The extract of *Celtis choseniana* Nakai alleviates testosterone-induced benign prostatic hyperplasia through inhibiting 5α reductase type 2 and the Akt/NF-κB/AR pathway

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**[ABSTRACT]** Benign prostatic hyperplasia (BPH) is a chronic male disease characterized by the enlarged prostate. *Celtis choseniana* Nakai (*C. choseniana*) is medicinally used to alleviate pain, gastric disease, and lung abscess. In this study, the effect of *C. choseniana* extract on BPH was investigated using testosterone-induced rats. Sprague Dawley rats were divided into five groups: control, BPH (testosterone 5 mg kg⁻¹), Fina (finasteride 2 mg kg⁻¹), and *C. choseniana* (50 and 100 mg kg⁻¹). After four weeks of TP treatment with finasteride or *C. choseniana*, prostate weights and DHT levels were measured. In addition, the prostates were histopathologically examined and measured for protein kinase B (Akt)/nuclear factor-xB (NF-xB)/AR signaling, proliferation, apoptosis, and autophagy. Prostate weight and epithelial thickness were reduced in the *C. choseniana* groups compared with that in the BPH group. The extract of *C. choseniana* acted as a 5α reductase inhibitor, reducing DHT levels in the prostate. Furthermore, the extract of *C. choseniana* blocked the activation of p-Akt, nuclear NF-xB activation and reduced the expression of AR and PSA compared with BPH. Moreover, the expression of Bax, PARP-1, and p53 increased, while the expression of bcl-2 decreased. The present study demonstrated that *C. choseniana* extract alleviated testosterone-induced BPH by suppressing 5α reductase and Akt/NF-xB activation, reducing AR signaling and inducing apoptosis and autophagy in the prostate. These results suggested that *C. choseniana* probably contain potential herbal agents to alleviate BPH.

**[KEY WORDS]** Apoptosis; Androgen receptor; Benign prostate hyperplasia; *Celtis choseniana* Nakai; 5α-Reductase type 2; NF-κB

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**Introduction**

Benign prostatic hyperplasia (BPH) is a chronic male disease that affects more than 50% of men over 60 years of age [1-3]. Patients with BPH present prostate enlargement that results in a series of uncomfortable symptoms, such as lower urinary tract symptoms and erectile dysfunction [4]. Although BPH is not a life-threatening disease, increased awareness of patients’ decreased quality of life sparked interest in the management of prostate hyperplasia and hypertrophy.

An imbalance between proliferation and apoptosis leads to BPH or prostate cancer [4]. Androgen is considered a risk factor for prostate enlargement. Dihydrotestosterone (DHT), which is converted from testosterone via 5α-reductase type 2, bound to the androgen receptor (AR), activating prostate hyperplasia of stromal and glandular epithelial cells in the prostate [5]. On the other hand, low testosterone levels induced cell death in the prostate by activating apoptosis [6]. Inhibiting the conversion from testosterone to DHT facilitated a reduction in prostate size, alleviating BPH symptoms in animal and clinical studies [7,8].

*Celtis choseniana* Nakai is an endemic herbal plant that belongs to the family Ulmaceae [9]. Traditionally, the genus *Celtis* has been used as a medicinal plant in China, Korea, and Japan. Its leaves and bark can be used to treat pain, gastric disease and lung abscesses [10,11]. In addition, *C. choseniana* extract exhibited anti-inflammatory activity during macrophage-mediated inflammatory responses [12]. The main phytomedicinal constituents of *C. choseniana* extract are quercetin, luteolin, and kaempferol, which are anti-inflammatory flavonoids [13,14]. Among them, luteolin exhibited the highest anti-inflammatory effects [12]. However, there is not much research concerning the phytomedicinal activity of the extract of *C. choseniana*. Therefore, in this study, the effect of *C. choseniana* extract on BPH was investigated...
using a testosterone-induced rat model of BPH in vivo.

