Regular Article

Quisqualis indica Improves Benign Prostatic Hyperplasia by Regulating Prostate Cell Proliferation and Apoptosis

Charith UB Wijerathne,[a,#] Hee-Seon Park,[a,#] Hye-Yun Jeong,[a] Ji-Won Song,[a] Og-Sung Moon,[b] Young-Won Seo,[b] Young-Suk Won,[b] Hwa-Young Son,[a] Jong-Hwan Lim,[c] Sung-Hum Yeon,[c] and Hyo-Jung Kwun*[,a]

[a] *Department of Veterinary Pathology, College of Veterinary Medicine, Chungnam National University; 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Republic of Korea:* [b] *Laboratory Animal Resource Center, Korea Research Institute of Bioscience and Biotechnology; Chungbuk 28116, Republic of Korea: and* [c] *HUONS Research Center, Hanyang University in ERICA campus; Ansan 15588, Republic of Korea.*

Received June 9, 2017; accepted September 4, 2017; advance publication released online September 22, 2017

Quisqualis indica **(QI) has been used for treating disorders such as stomach pain, constipation, and digestion problem. This study was aimed to evaluate the therapeutic efficacy of QI extract on treating benign prostatic hyperplasia (BPH) in LNCaP human prostate cancer cell line and a testosterone-induced BPH rat model. LNCaP cells were treated with QI plus testosterone propionate (TP), and androgen receptor (AR) and prostate specific antigen (PSA) expression levels were assessed by Western blotting. To induce BPH, the rats were subjected to a daily subcutaneous injection of TP (3 mg/kg) for 4 weeks. The rats in treatment group were orally gavaged with QI (150 mg/kg) together with the TP injection.** ***In-vitro*** **studies showed that TP-induced increases in AR and PSA expression in LNCaP cells were reduced by QI treatment. In BPH-model rats, the prostate weight, testosterone in serum, dihydrotestosterone (DHT) concentration and** ***5α-reductase*** **type 2 mRNA expression in prostate tissue were significantly reduced following the treatment with QI. TP-induced prostatic hyperplasia and the expression of proliferating cell nuclear antigen (PCNA) and cyclin D1 were significantly attenuated in QI-treated rats. In addition, QI induced apoptosis by up-regulating caspase-3 and -9 activity and decreasing the B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) ratio in prostate tissues of BPH rats. Further investigation showed that TP-induced activation of AKT and glycogen synthase kinase 3*β* (GSK3*β*) was reduced by QI administration. Therefore, our findings suggest that QI attenuates the BPH state in rats through anti-proliferative and pro-apoptotic activities and might be useful in the clinical treatment of BPH.**

Key words benign prostatic hyperplasia; dihydrotestosterone; *Quisqualis indica*; testosterone

Benign prostatic hyperplasia (BPH) is a common urogenital disorder that affects up to 85% of males who are over 50 years-old.[1)] BPH is characterized by the increased proliferation of epithelial and stromal cells of the prostate.[2)] BPH generally causes lower urinary tract symptoms (LUTS), such as incomplete bladder emptying, bladder obstruction, bloody urination, and frequent urination.[3)]

The etiology of BPH is not entirely clear. However, the development and incidence of BPH are associated with dysregulation of androgens, and with increased proliferation and decreased apoptosis of cells in the prostate gland.[4–9)] Testosterone and dihydrotestosterone (DHT) have key roles in the development and growth of the entire male genital tract, and they stimulate differentiation of the prostate gland.[10,11)] The adrenal glands and testes synthesize testosterone, and prostatic 5*α*-reductase type 2 converts it to DHT.[12)] DHT then binds to the androgen receptor (AR), which is transported to the nucleus, where it regulates genes important for prostate growth and differentiation.[4)] BPH development and progression are associated with activation of the AKT pathway, a major growth factor-mediated signal transduction pathways.[6)] Over-stimulation of the AKT pathway increases the activity of B-cell lymphoma 2 (Bcl-2) and cyclin D1, leading to increased proliferation and decreased apoptosis of cells, and transformation to the malignant state.[8,9,13,14)]

Quisqualis indica (QI), commonly known as Chinese honeysuckle or Rangoon creeper, is a vine of the Combretaceae family that grows in tropical Asian countries, such as China, the Philippines, Bangladesh, Myanmar, India, and Malaysia.[15)] Different parts of this plant have compounds such as quisqualic acid, the alkaloid trigonelline, the flavonoid rutin, the amino acids L-proline and L-asparagine, and two forms of the enzyme cysteine synthase (isoenzymes A and B).[15)] Practitioners of traditional Asian medicine have used QI to treat stomach pain, constipation, and digestion problems.[16)] Recent studies have shown that QI has anti-inflammatory, antioxidant, antimicrobial, cytotoxic, insecticidal, anti-obesity, antidiabetic, immunomodulatory, and hypolipidemic properties.[17–21)] However, the pharmacological effects of QI on BPH have not yet been established. We investigated the potential therapeutic effects of QI on BPH by using a human prostate cancer cell line and a rat model of testosterone propionate (TP)-induced BPH.

MATERIALS AND METHODS

Preparation of QI Extract Seeds of QI were obtained from a local herbal market in Ansan, Korea, authenticated by Dr. Yeon, and deposited at the herbarium of the HUONS Research Center (Voucher No. HU033/SKJA150427). The dried seeds were homogenized to a fine powder, and 100 g of this

