Saudi Pharmaceutical Journal 31 (2023) 1219–1228

HOSTED BY

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

# Neferine attenuates development of testosterone-induced benign prostatic hyperplasia in mice by regulating androgen and TGF-β/Smad signaling pathways

Chi-Ming Liu [a,*,1], ZiChen Shao [a,b,1], XuZhou Chen [a], HanWu Chen [a], MengQiao Su [a,b], ZiWen Zhang [a], ZhengPing Wu [c], Peng Zhang [a], LiJie An [a,b], YinJie Jiang [a], Ai-Jun Ouyang [d]

[a] School of Medicine, Yichun University, 576 XueFu Road, Yuanzhou District, Yichun 336000, Jiangxi Province, China
[b] College of Chemistry and Bio-engineering, Yichun University, 576 XueFu Road, Yuanzhou District, Yichun 336000, Jiangxi Province, China
[c] School of Aesthetic Medicine, Yichun University, 576 XueFu Road, Yuanzhou District, Yichun 336000, Jiangxi Province, China
[d] Department of Pharmacy, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Nanchang 330006, Jiangxi Province, China

ARTICLE INFO

*Article history:*
Received 18 February 2023
Accepted 6 May 2023
Available online 11 May 2023

*Keywords:*
Benign prostatic hyperplasia (BPH)
Androgen receptor (AR)
TGF-β/Smad
Neferine
Epithelial-mesenchymal transition (EMT)
Apoptosis

ABSTRACT

Benign prostatic hyperplasia (BPH) is a common urinary disease among the elderly, characterized by abnormal prostatic cell proliferation. Neferine is a dibenzyl isoquinoline alkaloid extracted from *Nelumbo nucifera* and has antioxidant, anti-inflammatory and anti-prostate cancer effects. The beneficial therapeutic effects and mechanism of action of neferine in BPH remain unclear.

A mouse model of BPH was generated by subcutaneous injection of 7.5 mg/kg testosterone propionate (TP) and 2 or 5 mg/kg neferine was given orally for 14 or 28 days. Pathological and morphological characteristics were evaluated. Prostate weight, prostate index (prostate/body weight ratio), expression of type Ⅱ 5α-reductase, androgen receptor (AR) and prostate specific antigen were all decreased in prostate tissue of BPH mice after administration of neferine. Neferine also downregulated the expression of procaspase-3, uncleaved PARP, TGF-β1, TGF-β receptor Ⅱ (TGFBR2), p-Smad2/3, N-cadherin and vimentin. Expression of E-cadherin, cleaved PARP and cleaved caspase-3 was increased by neferine treatment.

1–100 μM neferine with 1 μM testosterone or 10 nM TGF-β1 were added to the culture medium of the normal human prostate stroma cell line, WPMY-1, for 24 h or 48 h. Neferine inhibited cell growth and production of reactive oxygen species (ROS) in testosterone-treated WPMY-1 cells and regulated the expression of androgen signaling pathway proteins and those related to epithelial-mesenchymal transition (EMT). Moreover, TGF-β1, TGFBR2 and p-Smad2/3, N-cadherin and vimentin expression were increased but E-cadherin was decreased after 24 h TGF-β1 treatment in WPMY-1 cells. Neferine reversed the effects of TGF-β1 treatment in WPMY-1 cells. Neferine appeared to suppress prostate growth by regulating the EMT, AR and TGF-β/Smad signaling pathways in the prostate and is suggested as a potential agent for BPH treatment.



## 1. Introduction

Benign prostatic hyperplasia (BPH) is a common urinary condition in middle-aged and elderly men (Paniagua Cruz et al., 2020). Lower urinary tract symptoms (LUTS), including dysuria, urinary tract infection and occasional complications, such as obstructive nephropathy and impaired renal function are seen (Parsons et al., 2020). BPH progression depends on high androgen levels (Rastrelli et al., 2019). Testosterone (T) is converted into dihydrotestosterone (DHT) by 5α-reductase and acts on the prostate androgen receptor. On DHT binding, AR enters the nucleus and regulates gene expression (Heinlein and Chang, 2002; Roy and Chatterjee, 1995). Prostate specific antigen (PSA) is a marker of BPH, regulated by the androgen receptor (AR). BPH medications include α-adrenoceptor blockers and 5α-reductase inhibitors which reduce prostate volume and relieve LUTS.

The epithelial-mesenchymal transition (EMT) is involved in embryogenesis, cancer metastasis, fibrosis and BPH (Bakir et al., 2020; Lu et al., 2012; Marconi et al., 2021). Epithelial cells lose their polarity during the EMT, cell–cell adhesion is disrupted and a mesenchymal phenotype is acquired (Micalizzi et al., 2010) with

* Corresponding author at: School of Medicine, Yichun University, 576 XueFu Road, Yuanzhou District, Yichun 336000, Jiangxi Province, China.
*E-mail address:* beagleliu@gmail.com (C.-M. Liu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jsps.2023.05.004
1319-0164/

reduction of the epithelial marker, E-cadherin, and increase in the mesenchymal markers, N-cadherin and vimentin (Wang et al., 2021a). Reversing the EMT has been suggested as a BPH treatment target. The cytokine, TGF-β, participates in cell proliferation, differentiation, apoptosis, migration and the EMT (Larson et al., 2020; Wang et al., 2021b) and is highly expressed in BPH patients, causing tissue inflammation and angiogenesis (Afdal et al., 2019). Exogenous TGF-β induced migration, EMT of prostatic epithelial cells and promoted the proliferation of prostatic stromal cells (Huang and Lee, 2003; Zhu and Kyprianou, 2005).

