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Krill oil ameliorates benign prostatic hyperplasia by regulating G₁-phase cell cycle arrest and altering signaling pathways and benign prostatic hyperplasia-associated markers

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ABSTRACT

Krill oil (KO) exhibits various biological activities, such as anti-inflammatory and antitumor effects. However, the inhibitory effects of benign prostatic hyperplasia (BPH) *in vitro* and *in vivo* have not yet been studied. This study investigated the anti-BPH effects of KO extracted by an enzymatic hydrolysis method. KO treatment inhibited the proliferation of WMPY-1 and BPH-1 cells by induction of G₀/G₁ phase arrest through the modulation of positive and negative regulators in both prostate cell types. KO treatment stimulated phosphorylation of c-Jun N-terminal kinase (JNK) and p38 signaling. In addition, KO changed the expression of BPH-related markers (5α-reductase, androgen receptor, FGF, Bcl-2, and Bax) and the activity of the proliferation-mediated NF-κB binding motif. KO-induced levels of proliferation-mediated molecules of prostate cells were attenuated in the presence of siRNA-specific p-38 (si-p38) and JNK (si-JNK). Furthermore, the administration of KO alleviated prostate size and weight and the cell layer thickness of prostate glands in a testosterone enanthate-induced BPH rat model. KO treatment altered the level of dihydrotestosterone in serum and the expression levels of BPH-related markers in prostate tissues. Finally, KO-mediated inhibition of prostatic growth was validated by histological analysis. These results suggest that KO has an inhibitory effect on BPH in prostate cells *in vitro* and *in vivo*. Thus, KO might be a potential prophylactic or therapeutic agent for patients with BPH.

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1. Introduction

Benign prostatic hyperplasia (BPH) is one of the most common diseases in men aged over 60 years^[1]. It is the fourth most common

diagnosis in older men^[2]. The disease is non-malignant growth of the prostate, although BPH also triggers lower urinary tract symptoms and bladder outlet obstruction. These effects can interfere with activities of daily living, causing significant impairment of the disease-specific quality of life and interfering with sexual functioning^[3,4]. BPH treatment typically includes surgery, laser, and medication^[3,6], but can cause recurrence and side effects, such as erectile dysfunction, dizziness, hypotension, fatigue, weakness, insomnia, sexual

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dysfunction, or prostate cancer^[3-6]. Therefore, it is urgent to develop effective and safe treatments for BPH.

The development of BPH depends on functional androgen signaling. Testosterone is the biosynthetic precursor of dihydrotestosterone (DHT)^[7]. DHT is synthesized from testosterone by 5 α -reductase, a highly lipophilic enzyme found on intracellular membranes^[8]. The binding of DHT to the androgen receptor (AR) results in a cascade of events necessary for the formation of signaling factors that regulate cellular growth^[9]. In the adult prostate, DHT and other androgens are hypothesized to contribute to the maintenance of homeostasis between the processes of cell proliferation and cell death^[9]. Cell proliferation and apoptosis in prostate cells are regulated by androgen-dependent mechanisms affected by intermediaries induced by the binding of DHT to AR^[9-10]. DHT may stimulate the production and secretion of growth factors, such as epidermal growth factor (EGF), keratinocyte growth factor, insulin-like growth factor, and fibroblast growth factor (FGF), all of which modulate cellular proliferation^[9]. Likewise, DHT affects the activity of transforming growth factor-beta, which modulates apoptosis^[9,11].

Consequently, 5 α -reductase inhibition, and thus the prevention of the conversion of testosterone to DHT, might be a crucial target for the development of drugs to prevent or treat BPH^[12]. Various pharmacological inhibitors of 5 α -reductase, such as finasteride or dutasteride, have been used to treat patients with BPH^[5-6]. However, these treatments are known to have fatal side effects^[5-6]. Among the treatment strategies for BPH inhibition, studies of natural products, such as pumpkin seed diets, *Serenoa repens*, and *Pygeum africanum*, are actively underway^[13-15], but more effective novel therapies with inexpensive and minimal side effects are needed for these patients^[3-6].

Mammalian cells proliferate through the cell cycle. Therefore, cell cycle arrest is a critical step for the suppression of prostate cell proliferation^[16]. The cell cycle regulates division and replication and is divided into G₀/G₁, S, and G₂/M phases. G₀ is a resting phase, G₁ is the replication preparation phase before DNA replication, the S-phase is the DNA synthesis phase, G₂ is the preparation phase for mitosis after DNA replication, and the M-phase is the mitosis phase^[17]. The cell cycle in eukaryotic cells begins with the onset of the G₁ phase^[17]. The proliferation of animal cells activates the expression of cyclin proteins and their binding to cyclin-dependent kinases (CDKs). Progression of the cell cycle occurs due to the systemic changes in the cell cycle regulators, such as CDKs, cyclins, and CDK inhibitors (CDKIs), during the G₁- to S-phase transition^[18-19].

The phosphatidylinositol-3-kinase (PI3K)/ α -serine/threonine-protein kinase (AKT) signaling pathway is important for many aspects of cell growth and survival under both physiological and pathological conditions^[19]. Regarding cell survival, AKT can inactivate transcription factors that induce the expression of pro-Apoptotic factors, such as Bcl-2-associated death promoter, pro-Caspase-9, and other pro-apoptotic factors, such as Fas-ligand^[19-20]. AKT activity is associated with increased resistance of cells to apoptosis mediated by apoptosis-inducing ligand 2 (APO-2L), also known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)^[20-21]. Finally, AKT also activates the inhibitor of NF- κ B (I κ B) kinase (IKK), which is a positive regulator of survival NF- κ B^[19].

Mitogen-activated protein kinase (MAPK) is α -serine/threonine kinase that phosphorylates specific substrates at serine and/or

threonine residues upon stimulation. The MAPK signaling pathway regulates gene expression, mitosis, proliferation, and apoptosis under multiple stressful circumstances^[22]. MAPKs consist of three family members, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38^[23]. The ERK protein plays a central role in the stimulation of cell proliferation. Two isoforms of ERK (ERK1 and ERK2) are ubiquitous and represent convergence points for mitotic signals from diverse receptor arrays^[24]. The p38 family is involved in apoptosis activation in some cell types^[24]. JNK proteins are involved in positive and negative cell cycle regulatory pathways in response to various extracellular stimuli^[25].

Antarctic krill (*Euphausia superba*) is a type of crustacean found in the polar sea of Antarctica, and its biomass has been estimated at 379 million tons^[26]. Krill is a sustainable source of omega-3 polyunsaturated fatty acids (*n*-3 PUFAs), which contain eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), 2 particularly long-chain PUFAs^[27]. Krill oil (KO) consists of fatty acids bound to phospholipids, mainly phosphatidylcholine^[27]. KO is a source of various active biomolecules, such as astaxanthin, flavonoids, vitamin A, and α -linolenic acid^[28]. In addition, KO has better bioavailability compared to fish oil^[29]. KO has attracted attention as a notable nutritional resource due to its abundant biomass and nutrients, good bioavailability, and relatively clean environment source from the polar region. KO has been found to inhibit NF- κ B activity, EGF receptor (EGFR), and cyclin D1 in macrophage cells and colorectal cancer cells^[30-32]. BPH is affected by hyperlipidemia, oxidation, and inflammation^[33-35]. Moreover, KO displays many physiological activities, including anti-hyperlipidemia, anti-inflammatory, antioxidant, and anticancer effects^[31-32,36-37].

In our previous study, the KO extracted by enzymatic hydrolysis (Alcalase) exhibited a chemical composition profile that included long-chain PUFAs and showed potent antitumor efficacy against bladder cancer without adverse toxic effects *in vitro* and *in vivo*^[38]. However, the suppressive effect of KO on BPH has not yet been studied. Therefore, in the present study, we investigated the molecular effects of KO on BPH *in vitro* and *in vivo*.

2. Materials and methods

2.1 Materials

Antibodies against phospho-ERK (9101S), ERK (9102S), phospho-p38 MAPK (9211S), p38 MAPK (9212S), phospho-JNK (9251S), JNK (9258S), phospho-AKT (9271S), AKT (9272S), and p21WAF1 (2947S) were purchased from Cell Signaling (Danvers, MA, USA). Antibodies against AR (sc-7305), FGF-2 (sc-1360), EGF (sc-166779), CDK2 (sc-163), cyclin E (sc-377100), CDK4 (sc-23896), cyclin D1 (sc-8396), p53 (sc-1641), p27KIP1 (sc-1641), β -actin (sc-47778), Bcl-2 (sc-7382), and Bax (sc-20067) were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against SRD5A1 (26001-1AP) and Ki-67 (14-5698-82) were bought from Invitrogen (MA, USA).