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: hyojung@cnu.ac.kr

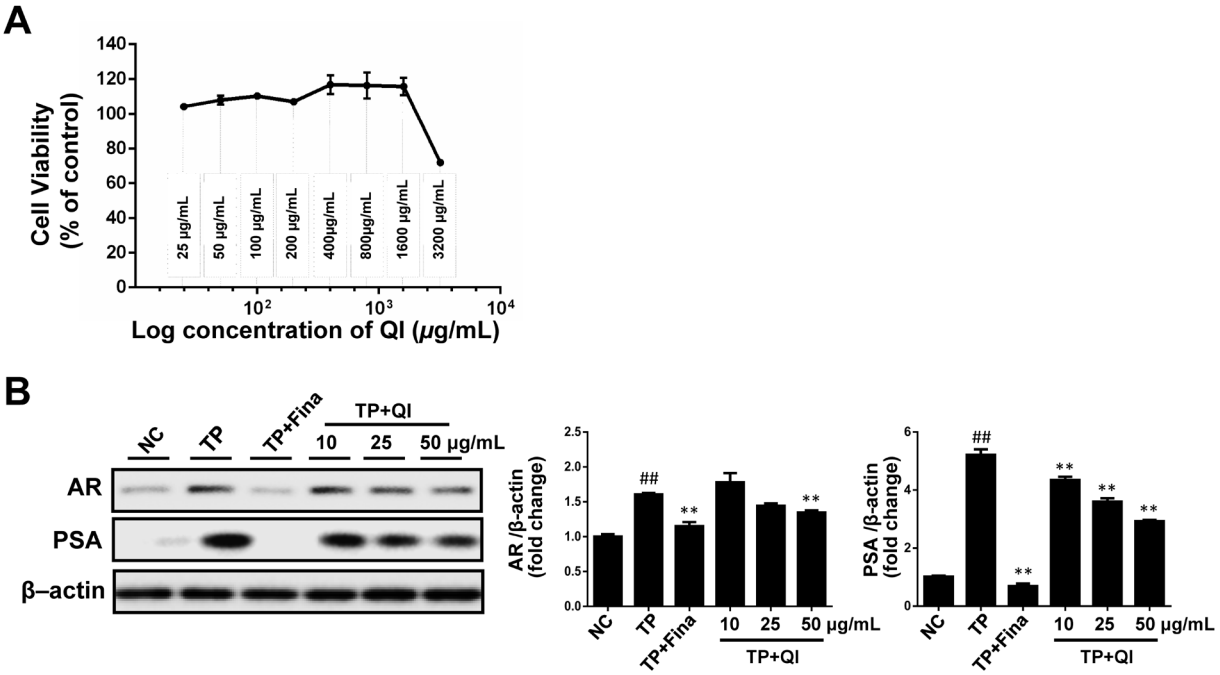

Fig. 1. Effects of QI on the LNCaP Human Prostate Cancer Cell Line

(A) Cell viability. LNCaP cells were treated with QI (25–3200 μg/mL) for 72 h, and cell viability was determined by MTT assay. (B) Western blot analysis of AR and PSA. LNCaP cells were incubated in medium containing testosterone (100 nmol), finasteride (10 μM) or QI (10, 25, or 50 μg/mL) for 72 h, and then cell lysates (30 μg) were assayed for expression levels of AR and PSA by Western blotting. NC, Vehicle-treated cells; TP, TP (100 nmol)- and vehicle-treated cells; TP+Fina, TP (100 nmol)- and finasteride (10 μM)-treated cells; TP+QI, TP (100 nmol)- and QI (10, 25, or 50 μg/mL)-treated cells. Data are representative of three independent experiments. Data are expressed as the means±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with the NC group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with the TP group.

powder was extracted by reflux with 1 L of 70% ethyl alcohol at 80°C for 1 h. The resulting fraction was vacuum-dried and stored at 4 °C until use.

For identification of amino acids, including quisqualic acid, all amino acids were subjected to pre-column derivatization with *O*-phthaldialdehyde and ethanethiol, as described previously.[22] Free amino acids were measured using HPLC, performed on an Agilent 1200 series system (Agilent Technologies, CA, U.S.A.). Separation of QI extract was performed at 30°C with a ZORBAX Eclipse Plus C_{18} column (4.6×250 mm, 5 μm particle size; Agilent Technologies). A sodium acetate buffer (solvent A; 0.01 M, pH 4.6) with methanol (solvent B) used as the mobile phase, and the programmed gradient runs were 0–5 min (20% B), 5–30 min (50% B), and 30–33 min (100% B), followed by a 1 mL/min flow rate equilibration with 20% B for 10 min. Absorbance was measured at 338 nm. The QI extract contained various amino acids, including asparagine, quisqualic acid, arginine, and glutamic acid (Supplementary Fig. 1). The administered extract was standardized by the quisqualic acid content (≥0.63%), using a validated HPLC assay, as described by the Hong Kong Chinese Materia Medica Standards (HKCMMS) Office.[23]

Cell Culture The human prostate cancer cell line (LNCaP) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). These cells were grown in RPMI-1640 medium (Gibco, CA, U.S.A.) with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco), and maintained in a 5% CO_2 incubator at 37°C.

Cell Treatments LNCaP cells were seeded onto 6-well plates (5×10^5 cells/well) in 1 mL of RPMI-1640 medium with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. One day later, the medium was removed and the cells were incubated in medium containing 100 nmol TP (Tokyo Chemical Ins. Co., Tokyo, Japan) and QI (10, 25 or 50 μg/mL) for an additional 72 h. Following two washes with phosphate-buffered saline (PBS), the cells were harvested for preparation of whole-cell extracts.

Cell Viability Assay The cytotoxic effect of QI extract on LNCaP cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[24] In brief, cells were plated at 1×10^4 cells per well in 96-well plates in 100 μL RPMI-1640 medium, and QI was added (concentration range: 25 to 3200 μg/mL). After incubation at 37°C for 72 h, the medium was replaced with 10 μL of the MTT reagent (100 μL/mL, Daeillab Service, Seoul, South Korea), followed by incubation under the same conditions for 2 h. Absorbance was determined at 450 nm using a microplate reader. Cell viability was calculated as: 100%×($OD_{450\,nm}$ of QI group/$OD_{450\,nm}$ of control group).

Animals Eight-week-old male Sprague-Dawley (SD) rats were purchased from Orient Bio (Seongnam, South Korea). Animals were maintained under standard laboratory conditions (22±2°C; relative humidity, 50±5%; 12 h light/dark cycle), and were allowed to consume a standard rodent chow and sterilized tap water *ad libitum*. All animal protocols were approved by the Animal Experimental Ethics Committee of Chungnam National University (Daejeon, South Korea).

Testosterone-Induced BPH Rats were acclimatized for 1 week prior to randomized division into 4 groups (6 rats/group): (a) normal control (NC) group (oral PBS and subcutaneous injection of corn oil); (b) BPH group (oral PBS and subcutaneous injection of TP [3 mg/kg body weight (BW); Tokyo Chemical Ins. Co.]; (c) positive control group (oral finasteride [10 mg/kg BW; Sigma, St. Louis, MO, U.S.A.] and subcutaneous injection of TP [3 mg/kg BW]; and (d) QI group

(oral QI [150 mg/kg BW] and subcutaneous injection of TP [3 mg/kg BW]). All rats received treatments once per day for 4 weeks, and body weights were measured once per week. The most recent body weight was used to determine the volume of QI delivered as 5 mL/kg BW. We conducted a preliminary trail with three doses of QI (50, 150 and 300 mg/kg) to identify the optimum dose for treatment of BPH. Based on these experiments, we chose a dose of 150 mg/kg, because this dose reduced BPH-related parameters (data not shown).

At the end of the experiment, rats were fasted overnight, anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg BW), and dissected to obtain blood from caudal vena cava. This blood was centrifuged, and serum was stored at −80°C until further analysis. The prostate of each rat was also carefully recovered and weighed. The prostatic index and percent inhibition were calculated as previously described: % inhibition=100−[(treated group−negative control)/(positive control−negative control)×100].[25] The ventral prostate of each rat was fixed overnight in 10% neutral buffered formalin (NBF), and the rest of the prostate was snap-frozen in liquid nitrogen for protein assays.

Hematoxylin and Eosin (H&E) Staining Prostate tissues of each rat were stained with H&E (Sigma) and examined under a microscope (Nikon ECLIPSE Ni-U, Tokyo, Japan) at 200×magnification. Images were captured from 10 randomly selected fields per rat, and epithelial thickness was measured using ImageJ software (ImageJ v46a; NIH, U.S.A.).

Immunohistochemistry (IHC) IHC was performed using a Vectastain Elite ABC Kit (Vector Laboratories, CA, U.S.A.) according to the manufacturer's instructions. After antigen retrieval processing, the sections were blocked in normal serum and then incubated with anti-proliferating cell nuclear antigen (PCNA; Abcam, MA, U.S.A.) antibody at 4°C. After overnight, the sections were incubated in biotinylated secondary antibody and developed using a diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories). PCNA-positive nuclei were counted in 10 randomly selected fields (×400) from each rat. Data are expressed as PCNA-positive nuclei per 100 cells.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Staining Prostate epithelial cell apoptosis was detected using an *in situ* apoptosis detection kit (Millipore, MA, U.S.A.). Positive cells were counted in 10 randomly selected fields (×400).

Determination of Testosterone and DHT Levels The serum and prostate concentrations of DHT and testosterone were determined using commercial ELISA (enzyme-linked immunosorbent assay) kits (ALPCO Diagnostics, NH, U.S.A.), according manufacturer's instructions.

