The traditional Korean herbal medicine Ga-Gam-Nai-Go-Hyan suppresses testosterone-induced benign prostatic hyperplasia by regulating inflammatory responses and apoptosis

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Abstract. Benign prostatic hyperplasia (BPH) is a pathological condition that affects the majority of men above the age of 50 years. Pharmacological agents are typically used to treat BPH; however, there are currently no pharmacological agents that are able to completely cure BPH without causing adverse side effects. As a result of these side effects, there is a great interest in developing effective herbal medicines that are able to inhibit the progression of BPH and are safe for long-term use. Ga-Gam-Nai-Go-Hyan (GGN) is a traditional Korean herbal medicine that has been widely used to treat BPH; however, no biological studies have been performed to elucidate the efficacy of GGN. The aim of the present study was to evaluate the efficacy of GGN as a treatment for BPH. GGN administration was demonstrated to significantly decrease prostate weight ($P<0.001$), the relative prostate weight ratio ($P<0.001$) and the ratio of prostate weight to body weight ($P<0.001$). In addition, GGN treatment was revealed to suppress testosterone and dihydrotestosterone serum levels ($P<0.001$) and the growth of prostatic tissue. GGN also decreased the levels of the two inflammatory proteins ($P<0.05$), inducible nitric oxide synthase and cyclooxygenase-2, decreased the levels of the two apoptotic suppressors ($P<0.05$) B-cell lymphoma (Bcl)-2 and Bcl-xL and increased the levels of the pro-apoptotic factors ($P<0.05$) Bcl-2-associated X protein, caspase-3, caspase-8, Fas, Fas ligand and Fas-associated protein with death domain. The results of the present study suggested that GGN may have suppressive effects on the development of BPH and therefore have the potential to be used for treating BPH.

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Introduction

Benign prostatic hyperplasia (BPH) is one of the most common chronic diseases in men (1). A previous epidemiological study has determined that >50% of men over the age of 50 years exhibit symptoms of BPH, such as urinary urgency and retention, and the incidence rate gradually increases with age (2). Aging and androgens are known to be the two main factors associated with the development of BPH, and it has previously been reported that inflammation may be another key factor in prostatic enlargement (3,4). In addition, repeated tissue damage due to chronic inflammation may provoke compensatory cellular proliferation, which increases the risk of hyperplastic growth (3,5). Chronic inflammation is able to induce proliferation in prostate tissue by affecting apoptotic protein expression. Cyclooxygenase (COX)-2 inhibition is able to significantly increase apoptotic activity in prostate cells via directly upregulating B-cell lymphoma (Bcl)-2 expression (6). Cell growth in the normal prostate is regulated via a delicate balance of cell death and proliferation; therefore, disruption of the molecular mechanisms that regulate these processes may lead to a state of epithelial and stromal hyperplasia (7,8).

At present, therapy for BPH is largely based on the use of α_1-andrenergic receptor blockers and 5α-reductase inhibitors, which relax prostatic smooth muscle and reduce prostatic volume, respectively (9,10). Data from a previous systematic review indicates improved urine flow, nocturia and quality of life resulting from these treatments when used in combination or as monotherapies (11). Recently, the use of phosphodiesterase type 5 (PDE5) inhibitors was also recognized as an effective treatment for BPH (12). These pharmacological agents are effective; however, they induce undesirable adverse effects, including blood loss, urinary incontinence, infection, sexual dysfunction and morphological changes in the prostate (13,14). Therefore, it is necessary to identify novel effective herbal products for the treatment of BPH that are also safe for long-term use.

Ga-Gam-Nai-Go-Hyan (GGN) is an oriental herbal blend that has been used for a long time in Korea (15). It is composed of nine herbs: *Morinda officinalis* Haw, *Cistanche salsa, Cornus officinalis, Cuscuta japonica*