2.2 Extraction of Antarctic KO

KO prepared by Alcalase treatment was supplied by SD Biotechnologies Co., Ltd. (Seoul, Korea). The exact method for KO

preparation is described in our previous study^[38]. Briefly, krill washed with tap water was pulverized and hydrolyzed with Alcalase (from *Bacillus licheniformis*) until liquefied. The enzyme was inactivated by heating, and then the sludge, including the shell and head of krill, was removed from the enzyme hydrolysates by decanter centrifugation. After the lipids and phospholipids were extracted from the enzyme hydrolysates by repeated centrifugation, they were purified by pressure filtration. After sterilization, the filtrates were placed on a rotary vacuum evaporator to reduce the water content below 2%. The filtrates were finally filtered through a 50-mesh sieve to obtain the KO product composed of 46.6% phospholipid, 130.72 mg/g EPA, 77.99 mg/g DHA, and 0.04 mg/g astaxanthin. Ethanol was used as a solvent to dissolve KO. The filtrates were finally filtered through a 50-mesh sieve to obtain the KO product composed of 46.6% phospholipid, 130.72 mg/g EPA, 77.99 mg/g DHA, and 0.04 mg/g astaxanthin. The contents of the phospholipids, omega-3 fatty acids (EPA and DHA), and astaxanthin in KO were determined using MNR microscopy, GC-FID analysis, and HPLC-UV, respectively, according to the methods described in Supplementary Materials and Methods. The original chromatograms and spectrum were provided in Figs. S1–3. For the following experiments, KO was first dissolved with absolute ethanol to prepare a stock solution of 100 mg/mL concentration and then diluted with medium to the concentrations of 100–200 µg/mL. Importantly, the final concentration of the ethanol was set at 0.2% (V/V) in all groups to exclude its possible cytotoxic effect. A 0.2% ethanol without KO was treated in the control cells and used as the control group.

2.3 Cell cultures

Human normal prostate stromal cells (WPMY-1) were purchased from the American Type Culture Collection (Baltimore, MD, USA). WPMY-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM). The medium was supplemented with 1% penicillin-streptomycin (Corning) and 5% fetal bovine serum (FBS). Cells were incubated in an incubator at 37 °C with an atmosphere of 5% CO₂ in air. Human benign hyperplasia cells (BPH-1) were purchased from Sigma-Aldrich (Burlington, MA, USA). BPH-1 cells were maintained in RPMI 1640 medium with L-glutamine. The medium was supplemented with 1% penicillin-streptomycin (Corning) and 5% FBS. Cells were incubated in an incubator at 37 °C with an atmosphere of 5% CO₂ in air.

2.4 Cell viability assay

Cell viability was assessed using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. Cells were seeded in 96-well plates (3×10^4 cells/well) and then incubated with KO (0, 100, 150, and 200 µg/mL) for 24 h. After incubation, the medium was aspirated, and a new medium containing 10 µL of 5 mg/mL MTT was added. After 4 h, the medium was aspirated, and dimethyl sulfoxide (DMSO) was added. Absorbance was measured at 540 nm using a fluorescent plate reader (Fisher Scientific, Inc., Rockford, IL, USA). The results were expressed as percentage cell viability compared with the control group.

2.5 Cell counting

WPMY-1 and BPH-1 cells were independently seeded at 2×10^5 cells/well in 6-well plates. The cells were treated with KO (0, 100, 150, and 200 µg/mL) for 24 h. A hemacytometer and microscope (Optika, Ponteranica, Italy) were used to count the cells.

2.6 Flow cytometric analysis

For cell cycle analysis, cells were collected and fixed in 70% ethanol at –20 °C for 4 h. Cells were washed with ice-cold PBS and the cell cycle kit (MCH100106, Luminex, Austin, TX, USA) containing DNA-intercalating dye was added. The cells were kept in a dark room for 2 h. The cell cycle stage was investigated using a Muse™ Cell Analyzer (EMD Millipore, CA, USA).

2.7 Immunoblot assay

The cells were washed using ice-cold PBS. The cell lysates were then prepared by incubating with lysis buffer on ice for 10 min and vigorously mixed by vortex 3 times. Then, the cell lysates were centrifuged at $23\,545 \times g$ at 4 °C for 10 min. The protein contents in the supernatant were normalized using a BCA method (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of cellular proteins were loaded onto 10% and 12% polyacrylamide gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% SDS under denaturing conditions. The proteins were transferred from the gel to nitrocellulose membranes (Hybond, Amersham Corp.). After blocking with TBS-T containing 5% bovine serum albumin (BSA; MP Biomedicals, OH, USA) or 5% skim milk, the membranes were incubated with primary antibodies for 24 h, followed by peroxidase-conjugated secondary antibodies for 120 min. The enhanced chemiluminescence (ECL) immunoblotting detection reagent (SuperSignal, Thermo Fisher Scientific) was used for visualization of the protein blots according to the manufacturer's recommendation.

2.8 Electrophoretic mobility shift assay (EMSA)

WPMY-1 and BPH-1 cells were incubated in the solution A buffer and were maintained on ice for 15 min. The cell lysates were collected using a cell scraper and transferred to a fresh tube. The cell lysates were vortexed gently in 0.5% Nonidet P-40 and then centrifuged at $23\,545 \times g$ for 2 min. The nuclear pellet was re-suspended in a buffer and maintained for 15 min at 4 °C. After adding solution B buffer to the samples, followed by incubation at 4 °C for 1 h, the samples were centrifuged at $23\,545 \times g$ for 5 min. Protein contents of the nuclear extracts were determined using the BCA assay. The probe of consensus oligonucleotide sequences for NF-κB was manufactured (AGTTGAGGGGACTTTCCCAGGC). The NF-κB oligonucleotide was end-labeled by incubation with T4 polynucleotide kinase (Promega, WI, USA) and [γ -³²P ATP] at 37 °C for 1 h. The nuclear extract (8 µg) was incubated in a 5× binding buffer with the ³²P-labeled oligonucleotide probe for 20 min at room temperature. The DNA-protein complexes were electrophoresed on a native 6% polyacrylamide gel using 0.5% TBE running buffer. Then,

the gel was washed, dried, and visualized with autoradiography using an X-ray film at -70°C overnight.

2.9 Transfection of small interfering RNA (siRNA)

Small interfering RNA (siRNA) for p38 (Cat # 6564), JNK (Cat # 6232), and scramble (Cat # 6568) were purchased from Cell Signaling Technology (Danvers, MA, USA). For transfection of siRNA, prostate cells were cultured into 6 well plates, and then transfected with scrambled siRNA (si-control), p38 siRNA (si-p38), and JNK siRNA (si-JNK) at 20% confluency using the Lipofectamine 2000 transfection reagent following the instruction provided by manufacturer (Invitrogen, MA, USA). After incubating the cells with KO for 24 h, subsequent experiments were performed via MTT assay, cell counting assay, immunoblot, and EMSA assay.

2.10 Generation of BPH rat model

All animal studies were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (approval number: 202000129). Six-week-old male SD rats (DBL, Chungbuk, Korea) were acclimated for 1 week in an animal room. To exclude the influence of intrinsic testosterone, the rats were castrated after anesthesia by inhalational anesthesia of enflurane (USP). BPH was induced by intraperitoneal injections of 6 mg/kg of testosterone enanthate (TE, mibe GmbH, Arzneimittel, Germany) for 28 days every week. Finasteride (ALAC, LLC, USA) was used as a positive drug in the BPH experiment. The rats were weighed weekly during the experiments.

2.11 Experiment of KO against BPH rat model

The rats were randomly divided into five groups ($n = 5$ per group). During the development of prostate hyperplasia, rats were orally injected with 50 or 100 mg/kg body weight (BW) of KO daily for 4 weeks: KO groups (testosterone + KO 50 mg/kg, testosterone + KO 100 mg/kg). BW was measured once per day. After 4 weeks, blood samples were obtained and kept at room temperature for 2 h. The sera were collected from blood samples by centrifugation ($3\,000 \times g$, 4°C , 40 min). Each of the bilateral ventral lobes of the prostates was immediately recovered and weighed. Relative prostate weight (PW) was calculated as the ratio of PW (mg) to BW (100 g) to obtain the PW/BW index. The prostate tissue of each rat was split in half; one half was fixed in 10% formalin for histological assays; the remaining half was stored at -80°C for immunoblot assays. Tween 80 (0.5%) was used as the vehicle for KO. Water was used as the vehicle for finasteride.

2.12 Enzyme-linked immunosorbent assay (ELISA)

Sera from BPH-induced rats were obtained. DHT was measured using an ELISA kit (MyBioSource, San Diego, CA, USA). Plates were washed, biotinylated antibody solution was added, and the plates were incubated at 37°C for 60 min. After washing the plates 3 times, the enzyme working solution was added and incubated at 37°C for 30 min. Then, the plates were washed 5 times and incubated in the color reagent solution at 37°C for 30 min. Finally, color reagent C

was added. The optical density was measured using a microplate reader within 10 min after adding color reagent C.

2.13 Histopathology

Prostate tissues from BPH-induced rats were obtained and fixed with a 10% formalin solution. Tissues were then dehydrated using ethanol, sectioned sequentially, and embedded into a paraffin block. The paraffin-embedded slide was stained with hematoxylin and eosin (H&E), and immunohistochemistry was performed to measure the expression of Ki-67. The tissue slides were observed using a microscope.

2.14 Statistical analysis

Where appropriate, data were expressed as mean $\pm$ standard deviation (SD) and analyzed by Student's *t*-test, ANOVA, and Tukey's least significant difference test. Statistical significance was set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3. Results

3.1 Inhibition effect of KO on the proliferation of WPMY-1 and BPH-1 cells

To investigate the suppression effect of KO on the proliferation of WPMY-1 and BPH-1 cells, the cells were treated with KO (0, 100, 150, and 200 $\mu\text{g/mL}$) for 24 h. Cell proliferation was investigated by the MTT assay and cell counting. At the highest concentration of KO examined, the viability of WPMY-1 and BPH-1 cells was decreased to 47% and 58%, as assessed by the MTT assay (Fig. 1A). Similar patterns were observed in the cell counting assay result (Fig. 1B). In both the MTT and the cell counting assay, cell proliferation was significantly reduced when the cells were treated with KO.