Chinese traditional medicine encompasses plant phytochemicals with various pharmacological activities against disease. Neferine is a bisbenzylisoquinoline alkaloid extracted from *Nelumbo nucifera* Gaerth (Lotus) and is used both as food and Traditional Chinese Medicine. Neferine has anti-cancer (Manogaran et al., 2022), antioxidant (Qi et al., 2021), anti-diabetic (Marthandam Asokan et al., 2018) and anti-inflammatory (Chiu et al., 2021) activities. Neferine, liensinine and isoliensinine inhibited prostate cancer growth by down-regulating the expression of AR, PSA and type II 5α-reductase in prostate cancer (Liu et al., 2021). However, the therapeutic benefits and mechanism of action of neferine on BPH remain unclear.

The present study examined the effects of neferine on BPH development *in vivo* and *in vitro* and its impact on the EMT and TGF-β/Smad signaling pathway. The aim was to clarify the effects of neferine in BPH to prevent disease development and progression.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Neferine of 95–99% purity (cat. no. BP0987) was purchased from Chengdu Biopurify Phytochemicals, Ltd (Chengdu, China) and finasteride (cat. no. F156753), testosterone (cat. no. T102169) and testosterone propionate (cat. no. T101368) from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). PSA ELISA kits (cat. no. JM-02752 M1) were purchased from Jiangsu Jingmei Biotechnology Co., Ltd (Jiangsu, China). Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% fetal bovine serum (FBS), both from Cellmax (Beijing, China), and penicillin (100 U/ml)-streptomycin (100 μg/ml) from Beyotime Institute of Biotechnology (Haimen, China). Reactive oxygen species assay kit (cat. no. BL714A) was obtained from Biosharp (Hefei, Anhui, China). Recombinant Human TGF-β1 (cat. no. P00121) was purchased from Solarbio (Beijing, China); antibodies against β actin from MilliporeSigma (cat. no. A5441; Massachusetts, USA); against androgen receptor (AR; cat. no. CY5030), TGF-β1 (cat. no. CY2179) and 5α-reductase type Ⅱ (cat. no. CY8576) from Abways Technology (Shanghai, China). Primary antibodies against PSA (cat. no. ab76113) were purchased from Abcam (Shanghai, China); against E-cadherin (cat. no. 3195), N-cadherin (cat. no. 13116), vimentin (cat. no. 5741), PARP (cat. no. 9542), caspase-3 (cat. no. 14220), cleaved caspase-3 (cat. no. 9664) and cleaved PARP (cat. no. 5625) from Cell signaling technology (Shanghai, China). Antibodies against TGFBR2 (cat. no. AF5449), Phospho-Smad2/3 antibody (cat. no. AF3367) and Smad2/3 (cat. no. AF6367) were purchased from Affinity Biosciences LTD (Shanghai, China). Anti-rabbit IgG HRP-linked antibody (cat. no. 7074) and anti-mouse IgG HRP-linked antibody (cat. no. 7076) were purchased from Cell Signaling Technology.

### 2.2. Animal experiments

7-week-old male Institute of Cancer Research (ICR) mice (Hunnan SJA Laboratory Animal Co., Ltd.) were acclimatized for 7 days prior to experimentation at 22 ± 2 °C with a 12-h light/12 h dark cycle. All experiments were approved and conducted according to the guidelines of the Animal Care and Ethics Committee of Yichun University (Approval No. 2022006). A dose of 7.5 mg/kg testosterone propionate was given to induce BPH (Gu et al., 2021; Huang et al., 2017; Solanki et al., 2021; Zou et al., 2017). A previous study indicated that neferine at 1 and 10 mg/kg has vasorelaxant and antihypertensive effects and intermediate doses of 2 and 5 mg/kg were selected for the current study (Wicha et al., 2020). Mice were randomly divided into five groups: (A) control (vehicle, n = 6); (B) testosterone propionate model (TP, 7.5 mg/kg, n = 6); (C) TP + neferine (2 mg/kg, n = 6) (D) TP + neferine (5 mg/kg, n = 6) (E) TP + finasteride (10 mg/kg, n = 6). The BPH model was induced by subcutaneously injecting 7.5 mg/kg testosterone propionate (TP) dissolved in corn oil. Neferine and finasteride were dissolved in corn oil and given orally once a day. Treatment was given for 14 or 28 days and the weights of mice were measured weekly. Mice were sacrificed at 14 or 28 days and the prostate/body weight ratio was calculated to give the prostate index. Prostate index = prostate wight of mice (g)/ body weight of mince (g) × 1000. Serum was collected for determination of PSA concentration by ELISA kit, according to the manufacturer's instructions.

### 2.3. Histopathological examination

Prostatic specimens were fixed in 10% formalin overnight, embedded in paraffin and sliced into 4 μm sections. Slides were stained with hematoxylin and eosin (H&E) solutions and morphology was examined under a 10x40 inverted microscope (Nikon TI DH).

### 2.4. Cell lines and cell culture

Immortalized normal prostate stromal cells (WPMY-1) were purchased from Shanghai Fuheng Biotechnology Co., Ltd. (Shanghai, China) and cultured in DMEM media supplemented with penicillin (100 U/ml)-streptomycin (100 μg/ml) and 10% FBS at 37C in a humidified atmosphere with 5% $CO_2$.

### 2.5. Determination of cell survival rate by MTT assay

WPMY-1 cells were plated in 96-well plates at a density of $1x10^4$ cells per well and allowed to reach 80% confluency before treatment with neferine (10, 100 μM) or finasteride (10, 100 μM) with/without 1 μM testosterone for 24 h or 48 h. 10 μl of 5 mg/ml MTT was added to the 100 μl well volume and incubated for 4 h at 37 °C. Medium was discarded and 100 μl/ well DMSO was added to dissolve formazan crystals with incubation at room temperature for 20 min. Absorbance was measured at 570 nm with a BIO-RAD microplate reader.