Protein Extraction and Western Blot Analysis LNCaP cells or tissues were homogenized in RIPA lysis buffer (Cell Signaling Technology, MA, U.S.A.) supplemented with protease inhibitors (Roche, Mannheim, Germany) and phosphatase inhibitors (Roche). For immunoblotting, proteins (30–40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, and then transferred to nitrocellulose membranes at 40 V for 2 h. For experiments using LNCaP cells, membranes were probed with anti-androgen receptor (AR; Cell Signaling Technology), anti-prostate specific antigen (PSA; Cell Signaling Technology), or anti-β-actin (loading control; Sigma) antibodies in Tris-buffered saline with 0.05% Tween-20 (TBST) and 5% skim milk. Prostate tissues were probed with antibodies against PCNA, cyclin D1, caspase-3, caspase-9, AKT, phosphorylated-AKT (p-AKT), glycogen synthase kinase 3β (GSK3β), phosphorylated-GSK3β (p-GSK3β; Cell Signaling Technology), B-cell lymphoma 2 (Bcl-2), Bcl-2-like protein (Bax; Santa Cruz Biotechnology, CA, U.S.A.), or β-actin (Sigma). After washing, membranes were developed using an enhanced chemiluminescence kit (Thermo Scientific, MA, U.S.A.).

RNA Isolation and RT-PCR The RNeasy mini kit (Qiagen, MD, U.S.A.) was used to extract total RNA from prostate tissues and the A_{260}/A_{280} ratio was used to determine RNA purity. A commercially available reverse transcription kit (Qiagen) was used to generate cDNA from 1 μg of total RNA. Then, PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, CA, U.S.A.), with the SYBR Green PCR Master Mix (Life Technologies). The PCR primer for 5α-reductase type 2 was 5′-ATT TGT GTG GCA GAG AGA GG-3′ (forward) and 5′-TTG ATT GAC TGC CTG GAT GG-3′ (reverse); the primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was 5′-TGA TTC TAC CCA CGG CAA GT-3′ (forward) and 5′-AGC ATC ACC CCA TTT GAT GT-3′ (reverse). The data were analyzed using Applied Biosystems 7500 Real-Time PCR System software, and mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with β-actin as the control.

Toxicity Study Eight-week-old female Fischer 344 (F344) rats were randomly divided into two groups (6 rats per group): the NC group was given oral PBS and the QI group was given oral QI at a dose of 2000 mg/kg BW for 4 weeks. After 4 weeks, rats were sacrificed by CO_2 inhalation, and blood was drawn from the vena cava. The major organs, including the kidneys, liver, lungs, and spleen, were rapidly dissected out, washed in PBS, and weighed. The isolated organs were then cut into pieces and fixed in 10% formalin for subsequent histopathological examination.

Statistical Analysis All data are expressed as the means±standard errors of the mean (S.E.M.). Data were analyzed using a one-way ANOVA. SPSS 16.0 software (IBM, NY, U.S.A.) was used for this analysis. Data were compared using a multiple comparison test, with Tukey adjustment. A p-value below 0.05 was considered significant.

RESULTS

Effect of QI on the Viability of Human Prostate Cancer Cells To examine the potential cytotoxicity of QI on LNCaP cells, the MTT assay was performed. The cell viability was not substantially different between cells treated with QI (25 to 3200 μg/mL) and control cells. However, the exposure to 3200 μg/mL concentration of QI significantly decreased the viability by 72.5% (Fig. 1A), indicating that the high concentration of QI inhibits cell growth by its toxicity. Based on these results, we used the low concentration of QI (≤1600 μg/mL) to avoid the cytotoxic effects.

Effect of QI on Expression of AR and PSA in Prostate Cancer Cells Androgens promote the development and progression of prostate cancer, and PSA is an androgen-responsive gene that is widely used to assess progression of human prostate cancer.[26] Finesteride is a type 2 5α-reductase inhibitor that downregulates the conversion of testosterone to DHT and

interferes the binding of DHT to AR.[27] Thus, we examined the effect of QI treatment on AR and PSA expression in LNCaP cells. The Western blotting results showed that TP treatment significantly increased the expression of AR and PSA, and that co-treatment with finasteride or QI (50 μg/mL) significantly attenuated this effect (Fig. 1B). These findings suggest that QI suppresses androgen signaling in prostate cancer cells.

Effect of QI on Prostate Weight in a Rat Model of BPH Next, we determined the therapeutic potential of QI for treatment of BPH by using a rat model of TP-induced BPH. In BPH group, treatment of TP resulted in a significant increase in prostate weights (absolute and relative) compared to the NC group. In comparison to the BPH group, finesteride and QI treatment significantly reduced the TP induced enlargement of the prostate gland of rats (Table 1). Further, the prostate growth inhibition ratio in the QI group was found to be 37.9%, a value similar to that (36.2%) for the finasteride-treated group (Table 1), indicating QI has potential for treatment of BPH.

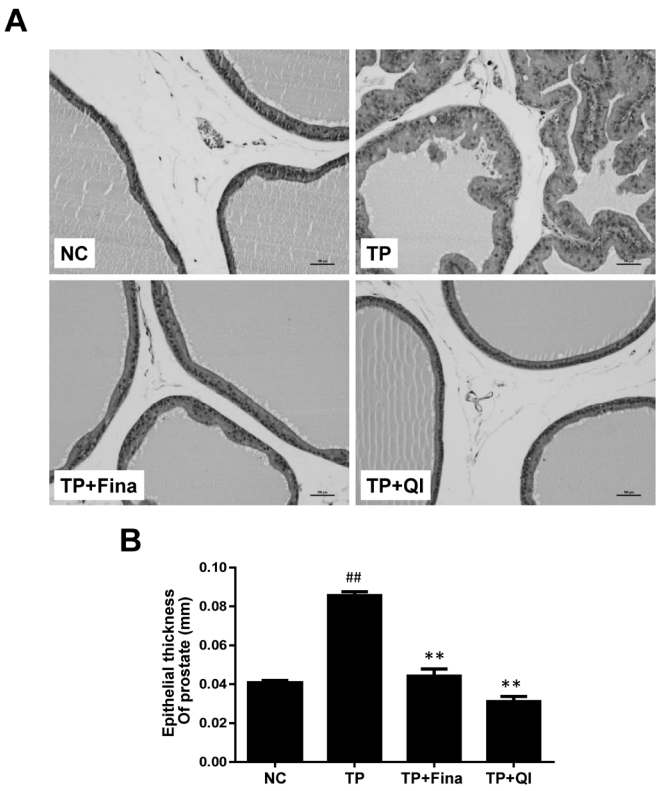

Fig. 2. Effects of QI on Histological Changes in Prostate Tissues in BPH-Model Rats

(A) Representative photomicrographs of H&E-stained prostate tissues (magnification, ×200). (B) The epithelial thickness of prostate tissues. Abbreviations: NC, corn oil-injected and PBS-treated rats; TP, TP (3 mg/kg)- and PBS-treated rats; TP+Fina, TP (3 mg/kg)- and finasteride (10 mg/kg)-treated rats; TP+QI, TP (3 mg/kg)- and QI (150 mg/kg)-treated rats. Data are expressed as the means±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with the NC group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with the TP group.

Effect of QI on Histological Changes in the Prostate of Rats with BPH Histological analyses of prostate tissues indicated the rats in the NC group had normal cell morphology, but those in the TP group had significant glandular hyperplasia, with multiple layers of epithelial cells, and a decreased glandular luminal area (Fig. 2A). Notably, finasteride and QI treatment reversed the effects of TP (Fig. 2A). As shown in Fig. 2B, rats in the TP-induced BPH group exhibited a significant increase in the epithelial thickness of their prostates compared with those in the NC group. However, treatment with finasteride or QI significantly reduced this increase in epithelial thickness. These findings indicate that the reduced prostate weight after QI treatment results from the attenuation of the abnormal histological effects in the prostate of BPH rats.