3.2 Arrested cell cycle of G_0/G_1 phase in WPMY-1 and BPH-1 cells

To investigate the effect of KO on cell cycle distribution, WPMY-1 and BPH-1 cells were treated with KO for 24 h, followed by cell cycle analysis. The results showed that KO treatment (0, 100, 150, and 200 $\mu\text{g/mL}$) significantly increased the percentage of G_1 phase cells (47.4%, 47.7%, 50.0%, and 57.6%) with a reduction in the percentage of G_2/M phase cells (31.6%, 32.4%, 31.2%, and 24.8%) in WPMY-1 cells (Figs. 2A and C). KO-treated BPH-1 cells (at 0, 100, 150, and 200 $\mu\text{g/mL}$ of KO) revealed cell population accumulation at G_0/G_1 phase (48.9%, 51.1%, 53.6%, and 59.5%) and a decline in the cell population at G_2/M phase (35.8%, 34.3%, 32.6%, and 27.5%) (Figs. 2B and D). The percentage of S-phase cells was not affected by KO treatment in both cell types (Figs. 2A-D). These results suggested that KO stimulated cell cycle arrest at G_0/G_1 in both WPMY-1 and BPH-1 cells.

3.3 KO regulated expression of cell cycle-related protein markers in WPMY-1 and BPH-1 cells

To determine the mechanism by which KO regulates the cell cycle in WPMY-1 and BPH-1 cell lines, we examined the expression

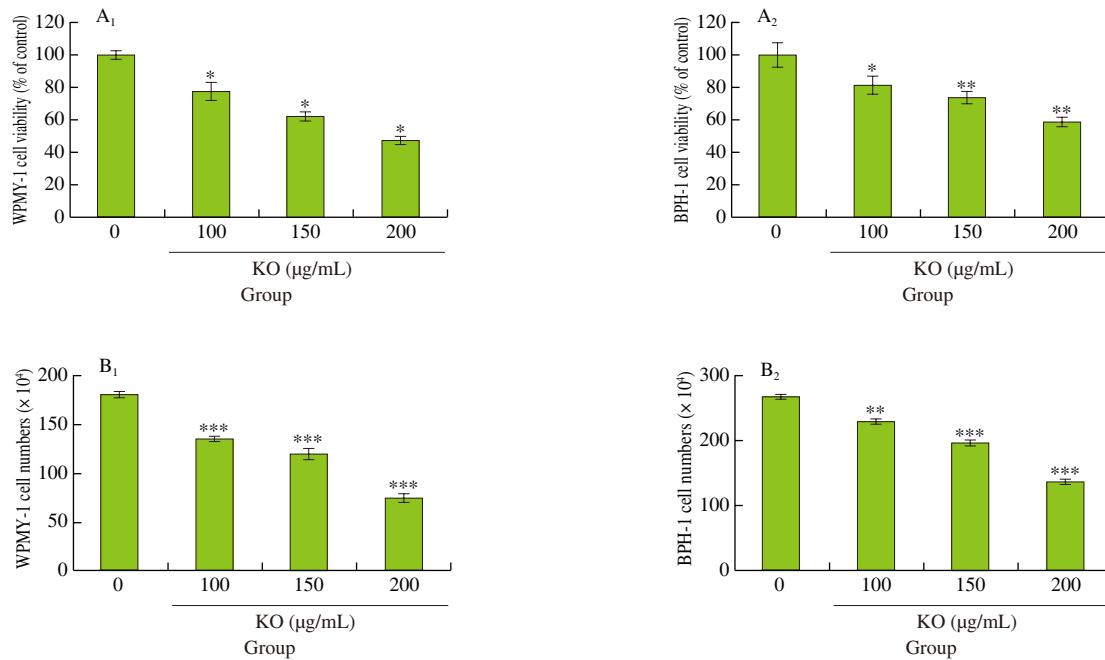


Fig. 1 Effect of KO on the proliferation of human prostate cells WPMY-1 and BPH-1. WPMY-1 and BPH-1 cells were treated with various concentrations (0, 100, 150, and 200 µg/mL) of KO for 24 h. (A) Cell viability was determined by the MTT assay. (B) The cell number was counted using a hemacytometer and microscope. Results represent mean $\pm$ SD of 3 independent experiments (* P < 0.05, ** P < 0.01, and *** P < 0.001).

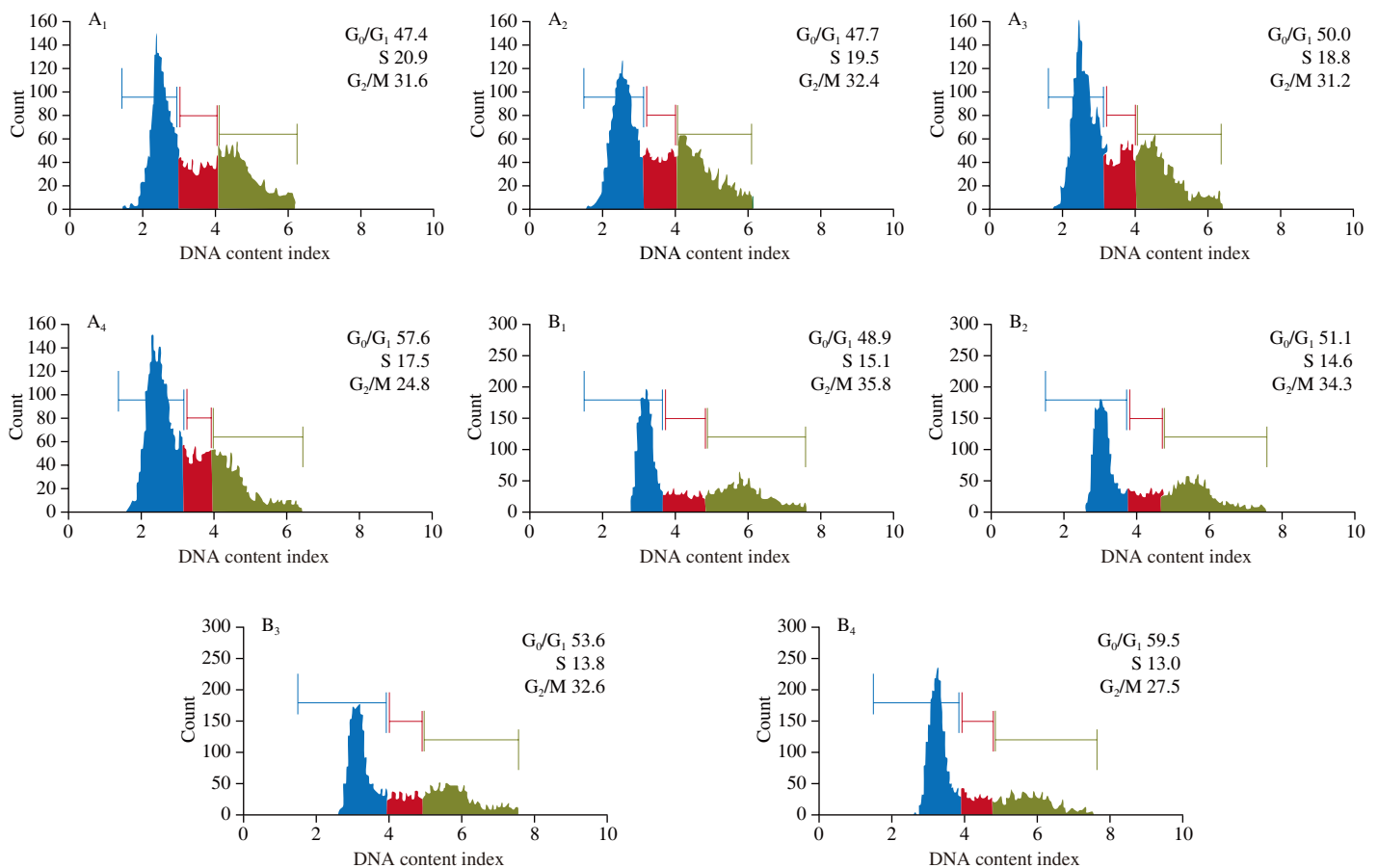


Fig. 2 KO induced cell cycle arrest at G₀/G₁ phase in WPMY-1 and BPH-1 cells. WPMY-1 and BPH-1 cells were treated with various concentrations (0, 100, 150, and 200 µg/mL) of KO for 24 h. Flow cytometry was performed to determine the cell cycle phase of (A) WPMY-1 cells and (B) BPH-1 cells. Cell population in G₀/G₁, S, and G₂/M phase of (C) WPMY-1 and (D) BPH-1. Results represent mean $\pm$ SD of 3 independent experiments (* P < 0.05, ** P < 0.01, and *** P < 0.001).