Material and Methods

Plant material

The plant, Celtis choseniana Nakai is an accepted name on the plant list (http://www.theplantlist.org). The 001–011 (3°) used in this research was obtained from Korea Plant Extract Bank in Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The plant was collected from Ulleung-gun, Gyeongsangbuk-do, Korea, in 2000. A voucher specimen (KRIP 0004939) was kept in the herbarium of Korea Research Institute of Bioscience and Biotechnology. The plant (96 g) dried in the shade and powdered was added to 1 L of 99.9% methyl alcohol (HPLC grade) and extracted through 30 cycles (40 kHz, 1500 W, 15 min, ultrasonication-120 min. standing per cycle) at room temperature using an ultrasonicator (SDN-900H, SD-ULTRASONIC Co., Ltd., Seoul, Korea). After filtration and drying under reduced pressure, C. choseniana extract (10.3 g) was obtained.

HPLC analysis of luteolin in C. choseniana

The constituents of Celtis choseniana Nakai were analyzed by high performance liquid chromatography (HPLC) with a 1260 Infinity II system (Agilent, CA, USA) equipped with a UV detector. Luteolin (Sigma, St. Louis, USA) was used as the reference compound [12]. The conditions are described in Table 1.

Experimental animals

The experimental protocols were approved (201906A-CNU-116) by the Institutional Animal Ethics Committee of Veterinary Medicine, Chungnam National University. Sprague-Dawley (SD) 6-week-old male rats were purchased from Orient Bio (Korea). Before experiments, the animals were acclimatized under stable conditions (22 ± 2 °C, 30%–35% relative humidity, 12 h light/dark cycle) for one week, with access to standard food and water ad libitum. This research was carried out according to the Guide for the Care and Use of Laboratory Animals (8th edition).

A total of 25 SD rats were randomly divided into five groups (n = 5). Except for the control rats, all rats were subcutaneously (SC) injected with testosterone propionate (TP, 10 mg·kg⁻¹, Tokyo Chemical Ins. Co., Tokyo, Japan) for four weeks daily. All rats were treated with their corresponding compound for four weeks as follows: a control group (10 mg·kg⁻¹ PBS, p.o.), a BPH group (10 mg·kg⁻¹ TP alone, s.c.), a Fina group (TP, s.c. + finasteride 10 mg·kg⁻¹, p.o.), a 50 group (TP, s.c. + C. choseniana 50 mg·kg⁻¹, p.o.), and a 100 group (TP, s.c. + C. choseniana 100 mg·kg⁻¹, p.o.). At the end of the experiment, the rats were fasted overnight and anesthetized with CO₂ in a chamber. The blood samples obtained from the heart were centrifuged at 3000 g for 15 min, and the serum was stored at −80 °C until later use. The dissected prostate tissues and body weight of SD rats were measured at the end of the experimental period. The relative prostate weights were calculated by dividing the prostate weights by the body weights. Half of the prostate gland was formalin-fixed and subjected to paraffin embedding and sectioning. The remaining prostate samples were frozen in liquid nitrogen and stored at −80 °C for Western blot analysis.

Measurement of DHT levels

DHT levels in serum and prostate tissue samples were measured using a commercial enzyme-linked immunosorbent assay kit (MyBiosource, California, USA) according to the manufacturer’s instructions.

Western blot

The collected cells and frozen prostate tissue samples were lysed and quantified as previously described [13]. For cytosolic and nuclear fractions, the prostates were homogenized in an extraction kit (Abcam, Cambridge, UK) according to the manufacturer’s instruments. Protein concentrations were determined by a bicinchoninic acid assay (BCA, Thermo Fisher Scientific, Massachusetts, USA). Western blot was performed as previously described [13]. The primary antibodies used are shown in Table S1. Proteins were visualized with an enhanced chemiluminescence detection kit (Amer sham Pharmacia Biotech, Buckinghamshire, UK) and quantified using Image J software (Image J v1.46a; NIH, USA).

