Echinacoside attenuates glandular fibrosis in benign prostatic hyperplasia via inhibiting MKK6/MK2 signaling pathway

Si Qin1,2, Jing-Lou Chen3,*, Xiao-Feng Zhou1, Cong-Yue Xu1, Jing Guo2

1Department of Pharmacy, Jianghan University, Wuhan 430056, China. 2Department of Clinical Medicine, Jianghan University, Wuhan 430056, China. 3Institutes of Biomedical Sciences, Jianghan University, Wuhan 430056, China. *Department of Basic Medicine, School of Medicine, Jianghan University, Wuhan 430056, China.

*Correspondence to: Jing-Lou Chen, Department of Pharmacy, School of Medicine, Jianghan University, No. 8, Sanjiaohu Road, Economic and Technological Development Zone, Wuhan 430056, China. E-mail: jinglouchen@126.com.

Author contributions
Qin S prepared tables/figures and wrote the final manuscript. Chen JL planned the experiments and conducted animal experiments. Qin S, Zhou XF, Xu CY, and Guo J carried out other experimental parts and were responsible for data analysis. All the authors participated in the final approval of the manuscript.

Competing interests
The authors declare no conflicts of interest.

Acknowledgments
This research is supported by the Research Fund of Jianghan University (grant number 2023KJZX23).

Peer review information
Traditional Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
BPH, benign prostatic hyperplasia; LUTS, lower urinary tract symptoms; ECH, echinacoside; TGF-β1, transforming growth factor-β1; EMT, epithelial-mesenchymal transition; BPH-1, benign prostatic hyperplasia epithelial-1; Veh, vehicle control; TP, testosterone propionate; PI, prostate index; ECM, extracellular matrix; Hyp, hydroxyproline; PSA, prostate specific antigen; IL, interleukin; TNF, tumor necrosis factor; GSH, reduced glutathione; E-cad, E-cadherin; qPCR, quantitative RT-PCR; p-, phosphorylated.

Citation

Executive editor: Jing-Yi Wang.

Received: 03 August 2023; Accepted: 07 December 2023.

Available online: 10 December 2023.

© 2024 By Author(s). Published by TMR Publishing Group Limited. This is an open access article under the CC-BY license. (https://creativecommons.org/licenses/by/4.0/)

Abstract
Background: Lower urinary tract symptoms commonly occur in the elderly population and seriously constrain the quality of life. Glandular fibrosis is an important pathobiological process in benign prostatic hyperplasia and is also a main inducing factor for benign prostatic hyperplasia-associated lower urinary tract symptoms. Cistanches species is an important herbal medicine resource and is traditionally used in ameliorating renal and prostatic defects. Methods: This study was to investigate the potential protective function of echinacoside (a bioactive compound from Cistanches) against prostatic fibrosis in mice and human benign prostatic hyperplasia epithelial-1 cell models. Results: It was found that echinacoside attenuated testosterone-induced prostatic hyperplasia and collagen deposition in mice, relieved prostate local inflammation and oxidative damage, and ameliorated prostatic epithelial-mesenchymal transition. Additionally, echinacoside inhibited the activation of the MKK6/MK2 signaling pathway both in vivo and in vitro. Conclusion: This study added new evidence for the anti-fibrotic function of echinacoside on the prostate and provided new insights for understanding its possible pharmacological mechanisms.

Keywords: benign prostatic hyperplasia; echinacoside; epithelial-mesenchymal transition; fibrosis
Medical history of objective
Cistanche deserticola Ma, a traditional Chinese herb used for kidney deficiency (a series of urinary disorders, such as urination weak and urinary incontinence) and white turbidity (a disease characterized by white liquid dripping during urination, which can be accompanied by painful urination), is derived from Li-Shizhen’s “Compendium of Materia Medica - Grass Department (1)” (started in the 31st year of the Jiajing reign of the Ming dynasty (1552 C.E.). Echinacoside is a natural phenylethanoid glycoside that exists in Cistanches species and has anti-fibrotic function.