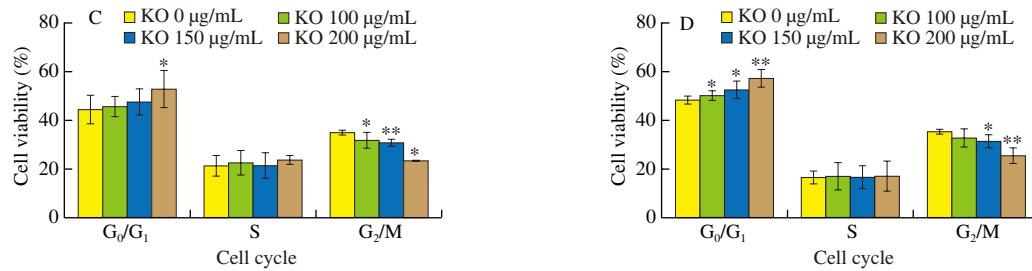


Fig. 2 (Continued)

of G₀/G₁ phase-related protein markers, such as CDKs, cyclins, p21, p27, and p53, using immunoblot analysis. In KO-treated WPMY-1 cells, the expression levels of CDK2, CDK4, cyclin D1, and cyclin E were decreased, and the level of p21 expression was increased without alteration of p27 and p53 levels (Figs. 3A and B). Contrary to WPMY-1 cells, in KO-treated BPH-1 cells, the expression levels of CDK2 and cyclin E decreased without reduction of CDK4 and cyclin D1 expression levels (Fig. 3A). In addition, KO treatment induced the expression levels of p21 and p27 in BPH-1 cells (Fig. 3B). However, the p53 level was not changed in the presence of KO (Fig. 3B). Our results indicated that KO treatment induced cell cycle arrest at G₀/G₁ phase through regulating the CDKs and cyclins by induction of p21 and p27 in prostate cells.

3.4 KO upregulated phosphorylation of JNK and p38 signaling in WPMY-1 and BPH-1 cells

The MAPKs and AKT signaling play an important role in cell growth, cell death, and cell division. Therefore, we investigated the phosphorylation level of MAPKs and AKT signaling in KO-treated prostate cells. KO treatment increased the phosphorylation levels of JNK and p38 in WPMY-1 cells (Fig. 4A), but the ERK1/2 and AKT phosphorylation levels remained constant (Figs. 4A and B). Similar results were obtained from KO-treated BPH-1 cells (Figs. 4A and B). These results demonstrated that KO reduced the proliferation of prostate cells via stimulation of phosphorylation of JNK and p38.

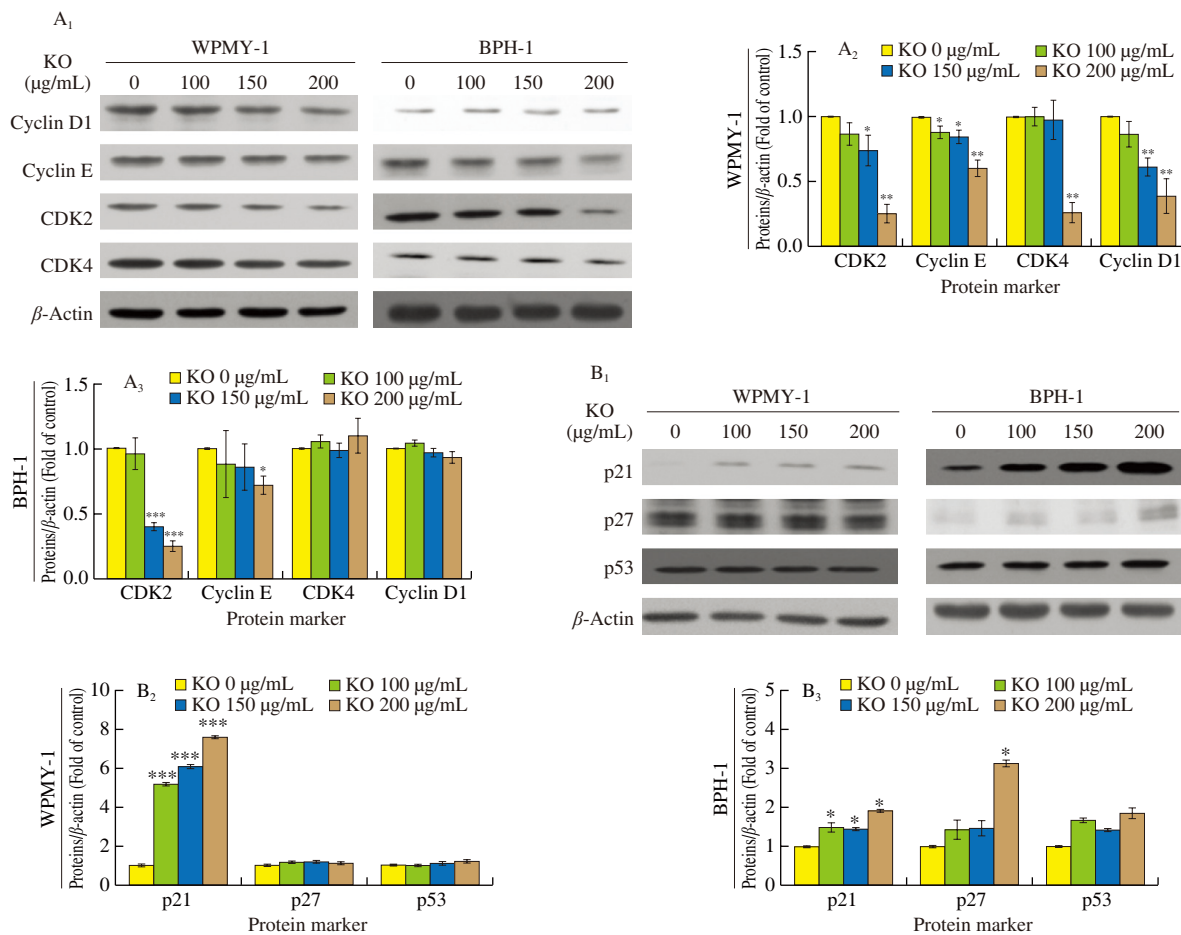


Fig. 3 Expression level of G₀/G₁ cell cycle regulatory protein markers in WPMY and BPH-1 cells treated with KO. WPMY-1 and BPH-1 cells were treated with various concentrations of KO (0, 100, 150, and 200 µg/mL) for 24 h. (A) Cyclin D1, Cyclin E, CDK2, CDK4; (B) p21, p27, p53. Immunoblot assay was performed to determine the changes in G₀/G₁ cell cycle-associated protein markers. β-Actin was used as a housekeeping protein. Results represent mean ± SD of three independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001).

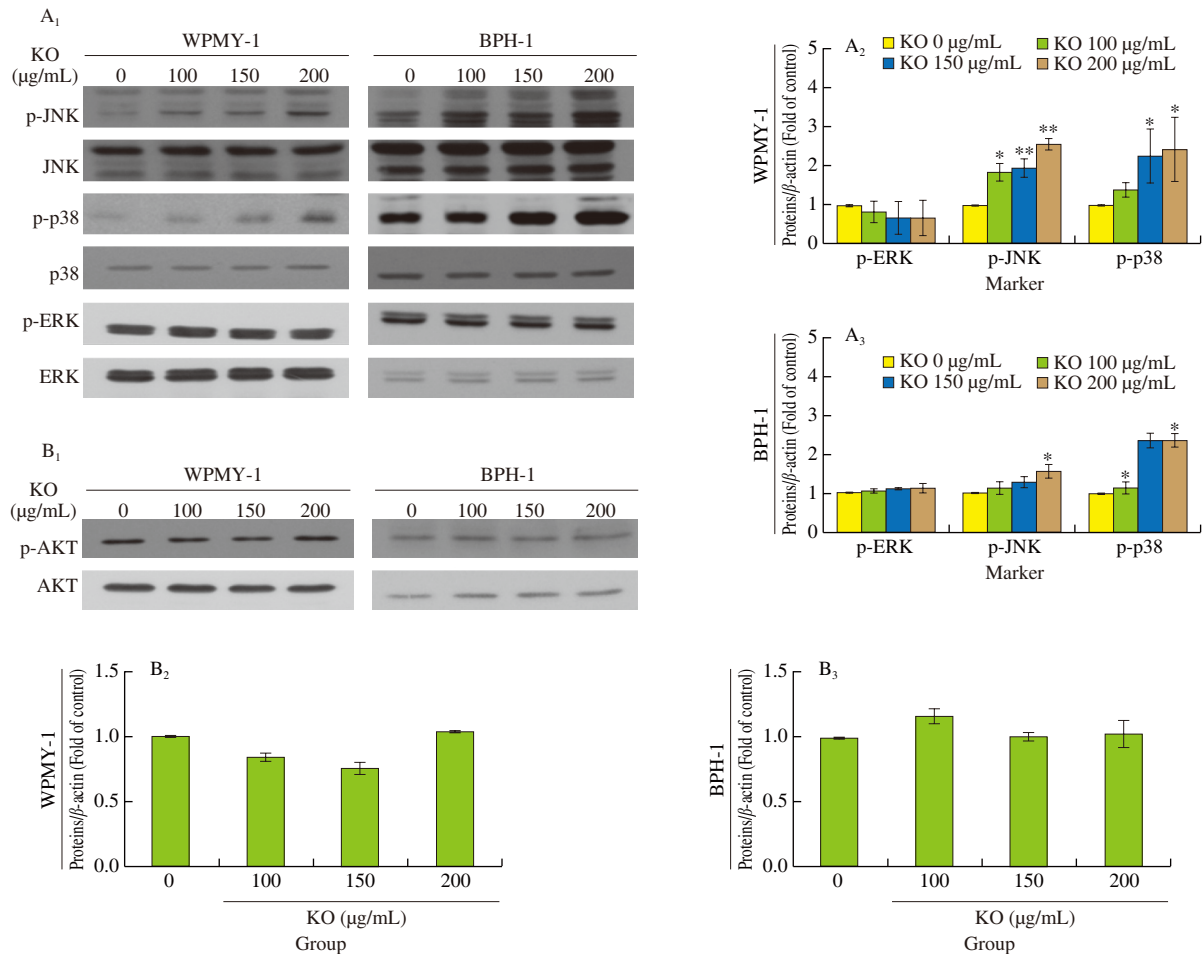


Fig. 4 KO induced p38 and JNK phosphorylation in WPMY-1 and BPH-1 cells. WPMY-1 and BPH-1 cells were treated with various concentrations of KO (0, 100, 150, and 200 µg/mL) for 24 h. Immunoblot assay was performed to determine phosphorylation level of (A) ERK1/2, JNK, p38, and (B) AKT. Results represent mean $\pm$ SD of 3 independent experiments (* P < 0.05, ** P < 0.01, and *** P < 0.001).