Histological study

The prostate was fixed in 10% neutral buffered formalin phosphate solution. Embedded in paraffin, the blocks were cut into 5-μm sections. Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed as previously described [13] using primary antibodies as follow; anti-5α-reductase type 2 (1 : 500, Santa Cruz). All stained sections were visualized under a light microscope (Nikon eclipse 80i, Nikon Corporation, Tokyo, Japan) at 200 × and 400 × magnification and images were captured with DP Controller software. Ten randomly selected images were acquired for each section, and the epithelial thickness was measured using Image J software.

TUNEL staining

TUNEL staining was performed using a peroxidase in situ apoptosis detection kit (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. The slides were deparaffinized and dehydrated according to our laboratory protocol. Color was developed using a 3,3’-diaminobenzid...
ine substrate, and Mayer’s hematoxylin was used for counterstaining. The apoptosis-positive area in the stained sections was visualized as brown under a light microscope (Nikon eclipse 80i) at 400 × magnification. Ten randomly selected images were acquired for each section, and apoptosis-positive cells were expressed as a number per 100 prostate cells.

Statistical analysis

All experiments were conducted in a double-blind manner. The results were randomly selected and are expressed as the mean ± SEM of double experiments. GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA) was used for data analysis. For comparisons, the Mann-Whitney U test was used to determine the statistical differences for nonparametric data between groups. A post hoc Tukey test was used for comparisons among multiple groups when relevant. A P value < 0.05 was considered statistically significant.

Results

HPLC analysis of the extract of C. choseniana

HPLC analysis for the quality control of C. choseniana extract was performed using standard anti-inflammatory flavonoid luteolin [12]. Luteolin in C. choseniana Nakai extract was detected at 40.1 min. The extract of C. choseniana Nakai was standardized with luteolin. Representative chromatograms of the reference standard (luteolin) and C. choseniana Nakai extract are shown in Fig. 1 and Table S2. The luteolin content in C. choseniana extract was 0.14%, and luteolin was used as a quality control for further study.

Effects of C. choseniana extract on prostate weights and histopathological changes

To induce the BPH model of rats, the animals were treated with both the extract of C. choseniana and testosterone. As shown in Table 2, the body weights of the BPH group increased compared with those of the control group. However, prostate weights significantly (P < 0.001) increased both absolutely and relatively. The Fina group showed a significant reduction in prostate weights (P < 0.01) compared with the BPH group. The prostate weights of the C. choseniana-treated groups also decreased, without statistical difference. Fig. 2 reflects the histopathological changes of the prostate. The difference in prostate tissue was pronounced as shown in Fig. 2B. The tubular glands of the control group were lined with cuboidal epithelium supported by stromal tissue. In contrast, the BPH group showed a columnar glandular epithelium with hyperplastic processes in multiple layers and a decrease in lumen volume. However, the groups treated with finasteride and C. choseniana showed reduced hyperplastic processes and increased lumen volume compared with the BPH group. The thickness of the prostate epithelium also significantly increased (P < 0.001) in the BPH group compared with those in the control group. However, the thickness of the prostate epithelium in the Fina and C. choseniana groups significantly decreased (P < 0.001, Fig. 2C).

Effects of C. choseniana extract on 5α-reductase in prostatic tissue and serum and prostatic DHT levels

The expression of 5α-reductase and DHT level were determined to evaluate the effects of C. choseniana extract. The BPH group presented significant increases in the % area of the 5α-reductase positive stained prostate (P < 0.001) compared with the control group. However, in both of the C. choseniana groups, the positive stained area decreased, compared with that in the BPH group. In particular, the % area of the positive stained prostate was more obvious in the 50 mg·kg⁻¹ C. choseniana group. In RWPE cells, the 5α-reductase was inhibited.
dactase concentration increased in testosterone-treated RWPE cells. However, the level of 5α-reductase was significantly reduced (P < 0.001) in C. choseniana and testosterone-cotreated RWPE cells (Fig. S1). The results of 5α-reductase expression were accompanied by DHT levels, as shown in Fig. 3. The BPH group demonstrated significantly (P < 0.001) increased levels of DHT in serum and prostatic tissue compared with the control group. In both the 50 and 100 mg·kg⁻¹ groups, DHT levels in serum and prostatic tissue significantly decreased (P < 0.05) compared with those in the BPH group.