Background
Lower urinary tract symptoms (LUTS) are usually concomitant with benign prostatic hyperplasia (BPH) in the elderly population and manifest as symptoms of urgency, weak stream, nocturia, frequency urination, etc [1]. Besides prostatic enlargement and smooth muscle contraction, glandular fibrosis is another important pathological process in BPH and is also a main inducing factor for BPH-associated LUTS [2]. Prostatic fibrosis can act alone or in combination with other factors to accelerate the progression of BPH, thus inducing the occurrence of LUTS [2, 3]. Aging is believed to be a prerequisite for BPH [4]. It is known that aging, characterized by progressive degradation of organ functions, is a spontaneous and irreversible process in the body [5]. However, the pathogenesis of the BPH still needs to be elucidated. In addition to hormones, several risk factors (including prostatic inflammation, growth factors, oxidative stress, and cell signaling pathways) are proposed to contribute to BPH [6, 7]. Among them, inflammation and oxidative stress are noticeable in age-related disorders [6].

Nowadays, 5α-reductase inhibitors (regulate androgen homeostasis) and α-blockers (relax prostatic smooth muscles) are the mainstream medications for treating BPH in clinical practice. Unfortunately, several side effects limit their application [8]. Therefore, novel therapeutic drugs still remain to be developed. Currently, botanical drugs and natural compounds with potential beneficial properties for the prostate have attracted more and more attention [9].

Cistanches species are widely distributed in arid or semi-arid areas (like the Xinjiang region). They are not only an important source of the traditional Chinese herb “Rou Cong Rong” but are also marketed as a tonic for the elderly. Traditional Chinese medicine believes that “Rou Cong Rong” has the activities of anti-aging, anti-oxidation, and anti-inflammation [5]. In traditional Korean medicine, Cistanthes salsas is used to ameliorate BPH [10]. Echinacoside (ECH) is a natural phenylethanoid glycoside that exists in Cistanches species. Evidence shows that ECH exerts anti-fibrotic function in the liver and kidney via inhibiting pro-fibrosis cytokines (such as transforming growth factor-β1 (TGF-β1)) and attenuating epithelial-mesenchymal transition (EMT) [11]. Fibrosis is one of the key pathological changes in both prostatic benign and malignant lesions [12]. However, the possible protective effects and pharmacological mechanisms of ECH on prostatic fibrosis remain unclear. So, the aim of this study was to investigate the anti-fibrotic ability of ECH in mice and human benign prostatic hyperplasia epithelial-1 (BPH-1) cell models.

Methods

Mice model of BPH and ECH treatment
Male C57BL/6 mice weighing 25 ± 2 g (number SCXX (e) 2018-0104, China) were obtained from Huazhong Agricultural University. They were fed in the animal experiment center of Jianghan University under a standard breeding environment (22 ± 3 °C, humidity 50 ± 10%, and 12 h light/12 h dark cycle). All the experimental operations of this animal study were performed in accordance with the ethical rules of NIH Guidelines for the Care and Use of Laboratory Animals (laboratory animal operator qualification number TY20190784). This study was approved by the Ethics Committee of Jianghan University (ethics approval number JDHXL2023-079). These mice were divided into 4 groups (n = 8) by random number table method: the vehicle control group (Veh), the ECH control group (ECH), the testosterone propionate-induced mice BPH model group (TP), and ECH treated mice BPH group (TP-ECH). The mice model of BPH was induced by subcutaneous injections of 3 mg/kg TP (batch 160822, Jinyao Pharmaceutical Co., Ltd., Tianjin, China) on alternate days for one month [9]. The mice from ECH and TP-ECH groups were orally given 20 mg/kg/d ECH (batch B137910, Alaidin Co., Ltd., Shanghai, China) [13]. Testosterone propionate was diluted with olive oil, and ECH was dissolved in purified water. At the end of this experimental period, mice prostate tissue samples were collected.

