, mainly fibroblasts are found, which produce components of the extracellular matrix (ECM). The ECM contains fibrillar components (collagen and elastin) embedded in a non-fibrillar ground substance. The quantity and quality of collagen and elastin are regulated through a precise equilibrium between synthesis, maturation and degradation. This equilibrium consists of a dynamic process of constant remodelling. The balance between synthesis and degradation of collagen and elastin is important for maintaining tissue integrity and tensile strength during tissue remodelling. Remodeling of vaginal connective tissue has an important role in the development and progression of POP [11]. Moreover, there is some support for the hypothesis that ECM protein turnover plays a role in the common pathophysiology of both POP and SUI [12, 13].

In our study, it was found that Type I and III collagen production increased in the puerarin group, and these are the most abundant collagens in the pelvic floor. Moreover, the increase in Type I collagen was greater than that for Type III collagen. The arrangement of the different types of collagen into fibrils of variable sizes confers strength or laxity depending on their ratio. Type I collagen is considered the strongest, being highly prevalent in the fascia, ligaments and fibrous tissues. Type III is found in the same tissues, but tends to be located on the surface of fibrils. Larger contribution of type III to a fiber will reduce its diameter and mechanical strength. It has been suggested that an alteration in the collagen type I to type III ratio may alter the pelvic connective tissues [14]. We found that fiber diameter and strength were increased after treatment, and based on the published results, this can be explained by the greater production of Type I collagen than Type III collagen.

Elastin expression also increased significantly in the puerarin group. Elastin is another component of the ECM and is responsible for the elasticity and recoil of tissues and organs in the body. Lesser amounts of elastin have been found in patients with POP [15]. Therefore, from all these findings, it is possible that treatment with puerarin stimulated the process of connective tissue remodeling.

It has been reported that in POP, there is degeneration of the pelvic floor and loss of innervation. The nerves innervating the pelvic floor are known to store and release a wide array of bioactive substances, including neuropeptides. Morphologic analysis of muscle specimens from women with third-degree genital prolapse and SUI has shown a marked decrease in neuropeptides, such as VIP and NPY, compared with controls [16]. Alteration in neuropeptide expression may lead to microcirculatory changes in the pelvic floor, followed by morphologic and functional changes in muscles and connective tissue [17]. Innervation has been identified as a very promising agent in the treatment of inflammatory and neurodegenerative diseases [18, 19]. VIP and NPY mediate vasodilation and smooth muscle relaxation, enhance pelvic and vaginal blood flow, and regulate vaginal lubrication [20, 21]. In our study, we found that NPY and VIP expression increased significantly after puerarin treatment, which indicates that this

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treatment resulted in the regaining of lost innervation. Thus, puerarin might prevent nerve damage or degeneration in postmenopausal and elderly women.

VIP and NPY have neuroprotective effects under inflammatory conditions. TGF- β has also been described as an anti-inflammatory cytokine. TGF- β and its receptors are widely expressed in all tissues, and the regulatory role played by this growth factor is of central importance in human diseases. It has been shown that TGF- β can modulate connective tissue [22]. In this study, the expression of TGF- β was significantly increased in patients treated with puerarin. It is agree with previous study.

In summary, POP women treated with puerarin had significantly increased collagen I, collagen III, elastin, NPY, VIP and TGF- β levels in the vaginal and surrounding tissues. Thus, administration of puerarin was associated with changes in connective tissue in patients with POP. Epidemiological and retrospective studies suggest that estrogens also exert a cardio-protective role. Therefore, puerarin could be a new choice for POP patients with cardiac disease. However, much remains to be understood of the complex dynamic interplay of enzymes, proteins and molecules that underlies the effects of puerarin.

Acknowledgements

This work is supported by the Chinese medicine guide class project of Shanghai science and Technology Commission (15401931400).

Disclosure of conflict of interest

None.

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