### 2.6. Determination of reactive oxygen species content

WPMY-1 cells were inoculated into a 6-well plate at a density of $1x10^5$ cells/well and incubated at 37 °C in a 5% $CO_2$ incubator for 48 h. Cells were treated with 1 or 10 μM neferine with or without 1 μM testosterone and incubated at 37C for 24 h before fixing with 10 μM H2DCFH-DA in the dark and washing twice with serum free medium. Cells were re-suspended in PBS and reactive oxygen species (ROS) were analyzed by flow cytometry (NovoCyte Flow Cytometer, Santa Clara, USA). NovoExpress software was used to interpret the data.

### 2.7. Western blot analysis

Cells were extracted by M-PER and prostate tissues by T-PER (Thermo Fisher Scientific, Inc.). Protein was quantified by Pierce Coomassie Protein Assay Kit (Thermo Scientific, Inc.) and 20 μg loaded per lane, separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with protein-free blocking solution (Shanghai epizyme Biotechnology Co., Ltd.) at room temperature for 30 min and incubated with primary antibodies raised against the following proteins: AR (1:1000), PSA (1:1000), Type Ⅱ 5α-reductase (1:1000), E-cadherin (1:1000), N-cadherin (1:1000), vimentin (1:1000), β-actin (1:8000), PARP (1:1000), caspase-3 (1:1000), cleaved caspase-3 (1:1000), cleaved PARP (1:1000), Smad2/3 (1:1000), p-Smad2/3 (1:1000), TGF-β1 (1:1000) or TGFBR2 (1:1000) at 4 °C overnight. Membranes were washed and incubated with secondary antibodies, anti-rabbit IgG HRP-linked antibody (1:1000) and anti-rabbit IgG HRP-linked antibody (1:1000) and anti-mouse IgG HRP-linked antibody (1:1,000) at room temperature for 1 h before detection of chemiluminescence by ultra-sensitive kit (ECL, Biosharp). Densitometry was performed by ImageJ 1.52a software (National Institutes of Health, USA).

### 2.8. Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). One-way analysis of variance was used to compare differences among more than two groups followed by Tukey's post hoc test. A p-value of<0.05 ($p < 0.05$) was considered to indicate statistical significance.

## 3. Results

### 3.1. Neferine attenuated prostatic hyperplasia in TP-induced BPH mice

Prostates were dissected (Fig. 1) and weights and prostate index (PI) were measured (Fig. 2A-D). TP treatment increased prostate weight, PI and serum PSA levels relative to the solvent control but these changes were reversed in a dose-dependent manner by treatment with 2 or 5 mg/kg neferine. Finasteride was used as a positive control and treatment with 5 mg/kg neferine vs 10 mg/kg finasteride attenuated the increase in prostate weight by 95.8% vs 80.0%, PI by 91.6% vs 63.7% and PSA by 99.9% vs 62.4% at 28 days. H&E staining revealed increased prostatic epithelial height and basal membrane folding in BPH mice compared with controls but daily treatment with 2 or 5 mg/kg neferine or 10 mg/kg finasteride improved the morphological changes (Fig. 3). Neferine ameliorated the changes to the histological structure, prostate weight and PI produced by BPH.

### 3.2. Effects of neferine on viability of WPMY-1 cells

1 μM testosterone was used in the current study as in several previous studies (Baek et al., 2022; Chen et al., 2021; Hong et al., 2020; Karunasagara et al., 2020). Cell survival rates of neferine or finasteride treated WPMY-1 cells decreased relative to controls in both the presence and absence of 1 μM testosterone (Fig. 4). Cell survival rates with 100 μM neferine were 31.98 ± 7.54 % of control rates and with 100 μM finasteride, 71.76 ± 2.01 %, in the absence of testosterone at 48 h (Fig. 4A, 4B). Moreover, the survival rates with neferine (1–10 μM) and finasteride (1–10 μM) treatments were also examined in the absence of testosterone at 24 h and 48 h (Fig. 4A, 4B). Testosterone increased the growth of WPMY-1 cells at 48 h. Neferine (10–100 μM) inhibited growth of WPMY-1 cells at 24 h and 48 h in the presence of testosterone. Cell survival rate after 100 μM neferine treatment was 15.10 ± 3.43 % of control rate

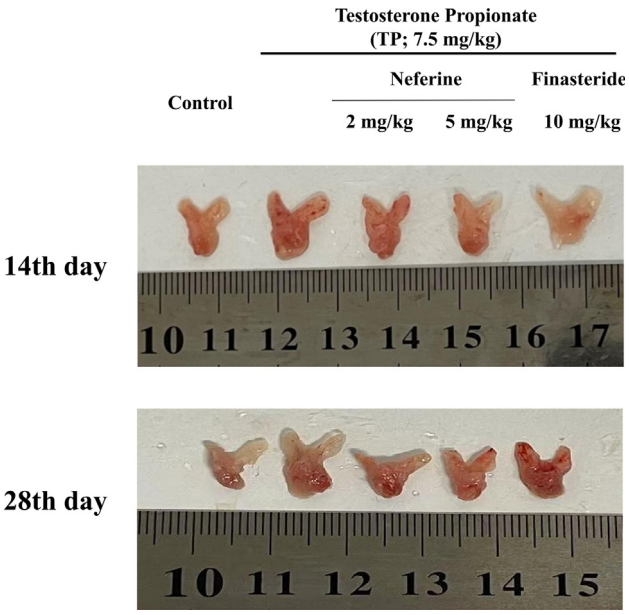


**Fig. 1.** Effects of neferine (2, 5 mg/kg) and finasteride (10 mg/kg) on prostate in testosterone propionate (7.5 mg/kg)-induced BPH mouse model at 14th and 28th days.

in the presence of testosterone at 48 h. Neferine thus antagonized the proliferative effect of testosterone on WPMY-1 cells (Fig. 4C, 4D).