Analyses for mice prostatic hyperplasia and glandular fibrosis
The mice’s prostatic hyperplasia was evaluated by testing prostate relative weight via calculating prostate index (PI). PI = prostate weight/body weight. The extracellular matrix (ECM) components changes and collagen deposition are important pathological phenomena in organ fibrosis. So, prostate glandular fibrosis was assessed via observing the positive area of Masson and Sirius red stain using mice prostate paraffin embedded sections, as well as by detecting the prostatic level of hydroxyproline (Hyp, the main characteristic component of ECM) according to the kit instruction (Nanjing, China). Morphological changes were scanned and photographed (× 20) by NanoZoomer S360 section scanner (Hamamatsu, Shizuoka, Japan). The positive Masson and Sirius red stained area was measured using Image-Pro Plus 6.0. Additionally, the serum level of prostate specific antigen (PSA) was measured in accordance with the instructions of the commercial kit (Yangji Biotechnology Co., Ltd., Shanghai, China).

Analyses of mice prostate local inflammation and oxidative damage
The prostate local inflammation was estimated by visualizing inflammatory cell foci via identifying macrophage infiltration through immunohistochemical analysis for F4/80 expression, as well as by measuring the prostatic levels of proinflammatory cytokines (interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α)). After being deparaffinized, rehydrated, and rinsed, the mice’s prostate sections were incubated with F4/80 (Abcam, Cambridge, MA, USA) antibody at 4 °C overnight. Then, they were incubated with IgG, color developed, scanned, and photographed (× 20) by NanoZoomer S360 section scanner (Hamamatsu, Shizuoka, Japan). The prostatic levels of IL-1β, IL-6, and TNF-α were measured using tissue homogenate in accordance with the instructions of the commercial kits (ELISA Lab, Shanghai, China).

The prostate’s local oxidative stress state was estimated by measuring mice’s prostatic antioxidant function (including biological enzymes with antioxidant effects, antioxidant compounds, and characteristic products of lipid peroxidation). All the prostatic levels of SOD, GPx and CAT, reduced glutathione, total sulphhydril, and malondialdehyde were detected using spectrophotometric kits (Nanjing jiangcheng Bioengineering Institute Co., Ltd., Nanjing, China).

Analysis for mice prostatic EMT
Mice prostatic EMT was evaluated by immunohistochemical analysis...
for epithelial biomarker E-cadherin (E-cad) and mesenchymal indicator α-SMA. The sections were scanned and photographed (×40) by NanoZoomer S360 section scanner (Hamamatsu, Shizuoka, Japan). The proportion of positive stained area was measured using Image-Pro Plus 6.0. In addition, the EMT modulators TGF-β1, Snail and ZEB-1 were evaluated by quantitative RT-PCR analysis. Relative levels were calculated based on the equation 2^(-ΔΔCt). GAPDH was used as the internal standard. The primer sequences are shown in Supplementary Table S1.

**BPH-1 cells incubated with ECH and flow cytometry assay**

Human benign prostatic hyperplasia epithelial-1 (BPH-1) cells were cultured in DMEM medium (10% fetal bovine serum and 100 U/mL penicillin/streptomycin) and divided into negative control group and ECH treated group. The cells of ECH treated group were incubated with 50 μM ECH for 48 h based on our previous study. Then, the cells were harvested for the analysis of cell cycle distribution by flow cytometry. These cells were fixed in precooled 70% (v/v) ethanol for 12 h, stained with 100 μg/mL PI for 0.5 h, and subsequently monitored the DNA content.

**Analysis for MKK6/MK2 signaling pathway in vivo and in vitro**

The activity of the prostatic MKK6/MK2 signaling pathway was evaluated by detecting the mRNA levels of MKK6 and MK2 in mice prostate via quantitative RT-PCR, as well as by testing the protein levels of MK2, phosphorylated MK2 (p-MK2), MKK6 and p-MKK6 in BPH-1 cells via western blot. The protein samples from BPH-1 cells were extracted, separated using gel electrophoresis, transferred to PVDF membrane, and incubated with MK2, p-MK2, MKK6, or p-MKK6 antibodies (Abcam, Cambridge, MA, USA). β-actin was used as the internal standard.