Effect of QI on Testosterone, DHT, and 5α-Reductase Type 2 in Rats with BPH Serum testosterone acts on the prostate, which produces growth factors that regulate cell growth, survival, and apoptosis.[28] In our study, TP injection significantly increased the level of serum testosterone, and that finasteride and QI partially reversed this effect (Fig. 3A). The prostate produces DHT, which has a major effect on BPH progression, by 5α-reductase-mediated conversion of circulating testosterone.[11] The level of DHT was significantly higher in BPH group compared to the NC group. However, treatment with finesteride and QI significantly reduced the DHT level in BPH induced rats (Fig. 3B). In addition, mRNA expression of *5α-reductase* type 2 was up-regulated in TP-induced BPH group compared to the NC group and this level was suppressed by both finasteride and QI treatment (Fig. 3C). These data suggest that QI reduces the androgen concentrations in BPH induced rats.

Effect of QI on Prostate Cell Proliferation in Rats with BPH Next, we investigated the anti-proliferative effect of QI by measuring expression of PCNA in the prostate. Rats in the NC group had very low expression of prostatic epithelial PCNA, but rats in the TP group had an increased number of PCNA-positive cells (Figs. 4A, B). Finasteride and QI partially reversed the effect of TP. Consistent with these results, Western blotting results indicated the TP group had significantly increased levels of PCNA and cyclin D1 compared to the NC group, and treatment with QI and finasteride significantly attenuated this effect (Figs. 4C, D). These data suggest that QI attenuates BPH through anti-proliferative activity.

Effect of QI on Apoptosis in Rats with BPH To determine whether the inhibitory effect of QI on prostate growth

Table 1. Effect of QI on Prostate Weights

Group	Treatment	Prostate weight		% inhibition
		Absolute (g)	Relative (%)	
NC	Corn oil/PBS	0.595±0.036	0.151±0.009	
TP	TP/PBS	1.532±0.089**	0.434±0.019**	
TP+Fina	TP/Finasteride	1.193±0.047$^{\#\#}$	0.339±0.013$^{\#\#}$	36.2
TP+QI	TP/QI	1.177±0.050$^{\#\#}$	0.334±0.017$^{\#\#}$	37.9

The % inhibition was calculated by using absolute prostate weight as follow: 100−[(treated group−negative control)/(positive control−negative control)×100]. Abbreviations: NC, corn oil-injected and PBS-treated rats; TP, TP (3 mg/kg)- and PBS-treated rats; TP+Fina, TP (3 mg/kg)- and finasteride (10 mg/kg)-treated rats; TP+QI, TP (3 mg/kg)- and QI (150 mg/kg)-treated rats. Results are expressed as the means±S.E.M. $^{\#\#}p<0.01$ compared with the NC group; $^{**}p<0.01$ compared with the TP group.

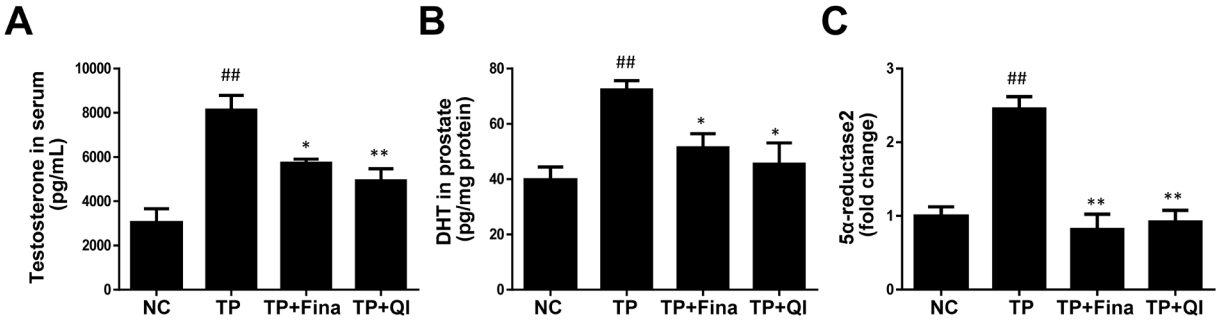

Fig. 3. Effect of QI Administration on Testosterone, DHT, and 5α-Reductase Type 2 in BPH-Model Rats

(A, B, C) The serum concentrations of testosterone (A) and the levels of DHT (B) in prostate tissue were examined by ELISA. (C) The mRNA levels of 5α-reductase type 2 in prostate tissue were analyzed by RT-qPCR. Abbreviations: NC, corn oil-injected and PBS-treated rats; TP, TP (3 mg/kg)- and PBS-treated rats; TP+Fina, TP (3 mg/kg)- and finasteride (10 mg/kg)-treated rats; TP+QI, TP (3 mg/kg)- and QI (150 mg/kg)-treated rats. Data are expressed as the means±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with the NC group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with the TP group.