### 3.3. Neferine inhibited testosterone-stimulation of the androgen receptor signaling pathway

Expression of type Ⅱ 5α-reductase, AR and PSA were measured *in vivo* and *in vitro*. Treatment with neferine (2, 5 mg/kg) or finasteride (10 mg/kg) inhibited expression of type Ⅱ 5α-reductase, AR and PSA in TP-induced BPH mice and expression of all three proteins was also inhibited by 24 h treatment of WPMY-1 cells with testosterone plus neferine or finasteride (Fig. 5A-F). The mechanism by which neferine regulates prostate growth may involve the downregulation of the AR signaling pathway.

### 3.4. Effects of neferine on apoptosis-related protein expression in TP-induced BPH mice

There have been previous reports of anti-apoptotic effects in the hyperplastic prostate. Expression of the apoptotic markers, pro-caspase-3 and uncleaved PARP, were upregulated in TP-induced BPH mice (Fig. 6A-B). Pro-caspase-3 and uncleaved PARP were decreased in neferine and finasteride treatment groups. However, cleaved caspase-3 and cleaved PARP were induced after neferine and finasteride treatment (Fig. 6A-B). These results indicate that neferine stimulates apoptosis in TP-induced BPH mice.

### 3.5. Neferine inhibited benign prostatic hyperplasia by regulating TGF-β/Smad signaling pathway and the EMT

The progression of BPH is androgen-dependent and TGF-β is known to interact with the hormone to promote the EMT and fibrosis in the prostate (Alonso-Magdalena et al., 2009). TGF-β1, TGFBR2 and p-Smad2/3 were upregulated in TP-induced BPH mouse prostate, as were the EMT-associated mesenchymal markers, N-cadherin and vimentin, while expression of the epithelial maker, E-cadherin, was decreased (Fig. 7A, 7C). The same changes were observed in 24 h testosterone-treated WPMY-1 cells (Fig. 7B,

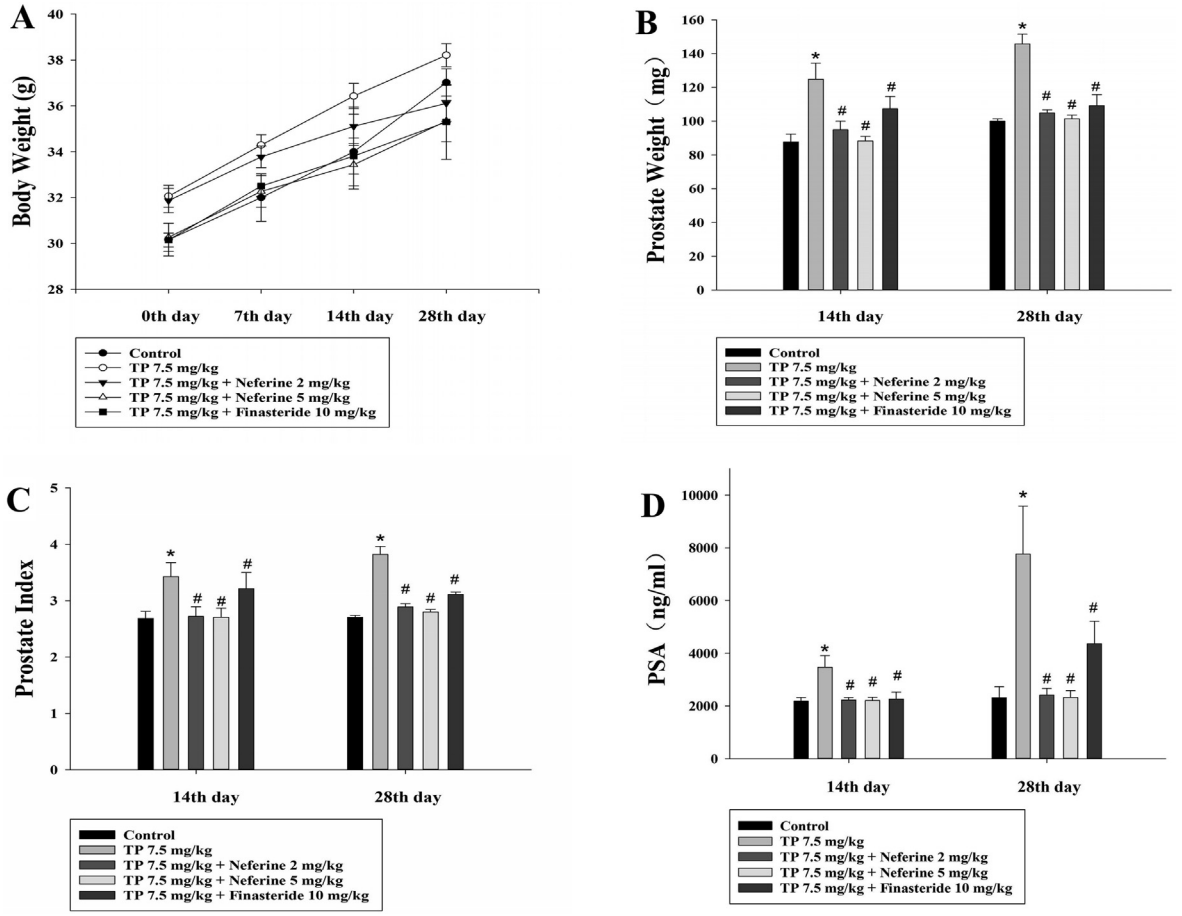


**Fig. 2.** Effects of neferine (2, 5 mg/kg) and finasteride (10 mg/kg) on the body weight (A), prostate weight (B), prostate index (C) and serum PSA level (D) in testosterone propionate (TP; 7.5 mg/kg)-induced BPH mouse model at 14th and 28th days. Data are presented as mean ± SEM (n = 6) ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with TP group).