**Statistical analysis**

The values were presented as mean ± standard deviation. Results were analyzed statistically by one-way ANOVA comparing means using SPSS (version 17.0; SPSS Statistics, Chicago, IL, USA). Differences were considered as significant at P < 0.05.

**Results**

**ECH ameliorated prostatic hyperplasia and glandular fibrosis**

When compared to the Veh group, ECH alone did not obviously (P > 0.05) affect the prostatic Hyp content (Figure 1A) and Col-I protein expression (Figure 1B). However, testosterone propionate significantly enhanced prostatic Hyp content and promoted prostatic Col-I expression compared to the Veh group. When compared to the TP group, co-treated with ECH significantly reduced prostatic Hyp content and inhibited prostatic Col-I expression. Additionally, as shown in Figure 2A, 2B, ECH significantly inhibited the enhancement of mice’s prostate positive Masson and Sirius red stained area induced by testosterone propionate. It can be concluded that ECH can ameliorate prostate glandular fibrosis.

Furthermore, ECH alone did not obviously (P > 0.05) affect prostate weight as well as the levels of PI and serum PSA (Figure 1C–1E). Testosterone propionate significantly increased prostate weight, the levels of PI and serum PSA compared to the Veh group. When compared to the TP group, co-treated with ECH significantly decreased the prostate weight as well as the levels of PI and serum PSA. Additionally, as indicated in Figure 3, ECH (10–200 μM) significantly decreased the viability of BPH-1 cells. Moreover, ECH (50 μM) significantly decreased the BPH-1 cell distribution in G2/M and S phase increased cell accumulation in G1/G0 phase compared to the negative control group. These results showed that ECH can ameliorate prostatic hyperplasia in vivo and in vitro.

![Figure 1](https://www.tmrjournals.com/tmr)

**Figure 1 Evaluation for mice prostatic collagen and hyperplasia.** (A) The mice prostatic level of hydroxyproline (Hyp). (B) The mice prostatic expression of Col-I. (C) The mice prostate weight. (D) The mice level of prostate index (PI). (E) The serum level of PSA. *P < 0.01, **P < 0.05 compared to the TP group. TP; testosterone propionate; Hyp, hydroxyproline; ECH, echinacoside; Veh, vehicle control; PI, prostate index; PSA, prostate specific antigen.
**Figure 2 Evaluation for mice prostatic collagen deposition.** (A) Masson and Sirius red staining for the evaluation of mice prostatic collagen deposition. Arrow, The positive stained area. (B) The positive stained percentage of mice prostate sections. \*P < 0.05 compared to the TP group. ECH, echinacoside; Veh, vehicle control; TP; testosterone propionate.

**Figure 3 Evaluation for viability and cell cycle of BPH-1 cells.** (A) The viability of BPH-1 cells. (B) Cell cycle of the negative control. (C) Cell cycle of the ECH treated group. (D) The data analysis for cycle distribution. \*P < 0.05 compared to the negative control. ECH, echinacoside.

**ECH relieved mice prostatic local inflammation and oxidative damage**

As can be visualized in Figure 4A, there was obvious macrophage infiltration (positive expression of F4/80) in the mice prostate of TP group compared to the Veh group. Co-treated with ECH significantly inhibited the testosterone propionate-induced macrophage infiltration. Similarly, Figure 4B indicated that the levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α were significantly increased in mice prostate of TP group compared to the Veh group. When compared to the TP group, co-treated with ECH significantly decreased the prostatic levels of IL-1β, IL-6, and TNF-α.

As shown in Figure 5, when compared to the Veh group, the antioxidant biological enzyme activities and antioxidant compounds levels were significantly reduced, while the lipid peroxidation biomarker content was significantly enhanced in the mice prostate of TP group. When compared to the TP group, co-treated with ECH significantly increased the activities of SOD, GPs, and CAT, enhanced the levels of reduced glutathione and total sulphhydril, as well as decreased the level of malondialdehyde. It suggested that ECH can relieve prostatic local inflammation and oxidative damage in BPH mice.