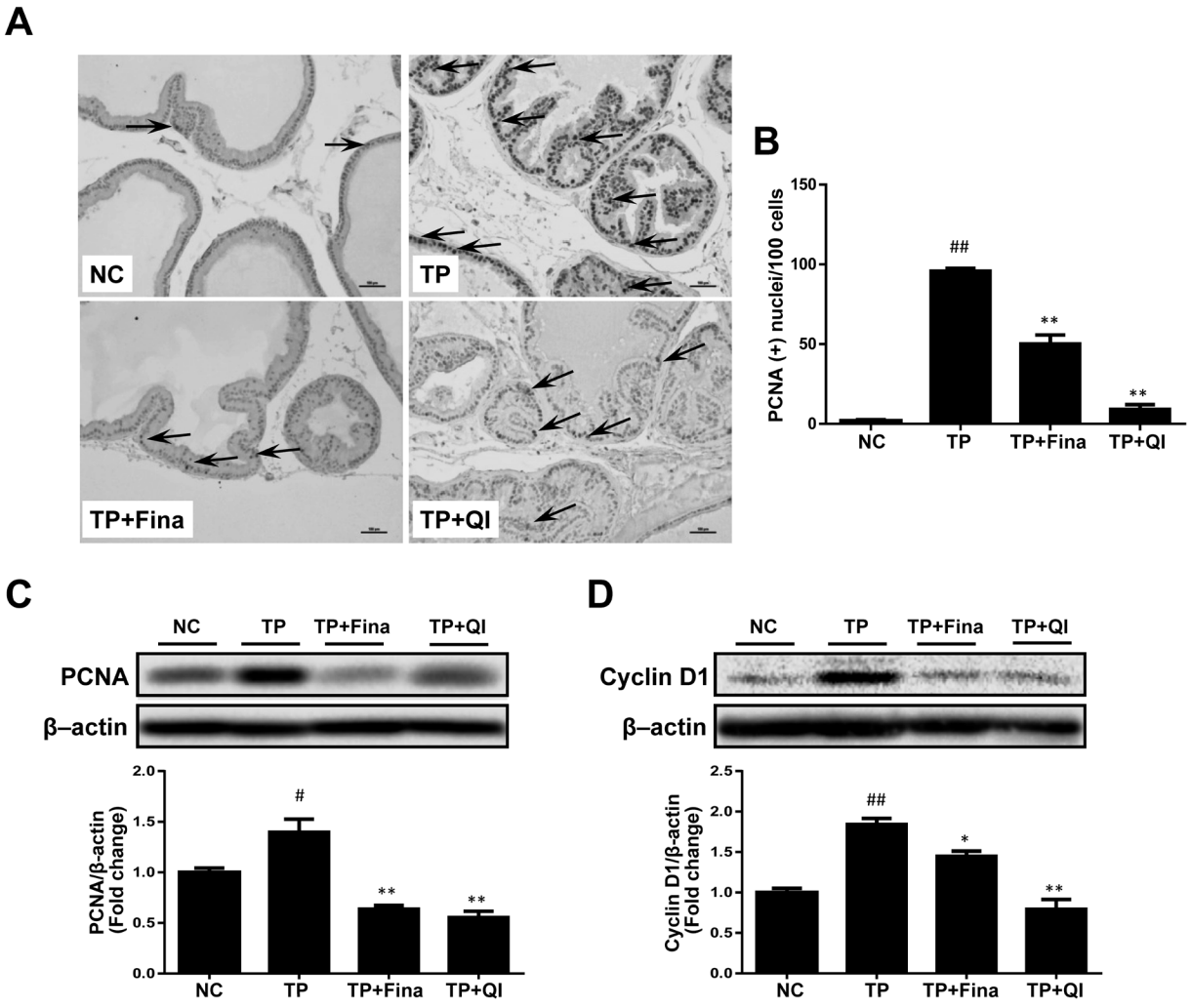

Fig. 4. Effect of QI Administration on Prostate Cell Proliferation in BPH-Model Rats

(A) Representative photomicrographs of prostate tissues immunostained with an anti-PCNA antibody (magnification, ×200). (B) Number of PCNA-positive cells (Arrows) in the prostate. PCNA-positive nuclei were counted in 10 randomly selected fields (magnification, ×400) from each rat; results are expressed as PCNA positive nuclei/100 cells. (C) The expression levels of PCNA and cyclin D1 (D) were determined by Western blotting; β-actin was used as an internal control. Abbreviations: NC, corn oil-injected and PBS-treated rats; TP, TP (3 mg/kg)- and PBS-treated rats; TP+Fina, TP (3 mg/kg)- and finasteride (10 mg/kg)-treated rats; TP+QI, TP (3 mg/kg)- and QI (150 mg/kg)-treated rats. Data are expressed as the means±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with the NC group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with the TP group.

was attributable to apoptosis, we examined its effect on apoptosis in BPH-model rats using TUNEL staining. The number of TUNEL-positive cells in the prostate was reduced in the BPH group compared with the NC group (Figs. 5A, B). However, an increased number of positive cells were observed in finesteride and QI treated groups (Figs. 5A, B). Moreover, Western blotting revealed significantly increased expression of cleaved caspase-3 and -9 with finasteride and QI treatment compared with the BPH group (Fig. 5C). In addition, the expression of anti-apoptotic protein, Bcl-2 was decreased,

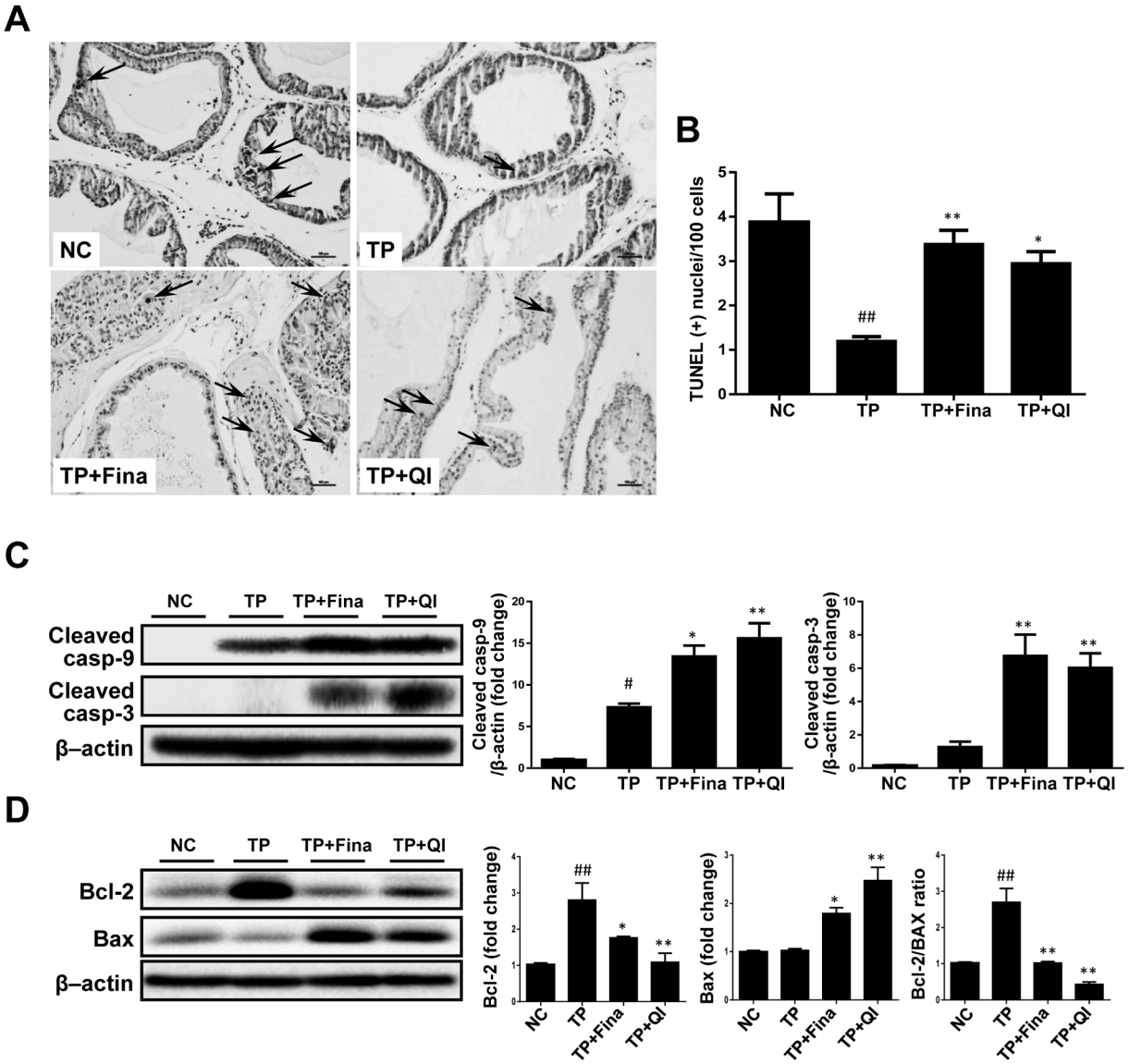

Fig. 5. Effect of QI Administration on Prostate Cell Apoptosis in BPH-Model Rats

(A) Representative photomicrographs of prostate tissue showing apoptotic cells (Arrows), determined by TUNEL staining (magnification, ×400). (B) Number of TUNEL-positive cells in the prostate. TUNEL-positive nuclei were counted in 10 randomly selected fields (magnification, ×400) from each rat; results are expressed as TUNEL-positive nuclei/100 cells. The expression levels of caspase-3 and -9 (C) and Bcl-2 and Bax (D) were determined by Western blotting; β-actin was used as an internal control. The results were obtained in two different gels by calculating the average of six independent prostates per group. Abbreviations: NC, corn oil-injected and PBS-treated rats; TP, TP (3 mg/kg)- and PBS-treated rats; TP+Fina, TP (3 mg/kg)- and finasteride (10 mg/kg)-treated rats; TP+QI, TP (3 mg/kg)- and QI (150 mg/kg)-treated rats. Data are expressed as the means±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with the NC group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with the TP group.