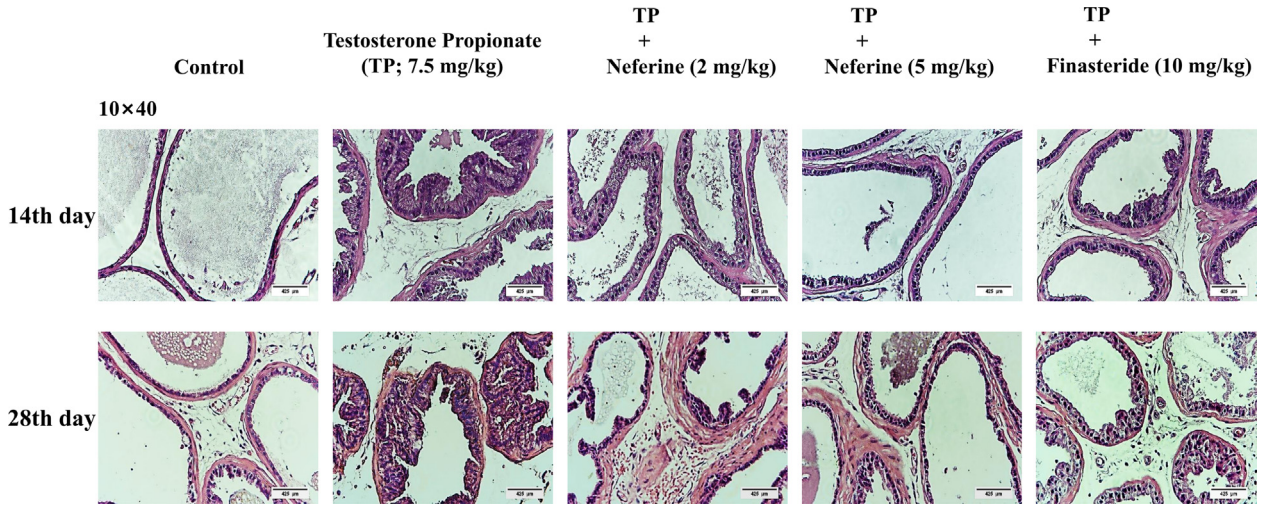


**Fig. 3.** The effects of neferine on prostate histology by H&E staining.

7D). Neferine and finasteride treatments reversed these effects both *in vivo* and *in vitro*. TGF-β1 promoted the EMT by regulating E-cadherin, N-cadherin and vimentin expression in WPMY-1 cells, effects which were prevented by treatment of the cells with neferine and finasteride (Fig. 8A-B). In conclusion, testosterone and TGF-β1 affect the EMT and prostate growth and neferine inhibited benign prostatic hyperplasia by regulating TGF-β/Smad signaling pathway and the EMT.

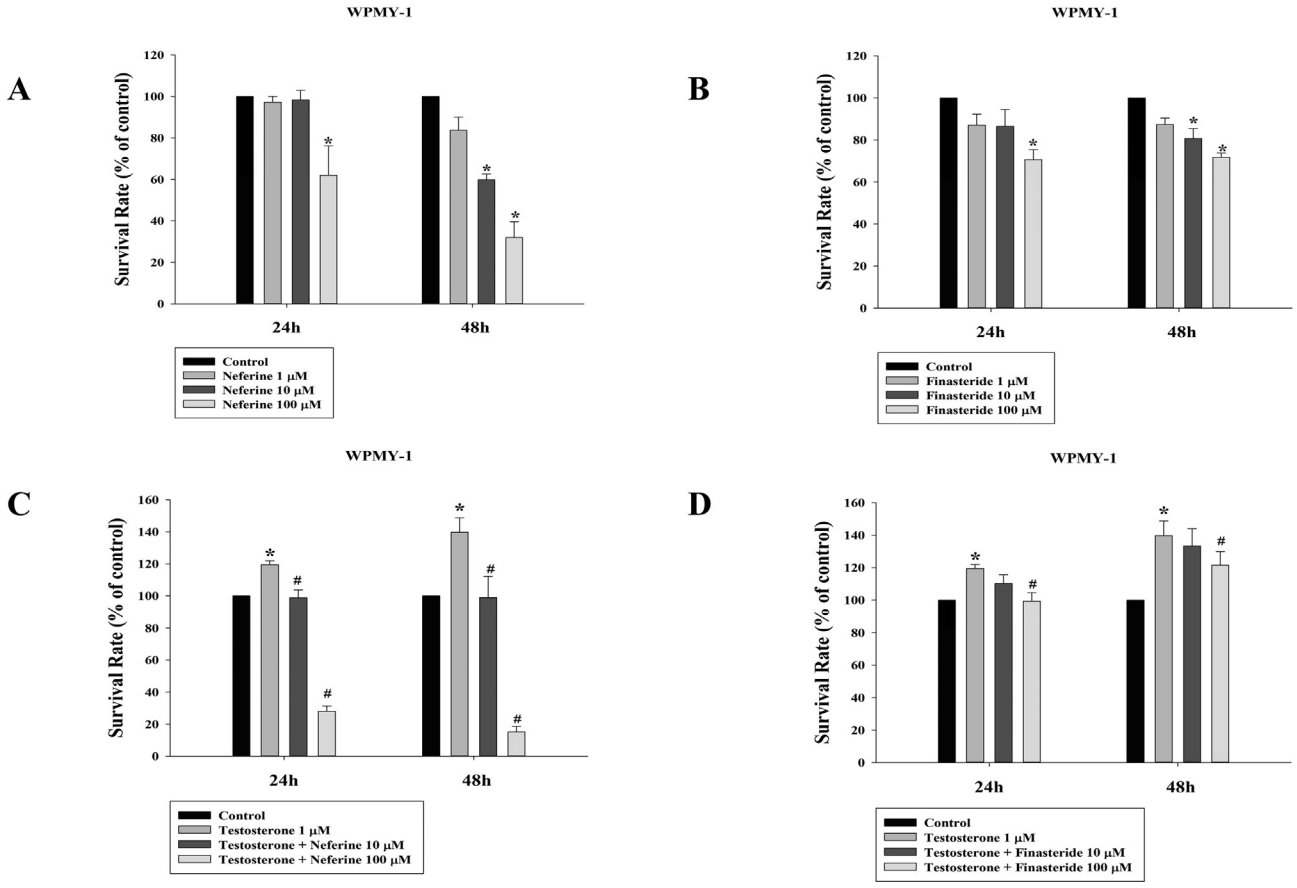


**Fig. 4.** Neferine and finasteride inhibited testosterone-stimulated growth in WPMY-1 cells. WPMY-1 cells were incubated with neferine (1–100 μM) or finasteride (1–100 μM) and testosterone (1 μM) for 24 h and 48 h. All values are presented as mean ± SEM for three separate experiments. ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with testosterone group).