**ECH attenuated mice prostatic EMT**

As can be seen from Figure 6A–6C, the proportion of positive E-cad stained area was significantly reduced, while the proportion of positive α-SMA stained area was significantly enhanced in mice prostate from TP group compared to those from Veh group. In addition, the prostatic mRNA levels of Snail, ZEB-1 and TGF-β1 were also significantly enhanced in mice of TP group compared to the Veh group.
group. When compared to the TP group, co-treated with ECH significantly increased the positive E-cad stained area, decreased the positive α-SMA stained area, as well as reduced the mRNA levels of Snail, ZEB-1 and TGF-β1. The results illustrated that ECH can ameliorate testosterone propionate-induced mice prostatic EMT.

**ECH inhibited prostatic MKK6/MK2 signaling pathway**

As summarized in Figure 7, when compared to the Veh group, the prostatic mRNA levels of MKK6 and MK2 in the mice prostate of TP group were significantly enhanced. Co-treated with ECH significantly reduced the prostatic mRNA levels of MKK6 and MK2 compared to the TP group. When compared to the negative control group, ECH significantly decreased the levels of p-MKK6 and p-MK2 in BPH-1 cells. It can be concluded that ECH had the activity of inhibiting the prostatic MKK6/MK2 signaling pathway.

**Discussion**

BPH is a kind of benign solid tumor lesion that is highly prevalent in middle-aged and elderly men [8]. Increased prostate stromal/epithelial ratio is a symptom of prostatic fibrosis. This is also the key reason why some patients find it difficult to improve their LUTS induced by BPH [4]. Organ fibrosis is often triggered by abnormal body repair, which is caused by persistent external harmful stimuli. Furthermore, organ fibrosis usually shows an irreversible process. As a result, the mortality rate ultimately caused by fibrosis accounts for about half of the total death toll in industrial society [14]. So, the aim of this study was to investigate the anti-fibrotic ability of ECH, a phenylethanoid glycoside from traditional Chinese herbal medicine resources Gúshénchí species, in BPH mice and BPH-1 cells.

Changes in the composition of ECM, especially excessive synthesis and accumulation of collagen, contribute to the formation of organ fibrosis. Among the constituent cells of the prostate gland, activated stromal fibroblasts are mainly responsible for the secretion of collagen fibers [15]. During the activation of stromal fibroblasts in BPH, over-expression of α-SMA is a landmark event and leads to prostatic cell proliferation and collagen deposition [4]. Both our previous study and relevant reports demonstrated that the transcellular signaling communication between epithelial and stromal cells played an essential role in BPH [16]. In the pathological progress of glandular fibrosis in BPH, EMT is an important promotional event. Prostate epithelial cells lose their polarity and adhesion characteristics and obtain motor ability similar to mesenchymal cells [17]. A considerable proportion of stromal fibroblasts, which highly expressed the marker protein α-SMA, originated from epithelial cells during the EMT process [18].

![Figure 4 Evaluation for mice prostate local inflammation.](image1)

Figure 4 Evaluation for mice prostate local inflammation. (A) Immunofluorescence analysis for macrophage marker F4/80. Arrow, The positive stained area. (B) The prostatic levels of proinflammatory cytokines IL-1β, IL-6 and TNF-α. *P < 0.01 compared to the TP group. ECH, echinacoside; Veh, vehicle control; TP, testosterone propionate. IL, interleukin; TNF, tumor necrosis factor.

![Figure 5 Evaluation for mice prostatic oxidative damage.](image2)