Chois, *Psoralea corylifolia* L, *Dendrobium nobile* Lindley, *Trigonella foenumgraecum* L, *Foeniculum vulgare* Mill and *Aconitum carmichaeli* Debeaux (Table I). It has previously been reported that GGN is effective in the treatment of pallor, dizziness, chronic prostatitis, impotence and BPH (15). In addition, GGN has been used to treat patients with genital herpes, which may support its use in the treatment of GGN, as *Trichomonas vaginalis* has been detected in the urine of patients with BPH, which may indicate an association between infections of the genital system and BPH (16). A Korean medicine book entitled The Treasured Mirror of Eastern Medicine reported that the primary therapeutic facets of herbs used in GGN are similar to those of therapeutic agents used in the treatment of BPH (17). Certain major active components of GGN, including quercetin, kaempferol, coumarins and lignin glycosides, have been previously reported to exhibit anti-inflammatory and anti-oxidative qualities (18-21). Furthermore, a recent study by our group revealed that *Cistanche salsa* extract elicits an anti-proliferative effect on the prostate tissue of rats with BPH (22). Although studies on the physiological functions of the major active components of GGN have been performed, the molecular mechanism(s) underlying the effect of GGN on BPH have not yet been investigated; therefore, the aim of the present study was to assess the anti-proliferative effects of GGN in a testosterone-induced rat model of BPH, and to demonstrate that it functions through regulation of the inflammatory response and apoptotic protein expression.

Table I. Recipe of Ga-Gam-Nai-Go-Hyan formulation used.

Species	Parts used	Weight (g)
Morinda officinalis Haw	Roots	120
Cistanche salsa	Context stem	120
Cornu sofficinalis	Fruit	120
Cuscuta japonica Chois	Seed	120
Psoralea corylifolia L	Seed	100
Dendrobium nobile Lindley	Above ground	80
Trigonella foenumgraecum L.	Seed	80
Foeniculum vulgare Mill.	Fruit	40
Aconitum carmichaeli Debeaux	Roots	20

Materials and methods

Materials and reagents. All herbs used to prepare GGN were purchased from Omniherb (Dong Woo Dang Pharmacy Co. Ltd., Yeongcheon, Korea). Finasteride was obtained from Merck & Co., Inc. (Whitehouse Station, NJ, USA). Antibodies against inducible nitric oxide synthase (iNOS; M-19; sc-650), COX-2 (C-20; sc-1745), procaspase-3 (E-8; sc-7272), procaspase-8 (C-20; sc-6136), B-cell lymphoma-2 (Bcl-2; C-2; sc-7382), Bcl-extra large (Bcl-xL; H-5; sc-8392), Bcl-2-associated X protein (Bax; B-9; sc-7480), p53 (FL-393; sc-6243), Fas (A-20; sc-1023), Fas ligand (Fas-L; C-178; sc-6237) and β-actin (ACTBD11B7; sc-81178) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). An antibody against Fas-associated protein with death domain (FADD; ab24533) was purchased from Abcam (Cambridge, UK). The horseadish peroxidase-conjugated secondary antibodies (goat anti-rabbit, 111-035-003; rabbit anti-mouse, 315-035-003; and donkey anti-goat, 705-035-003) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other reagents were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

Preparation of GGN. GGN consists of nine different herbs; *Morinda officinalis How* (120 g), *Cistanche deserticola* Y. C. Ma. (120 g), *Cornus officinalis* Sieb. et. Zucc. (120 g), *Cuscuta chinensis* Lamark (120 g), *Psoralea corylifolia* L. (100 g), *Dendrobium nobile Lindl*. (80 g), *Trigonella foenum-graecum* L. (80 g), *Foeniculum vulgare* Mill. (40 g) and *Aconitum carmichaeli* Debx. (20 g). The herbs had a moisture content of <13% by weight and were air-dried. The combination of herbs was extracted with 50% (v/v) ethanol-water at 60˚C for 8 h. The extracts were then filtered through 15-μm cartridge paper and ethanol was removed via vacuum rotary evaporation (Eyela; Tokyo Rikakikai Co., Ltd., Tokyo Japan). The concentrates were freeze-dried and the yield was 12%. Powders were dissolved in distilled water prior to experiments and residual powders were stored at -20˚C.