3.5 KO inhibited binding activity of NF- κ B in WPMY-1 and BPH-1 cells

Stimulation of NF- κ B activation is involved in the regulation of proliferation, differentiation, and signaling pathway in mammalian cells^[39–40]. Therefore, we investigated whether KO regulates the binding activity of NF- κ B in WPMY-1 and BPH-1 cells using EMSA (Fig. 5). Both cell types were treated with KO for 24 h, and the nuclear extract was obtained from the cell lysates. The EMSA result indicated that KO blocked the binding activity of the NF- κ B motif in WPMY-1 and BPH-1 cells. These results demonstrated that reduced transcriptional activation of NF- κ B is attributed, in part, to the KO-induced inhibition of prostate cell proliferation.

3.6 Knockdown of p38 and JNK abolished the KO-mediated inhibition of prostate cell proliferation

To determine whether p38 and JNK signaling is involved in the KO-induced decrease in proliferation of prostate cells, we employed siRNA-specific p38 (si-p38) and JNK (si-JNK) (Fig. 6A). Knockdown of p38 and JNK prevented the decreased proliferation of prostate cells in the presence of KO (Fig. 6B). Decreased expression of CDK2, CDK4, cyclin D1, and cyclin E by KO was blocked in the WPMY

cells transfected with si-p38 (Fig. S4A). The siRNA inhibition of p38 attenuated upregulation of p21WAF1 expression in KO-treated WPMY cells (Fig. S4A). In addition, transfection of BPH-1 cells with si-p38 attenuated a KO-induced decrease in the expression level of CDK2 and cyclin E (Fig. S4A). The increased expression of p21WAF1 and p27KIP1 in KO-treated BPH-1 cells was prevented by si-p38 transfection (Fig. S4A). Furthermore, KO-mediated suppression of NF- κ B binding activity was abolished in both prostate cells transfected with si-p38 (Fig. S5A). Similar results were detected in the si-JNK transfected prostate cells (Figs. S4B and S5B). These results demonstrated that p38 and JNK signaling might be contributed to the regulation of KO-mediated proliferation of prostate cells.

3.7 KO regulated expression level of BPH-associated protein markers in WPMY-1 and BPH-1 cells

To investigate the expression levels of BPH-associated protein markers, such as 5 α -reductase, AR, FGF, Bcl-2, and Bax, prostate cells were treated with KO for 24 h. Immunoblot results showed that KO treatment reduced the expression levels of 5 α -reductase, AR, EGF, and FGF-2 proteins in WPMY-1 and BPH-1 cells (Fig. 7A). In addition, the anti-apoptotic protein marker Bcl-2 level was decreased

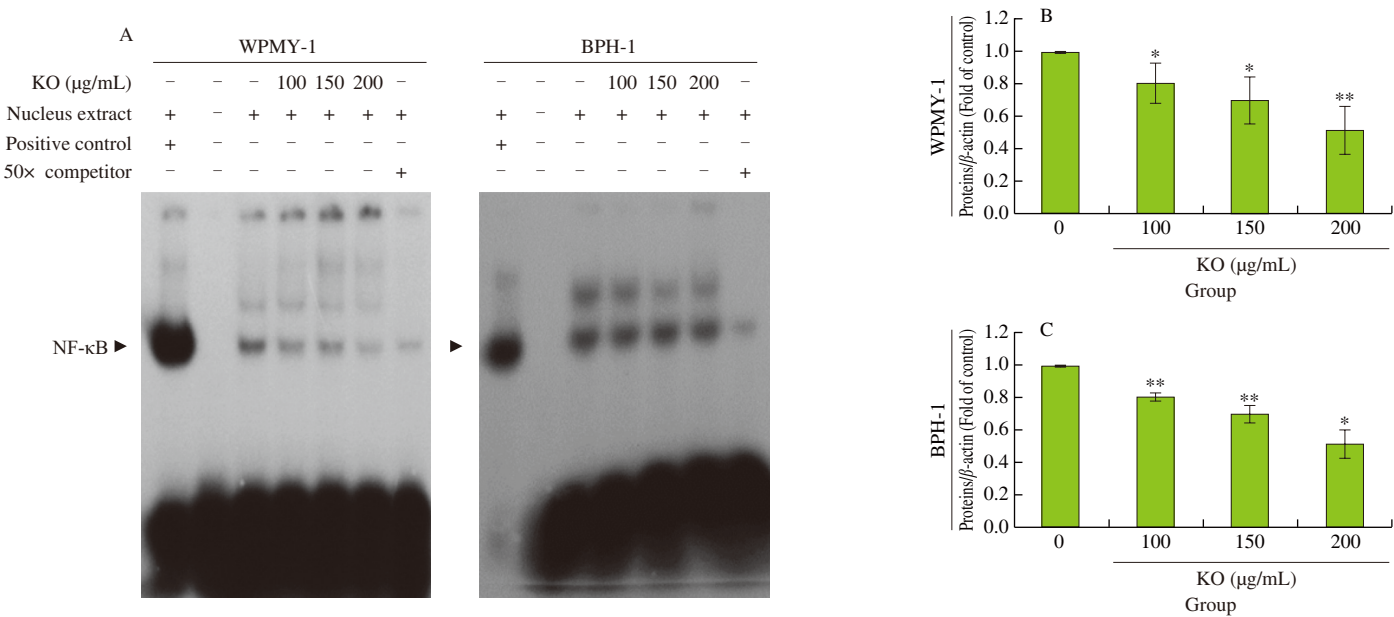


Fig. 5 Inhibition of NF-κB binding activity in WPMY and BPH-1 cells treated with KO. WPMY-1 and BPH-1 cells were treated with various concentrations of KO (0, 100, 150, and 200 μg/mL) for 24 h. After isolation of the nuclear extract, EMSA was performed to determine the change in binding activity of NF-κB in KO-treated prostate cells. Results represent mean ± SD of 3 independent experiments (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