C. choseniana extract inhibited the NF-κB signaling in BPH tissues

As shown in Fig. 4, protein kinase b (AKT) phosphorylation increased in the BPH group compared with that in the control group. Interestingly, in the Fina and C. choseniana groups, AKT phosphorylation decreased compared with that in the BPH group. Furthermore, the expression of the nuclear factor kappa light chain enhancer of activated B cell (NF-κB) and the I-kappa B kinase (I-κB) was investigated in the prostatic nuclei and cytosols. The expression of cytosolic I-κB significantly increased in the BPH group compared with that in the control group. However, the expression of I-κB decreased in the Fina and C. choseniana groups, especially the expression of I-κB in the 100 mg·kg⁻¹ C. choseniana group. Similarly, the expression of NF-κB in the prostatic nuclei significantly increased in the BPH group compared with that in the control group. However, the expression of NF-κB decreased in the Fina and C. choseniana groups. (Fig. 4).

Effects of C. choseniana extract on BPH proliferation in prostatic tissues

As shown in Fig. 5, the expression of AR significantly increased (P < 0.001) in the BPH group compared with that in the control group. However, the expression of AR in the Fina group decreased. In particular, the C. choseniana extract groups showed significant decreases (P < 0.001) in AR expression compared with the control group. Unlike the changes in AR, estrogen receptor (ER)-α did not decrease in
the Fina and C. choseniana groups. In addition, there was no significant difference in the expression of ER-β. Along with the AR expression results, the BPH group demonstrated a markedly increased level of prostatic prostate specific antigen (PSA) compared with the control group. However, the Fina group and both C. choseniana groups exhibited significant decreases ($P < 0.001$) in PSA level compared with the BPH group. Similarly, the proportion of proliferating cell nuclear antigen (PCNA) significantly increased ($P < 0.001$) in the BPH group compared with that in the control group, but was reduced in the Fina and C. choseniana groups, as shown in Fig. 5.

**Effects of C. choseniana extract on apoptosis in BPH tissues**

To evaluate apoptosis, the levels of the apoptosis-related proteins, B cell lymphoma associated X (Bax), B cell lymphoma 2 (Bcl-2), and poly (ADP-ribose) polymerase (PARP-1) were examined. As shown in Fig. 6, the ratio of Bax to Bcl-2 significantly decreased ($P < 0.005$) in the BPH group. However, this ratio increased in the Fina and C. choseniana groups and significantly increased in the 50 mg·kg$^{-1}$ C. choseniana group. According to TUNEL staining, the number of positive cells decreased in the BPH group but increased in the Fina and C. choseniana groups.

**Effects of C. choseniana extract on autophagy in BPH tissues**

As shown in Fig. 7, AMP-activated protein kinase (AMPK) phosphorylation was reduced in the BPH group compared with that in the control group. However, the phosphorylation of AMPK significantly increased ($P < 0.001$) in the Fina and C. choseniana extract-treated groups. To evaluate autophagy, the expression of the microtubule-associated protein 1A/1B light chain (LC3) and sequenstrosome-1 (p62) was examined by Western blot. The conversion of LC3-I to LC3-II decreased in the BPH group, while the expression of p62 in the BPH group significantly increased ($P < 0.001$) compared with those in the control group. However, in the groups treated with Fina or C. choseniana extract, LC3-II conversion increased, and p62 expression was significantly reduced.