Animals. Ten-week-old male Wistar rats (n=24; weight, 200±20 g) were purchased from Daehan Biolink Co., Ltd., (Republic of Korea). Rats were housed under constant conditions (temperature, 22±2˚C; humidity, 55±9%; 12 h light/dark cycle; *ad libitum* access to food and water) in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Ethics Committee for Animal Care and the Use of Laboratory Animals at Sang-ji University (Wonju, Korea; approval documents, 2013-03;) and conducted in accordance with University guidelines. Rats were randomly distributed into four groups (n=6 in each); the sham-operated group (Con; administered with 200 μl distilled water orally), the BPH model group (BPH), the BPH-induced group administrated with finasteride (Fina; 5 mg/kg/day; p.o. Merck & Co., Inc.) and the BPH-induced group administrated with GGN (GGN; 100 mg/kg/day; p.o.). BPH was induced in rats via castration and subsequent subcutaneous injections of 100 μl testosterone propionate (Waco Pure Chemical Industries, Ltd., Osaka, Japan; 10 mg/kg/day), as previously reported (23). At the end of the four-week period, body weight was recorded and rats were fasted for 12 h. The following day, all rats were sacrificed by cervical dislocation following anesthetization with Zoletil 50 (20 mg/kg intraperitoneally; Virbac, Carros, France), and blood samples were obtained via cardiac puncture. Prostatic tissue was excised, rinsed, weighed and stored at -70˚C until use.

Prostate weight to body weight ratio. Prostatic tissues were harvested, rinsed, and weighed immediately following sacrifice. The prostate weight to body weight (PW/BW) ratio was calculated. The relative prostate weight ratio was calculated as follows: Relative prostate weight ratio=prostate weight of rats in the experimental groups (BPH, Fina, GGN)/prostate weight of rats in the Con group.

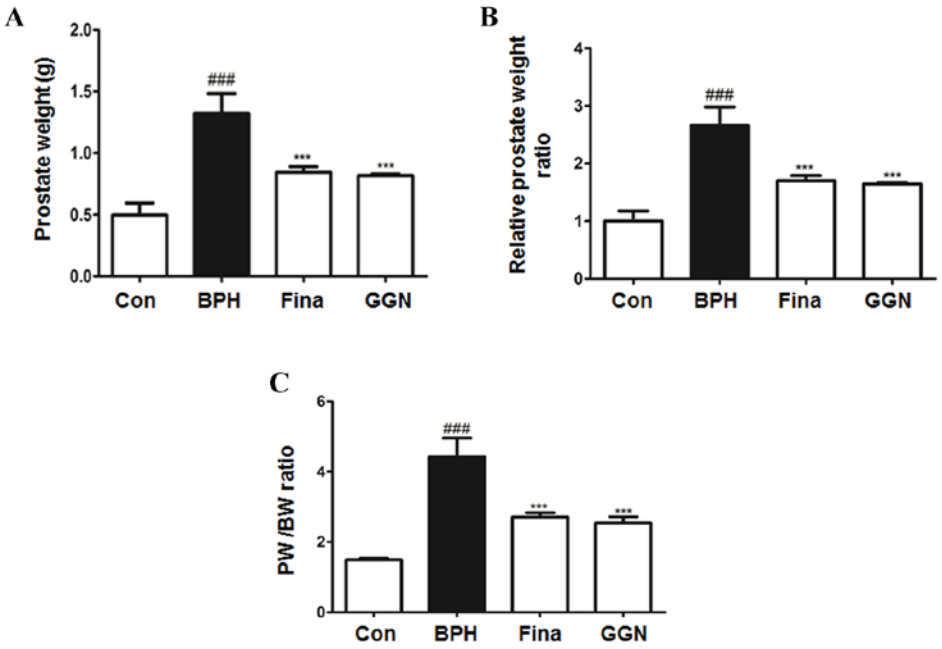

Figure 1. Effect of GGN administration on prostate weight in BPH-induced rat models. (A) Prostate weight; (B) relative prostate weight ratio; and (C) prostate weight per body weight ratio. Values are expressed as the mean ± standard error of six rats per group. Experiments were performed once per sample. ###P<0.001 vs. Con group; ***P<0.001 vs. BPH group. GGN, Ga-Gam-Nai-Go-Hyan; BPH, benign prostatic hyperplasia; fina, finasteride-administered group; Con, sham-operated group; PW, prostate weight; BW, body weight.

Serum analysis. Serum concentrations of testosterone were determined using a testosterone enzyme immunoassay kit (582701; Cayman Chemical Co., Ann Arbor, MI, USA). Serum concentrations of dihydrotestosterone (DHT) were determined via enzymatic methods using a commercially available assay kit (11-DHTHU-E01; ALPCO, Salem, NH, USA) according to the manufacturer's protocol.

