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Original Research Article

Rauwolfia vomitoria extract suppresses benign prostatic hyperplasia by reducing expression of androgen receptor and 5α-reductase in a rat model

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ABSTRACT

Objective: Herbal medicine is an important therapeutic option for benign prostatic hyperplasia (BPH), a common disease in older men that can seriously affect their quality of life. Currently, it is crucial to develop agents with strong efficacy and few side effects. Herein we investigated the effects of the extract of *Rauwolfia vomitoria*, a shrub grown in West Africa, on BPH.

Methods: Rats with testosterone-induced BPH were treated with *R. vomitoria*. Prostates were histologically analyzed by Hematoxylin and eosin staining. Proliferation index and the expression levels of androgen receptor and its associated proteins were quantified through immunohistochemistry and immunoblotting. Androgen receptor target genes were examined by quantitative real-time polymerase chain reaction. The sperm count and body weight of rats were also measured.

Results: The oral administration of *R. vomitoria* extract significantly reduced the prostate weight and prostate weight index in BPH rats, supported by the decreased thickness of the prostate epithelial layer and increased lumen size. Similar effects were observed in the BPH rats treated with the reference drug, finasteride. *R. vomitoria* extract significantly reduced the testosterone-induced proliferation markers, including proliferating cell nuclear antigen and cyclin D1, in the prostate glands of BPH rats; it also reduced levels of androgen receptor, its associated protein steroid 5α-reductase 1 and its downstream target genes (*FK506-binding protein 5* and *matrix metalloproteinase 2*). Notably, compared with the finasteride group, *R. vomitoria* extract did not significantly reduce sperm count.

Conclusion: R. vomitoria suppresses testosterone-induced BPH development. Due to its milder side effects, *R. vomitoria* could be a promising therapeutic agent for BPH.

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1. Introduction

Benign prostatic hyperplasia (BPH) is a common chronic disease among men, and its incidence increases with age [1,2]. The progressive hyperplasia in the epithelial and stromal compartments of the prostate gland are two characteristics of BPH, and they contribute to an increase in size and weight of the prostate gland [3]. Although aging and related changes in hormone balance are two causes of BPH, the molecular pathological mechanisms leading to BPH are still

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unclear [4]. At present, testosterone and dihydrotestosterone are known to be highly correlated with BPH. Testosterone, which is produced in the testis and spreads to the prostate, is converted into dihydrotestosterone by the action of 5α-reductase [4,5]. Possessing two to three times greater affinity for androgen receptor than testosterone, dihydrotestosterone is an acute mediator of BPH [6]. Though serum concentration of testosterone decreases with age, high activity of 5α-reductase increases the amount of dihydrotestosterone [7]. High levels of dihydrotestosterone increase the levels of androgen receptor's downstream target genes, including *cyclin D1*, *FK506-binding protein 5* (*FKBP5*) and *matrix metalloproteinase 2* (*MMP2*), by binding to intracellular androgen receptor and inducing its transcriptional activity [8,9]. As one of the major cell cycle regulators, *cyclin D1* promotes cell proliferation through its interaction with cyclin-dependent kinase 4 and cyclin-dependent kinase 6 [10]. *FKBP5*, another well-known androgen receptor target gene, can form a heat-shock protein 90-based super-chaperone complex, which eventually enhances androgen receptor transcriptional activity [11,12]. *MMP2*, which functions in remodeling the extracellular matrix, is expressed during prostate glandular morphogenesis [13]. Taken together, the activation of androgen receptor signaling maintains and enhances prostatic cell proliferation and survival, contributing to increased prostate volume and weight during BPH development [14,15].

Currently, the most common medications used for BPH are 5α-reductase inhibitors and α-blockers [16]. The 5α-reductase inhibitors can reduce dihydrotestosterone level, while the α-blockers relieve BPH symptoms by relaxing the smooth muscles in the prostate [16]. However, 5α-reductase inhibitors and α-blockers show several side effects, such as dizziness, erectile dysfunction and cardiovascular risks [17]. Therefore, herbal medicines provide a complementary and alternative method for the treatment of BPH, because they are believed to possess milder side effects in general.

Derived from the root bark of a plant from the tropical forests of Africa, *Rauwolfia vomitoria* Afz. extract is a traditional folk medicine used to treat a variety of conditions, including hypertension, fever and cancer [18–21]. Currently, *R. vomitoria* extract is widely used as a health supplement that is rich in bioactive β-carboline and indole alkaloids [22,23]. In view of the anti-cancer effects of *R. vomitoria* extract against prostate cancer cells [18], we hypothesized that *R. vomitoria* extract may be used for BPH, another common prostate disease characterized by increased proliferation of prostate epithelial and stromal cells. In the present study, we investigated the activities of *R. vomitoria* extract against BPH in a testosterone-induced rat BPH model.