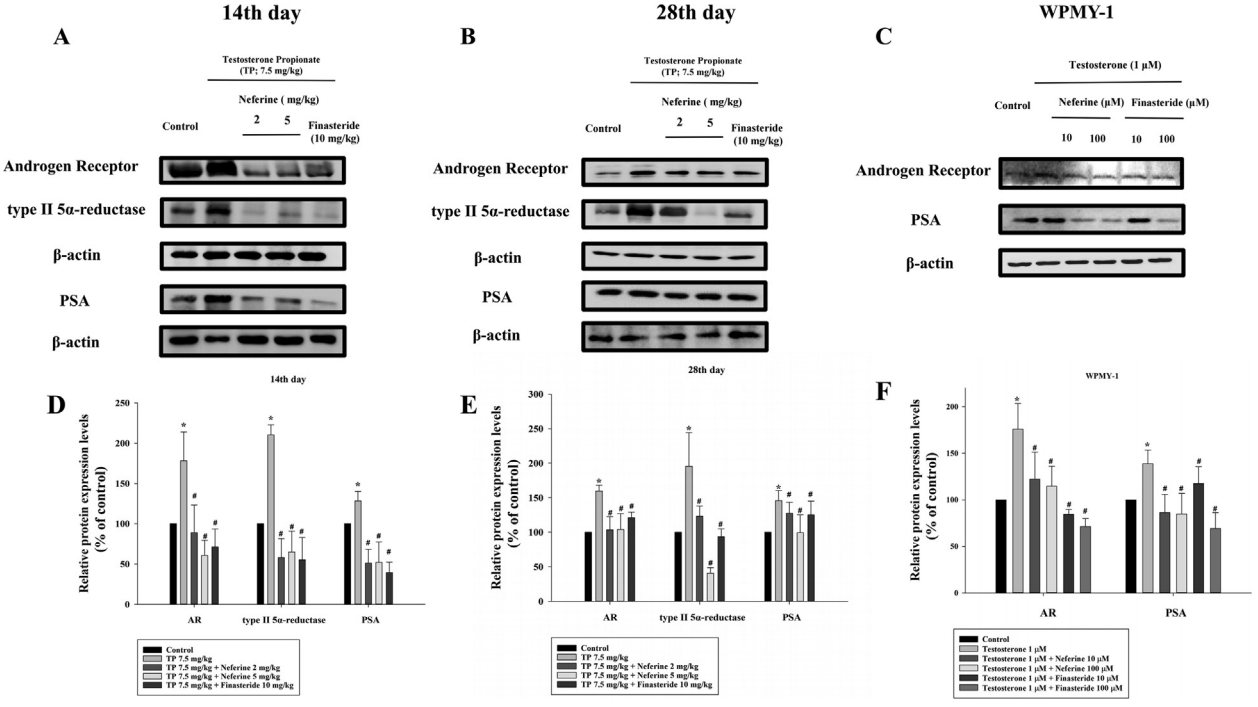


**Fig. 5.** Effects of neferine (2, 5 mg/kg; 10–100 μM) and finasteride (10 mg/kg; 10–100 μM) on the expression of 5α-reductase and androgen receptor signaling pathway proteins in testosterone propionate (TP; 7.5 mg/kg)-induced BPH mouse model after 14 days (A), 28 days (B) and testosterone-stimulated growth in WPMY-1 cells at 24 h (C). Western blot analysis of expression of type Ⅱ 5α-reductase, androgen receptor (AR) and PSA (D-F). Data are presented as mean ± SEM. ($^{*}p < 0.05$ compared with control group, $^{\#}p < 0.05$ compared with TP or testosterone group).