Histological analysis. The prostatic tissue was fixed in 4% buffered formalin and embedded in paraffin, and 4-μm thick sections were cut. The sections were stained with hematoxylin and eosin for histological examination, and images were acquired using an SZX10 microscope (Olympus Corp., Tokyo, Japan). The thickness of the epithelium in the prostate tissue (TETP) was measured using the Leica Application Suite software (LAS ver. 3.3.0; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Western blot analysis. The prostatic tissue from each rat was homogenized in PRO-PREP lysis buffer (Intron Biotechnology Inc., Seongnam, Korea). Tissue extracts were centrifuged at 16,000 x *g* (4˚C) for 20 min, and the resulting supernatant was transferred to a clean tube. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Aliquots of each protein sample (30 μg) were separated by 10-12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Immobilion-P transfer membrane; Merck Millipore). The membranes were blocked with 2.5% skimmed milk at 4˚C for 30 min and incubated overnight with 1:1,000 dilutions of the primary antibodies (anti-iNOS, anti-COX-2, anti-caspase-3, anti-caspase-8, anti-Bcl-xL, anti-Bax, anti-P-53, anti-Fas, anti-Fas-L, anti-FADD and anti-β-actin). The blots were washed three times with Tween-20/TBS and then incubated with 1:2,000 dillutions of corresponding secondary antibodies (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Blots were again washed three times with Tween-20/TBS and the immunoreactive protein bands were visualized via enhanced chemiluminescence, and the developed blots were exposed to X-ray film (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Each blot was performed in triplicate and densitometric analysis was performed using Bio-rad Quantity One Software (version 4.6.3; Bio-Rad Laboratories, Inc.).

Statistical analysis. Values are expressed as the mean ± standard error of the mean for six rats. Data were analyzed using one-way analysis of variance with Dunnett's test and $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS statistical analysis software (version 19.0; International Business Machines, Armonk, NY, USA).

Results

GGN decreases prostate weight in a rat model of BPH. The mean prostate weight of rats in the BPH group was significantly higher than that of rats in all other groups ($P<0.001$). Compared with BPH rats, the prostate weight of rats in the Fina and GGN groups were significantly decreased ($P<0.001$; Fig. 1A). The relative prostate weight ratio in the BPH group was 2.66 times that in the Con group, which was a significant increase ($P<0.001$), whereas in the GGN group, prostate weight was only 1.64 times that in the Con group (Fig. 1B). In addition, the PW/BW ratio in the BPH group was also significantly higher than that in the other groups ($P<0.001$). Compared with

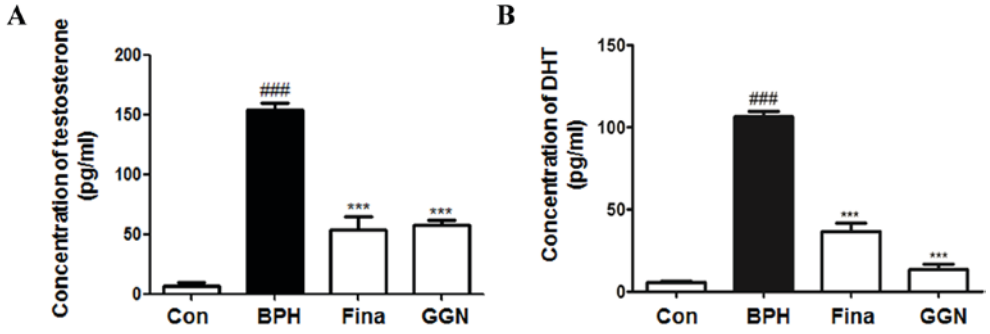

Figure 2. Effect of GGN administration on the concentration of (A) serum testosterone and (B) DHT production in prostate tissues of BPH-induced rat models. Values are expressed as the mean ± standard error of six rats per group. Experiments were performed once per sample. ###P<0.001 vs. Con group; ***P<0.001 vs. BPH group. GGN, Ga-Gam-Nai-Go-Hyan; DHT, dihydrotestosterone; BPH, benign prostatic hyperplasia; fina, finasteride-administered group; Con, sham-operated group.