2. Materials and methods

2.1. R. vomitoria extract preparation

R. vomitoria extract was supplied in powdered form from Natural Source International, Ltd. (New York, USA), and was the product of single extraction batch. The extract was prepared from root bark of *R. vomitoria* using alcohol extraction. Reserpine was removed by chloroform extraction. Quality control was conducted using high-performance liquid chromatography analysis. The extract was completely dissolved in sterile phosphate-buffered saline as a 50 mg/mL stock solution and stored at –80 ℃ in aliquots until needed.

2.2. Experimental animals and maintenance conditions

All the male Sprague-Dawley rats, with initial body weight of 180–200 g, were purchased from the Beijing Vital River Laboratory and Animal Technology Co., Ltd. (Beijing, China). The animal protocol was approved by the Administrative Panel on Laboratory Animal Care of the Clinical College of Nanjing University (confirmation number: 2018GKJDWLS-03-002) and followed the International Ethical Guidelines. The Sprague-Dawley rats were acclimated for about one week before experiments began and allowed access to food and water *ad libitum* during the experiment period.

2.3. Experimental procedures

The BPH rat model was designed based on a previous study [24]. In brief, 15 rats were castrated to exclude the influence of intrinsic testosterone, followed by the induction of BPH through a four-week program of daily intraperitoneal injection of 1.2–2.3 mL testosterone propionate (1 mg/mL) dissolved in corn oil (5 mg/kg; T101368, Aladdin Industrial Corporation, Shanghai, China) in the inguinal region. Five rats were given sham operations and received corn oil instead of testosterone propionate to serve as a control group. The rats with testosterone propionate-induced BPH were randomly divided into three groups, and were given daily doses of testosterone propionate (5 mg/kg) accompanied by vehicle, finasteride (a reference drug for BPH; 5 mg/kg; HY-13635, MedChemExp, China) or *R. vomitoria* extract (20 mg/kg), by oral gavage for 28 days. Every week the rats were weighed. On day 29, the animals were euthanized, and their prostates were harvested, weighed and subjected to histological analysis. The weights of prostates were used to calculate the prostate weight index, using the following formula: prostate weight index = prostate weight/body weight × 100%.

2.4. Hematoxylin & eosin and immunohistochemistry staining

The 5 μm thick sections of the paraffin-embedded ventral prostate tissues were stained with hematoxylin and eosin. Epithelial thickness was measured using ImageJ software (1.47v, National Institutes of Health, USA). The epithelium thickness was calculated by measuring the distance between the basal pole and the apical pole of the prostate epithelium. Each dot in each group was the average value of epithelium thickness in a field containing three lumens under 200× magnification. There were five rats in each group and five independent fields were taken from each rat's ventral prostate. For lumen area, representative 400× magnification images from each rat were selected and at least five lumen areas in the photos were calculated. For immunohistochemistry analysis, 5 μm thick sections were stained with primary antibodies against proliferating cell nuclear antigen (PCNA; sc-56, 1:1000, Santa Cruz Biotechnologies, USA) and androgen receptor (A9853, 1:2000, Sigma-Aldrich, USA), followed by incubation with the secondary antibody and visualization with 3,3′-diaminobenzidine. The method for calculating the percentage of androgen receptor/PCNA-positive cells was to randomly select three lumens from each field/section and calculate the ratio of positive cells to the total number of cells in the lumens. Three fields were chosen for each sample.

2.5. Immunoblotting

Protein lysates were prepared by homogenizing the prostate tissues in a cold radioimmunoprecipitation assay buffer, and were quantified using the Bradford method, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were incubated with primary antibodies against androgen receptor (N-20; sc-816, 1:200, Santa Cruz Biotechnology, Inc, USA), cyclin D1 (sc-753, 1:1000, Santa Cruz Biotechnology, Inc, USA), steroid

5α-reductase 1 (26001-1-AP, 1:1000, Proteintech Group, Inc., Rosemont, USA) or β-actin (A1978, 1:5,000, Sigma, USA) at 4 °C overnight. After washing with phosphate-buffered saline with 0.5% Tween 20 thrice, the membranes were incubated with secondary antibodies and visualized by a Tanon high-sig Western blotting substrate (180-5001, Tanon Company, China). β-Actin was used as an internal control. The chemiluminescence intensity of protein signals was semi-quantified by the ImageJ software (1.47v, National Institutes of Health, USA).