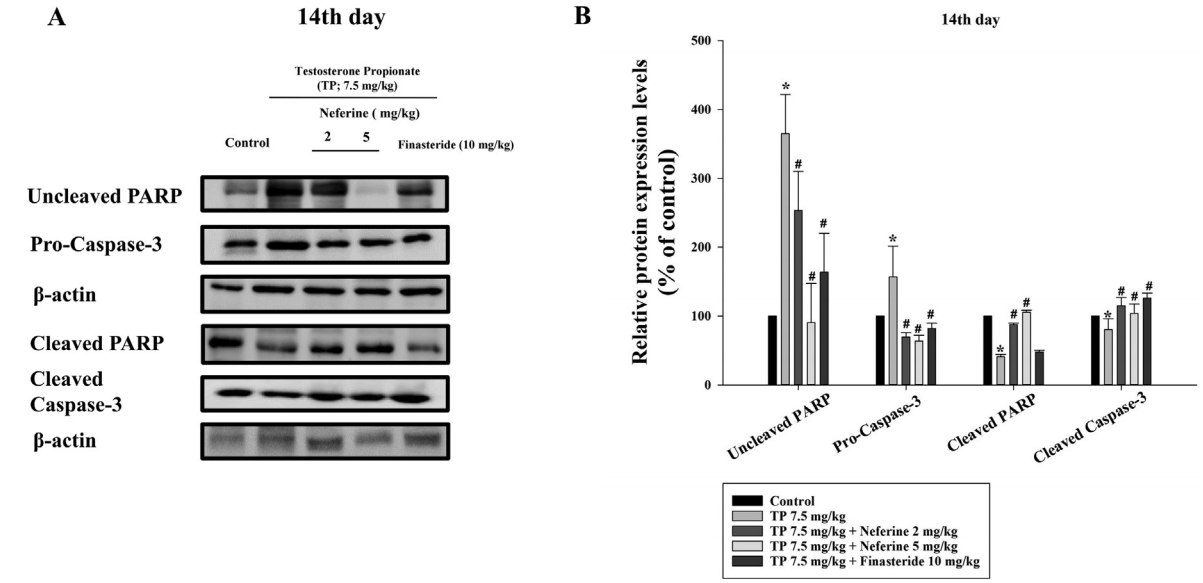


**Fig. 6.** Effects of neferine (2, 5 mg/kg) and finasteride (10 mg/kg) on the expression of apoptosis-related proteins in testosterone propionate (TP; 7.5 mg/kg)-induced BPH mouse model at 14th day (A). Western blot analysis of expression of pro-caspase-3, cleaved caspase-3, uncleaved PARP, and cleaved PARP (B). Data are presented as mean ± SEM. ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with TP group).

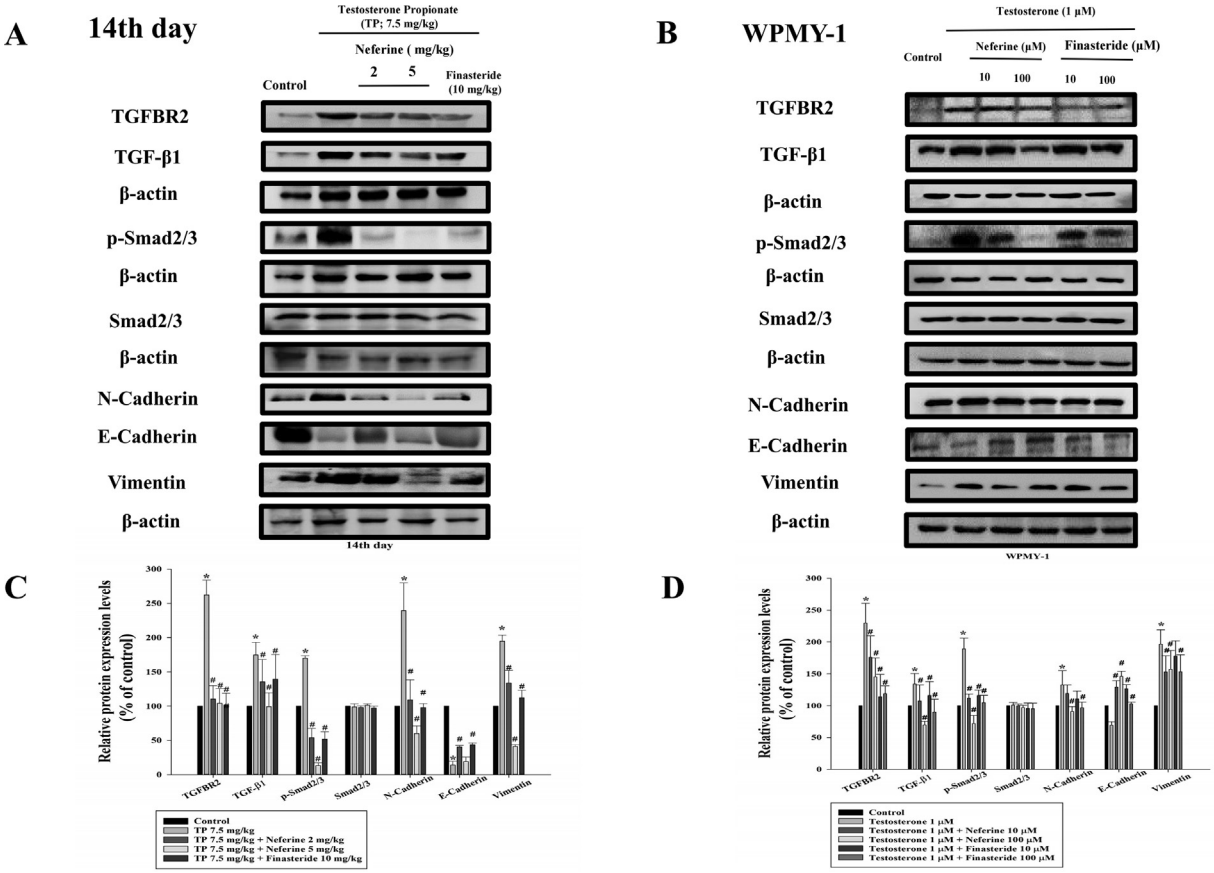


**Fig. 7.** Effects of neferine (2, 5 mg/kg; 10–100 μM) and finasteride (10 mg/kg; 10–100 μM) on the expression of TGFBR2, TGF-β1, p-Smad2/3, Smad2/3, N-cadherin, E-cadherin and vimentin in prostate tissues at 14 days (A) and WPMY-1 cells at 24 h (B). (C-D) Data are presented as mean ± SEM. ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with TP or testosterone group).