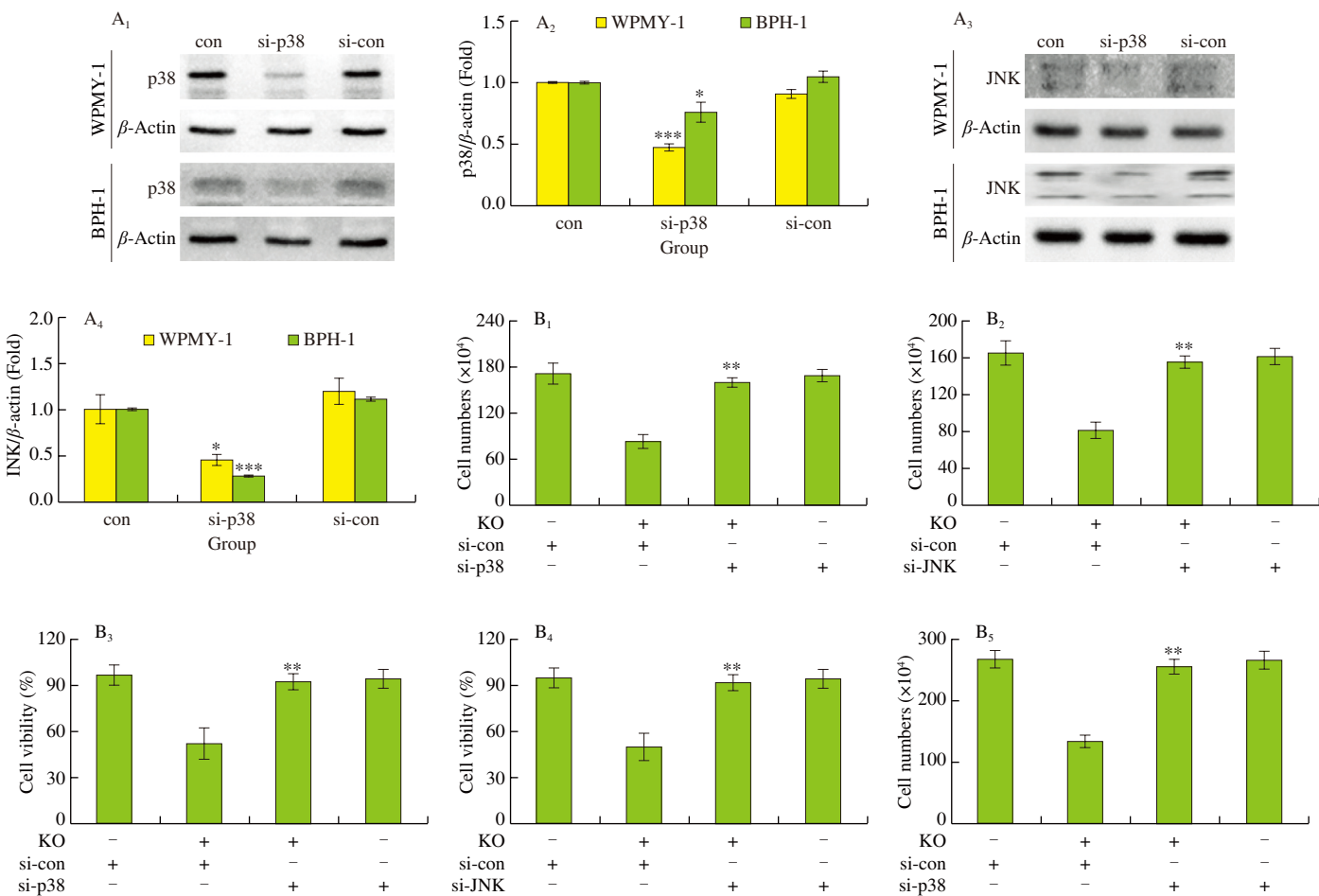


Fig. 6 Knockdown of p38 and JNK by siRNA suppressed KO-mediated growth inhibition of prostate cells. Prostate cells were transfected with si-con, si-p38 or si-JNK, then followed by KO (200 μg/mL) treatment for 24 h. (A) The efficacy of si-p38 and si-JNK was validated by immunoblot. β-Actin was used as an internal control protein. Results from three independent experiments are represented as means ± SD. (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with si-con). (B) MTT and cell counting assay was performed after transfection of si-p38 and si-JNK in KO-treated prostate cells; (B₁-B₄) WPMY-1, (B₅-B₈) BPH-1. Results represent mean ± SD of 3 independent experiments (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with si-con + KO treatment).

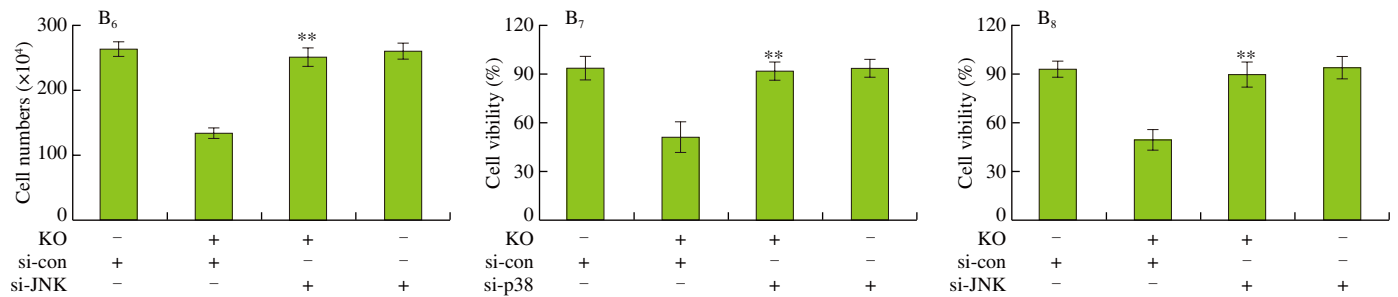


Fig. 6 (Continued)

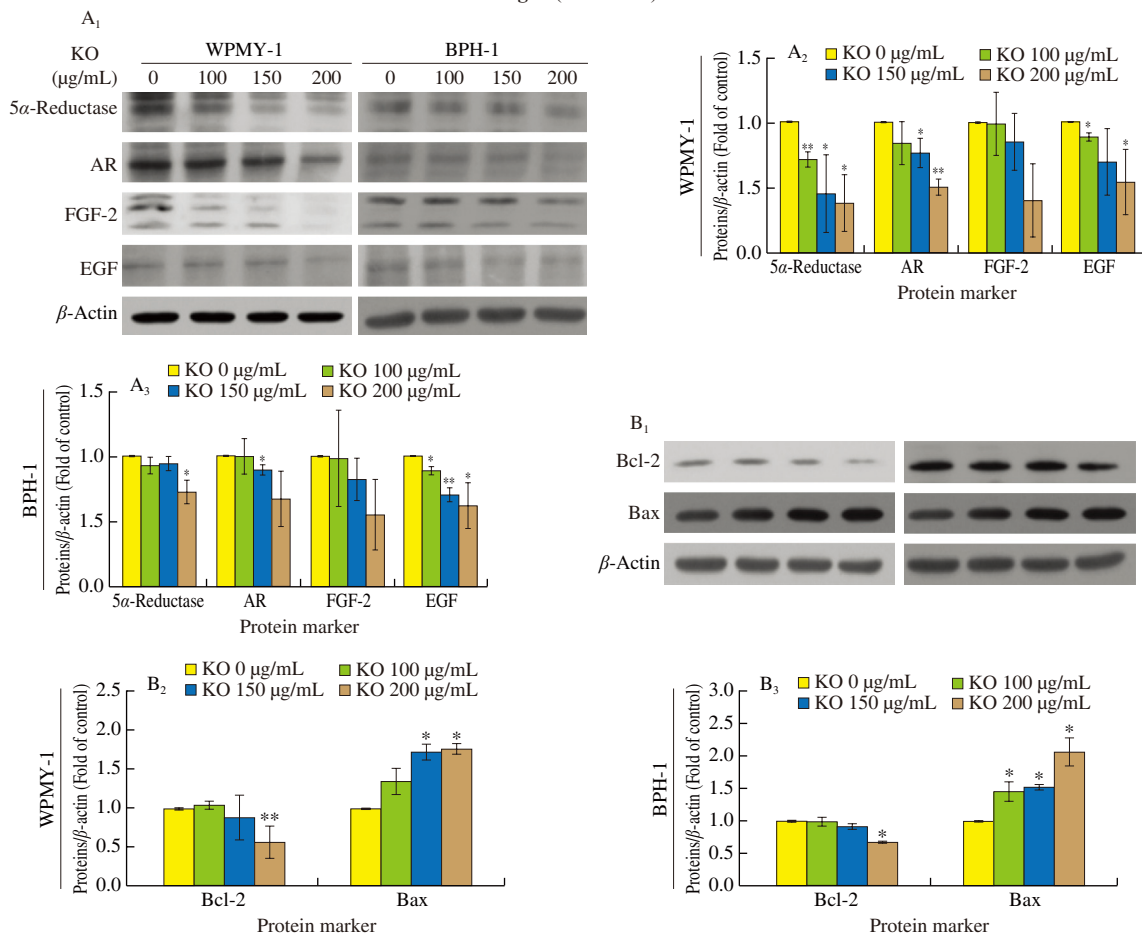


Fig. 7 Expression levels of BPH-associated protein markers in WPMY and BPH-1 cells treated with KO. WPMY-1 and BPH-1 cells were treated with various concentrations of KO (0, 100, 150, and 200 μ g/mL) for 24 h. Immunoblot assay shows the level of BPH-associated markers; β -actin was used as a housekeeping protein. Results represent mean $\pm$ SD of 3 independent experiments (* P < 0.05, ** P < 0.01, and *** P < 0.001).

in both cell types treated with KO (Fig. 7B). The expression level of Bax, an apoptotic protein marker, was increased in the presence of KO (Fig. 7B). These results showed that KO-induced inhibition of prostate cell proliferation might be involved in the changed expression levels of prostatic hypertrophy markers.

3.8 KO impeded BPH in TE-induced rat model

To investigate the effect of KO on the reduction of BPH *in vivo*, a TE-stimulated rat model was obtained. Finasteride was used as a positive control (Figs. 8A, D, and E). No significant change in BW was observed between the control and KO-treated groups (Fig. 8A). The prostate size of KO-administered rats was reduced compared with the control group (Fig. 8B). The PW/BW index in the KO-treated

groups was notably decreased compared to that in the control group (Fig. 8C). In addition, the amount of DHT in the serum of each group was determined through an ELISA assay. The amount of DHT was expressed relative to the total testosterone. The DHT index was lower in the treated groups than in the control group in a dose-dependent manner (Fig. 9A). Furthermore, the highest decrease in DHT level was observed in the finasteride group (Fig. 9B). Subsequently, histological analysis of prostate tissues was performed through H&E and Ki-67 staining (Fig. 9C). In comparison with the control group, TE-induced thickness of the epithelial tissue and muscle layer was significantly alleviated in the KO-treated groups (Fig. 9C). The histological effect of KO on the prostatic change of the TE-treated group was similar to the finasteride efficacy (Fig. 9C). These results demonstrate that KO attenuated TE-induced prostatic hyperplasia *in vivo*.