2.6. RNA isolation and quantitative real-time polymerase chain reaction

The isolation of total RNA from rat ventral prostate tissues was carried out using TRIzol (Invitrogen, Carlsbad, USA), and the first strand complementary DNA was synthesized using Hifair II 1st strand complementary DNA synthesis supermix (11123ES60, YEASEN, Shanghai, China), according to the manufacturer's instructions. Total RNA from five rat ventral prostate glands from each group was equally mixed and 1.0 μg of mixed total RNA from each group was used in each complementary DNA synthesis reaction. ChamQ Universal SYBR quantitative polymerase chain reaction master mix (Q711-02, Vazyme, Nanjing, China) and primer mixtures were used for the real-time polymerase chain reaction. We used the ΔΔCt method to analyze the data. Fold change was determined in relative quantification units using the *Actb* gene for normalization. The sequences of the primers were as follows: rat *Actb* forward primer, 5′-ACTCTGTGTGGATTGGTGGC-3′; rat *Actb* reverse primer, 5′-CGCAGCTCAGTAACAGTCCG-3′; rat *Fkbp5* forward primer, 5′-CAGCCTCCCGAAAATTCCCT-3′; rat *Fkbp5* reverse primer, 5′-CAGCCTTCCAGGTGGACTTC-3′; rat *Mmp2* forward primer, 5′-AGAAGGCTGTGTTCTTCGCA-3′, rat *Mmp2* reverse primer, 5′-AAAGGCAGCGTCTACTTGCT-3′.

2.7. Epididymal sperm count analysis

The eight-week-old rats (n = 15) were randomly categorized into three groups, and were administered vehicle (group 1), *R. vomitoria* extract (20 mg/kg; group 2) or finasteride (5 mg/kg; group 3), daily for four weeks by oral gavage. After the final intragastric administration, the rats were sacrificed. The cauda epididymis was collected, a sperm suspension was prepared in phosphate-buffered saline and diluted 100-fold and the sperm was counted using an optical microscope (Olympus Co., Japan).

2.8. Statistical analysis

Data values are presented as mean ± standard deviation. Figures were plotted using GraphPad Prism software (Version 6.02, GraphPad Software Inc., USA). Differences in mean values were analyzed by GraphPad Prism software (Version 6.02, GraphPad Software Inc., USA). Statistical analysis was performed among groups using one-way analysis of variance and values with $P < 0.05$ were considered to be statistically significant. Semi-quantification was carried out using Image J software (Version 1.8.0, National Institutes of Health, USA).

3. Results

3.1. R. vomitoria extract suppresses BPH development

After the 28-day treatment, compared to the animals in the sham-operation group, the sizes of the prostates in the testosterone propionate-induced BPH model group significantly increased, and this was reversed by the *R. vomitoria* extract treatment (Fig. 1A). Since finasteride is currently used for BPH patients, it served as a positive control. As expected, the finasteride treatment also reduced prostate size. The mean prostate weight and prostate weight index of rats in the BPH/vehicle group were significantly higher than those of rats in vehicle, BPH/*R. vomitoria* and BPH/finasteride groups ($P < 0.001$; Fig. 1B and C). Interestingly, the reduced prostate weight and prostate weight index in the BPH/*R. vomitoria* group were comparable with those in the finasteride treatment group. Lastly, we also observed that the body weights of rats did not display significant differences among three groups within the experimental period (Fig. 1D).

3.2. Reduction of prostate epithelial cells in the BPH rat model

As shown in Fig. 2A, histological analysis showed that the BPH/vehicle group had more epithelial nodules than the control group. In the BPH/vehicle group, the thickness of the prostate epithelial layer increased by about 3 times compared to the sham-operation control group, and the lumen area decreased by more than 50%. As we expected, finasteride treatment successfully reduced BPH phenotypes. Notably, we also observed the reduction of epithelial thickness and increase of lumen area in the BPH/*R. vomitoria* group; this effect was greater in the BPH/*R. vomitoria* group than in the BPH/finasteride group ($P < 0.001$, Fig. 2A–C). Furthermore, we performed immunohistochemistry staining to detect the level of a proliferative biomarker, PCNA. Compared with the sham-operation control group, the percentage of PCNA-positive cells in the prostate of the BPH/vehicle group nearly doubled, but this change was significantly reversed by the *R. vomitoria* extract (Fig. 2D and E). In addition, the immunoblotting assay demonstrated that another proliferative marker, cyclin D1, which increased in the BPH/vehicle group compared to the sham-operation control, decreased in BPH/*R. vomitoria* and BPH/finasteride groups (Fig. 2F and G). These results clearly demonstrate that *R. vomitoria* extract can restrict the proliferation of prostate epithelial cells in the rats with testosterone propionate-induced BPH.