**Discussion**

BPH is the most common disease characterized by glandular and stromal tissue hyperplasia. Androgen signaling and apoptotic processes are the major factors related to the development and progression of BPH [16, 17]. Although there are many therapeutic agents, such as 5α-reductase inhibitors or α-adrenergic blockers, research is being carried out on novel therapeutic substances due to drug side effects, such as loss of libido, erectile dysfunction, and upper respiratory tract infection [18]. In the present study, we evaluated the therapeutic effects of C. choseniana on testosterone-induced BPH development. Luteolin is one of the phytomedicinal constituents of C. choseniana and exerted anti-inflammatory activity [19]. When prostate inflammation develops into chronic disease, an imbalance between proliferation and apoptosis occurs in prostate tissue, leading to a prostate volume increase and a higher international prostate symptom score [19, 20].
effects of C. choseniana on BPH have yet to be elucidated. In this study, we investigated the effects of C. choseniana on BPH, which was related to the decline of androgen receptor signals.

Enlarged prostate and proliferation of the stromal and epithelium are important markers of BPH [21]. In the present study, prostate size and weight increased in testosterone-induced BPH rats. In addition, histopathological BPH showed alterations in the prostate, with increased epithelial thickness and stromal cells and reduced lumens. However, the Fina and C. choseniana groups presented reduced relative prostate weights and epithelium thickness.

The balance of testosterone and DHT hormones is the major factor during the development of BPH. Testosterone is converted to DHT by 5α-reductase, and DHT promotes prostate cell proliferation and survival by binding AR [2, 22, 23]. Recently, estrogen was implicated as a factor in the development of BPH [24]. Among the two estrogen receptors, ERα and ERβ, ERα mediates cell proliferation, whereas ERβ mediates cell apoptosis in prostate cells [24]. Our study showed reduced 5α-reductase expression in the prostate tissue of the Fina and C. choseniana groups compared with those in the BPH group. Furthermore, an increase in DHT level in serum and prostate tissue was found in the BPH group. In contrast, a remarkable reduction in serum and prostatic DHT level were seen in both C. choseniana groups. It was supported by the result that 5α-reductase was reduced in cells treated with C. choseniana and testosterone compared with those treated with testosterone alone. Moreover, AR was also markedly reduced in the C. choseniana groups compared with that in the BPH group. Therefore, the extract of C. choseniana suppressed 5α-reductase activity, resulting in a reduction in DHT level and AR. Unlike changes in the AR expression, there were no significant difference in ERs. The shrinkage rate of prostate volume was limited to just 20% or so after 5α-reductase inhibition [26]. Despite changes in androgen receptors, prostate weight did not significantly decrease as the expression of BPH [29]. Among the two estrogen receptors, ERα and ERβ, ERα mediates cell proliferation, whereas ERβ mediates cell apoptosis in prostate cells [24]. Our study showed reduced 5α-reductase expression in the prostate tissue of the Fina and C. choseniana groups compared with those in the BPH group. Furthermore, an increase in DHT level in serum and prostate tissue was found in the BPH group. In contrast, a remarkable reduction in serum and prostatic DHT level were seen in both C. choseniana groups. It was supported by the result that 5α-reductase was reduced in cells treated with C. choseniana and testosterone compared with those treated with testosterone alone. Moreover, AR was also markedly reduced in the C. choseniana groups compared with that in the BPH group.
ERα was not reduced, and the expression of ERβ did not significantly increase.

Akt and NF-κB were up-regulated during BPH progression and development [27], but relatively low in the normal prostate [28]. Activation of the Akt pathway resulted in IκB phosphorylation, degradation of IκB and liberated NF-κB accompanied by nuclear translocation [29]. Activation of nuclear NF-κB was related to AR transcriptional activity [30]. In vivo and in vitro, continuous activation of NF-κB maintained high nuclear AR levels, resulting in AR signaling. Additionally, NF-κB was shown to activate the transcription regulatory element of the PSA gene, and bind the sites located in

Fig. 5 Effects of C. Choseniana extract on the expression of AR, ER-α, ER-β, PSA, and PCNA in prostate tissue. Western blotting analysis of AR, ER-α, ER-β, PSA, and PCNA and β-actin in rat prostate tissue samples. BPH in rats was generated through daily subcutaneous injection of TP for 28 days. Control: PBS; BPH: TP; Fina: TP + Finasteride (10 mg·kg⁻¹, p.o.); the 50 group: TP + C. choseniana (50 mg·kg⁻¹, p.o.); and the 100 group: TP + C. choseniana (100 mg·kg⁻¹, p.o.). Data are presented as mean ± SEM (n = 5). ***P < 0.001 vs the control group; **P < 0.01 vs the control group; *P < 0.05 vs the BPH group.