Figure 5 Evaluation for mice prostatic oxidative damage. (A) The prostatic activities of antioxidant enzymes SOD, GPx and CAT. (B) The prostatic level of non-enzymatic antioxidant GSH. (C) The prostatic level of non-enzymatic antioxidant T-SH. (D) The prostatic level of lipid peroxidation product MDA. *P < 0.01 compared to the TP group. ECH, echinacoside; Veh, vehicle control; TP, testosterone propionate. CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; T-SH, total sulfhydryl; MDA, malondialdehyde.
In this EMT event of prostatic fibrosis, TGF-β1 attracts a lot of attention. TGF-β1 itself is not only a powerful fibrogenic cytokine but also an inducing factor for prostate stromal cell differentiation and activation [4]. Actually, TGF-β1 has numerous biological functions. Firstly, it activated EMT transcription factors (like Snail and ZEB1) and thus induced EMT in several organs. Secondly, TGF-β1 can stimulate cell proliferation. Last but not least, TGF-β1 is a mediator for oxidative damage and inflammation in vivo [15, 19, 20]. Oxidative stress is an important underlying mechanism of organ damage caused by aging or external stimuli [4]. Under normal conditions, the human body maintains a balance of reactive oxygen species (ROS) scavenging and generation. When the oxidative-antioxidant homeostasis is broken, cell injuries and organ fibrosis emerge [5, 15]. Moreover, ROS is downstream of TGF-β1 and also has the activity of promoting EMT [4, 20]. In our present study, ECH significantly ameliorated prostatic fibrosis, EMT and oxidative damage in BPH mice.

Another risk factor for prostatic fibrosis is the local inflammation in BPH. It observed that prostate tissue hyperplasia is often accompanied by chronic inflammation, which is believed to be a critical etiological factor for the emergence of LUTS in BPH population [6, 16]. Especially in age-related human organ dysfunctions and chronic diseases, fibrotic changes caused by chronic inflammation relished attention [21]. At the cellular level, local inflammation can not only activate fibroblasts but also recruit macrophages. The interaction between these cells produces a synergistic effect in promoting collagen deposition and accelerating organosclerosis [14]. On the one hand,
the activated stromal fibroblasts contribute to prostatic EMT [18, 22]. On the other hand, the macrophages infiltrated in prostate tissue will secrete a series of pro-inflammatory cytokines such as TGF-β, IL-1β, IL-6, and TNF-α [9, 16]. These pro-inflammatory cytokines drive immune response, stimulate immune cell proliferation and differentiation, and result in a vicious cycle. Ultimately, organ hyperplasia and fibrosis emerge [8, 23]. In this study, ECH inhibited prostatic macrophage infiltration and reduced the levels of pro-inflammatory cytokines in BPH mice. The cellular signaling pathways are responsible for maintaining the microenvironment homeostasis in the body. Their abnormal activation or inhibition by external stimuli or other factors will cause various adverse effects. The mitogen-activated protein kinases (MAPKs) pathway has the functions of modulating cell survival and apoptosis, cycle, differentiation, etc. Because MAPKs pathway participates in inflammation and oxidative damage, studies for the potential active compounds targeting MAPKs system are under the media spotlight [24]. MKK6 and MK2 are important node proteins in the MAPKPs38 signaling pathway (one component of MAPKs system). MKK6 is located in the upstream, while MK2 acts as a downstream mediator [25, 26]. In a study of experimental cardiac fibrosis, MKK6 is found to be positively correlated with the severity of the disease [27]. Other reports found that MKK6-MK2 pathway is involved in the maintenance of normal cell cycle and the modulation of local inflammation [25]. Over-activated MKK6-MK2 pathway can increase the levels of pro-inflammatory cytokines and induce cell proliferation [26]. In the human prostate, MKK6 initiates the changes in epithelial characteristics and contributes to prostatic EMT [28]. MKK6 is found to be positively correlated with organ fibrosis, such as cardiac fibrosis [27]. MKK6 is also found to be highly expressed in BPH and participates in prostatic epithelial immune responses and inflammation [29].

Conclusions

The results of this work demonstrated that ECH can inhibit mice BPH and collagen deposition, relieve local inflammation and oxidative damage, ameliorate prostatic EMT, and finally attenuate glandular fibrosis. This study added evidence for the anti-fibrotic ability of ECH in BPH with the possible underlying mechanisms of regulating MKK6/MK2 signaling pathway. The whole results indicated that ECH had the potential to delay the clinical progression of BPH via ameliorating glandular fibrosis. The improvement of ECH on urinary functions, as well as whether ECH has the effect of reversing the progression of prostatic fibrosis, should be explored in future research.

References

Submit a manuscript: https://www.tmrjournals.com/tmr