3.3. R. vomitoria extract inhibits androgen receptor and its associated protein levels in BPH rat model

Androgen receptor, which plays an essential role in prostate development, was examined by immunohistochemistry staining. As shown in Fig. 3A and B, nuclear androgen receptor levels in prostate epithelial cells nearly doubled in the BPH/vehicle group, compared to the sham-operation group, while BPH/finasteride and BPH/*R. vomitoria* groups reduced nuclear androgen receptor levels to the level present in the sham-operation group. Consistently, the steroid 5α-reductase 1 and androgen receptor were elevated by testosterone propionate treatment in the BPH/vehicle group (Fig. 3C–E). We also detected the mRNA levels of two well-known androgen receptor target genes, *FKBP5* and *MMP2*. As shown in Fig. 3F and G, the levels of these two androgen receptor target genes increased strikingly in the prostate glands of rats with testosterone propionate-induced BPH. However, such increases were significantly reversed by treatment with *R. vomitoria* extract and finasteride (Fig. 3C–G). Overall, *R. vomitoria* extract was able to restrict the androgen receptor-associated pathway in the prostate epithelial cells in BPH model.

3.4. Effect of R. vomitoria on epididymal sperm numbers

Reduced sperm count is one of the serious side effects of finasteride and has been observed in finasteride-treated rats and human patients [17]. To examine whether *R. vomitoria* extract has milder side effects than finasteride, we evaluated the sperm count in the