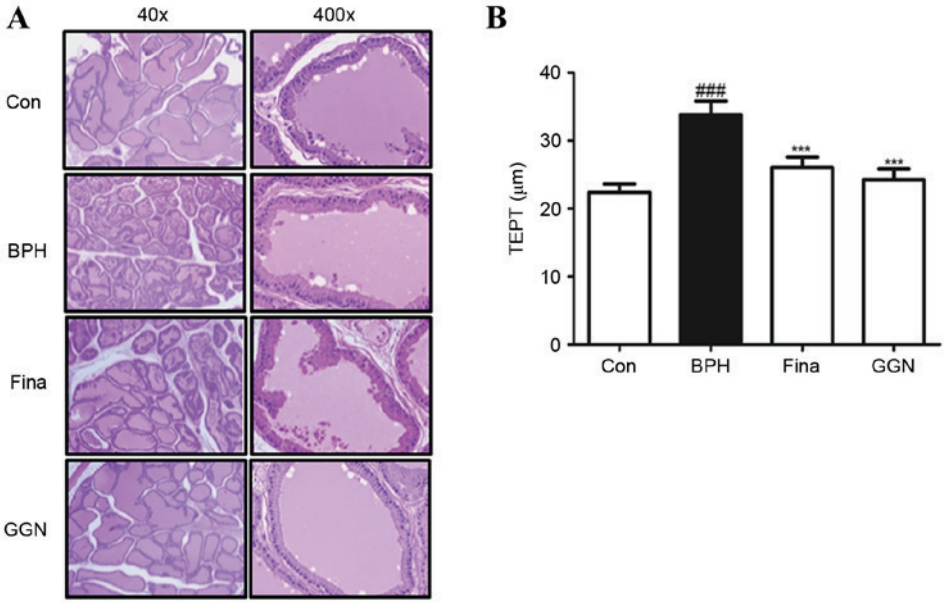

Figure 3. Effects of GGN administration on prostatic cell proliferation. (A) Hematoxylin and eosin staining of prostatic tissue from BPH-induced rat models (original magnification, x40 and x400, respectively). (B) Quantified TEPT expressed as the mean ± standard error of six rats per group. Experiments were performed once per sample. ###P<0.001 vs. Con group; ***P<0.001 vs. BPH group. Con, control; Fina, finasteride-administered group; GGN, Ga-Gam-Nai-Go-Hyan; BPH, benign prostatic hyperplasia; TETP, thickness of epithelium tissue from prostate; SEM, standard error of the mean.

the BPH group, the PW/BW ratio was significantly lower in the Fina and GGN groups (P<0.001), whereas no significant difference was observed between the PW/BW ratios of the GGN group (2.54) and the Fina group (2.71).

GGN decreases serum testosterone and DHT levels in a rat model of BPH. The serum testosterone and DHT levels of the rats in each group are displayed in Fig. 2. The concentrations of the testosterone and DHT in the BPH group were significantly higher than those in all other groups (P<0.001). In the Fina and GGN groups, serum testosterone and DHT levels were significantly lower than those in the BHP group (P<0.001). Of note, DHT levels in the GGN group were markedly lower than those in the Fina group.

GGN attenuates testosterone-induced morphological changes of the prostate gland. The effect of GGN on prostate gland morphology was investigated by histological analysis (Fig. 3). Rats in the BPH group exhibited histological changes typical of prostatic hyperplasia: Thickened glandular epithelium, vacuolated cytoplasm pointing into the glandular lumen and a decreased glandular luminal area (Fig. 3A). Administration of GGN for four weeks suppressed these typical histological patterns. The TETP was significantly higher in the BPH-induced group than in the other groups (P<0.001; Fig. 3B). In brief, histologic examination revealed that administration of finasteride or GGN ameliorated the major features of prostatic hyperplasia observed in the BPH group, although certain minor features of prostatic hyperplasia remained.