Fig. 6 Effects of C. Choseniana extract on apoptosis in prostate tissue. A. Western blotting of PARP-1, Bax, Bel-2, and β-actin and Bax/Bel-2 ratio in rat prostate tissue. B. TUNEL staining and positive cells per 100 cells in prostate tissue. Arrows indicate positive-stained cell in the prostate epithelial cells. BPH in rats was generated through daily subcutaneous injection of TP for 28 days. Control: PBS; BPH: TP; Fina: TP + Finasteride (10 mg·kg⁻¹, p.o.); the 50 group: TP + C. choseniana (50 mg·kg⁻¹, p.o.); and the 100 group: TP + C. choseniana (100 mg·kg⁻¹, p.o.). Data are presented as mean ± SEM (n = 5). ***P < 0.001 vs the control group; **P < 0.01 and *P < 0.05 vs the BPH group.
the PSA core enhancer [27,31]. PSA is a key marker for the diagnosis of BPH, which is an acidic nuclear protein mainly expressed in the S phase of the cell cycle using a cell proliferation marker. The levels of PSA and PCNA increased in BPH and prostatic cancer [32,33].

In our study, BPH activated phospho-Akt and nuclear NF-κB after dissociation from IκB, increasing the expression of AR, PSA and PCNA compared with the control. In particular, the expression of phospho-Akt, nuclear NF-κB, and cytosolic IκB was reduced in the C. choseniana-treated group. Following NF-κB activation, the C. choseniana groups showed significant decreases in PSA and PCNA levels compared with the BPH group. The reason for reducing AR signaling in C. choseniana is that C. choseniana suppresses the activation of Akt phosphorylation and nuclear NF-κB.

Moreover, NF-κB promoted cell survival by inducing antiapoptotic molecules, including Bcl-2 [34]. Catz and Johnson [39] reported that NF-κB/p65 and p50 complexes bound to the Bcl-2 promoter and induced the expression of Bcl-2 in LNCaP cells. In fact, in our study, parallel activation of NF-κB increased the expression of pro-apoptotic markers, including Bax, and decreased the expression of anti-apoptotic markers, including Bcl-2. Furthermore, the Bax/Bcl-2 ratio increased in the C. choseniana groups, indicating the induction of apoptosis in the C. choseniana groups. TUNEL staining supported our Western blot results.

Furthermore, recent studies have shown that AR played a differential role in various cell death signaling pathways, inhibiting apoptosis, necrosis, and autophagy [36,37]. Androgen deprivation and blocking AR induced AMPK activation, promoting autophagy [38]. During the autophagy process, LC3, which is present on the inner membrane of autophagosomes, binds to p62 to form autophagosome, and is then preferentially degraded during autophagy. Generally, an increase in LC3 and a decrease in p62 were characterized by autophagy flux [39,40]. Our results showed that AMPK phosphorylation increased in the C. choseniana-treated groups inducing autophagy. According to the AMPK phosphorylation, the expression of LC3 increased and the expression of p62 decreased, indicating autophagic flux induction in the C. choseniana-treated groups. In summary, C. choseniana induces apoptosis and autophagy by suppressing AR activation.

**Conclusions**

In conclusion, the present study demonstrates that C. choseniana extract alleviates testosterone-induced BPH, by suppressing 5α reductase and Akt/NF-κB/AR signaling and inducing apoptosis and autophagy. These results suggest that C. choseniana probably contain potential phytomedicinal herbal agents to alleviate BPH.

**References**


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