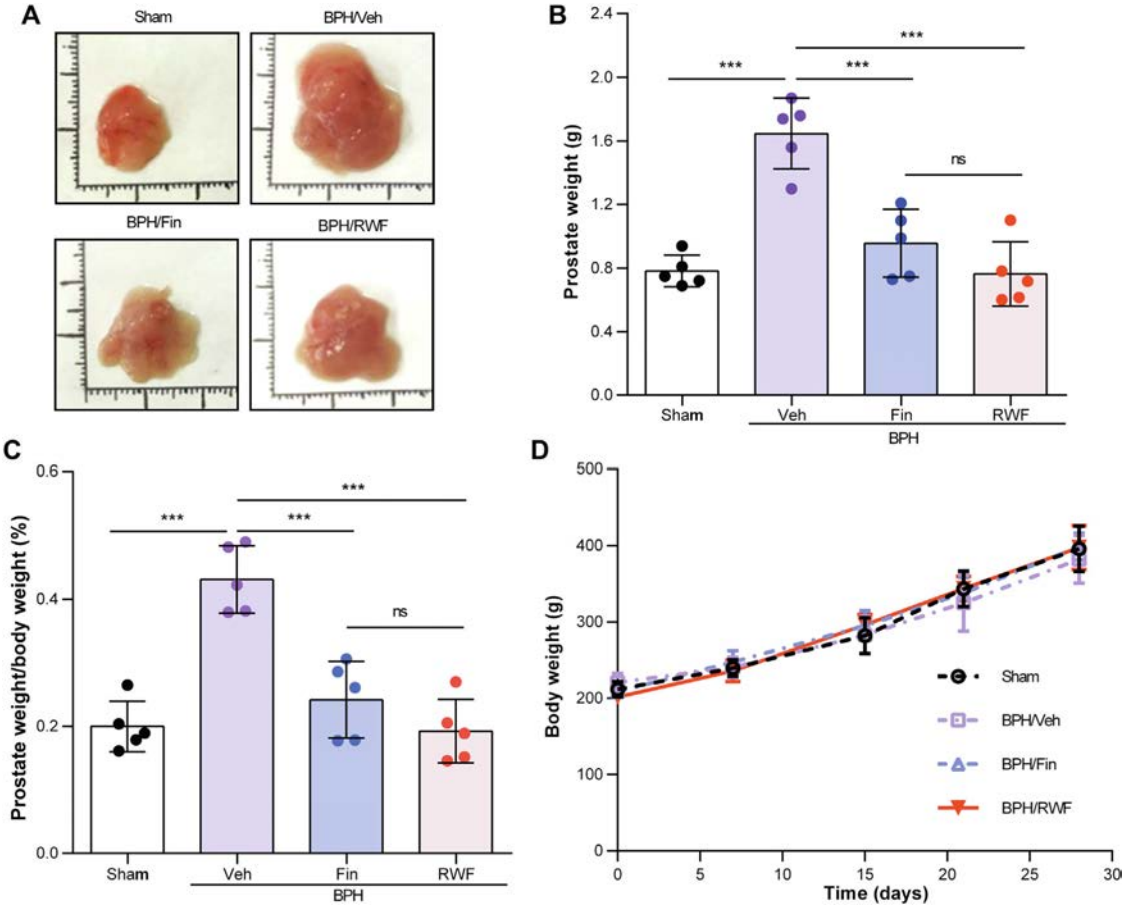

Fig. 1. Inhibition effects of *Rauwolfia vomitoria* extract on testosterone propionate-induced BPH rat model. After the model was established, rats were treated with Veh (BPH/Veh), 5 mg/kg Fin (BPH/Fin) and 20 mg/kg *R. vomitoria* extract (BPH/RWF). The sham-operated rats were used as control. (A) The dissection of prostates. (B) The total prostate weight of the rats. (C) The prostate weight index of the rats in four groups. (D) The body weights of four groups within 28-day treatment. ***$P < 0.001$; ns: no significant difference. BPH: benign prostatic hyperplasia; Fin: finasteride; RWF: *Rauwolfia vomitoria*; Veh: vehicle.

epididymis of *R. vomitoria*-, finasteride- and vehicle-treated rats. As shown in Fig. 4, finasteride treatment resulted in a significant reduction in sperm count, compared to untreated rats, while *R. vomitoria* treatment did not significantly change sperm counts.

4. Discussion

Phytomedicine is one of the important therapeutic options for BPH, which is characterized as the hyperproliferation of prostate cells. Derived from a shrub from tropical African forests, *R. vomitoria* extract is widely used as a health supplement and contains bioactive β-carboline and indole alkaloids [22,23]. Previous studies revealed its anti-tumor effects through inhibiting survival and proliferation of cancer cells [18,25]. In the present study, we found that *R. vomitoria* extract can inhibit BPH in a rat model by reducing the proliferation of prostate epithelial cells. Further, the proliferation index of prostate epithelial cells, as scored by two proliferative markers, PCNA and cyclin D1, was reduced. *R. vomitoria* treatment also restored the larger lumen size of BPH tissues.

Currently it is believed that the development of BPH is related to the accumulation of dihydrotestosterone in the prostate gland, and the presence of functional testicles is a necessary condition for the occurrence of prostatic hyperplasia [26,27]. When entering prostate cells, testosterone is usually converted by 5α-reductase in the microsomes into dihydrotestosterone, which is 2–3 times more active than testosterone [7]. Dihydrotestosterone binds to androgen receptor, promotes its activity and increases the levels of androgen receptor target genes (e.g., *FKBP5* and *MMP2*) and proliferation associated genes, such as *cyclin D1*.

Steroid 5α-reductase 1 and androgen receptor are both indicators that help to determine the severity of BPH and the degree of malignancy in prostate cancer. In particular, effects on steroid 5α-reductase 1 have gradually become a key therapeutic indicator for the development of new BPH drugs in recent years [26]. Previous studies have shown that alkaloids and phenolic compound-enriched plant extracts such as *Adina rubella* and *Pao pereira* have excellent therapeutic effects on BPH through their suppression of steroid 5α-reductase 1 [24,27,28]. In this study, *R. vomitoria* extract, rich in bioactive β-carboline and indole alkaloids [22,23], was able to strongly inhibit the induction of steroid 5α-reductase 1 levels and downstream the androgen receptor signaling pathway in the BPH/vehicle group. Recent study has shown that *R. vomitoria* contains high levels of monoterpene indole alkaloids, such as ajmaline, serpentine and yohimbine [29]. Among them, yohimbine has been reported to reduce nuclear androgen receptor by 47% in the preoptic area of intact male rat, as well as castrated, testosterone-treated male rats [30]. The competitive binding of yohimbine with R1881 (methyltrienolone) to androgen receptor was ruled out, suggesting yohimbine may function through other mechanisms to regulate androgen receptor activity. Future research should identify the key components in *R. vomitoria* and dissect the mechanism by which it regulates the androgen receptor pathway.