GGN decreases inflammatory proteins in prostatic tissue of rats with BPH. Inflammation is associated with the increased proliferation of prostatic epithelial cells (24). In patients with BPH, iNOS, which provides a sustained release of reactive nitrogen species that may induce cell damage, is typically present (25). In addition, COX-2, which generates

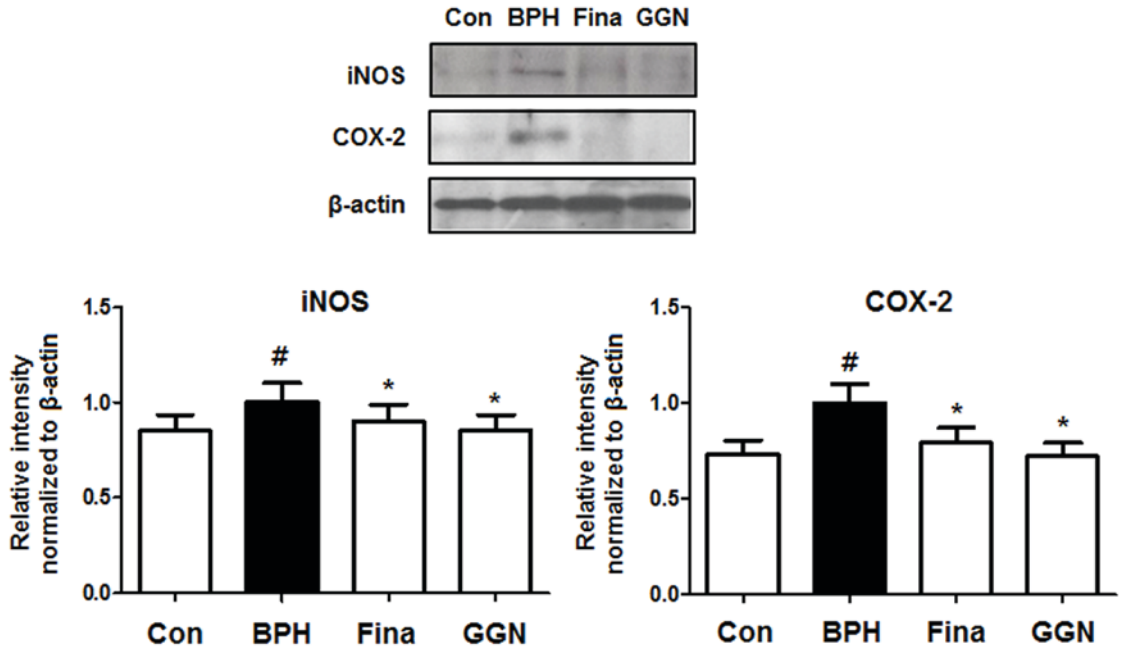

Figure 4. Effect of GGN administration on the expression of iNOS and COX-2 in prostate tissues of BPH-induced rat models. The expression levels of iNOS and COX-2 protein were determined via western blotting using specific antibodies. β-actin was used as an internal control. The data shown represent mean ± standard error of 6 rats per group. #P<0.05 vs. Con group; *P<0.05 vs. BPH group. GGN, Ga-Gam-Nai-Go-Hyan; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; BPH, benign prostatic hyperplasia; fina, finasteride-administered group; Con, sham-operated group.

pro-inflammatory prostaglandins, is detected in inflammatory cells (4). In the present study, the levels of iNOS and COX-2 in the prostatic tissue of BPH-induced rats were analyzed by western blotting to investigate the effects of GGN on inflammation. Compared with the Con group, treatment with testosterone increased the levels of iNOS and COX-2 in the BPH-induced group. By contrast, the Fina and GGN groups showed markedly reduced levels of these inflammatory proteins compared with the BPH group (Fig. 4).

GGN enhances apoptotic signaling in prostatic tissue of rats with BPH. BPH arises from an imbalance between cell proliferation and apoptosis (3). The two main apoptotic pathways are the intrinsic and extrinsic pathways. In the extrinsic pathway, apoptosis is induced by death activators binding to receptors at the cell surface and accumulation of the adaptor molecule FADD, which leads to the activation of initiator caspase (26). In the present study, the expression levels of proteins in the extrinsic pathway were assessed (Fig. 5A), and the Fina and GGN-treated group exhibited higher protein levels of Fas-L, Fas, FADD and p53, and lower protein levels of procaspase-8 and procaspase-3 compared with the levels in the BPH group.