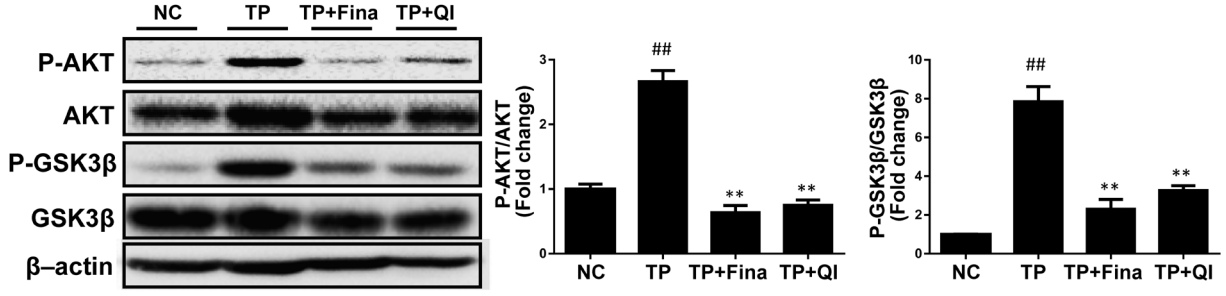

Fig. 6. Effects of QI on the AKT/GSK3β Pathway in Prostate Tissues

The results were obtained in two different gels by calculating the average of six independent prostates per group. Abbreviations: NC, corn oil-injected and PBS-treated rats; TP, TP (3 mg/kg)- and PBS-treated rats; TP+Fina, TP (3 mg/kg)- and finasteride (10 mg/kg)-treated rats; TP+QI, TP (3 mg/kg)- and QI (150 mg/kg)-treated rats. Data are expressed as the means±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with the NC group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with the TP group.

Table 2. Body Weights of Rats in 4-Week Oral Toxicity Study

Group	Week				
	0	1	2	3	4
NC	128.25±1.60	139.50±2.33	151.10±2.75	160.73±3.37	162.05±2.74
QI	129.00±2.00	138.80±2.14	149.34±1.79	159.00±1.53	161.00±1.51

NC, PBS-treated rats; QI; QI (2000 mg/kg)-treated rats. Results are expressed as the means±S.E.M.

Table 3. Relative Organ Weights after a 4-Week Oral Toxicity Study

Group	Relative organ weight			
	Liver	Lung	Kidney (right)	Spleen
NC	3.174±0.153	0.579±0.033	0.389±0.014	0.275±0.007
QI	3.105±0.042	0.648±0.030	0.389±0.004	0.297±0.007

NC, PBS-treated rats; QI; QI (2000 mg/kg)-treated rats. Results are expressed as the means±S.E.M.

Table 4. Clinical Chemistry Values Following a 4-Week Oral Toxicity Study

Parameters	Group	
	NC	QI
ALT (IU/L)	56.00±1.55	50.00±3.42
AST (IU/L)	104.75±3.04	103.00±2.52
ALP (IU/L)	578.50±22.59	589.86±12.41
TG (mg/dL)	58.75±7.16	53.43±7.33
CHO (mg/dL)	114.75±5.26	96.67±4.80
BUN (mg/dL)	20.15±0.65	19.89±0.84
Crea (mg/dL)	0.40±0.00	0.37±0.02
ALB (g/dL)	5.20±0.08	4.79±0.08

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TG, triglyceride; CHO, cholesterol; BUN, blood urea nitrogen; Crea, creatinine; ALB, albumin. Results are expressed as the means±S.E.M.

whereas pro-apoptotic Bax was increased in QI treated rats. Subsequently, QI treatment significantly reduced Bcl-2/Bax ratio in BPH induced rats (Fig. 5D), indicating QI prevents BPH by promoting apoptosis in the prostate.

Effect of QI on AKT/GSK3 Signaling in Rats with BPH The AKT/GSK3β pathway is important for regulation of cell proliferation and apoptosis.[29] As shown in Fig. 6, TP administration substantially increased the phosphorylation of prostatic AKT and GSK3β, and that finasteride and QI treatment reversed this effect. These results demonstrate that QI suppresses the development of BPH by regulating AKT/GSK3 signaling in rats.

Toxicity of QI Treatment of F344 rats with QI alone (2000 mg/kg) had no significant effect on body weight after 4 weeks (Table 2). QI-treated rats also had no changes in clinical signs or symptoms, mortality, or gross pathology (data not shown). In addition, QI treatment had no significant effect on the weights of 4 different organs (Table 3) or blood chemistry (Table 4) of these rats. H&E staining of the organs indicated no histopathological abnormalities associated with QI treatment (data not shown). These data indicate that QI causes no evident toxicities in rats at a dose of 2000 mg/kg.

DISCUSSION

Despite significant progress in available treatments, BPH remains the most common prostatic disease affecting older men.[3] Given the many side effects of surgery and pharmacological therapy, and the long latency of BPH, natural products from plants have emerged as alternative treatments because they may provide therapeutic efficacy with fewer adverse effects. The present study assessed the protective role of QI on BPH using a human prostate cancer cell line and a rat model of BPH. Our *in-vitro* results showed that QI treatment significantly inhibited the TP-induced increases of AR and PSA expression in LNCaP cells. In agreement, the TP-induced increases in prostate weight, serum testosterone, prostatic DHT concentration, and *5α-reductase* type 2 mRNA expression in prostate tissue were significantly attenuated in QI-treated rats. A 4-week toxicity study in rats further showed that QI had no adverse effects at a dose of 2000 mg/kg.

Overall enlargement of the prostate is a sign of BPH,[30,31] which is characterized by increased proliferation of epithelial and stromal cells in the prostate. Hyperplasia of these cells leads to increased prostate weight and compression of the urethral canal, thereby blocking urine flow.[32] Previous studies indicated that animals with experimentally induced BPH have significantly greater prostate weight than controls, and that finasteride treatment decreases BPH.[30,33] In line with these previous reports, our present findings revealed that TP injection contributed to a significant increase in prostatic weights of BPH induced rats compared to the control rats. In contrary, the rats treated with QI showed significantly decrease prostatic weights compared with BPH induced rats. Our histopathological examinations of rat prostate tissues are consistent with this result. More specifically, rats in the TP group had greater epithelial hyperplasia and epithelial thickness than rats in the TP/QI group. These findings suggest that QI effectively inhibited the development of prostatic hyperplasia and BPH.

Prostate DHT levels increase as BPH progresses, and prostatic 5α-reductase converts testosterone into DHT.[10,11] DHT increases hyperplasia of prostatic stromal and epithelial cells, resulting in the enlargement of prostate.[10] Therefore, 5α-reductase inhibitors are effective treatments for men with BPH. Finasteride is a type 2 5α-reductase inhibitor that down-regulates plasma and intra-prostatic DHT concentrations, and reduces prostate size, epithelial cell height, and the synthesis of DHT.[34,35] In agreement with previous studies, our results indicate that finasteride significantly reduced testosterone and

DHT levels in the serum and prostates of rats, and that QI had a similar effect. In addition, QI and finasteride also decreased the expression of *5α-reductase* type 2 mRNA in the prostate. These results indicate that the QI-induced reduction of prostatic epithelial hyperplasia is closely associated with the reduced DHT level.