In addition to 5α-reductases, the inhibition of type 5 phosphodiesterase is another important therapeutic target to alleviate

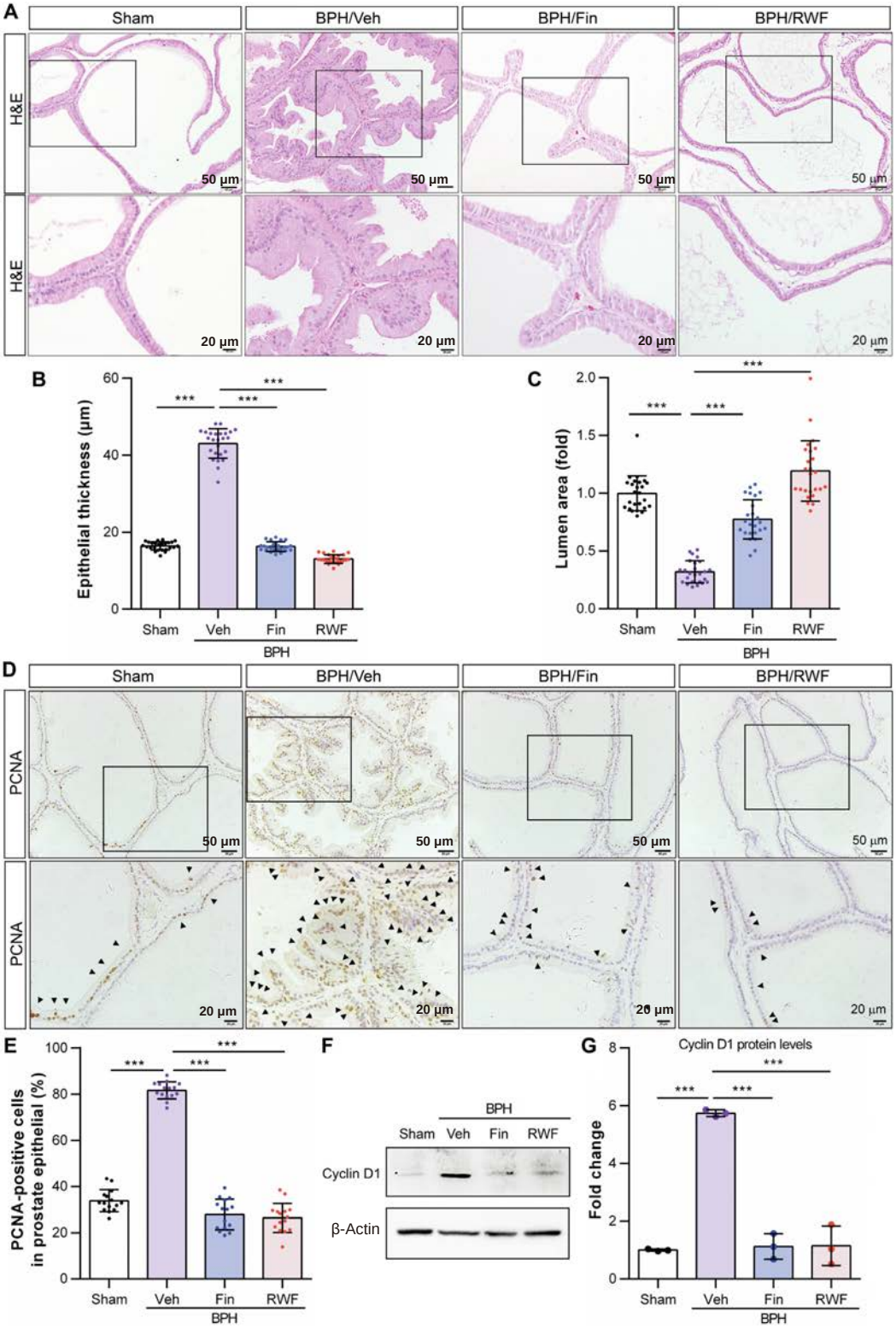

Fig. 2. The treatment of *Rauwolfia vomitoria* extract reduced the proliferation of prostate epithelial cells. (A) Representative H&E staining of the prostate tissue. (B and C) Quantification of the fold changes of thickness of epithelial layers (B) and lumen areas (C) in each rat (n = 5). (D and E) Representative PCNA immunohistochemistry staining (D) and quantification of percentage of PCNA-positive staining cells (arrowheads, E) in rat ventral prostate (n = 5). (F and G) The immunoblotting of cyclin D1 protein level in rat prostate tissues (F) and their quantification results (G) from three independent experiments. The values were presented as mean ± standard deviation. ***P < 0.001. BPH: benign prostatic hyperplasia; Veh: vehicle; Fin: finasteride; RWF: *Rauwolfia vomitoria*; H&E: Hematoxylin and eosin staining; PCNA: proliferating cell nuclear antigen.