In the intrinsic pathway, apoptosis, including disruption of the mitochondrial membrane potential, is critically regulated by Bcl-2 family proteins (27). As demonstrated in Fig. 5B, the Fina and GGN group exhibited lower levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL, and higher levels of the pro-apoptotic protein Bax than the BPH group. Therefore, although the ratio of Bcl-2 to Bax was significantly increased in the BPH group compared with the control group (P<0.05), the ratio decreased significantly following treatment with Fina and GGN (P<0.001), suggesting that GGN-induced apoptosis is regulated by the Bcl-2 family of proteins, which are important mediators of apoptosis (Fig. 5C).

Discussion

BPH is the most prevalent urologic health concern in the male population, with a histological prevalence at autopsy of 50% in men aged 50-60 years and 90% in men aged >80 years (28). It is characterized by the non-malignant overgrowth of prostatic tissue surrounding the urethra, and is usually present with one or more co-morbidities, including bladder dysfunction and hypertrophy, which may lead to acute urinary retention (2). Previously, androgens and age have been considered as the main determinants of prostate enlargement; however, the potentially important role of chronic inflammation in the pathogenesis of BPH has recently been identified (29).

Hormonal imbalance has an important role in the pathogenesis of BPH via induction of inflammatory responses and reduction of apoptosis, and testosterone has been shown to be associated with BPH (4). In the present study, rats were administered testosterone for four weeks to induce BPH. Prostate weight ratios and DHT levels in the BPH group were higher than those in the Con group, indicating that testosterone administration successfully induced BPH. However, four weeks of GGN treatment effectively inhibited BPH, and these effects were comparable to those observed in the Fina group.

In the inflammatory cells of the prostate, iNOS is the factor that predominantly activates reactive nitrogen, which is able to damage cells (30). Hyperplastic prostate tissue is characterized by increased NOS expression in the epithelial cells compared with normal tissue (31). In addition, nitric oxide may also be converted by COX enzymes to proinflammatory