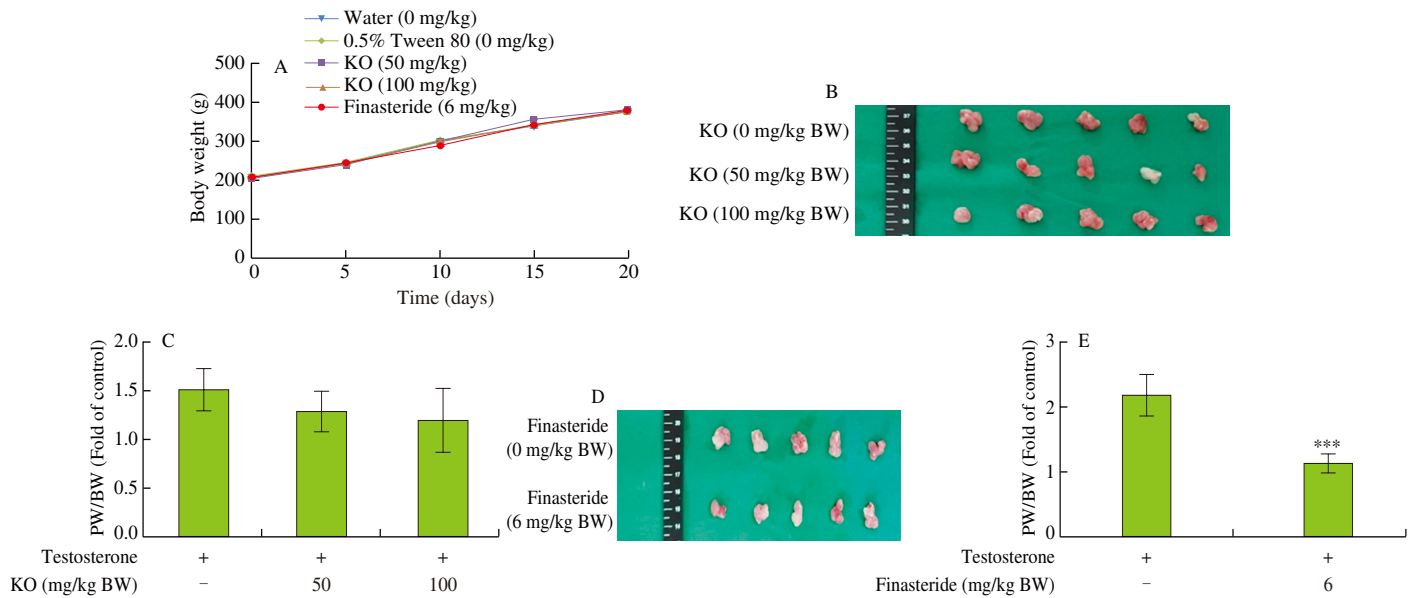


Fig. 8 Effect of KO in a TE-induced BPH rat model. (A) Changes in BW in TE-induced rat model during the experimental period. (B-C) Prostate tissue images and PW/BW index in control group (0.5% Tween 80) and KO-treated group. (D-E) Prostate tissue images and PW/BW index for control group (H₂O) and finasteride-injected group. Results represent mean $\pm$ SD of 3 independent experiments (** P < 0.001).

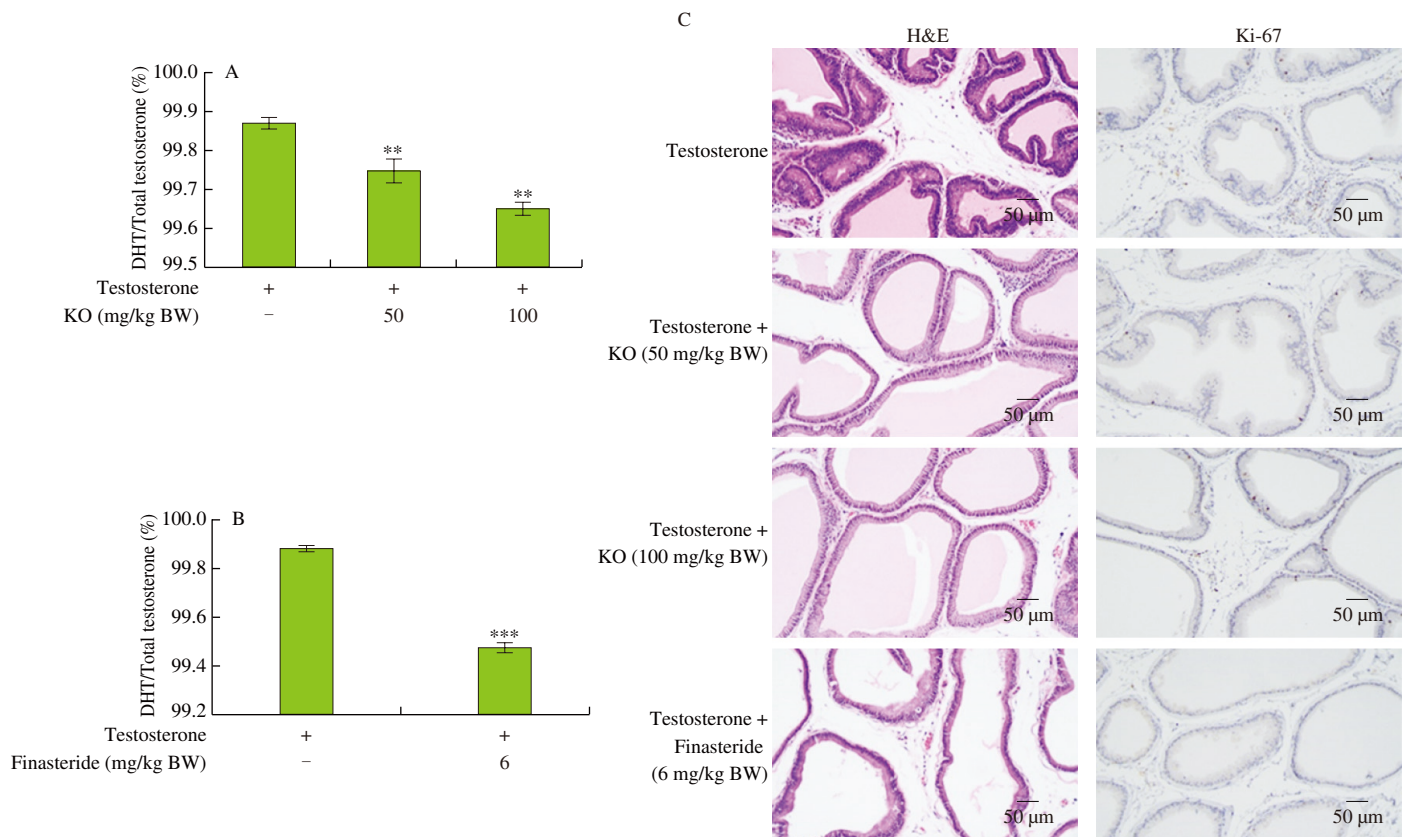


Fig. 9 Effect of KO on BPH in TE-induced BPH rat model. (A) DHT levels between control group and KO-treated groups were determined in serum using the ELISA kit. (B) DHT levels between control group and finasteride-injected group were analyzed in serum using the ELISA kit. (C) Prostate tissues were fixed and stained with H&E and IHC for Ki-67 and then visualized using a fluorescence microscope. Results represent mean $\pm$ SD of 3 independent experiments (** P < 0.01 and *** P < 0.001).

3.9 KO diminished the prostatic hyperplasia via altered levels of BPH-associated protein markers in a TE-induced BPH rat model

The expression levels of BPH-associated protein markers in prostate tissues were investigated using immunoblot analysis. The

expression levels of 5 α -reductase, AR, EGF, and FGF-2 proteins in KO-treated tissues were decreased compared with tissues from the control group (Fig. 10A). In addition, the Bcl-2 level was decreased, and that of Bax was increased in KO-treated prostate tissues (Fig. 10B). Overall, the *in vitro* experimental results compared favorably with the *in vivo* system.

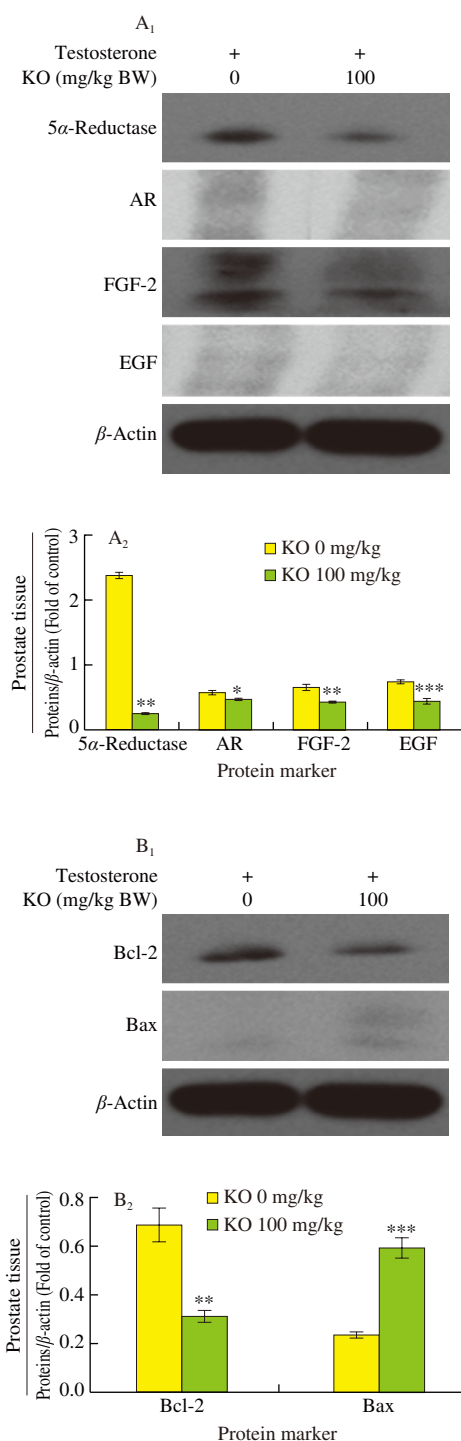


Fig. 10 Expression level of BPH-associated protein markers in prostate tissues from KO-treated BPH rat model. Immunoblot assay was performed to determine the expression levels of 5α-reductase, AR, FGF, Bcl-2, and Bax in prostatic tissues. β-Actin was used as a housekeeping protein because it was from the same tissue. Results represent mean ± SD of 3 independent experiments (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