lower urinary tract symptoms, which are associated with BPH. Type 5 phosphodiesterase is reported to be overexpressed in the prostate stroma of BPH samples, compared with its weak expression in the epithelial and stromal compartments of normal human prostates [31]. The inhibition of type 5 phosphodiesterase by tadalafil or sildenafil mainly relieves smooth muscle cells and reduces

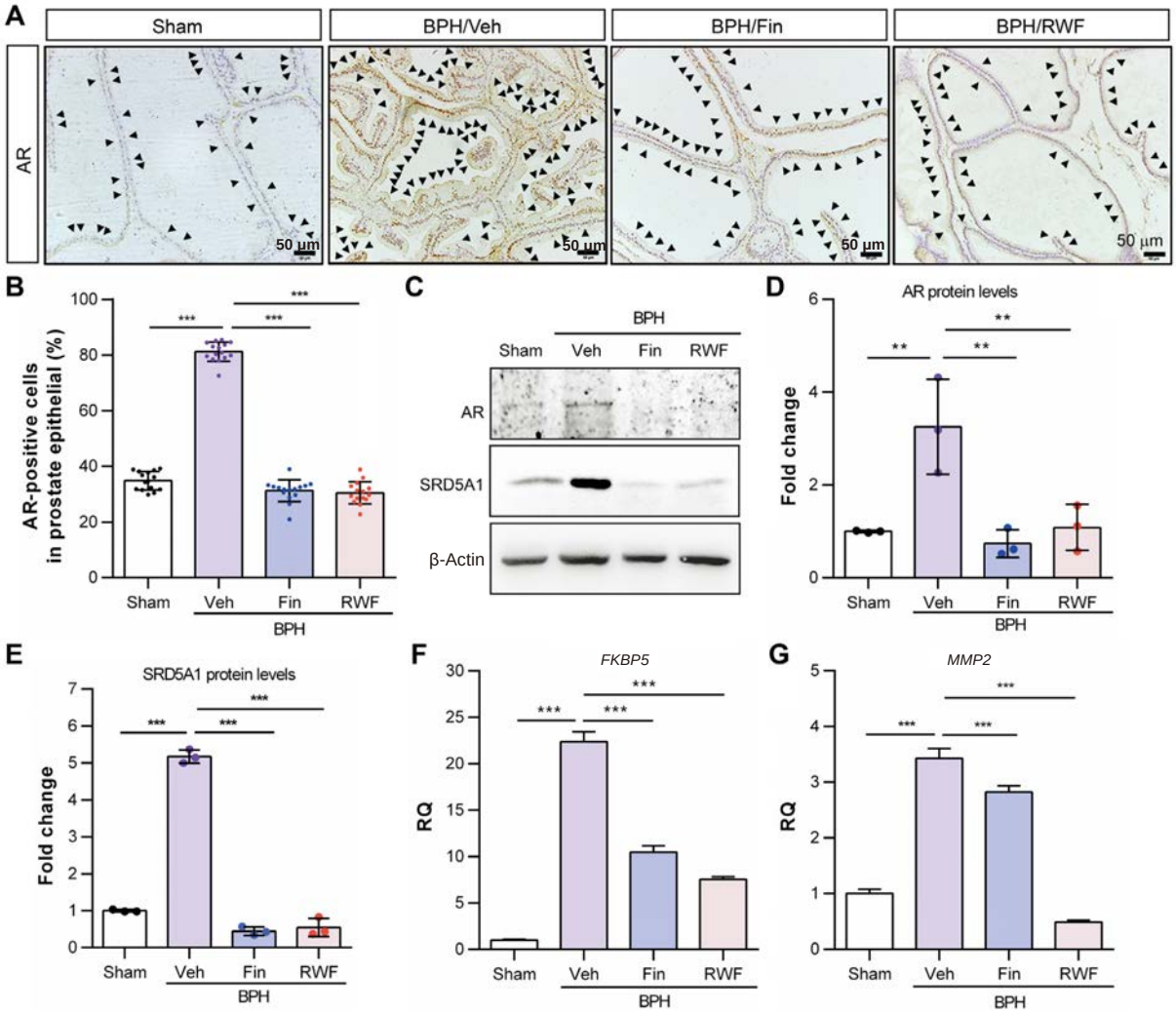

Fig. 3. *Rauwolfia vomitoria* extract inhibited the expression levels of AR, SRD5A1 and AR target genes in rat ventral prostates. (A and B) The representative immunohistochemistry staining on AR protein and quantification of percentage of AR-positive staining cells (arrowheads, B) in prostate epithelial cells of rats (n = 5) from each group. (C–E) The immunoblotting analysis of AR and SRD5A1 protein expression (C) and quantification of AR (D) and SRD5A1 (E) levels in rat prostate tissues (n = 3). (F and G) Quantification of the mRNA levels of two AR target genes *FKBP5* (F) and *MMP2* (G) by quantitative real-time polymerase chain reaction. Reactions were performed in triplicate. The values are presented as the mean ± standard deviation. $^{**}P < 0.01$; $^{***}P < 0.001$. AR: androgen receptor; BPH: benign prostatic hyperplasia; Fin: finasteride; RWF: *Rauwolfia vomitoria*; SRD5A1: steroid 5α-reductase 1; RQ: relative quantitation; Veh: vehicle.

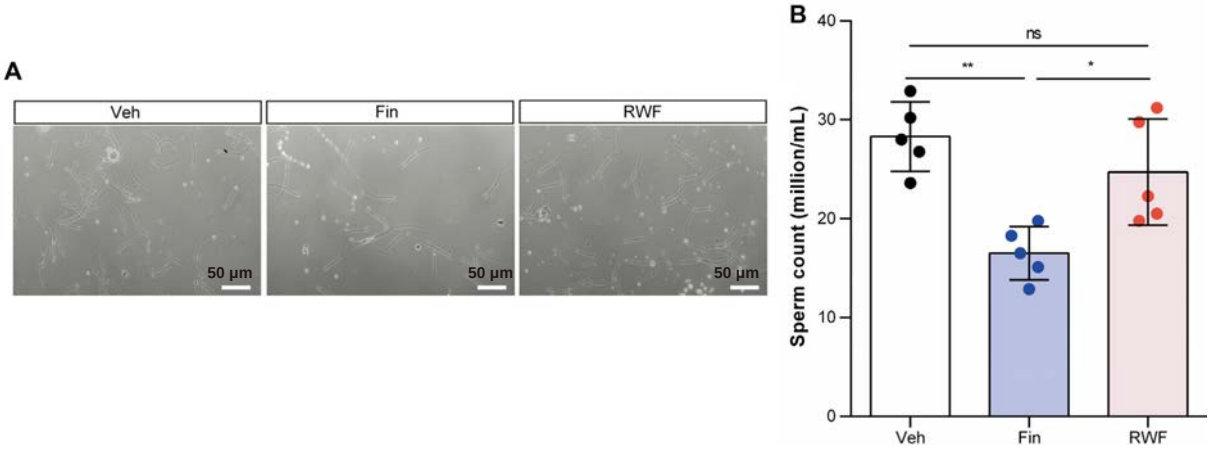

Fig. 4. *Rauwolfia vomitoria* extract did not reduce sperm number in rats. (A) The sperm numbers were evaluated after daily administration with Veh, Fin (5 mg/kg) or *R. vomitoria* extract (20 mg/kg) for 4 weeks. (B) The quantification of sperm numbers in three groups. Results are presented as mean ± standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; ns: no significant difference. Fin: finasteride; RWF: *Rauwolfia vomitoria*; Veh: vehicle.