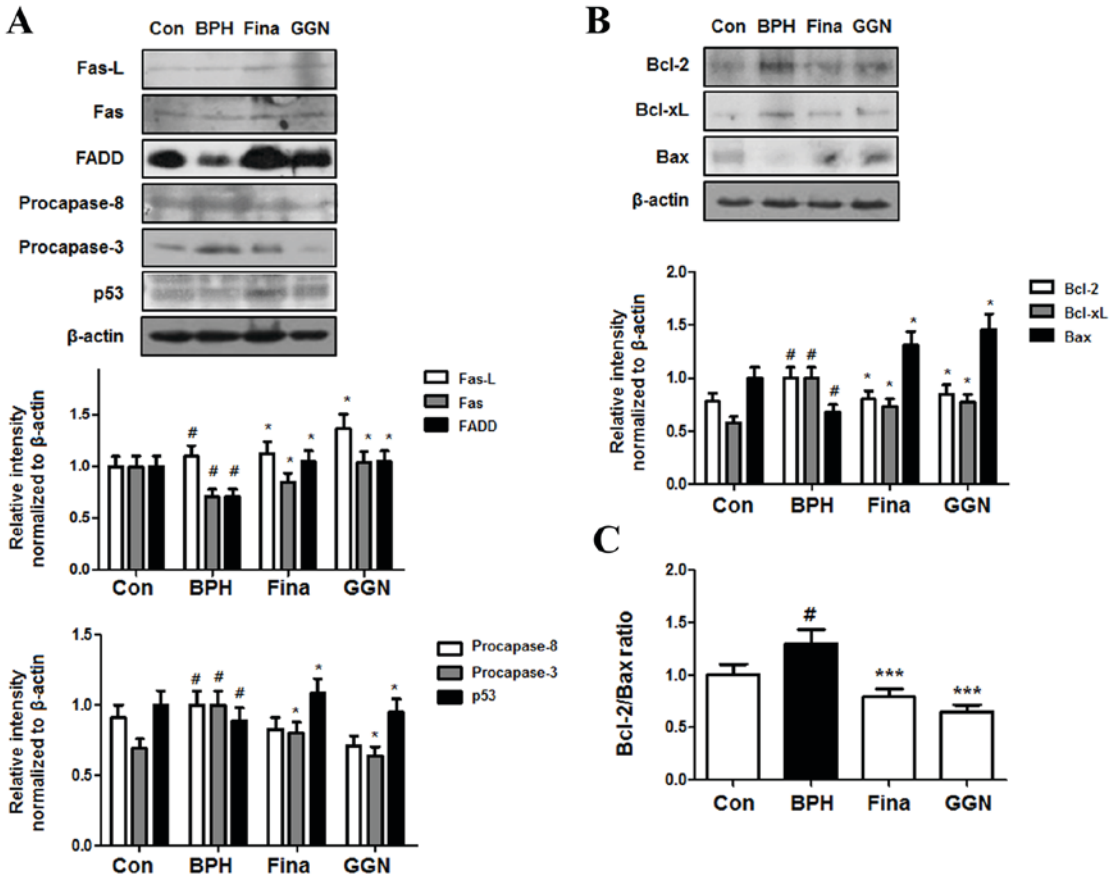

Figure 5. Effect of GGN administration on the expression of apoptotic proteins. (A) The expression levels of P-53, death receptor proteins and procaspase-3 were determined via western blotting using specific antibodies. (B) The expression levels of Bcl-2 family proteins were determined by western blotting using specific antibodies. β-actin was used as internal controls. (C) Densitometric analysis of Bcl-2 and Bax bands was performed, and the data (relative density normalized to β-actin) were plotted as the Bcl-2/Bax ratio. The data shown represents mean ± standard error of six rats per group. $^{\#}$P<0.05 vs. Con group; *P<0.05, ***P<0.001 vs. BPH group. GGN, Ga-Gam-Nai-Go-Hyan; Bcl, B-cell lymphoma; Bax, bcl-2-associated X protein; BPH, benign prostatic hyperplasia; fina, finasteride-administered group; FADD, Fas-associated protein with death domain; Fas-L, Fas ligand.

prostaglandins (32), and COX-2 has been detected in the epithelium and interstitial spaces of inflammatory cells (30). In the present study, the protein expression levels of COX-2 and iNOS were analyzed in a rat model of BPH using western blotting. The levels of COX-2 and iNOS proteins in the BPH group were higher than those in the Con group. By contrast, COX-2 and iNOS levels in the GGN group were lower than those in the BPH group. These results indicated that GGN may suppress the growth of prostatic cells through its anti-inflammatory effects.

In mammalian cells, apoptosis proceeds via the extrinsic and intrinsic pathways. The extrinsic pathway is triggered by the binding of extracellular signaling factors to death receptors at the plasma membrane. Fas is a cell-surface receptor protein belonging to the tumor necrosis factor receptor superfamily, and its physiological ligand, Fas-L, is a member of the corresponding tumor necrosis factor cytokine family (33). Fas-L-Fas signaling triggers apoptosis through FADD, and activation of the aspartate-specific cysteine protease, caspase-8, initiating a cascade of caspase activation leading to phagocytosis of the cell (34). The intrinsic pathway is activated by intracellular damage, such as oxidative stress, and is controlled by the mitochondria, which are regulated by the Bcl-2 protein family. Bcl-2 proteins are categorized based on their structure and function; this family includes anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and proapoptotic proteins, such as Bax (27). In various systems, the Bcl-2 family modulates apoptosis, with the Bcl-2/Bax ratio serving as a rheostat to determine a cell's susceptibility to apoptosis (35). p53 induces the release of various proapoptotic factors from the intermembrane space of the mitochondria (36). p53 interacts with Bax by binding to its Bcl-2 homolog 3 domains, leading to cytochrome *C* release by mitochondria into the cytoplasm (37). Cytochrome *C* activates caspase-9, which in turn activates a series of caspases, including caspase-3 (38). In the present study, it was demonstrated that administration of GGN increased the levels of Fas, Fas-L, FADD and p53, and decreased the levels of procaspase-3 and procaspase-8. In addition, GGN reduced the levels of Bcl-2 and Bcl-xL and increased the levels of Bax protein. These results indicated that GGN-induced apoptosis in a rat model of BPH is most likely due to stimulation of death receptors and the mitochondrial pathway.

In conclusion, the findings of the present study suggested that GGN is able to prevent BPH by regulating inflammatory responses and apoptosis. Based on this hypothesis, GGN may

have applications as a therapeutic agent for the treatment of patients with BPH.

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