4. Discussion

It is well known that KO is rich in phospholipids and fatty acids, such as EPA and DHA^[26–27]. Previous studies have shown that KO inhibits chronic prostate inflammation and various cancers. However, the efficacy of KO against BPH has not yet been reported. The

present study examined the molecular mechanism of action of KO on prostatic hyperplasia *in vitro* and *in vivo*.

A main cause of BPH is excessive prostate cell proliferation generated by an imbalance between cell growth and cell death^[9,41]. In the present study, treatment with KO inhibited cell proliferation in a dose-dependent manner in WPMY-1 and BPH-1 cells, as demonstrated by the MTT and cell counting assay. To confirm the molecular mechanism of prostate cell proliferation inhibition, prostatic proliferation markers associated with BPH, such as 5α-reductase, AR, FGF-2, EGF, Bcl-2, and Bax, were investigated. The expression levels of 5α-reductase, AR, FGF-2, EGF, and Bcl-2 were decreased, and the Bax protein level was increased. This result confirmed that the levels of 5α-reductase (which converts testosterone to DHT), AR, the growth factors FGF-2 and EGF, and the apoptosis-related proteins Bcl-2 and Bax were regulated in response to KO treatment. These results suggested that KO treatment inhibited prostate cell proliferation by modulating prostate proliferation markers and apoptosis-related markers.

Mammalian cells proliferate through the cell cycle, an essential element for cell proliferation^[16]. Having established that KO inhibits cell proliferation, we conducted cell cycle analysis to investigate the role of KO in cell cycle distribution. Flow cytometry revealed that KO treatment induced cell cycle arrest at G₀/G₁ phase. To further investigate the systemic regulatory mechanism involved in the KO-induced cell cycle arrest at G₀/G₁, the expression levels of G₀/G₁-associated protein regulators were investigated. The G₀/G₁ phase is regulated by the formation of CDK–cyclin complexes, such as CDK2–cyclin E and CDK4–cyclin D1, and the CDKs, such as p21, p27, and p53^[42]. In the present study, KO treatment significantly decreased the expression levels of CDK2, cyclin E, CDK4, and cyclin D1 in WPMY-1 cells. Downregulated levels of CDK2 and cyclin E were observed in KO-treated BPH-1 cells. KO treatment significantly increased the p21 level in WPMY-1 cells and increased the levels of p21 and p27 in BPH-1 cells. Our results demonstrated that different cell cycle regulators were associated with KO-stimulated suppression of prostate cell proliferation, suggesting that KO may affect cell cycle dysregulation depending on cell types and species. These results indicated that KO-induced inhibition of proliferation is attributed to the induction of G₀/G₁ phase arrest via reduction of CDKs and cyclins by stimulating CDKs in prostate cells.

The activation of transcription factors modulates many types of gene expression responsible for cellular stress, cell proliferation, pathological conditions, and immune responses^[43]. NF-κB is a key molecule correlated with cell proliferation regulation and cell cycle progression^[39,44]. We investigated whether NF-κB activation is responsible for the inhibition of KO-treated prostate cell proliferation using EMSA, which showed that KO decreased the binding activity of NF-κB in both prostate cell lines. Our result suggested that KO-induced suppression of prostate cell proliferation might involve the reduction of transcriptional NF-κB binding activity.

The MAPK signaling pathway plays an important role in apoptosis, cell proliferation, and cell division^[39]. The 3 major MAPK pathways in cells are the ERK1/2, p38 kinase, and JNK

pathways^[23]. The PI3K/AKT pathway affects a number of cellular biological processes, such as proliferation, growth, apoptosis, and cytoskeletal rearrangement^[19]. Phosphorylation of AKT and MAPKs was investigated in WPMY-1 and BPH-1 cells treated with KO. KO treatment induced phosphorylation of JNK and p38 in both prostate cell lines. However, ERK1/2 and AKT phosphorylation was not affected by treatment with KO. These results suggested that KO suppresses the proliferation of prostate cells through the JNK and p38 signaling pathways. In addition, genetic silencing studies indicated that p38 and JNK signaling is closely associated with KO-mediated inhibition of prostate cell proliferation via regulation of G₁-phase cell cycle regulators and NF- κ B activation. Our results provide a direct evidence of crosslink between signaling pathways (p38 and JNK) and growth inhibition in KO-treated prostate cells.

To confirm the inhibitory effect of KO on BPH *in vivo*, a TE-induced BPH rat model was obtained. Oral administration of KO reduced the size and weight of the prostate gland compared to the control group. In addition, the thickness of prostate tissues was significantly alleviated in KO-treated rats compared with the control group. The DHT index was also decreased in the KO-treated BPH rat model. The efficacy of KO on TE-induced proliferation of prostatic cells was validated by H&E and Ki-67 staining in the BPH rat model. Finally, the expression levels of BPH-associated molecular markers (5 α -reductase-AR axis, FGF, Bcl-2, and Bax) in the pathological growth of prostatic cells in the BPH rat model injected with KO were determined. The findings indicated that KO might inhibit the DHT index level via suppression of the 5 α -reductase-AR axis, resulting in the interruption of BPH enlargement. Collectively, our results demonstrate that KO exhibits inhibitory effects against BPH through dysregulation of the 5 α -reductase-AR-DHT axis, apoptosis-related molecules, and growth factors.

Sterols and fatty acids in several natural products have demonstrated anti-BPH effects by impeding enzyme 5 α -reductase, thus inhibiting the conversion of testosterone to DHT, a major growth inducer of the prostate gland^[45-46]. Fatty acids, phytosterols, and vitamins extracted from pumpkin seed oil inhibited BPH by suppressing the expression of 5 α -reductase and modulating the balance between cell apoptosis and proliferation in rats^[47]. In addition, the bark of *P. africanum*, comprising phytosterols and fatty acids, such as palmitic acid, resulted in the inhibition of prostatic fibroblast proliferation in response to growth factors, anti-inflammatory activity, inhibition of 5 α -reductase, and inhibition of prolactin levels^[13,45-46]. The sterol glycosides, polysaccharides, and fatty acids in the extracts of *Urtica dioica* roots induced alleviation of the symptoms of an enlarged prostate^[45-46,48]. *Quisqualis indica* extracts containing quisqualic acid (alkaloid trigonelline), rutin (flavonoid), and fatty acids ameliorated BPH by regulating prostate cell proliferation and apoptosis^[49]. The ethanol extract of Eijitsu (dried fruits of *Rosa multiflora*) revealed strong anti-androgenic activity, which was associated with the unsaturated fatty acids (oleic, linoleic, and γ -linolenic acids)^[50]. Moreover, oral injection with astaxanthin suppressed testosterone-induced BPH in rats via the regulation of superoxide dismutase (SOD) activity^[51]. Therapeutic effects of

DHA on BPH have been reported *in vitro* and animal model^[52]. A significant reduction of 5 α -reductase was also observed in prostatic cells after EPA treatment^[53]. It has been shown that most of the EPA and DHA in KO are present in the form of phospholipids, which contributed to a remarkably higher bioavailability than the form of triacylglycerides in fish oils^[54-55]. Previous studies identified the main physiologically effective constituents of KO as DHA, EPA, and the antioxidant astaxanthin, with no harmful toxic effects^[38,56]. In this study, we found an effective anti-BPH benefit via use of a KO product composed of phospholipid (46.6%), EPA (130.72 mg/g), DHA (77.99 mg/g), and astaxanthin (0.04 mg/g). However, further studies are needed to elucidate the association between the composition of KO and anti-BPH effect for clinical trials. Collectively, it is plausible that the phospholipids, astaxanthin and fatty acids, such as *n*-3 PUFAs (DHA and EPA), in KO might play a vital role in the potent anti-BPH efficacy observed in our experimental result.

5. Conclusion

In conclusion, KO suppressed the proliferation of both WPMY-1 and BPH-1 cells, and this correlated with CDKI-mediated G₀/G₁ cell cycle arrest, dysregulation of signaling pathways, decreased binding activity of NF- κ B, and the modulated level of BPH-associated molecular markers. In the TE-induced BPH rat model, KO administration inhibited pathological prostatic observations. The present results demonstrate the beneficial effects of KO against BPH and provide valuable information for the development of novel functional agents to treat or prevent BPH. A clinical study will be required in the future.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2023.9250017>.

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