BPH is characterized by an increased number of prostatic stromal and epithelial cells due to uncontrolled proliferation, and increased prostate volume.[36] Our *in vitro* data showed that QI inhibited cell viability in prostate cancer cells at the high concentration (≥3200 μg/mL). We further evaluated the mechanism responsible for the inhibitory effects of QI on BPH by measuring the expression of PCNA in the prostate tissue of TP-treated rats. PCNA expression is significantly elevated in patients with BPH or prostate cancer, and the extent of cell proliferation is related to the clinical grade of prostate cancer.[37] Animals with experimentally induced BPH also have significantly increased prostatic PCNA expression.[38,39] We found that rats with BPH that were given QI treatment had significantly reduced expression of PCNA and cyclin D1, as well as fewer PCNA-positive cells. This indicates that QI protected against BPH development due to its anti-proliferative activity. Our data also revealed that QI increased the apoptosis of prostate epithelial cells. Moreover, treatment with QI decreased the expression of the anti-apoptotic protein, Bcl-2, and increased the expression of the pro-apoptotic protein, Bax. Hence, the QI-treated rats have a decreased Bcl-2/Bax ratio, as well as increased levels of prostatic caspase-3 and -9. Various diseases, including BPH, are characterized by disruption of processes that regulate cell proliferation and cell death.[40,41] Epithelial cells in normal prostate tissue express little or no Bcl-2 or caspase-3, but hyperplastic changes are associated with increased levels of Bcl-2 and a decreased levels of Bax in the prostate.[42,43] The Bcl-2/Bax ratio is an important measure of cell fate, and an increased ratio (resulting from aberrant expression of these proteins) is common in BPH. Therefore, agents that promote prostate cell apoptosis have potential as treatments for BPH. We hypothesize that the pro-apoptotic activity of QI, together with its anti-proliferative activity, were responsible for the suppression of prostate cell proliferation and reduction in prostate volume.

We also investigated the effects of QI on the AKT/GSK3β pathway to elucidate the mechanism by which QI inhibits TP-induced cell proliferation in the prostate. Activation of the AKT pathway is known to play key roles in cell proliferation, apoptosis and survival in various cell types. AKT increases cyclin D1 levels by stimulating its mRNA translation.[44] Phosphorylation/inactivation of GSK3β by AKT also results in elevated levels of cyclin D1, thus allowing progression through the G1 phase of the cell cycle.[8,9] Additionally, AKT inactivates Bad and caspase-9, two pro-apoptotic proteins,[13] whereas inhibition of AKT signaling induces cell death by reducing the expression of Bcl-2 and enhancing the expression of Bax or Bad.[14] Our results show that QI treatment decreased the TP-induced activation of AKT and GSK3β. We also found that these QI-induced decreases in p-AKT and p-GSK3β levels paralleled reduced cyclin D1 expression and a decrease in the Bcl-2/Bax ratio. These results suggest that the QI-induced increase in apoptosis and suppression of prostate cell proliferation may be mediated *via* alterations in the AKT pathway.

The two most widely accepted classes of medications for treatment of BPH are 5α-reductase inhibitors and alpha −1 (α1)-adrenergic receptor antagonists.[45] However, the utility of these drugs is limited by their side effects, which include decreased libido, ejaculatory or erectile dysfunction, and nasal congestion.[46] Our results show that oral administration of QI to rats, at a dose of 2000 mg/kg/d for 4 weeks, produced no clinical signs or evident treatment-related adverse effects. However, further studies of potential chronic toxicity and genotoxicity are needed to establish the safe oral dose of QI.

In conclusion, the present study provides the first evidence that QI protects against TP-induced BPH in rats, as indicated by its ability to reduce prostate size and hyperplasia. These effects can be attributed, at least in part, to the decreased prostatic levels of DHT and the anti-proliferative and pro-apoptotic activities of QI. Use of QI as a therapeutic agent for the treatment of BPH warrants further exploration.

Acknowledgment This work was supported by a Grant from the Korean Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio Industry Technology Development Program (2016190262), funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Arruzazabala ML, Molina V, Mas R, Carbajal D, Marrero D, Gonzalez V, Rodriguez E. Effects of coconut oil on testosterone-induced prostatic hyperplasia in Sprague-Dawley rats. *J. Pharm. Pharmacol.*, **59**, 995–999 (2007).
2) Caballero SM, Fernandez CC, Perez-Fernandez R. Effect of an integral suspension of *Lepidium latifolium* on prostate hyperplasia in rats. *Fitoterapia*, **75**, 187–191 (2004).
3) Barkin J. Benign prostatic hyperplasia and lower urinary tract symptoms: evidence and approaches for best case management. *Can. J. Urol.*, **18** (Suppl.), 14–19 (2011).
4) Mizokami A, Koh E, Izumi K, Narimoto K, Takeda M, Honma S, Dai J, Keller ET, Namiki M. Prostate cancer stromal cells and LNCaP cells coordinately activate the androgen receptor through synthesis of testosterone and dihydrotestosterone from dehydroepiandrosterone. *Endocr. Relat. Cancer*, **16**, 1139–1155 (2009).
5) Descazeaud A, Weinbreck N, Robert G, Vacherot F, Abbou CC, Labrousse F, Allory Y, Rubin MA, De la Taille A. Transforming growth factor beta-receptor II protein expression in benign prostatic hyperplasia is associated with prostate volume and inflammation. *BJU Int.*, **108** (2b), E23–E28 (2011).
6) Wadsworth TL, Carroll JM, Mallinson RA, Roberts CT Jr, Roselli CE. Saw palmetto extract suppresses insulin-like growth factor-I signaling and induces stress-activated protein kinase/c-Jun N-terminal kinase phosphorylation in human prostate epithelial cells. *Endocrinology*, **145**, 3205–3214 (2004).
7) Wang Z, Olumi AF. Diabetes, growth hormone-insulin-like growth factor pathways and association to benign prostatic hyperplasia. *Differentiation*, **82**, 261–271 (2011).
8) Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.*, **12**, 3499–3511 (1998).

9) Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N. Cyclin D expression is controlled post-transcriptionally *via* a phosphatidylinositol 3-kinase/Akt-dependent pathway. *J. Biol. Chem.*, **273**, 29864–29872 (1998).
10) Andriole G, Bruchovsky N, Chung LW, Matsumoto AM, Rittmaster R, Roehrborn C, Russell D, Tindall D. Dihydrotestosterone and the prostate: the scientific rationale for 5alpha-reductase inhibitors in the treatment of benign prostatic hyperplasia. *J. Urol.*, **172**, 1399–1403 (2004).
11) Miller J, Tarter TH. Combination therapy with dutasteride and tamsulosin for the treatment of symptomatic enlarged prostate. *Clin. Interv. Aging*, **4**, 251–258 (2009).
12) Carson C 3rd, Rittmaster R. The role of dihydrotestosterone in benign prostatic hyperplasia. *Urology*, **61** (Suppl. 1), 2–7 (2003).
13) Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**, 231–241 (1997).
14) Zheng D, Zhu G, Liao S, Yi W, Luo G, He J, Pei Z, Li G, Zhou Y. Dysregulation of the PI3K/Akt signaling pathway affects cell cycle and apoptosis of side population cells in nasopharyngeal carcinoma. *Oncol. Lett*, **10**, 182–188 (2015).
15) Sahu J, Patel PK, Dubey B. *Quisqualis indica* Linn.: a review of its medicinal properties. *Int. J. Pharm Phytopharmacol. Res.*, **1**, 313–321 (2012).
16) Lin TC, Ma YT, Wu J, Hsu FL. Tannins and related compounds from *Quisqualis indica*. *J. Chin. Chem. Soc.*, **44**, 151–155 (1997).
17) Kambar Y, Asha MM, Chaithra M, Prashith KTR. Antibacterial activity of leaf and flower extract of *Quisqualis indica* Linn. against clinical isolates of Staphylococcus aureus. *Research J. Sci. Tech.*, **6**, 23–24 (2014).
18) Singh N, Khatri P, Samantha KC, Damor R. Antipyretic activity of methanolic extract of leaves of *Quisqualis indica* Linn. *Int. J. Pharm. Res. Dev*, **2**, 122–126 (2010).
19) Kaisar MA, Islam MR, Rahman MS, Hossain MK, Rashid MA. Total phenolic content, free radical scavenging activity and reducing power of *Quisqualis indica* Linn. *Dhaka. Univ. J. Pharm. Sci*, **8**, 173–175 (2009).
20) Sagrawat H, Khan MY. Immunomodulatory plants: a phytopharmacological review. *Pharmacogn. Rev.*, **1**, 248–260 (2007).
21) Jahan FN, Rahman MS, Rahman MM, Gibbons S, Masud MM, Sadhu SK, Hossain M, Hasan CM, Rashid MA. Diphenylpropanoids from *Quisqualis indica* Linn. and their anti-staphylococcal activity. *Lat. Am. J. Pharm.*, **28**, 279–283 (2009).
22) Fernstrom MH, Fernstrom JD. Rapid measurement of free amino acids in serum and CSF using high-performance liquid chromatography. *Life Sci.*, **29**, 2119–2130 (1981).
23) HKCMMS. "Hong Kong Chinese Materia Medica Standards 2015.": ‹http://www.cmd.gov.hk/hkcmms/vol6/main.html›, cited 29 March, 2017.
24) Jiang R, Xu XH, Wang K, Yang XZ, Bi YF, Yan Y, Liu JZ, Chen XN, Wang ZZ, Guo XL, Zhao DQ, Sun LW. Ethyl acetate extract from *Panax ginseng* CA Meyer and its main constituents inhibit α-melanocyte-stimulating hormone-induced melanogenesis by suppressing oxidative stress in B16 mouse melanoma cells. *J. Ethnopharmacol.*, **208**, 149–156 (2017).
25) Patil AA, Veeresh B, Yadav A. Combination of lauric acid and myristic acid prevents benign prostatic hyperplasia (BPH) symptoms in animal model. *Afr. J. Pharm. Pharmacol*, **10**, 101–106 (2016).
26) Lamb DJ, Weigel NL, Marcelli M. Androgen receptors and their biology. *Vitam. Horm.*, **62**, 199–230 (2001).
27) Chhipa RR, Halim D, Cheng J, Zhang HY, Mohler JL, Ip C, Wu Y. The direct inhibitory effect of dutasteride or finasteride on androgen receptor activity is cell line specific. *Prostate*, **73**, 1483–1494 (2013).
28) Marinese D, Patel R, Walden PD. Mechanistic investigation of the adrenergic induction of ventral prostate hyperplasia in mice. *Prostate*, **54**, 230–237 (2003).
29) Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat. Rev. Cancer*, **2**, 489–501 (2002).
30) Veeresh Babu SV, Veeresh B, Patil AA, Warke YB. Lauric acid and myristic acid prevent testosterone induced prostatic hyperplasia in rats. *Eur. J. Pharmacol.*, **626**, 262–265 (2010).
31) Arruzazabala ML, Mas R, Molina V, Noa M, Carbajal D, Mendoza N. Effect of D-004, a lipid extract from the Cuban royal palm fruit, on atypical prostate hyperplasia induced by phenylephrine in rats. *Drugs R D.*, **7**, 233–241 (2006).
32) Roehrborn CG. Male lower urinary tract symptoms (LUTS) and benign prostatic hyperplasia (BPH). *Med. Clin. North Am.*, **95**, 87–100 (2011).
33) Shin IS, Lee MY, Ha HK, Seo CS, Shin HK. Inhibitory effect of Yukmijihwang-tang, a traditional herbal formula against testosterone-induced benign prostatic hyperplasia in rats. *BMC Complement. Altern. Med.*, **12**, 48 (2012).
34) Tarter TH, Vaughan ED Jr. Inhibitors of 5alpha-reductase in the treatment of benign prostatic hyperplasia. *Curr. Pharm. Des.*, **12**, 775–783 (2006).
35) Aggarwal S, Thareja S, Verma A, Bhardwai TR, Kumar M. An overview on 5alpha-reductase inhibitors. *Steroids*, **75**, 109–153 (2010).
36) Lobaccaro JM, Boudan C, lechevallier E, Mottet N, Rebillard X, Lumbroso S, Gibelin B, Sultan C. Effect of finasteride (Proscar) on the proliferation of cultured epithelial and stromal cells from normal and hyperplastic human prostates. *Cell. Mol. Biol.*, **42**, 511–518 (1996).
37) Zhong W, peng J, He H, Wu D, Han Z, Bi X, Dai Q. Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia. *Clin. Invest. Med.*, **31**, E8–E15 (2008).
38) Lee MY, Shin IS, Seo CS, Lee NH, Ha HK, Son JK, Shin HK. Effects of Melandrium firmum methanolic extract on testosterone-induced benign prostatic hyperplasia in Wistar rats. *Asian J. Androl.*, **14**, 320–324 (2012).
39) Park E, Lee MY, Jeon WY, Lee N, Seo CS, Shin HK. Inhibitory effect of Yongdamsagan-tang water extract, a traditional herbal formula, on testosterone-induced benign prostatic hyperplasia in rats. *Evid. Based Complement. Alternat. Med.*, **2016**, 1428923 (2016).
40) Angelucci C, Lacopino F, Lama G, Zelano G, Gianesini G, Sica G, Bono AV. Reverse transcriptase-PCR analysis of apoptosis-regulating gene expression in human benign prostatic hyperplasia. *Anticancer Res.*, **25** (6B), 3937–3941 (2005).
41) Minutoli L, Rinaldi M, Marini H, Irrera N, Crea G, Lorenzini C, Puzzolo D, Valenti A, Pisani A, Adamo EB, Altavilla D, Squadrito F, Micali A. Apoptotic pathways linked to endocrine system as potential therapeutic targets for benign prostatic hyperplasia. *Int. J. Mol. Sci.*, **17**, E1311 (2016).
42) Chung KS, An HJ, Cheon SY, Kwon KR, Lee KH. Bee venom suppresses testosterone-induced benign prostatic hyperplasia by regulating the inflammatory response and apoptosis. *Exp. Biol. Med.* (Maywood), **240**, 1656–1663 (2015).
43) Chung KS, Shin SJ, Lee NY, Cheon SY, Park W, Sun SH, An HJ. Anti-proliferation effects of garlic (*Allium sativum* L.) on the progression of benign prostatic hyperplasia. *Phytother. Res.*, **30**, 1197–1203 (2016).
44) Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp. Cell Res.*, **253**, 210–229 (1999).
45) Bullock TL, Andriole GL Jr. Emerging drug therapies for benign prostatic hyperplasia. *Expert Opin. Emerg. Drugs*, **11**, 111–123 (2006).
46) Traish AM, Hassani J, Guay AT, Zitzmann M, Hansen ML. Adverse side effects of 5alpha-reductase inhibitors therapy: persistent diminished libido and erectile dysfunction and depression in a subset of patients. *J. Sex. Med.*, **8**, 872–884 (2011).