the contractility of the prostate gland [32]. Interestingly, a recent study revealed that *R. vomitoria* extract possesses an inhibitory effect on type 5 phosphodiesterase activity *in vitro* [33]. Further study on the detailed mechanisms how *R. vomitoria* extract can attenuate BPH development is needed.

5. Conclusions

Considering that *R. vomitoria* extract possesses many functions to treat cancer, neurological conditions, such as anxiety and insomnia, and obesity-related conditions, such as diabetes [25,34–36], our study provided a new piece of evidence, showing it as a potential phytomedicine for BPH patients. Given that *R. vomitoria* extract possesses similar effects on BPH, but less toxicity to sperm development, compared to finasteride, we believe that it may be an equally effective and relatively safer treatment for BPH. Further studies are warranted to examine the molecular mechanisms underlying the inhibitory effects of *R. vomitoria* extract on BPH.

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Authors' contributions

TF, ZSX, JXL and JKL contributed equally to this work: JY and SFY developed the hypotheses and experiments; TF performed animal treatment studies; ZSX and JXL performed histological and immunohistochemical analyses and JKL performed Western blotting assays. TF, ZSX, JXL and JKL performed statistical analysis, wrote the initial manuscript and were responsible for data collection, and preparation of the tables and figures. MQL and YTS participated in Western blotting. DW participated in data analysis. All authors participated in manuscript review. JY finished and approved the final version of the manuscript.

Declaration of competing 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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