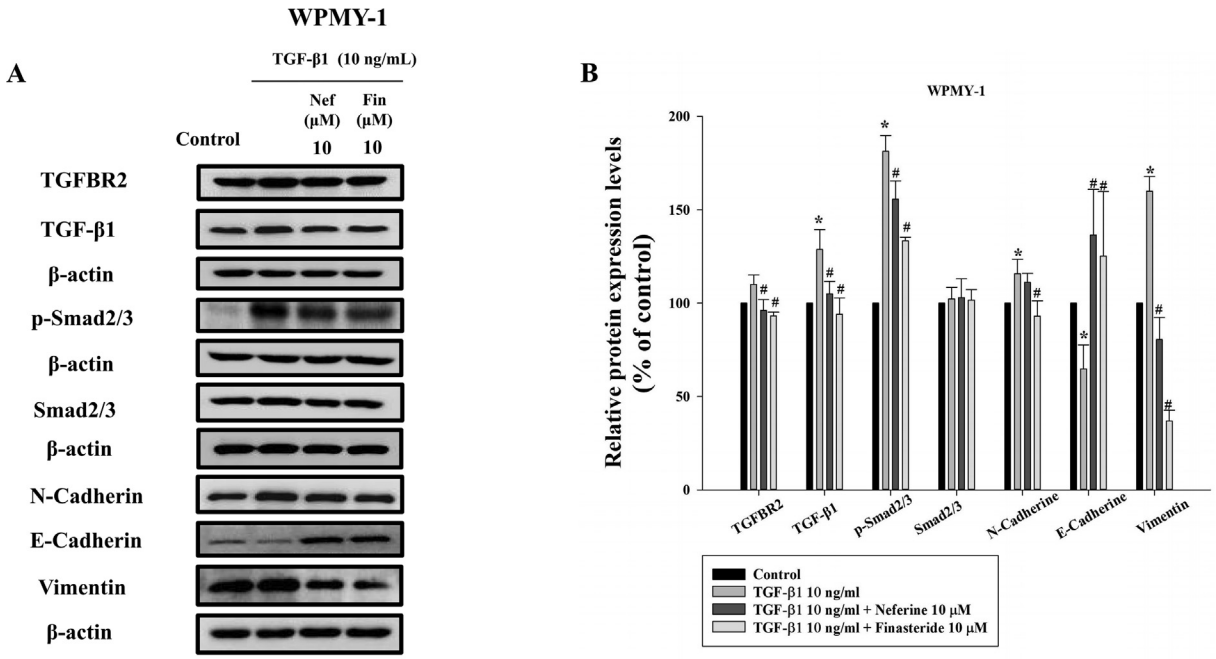


**Fig. 8.** Expression of TGFBR2, TGF-β1, p-Smad2/3, Smad2/3, N-cadherin, E-cadherin and vimentin proteins in WPMY-1 cells at 24 h after neferine (Nef; 10 μM), finasteride (Fin; 10 μM) and TGF-β1 (10 ng/mL) treatments (A). (B) Data are presented as mean ± SEM. ($^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with TGF-β1 group).

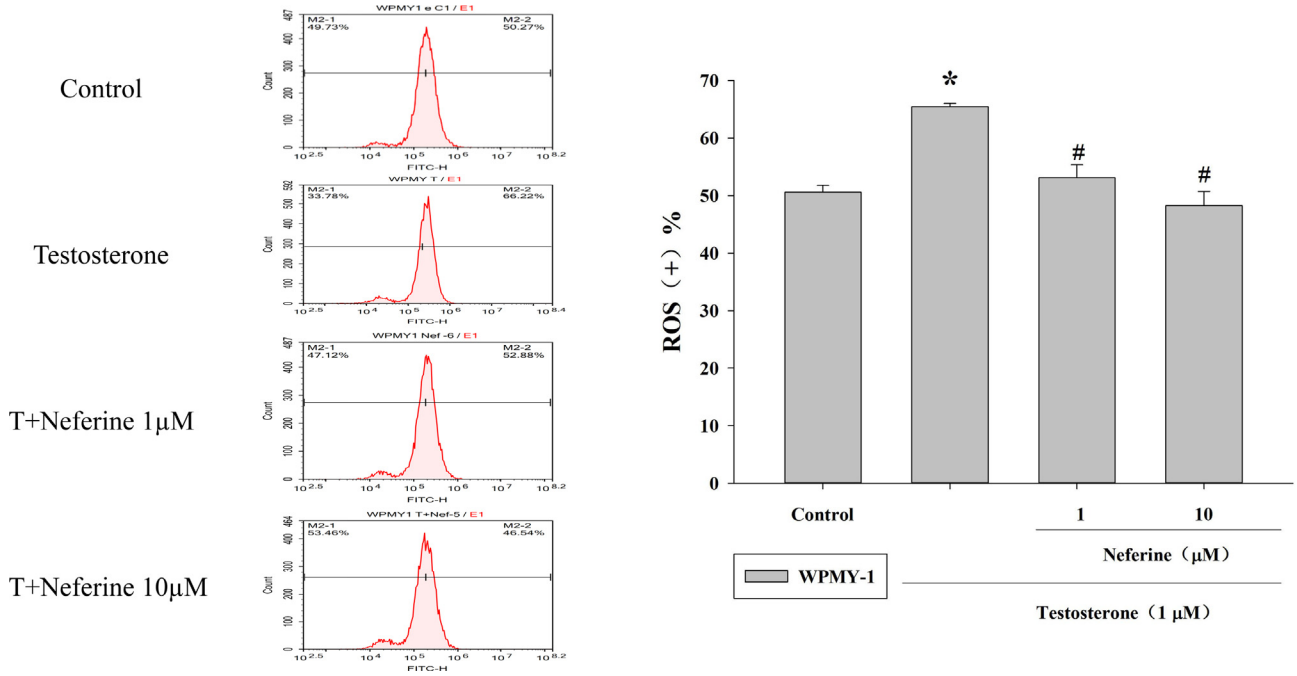


**Fig. 9.** Neferine (1–10 μM) reduced testosterone (1 μM)-induced ROS production in WPMY-1 cells. Data are presented as mean ± SEM (n = 3) ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with testosterone group).

### *3.6. Neferine prevented ROS-production in WPMY-1 cells*

Oxidative stress and ROS production were detected in BPH tissue or cells. During the current study by DCFH-DA staining, 24 h treatment with 1 μM testosterone increased ROS production in WPMY-1 cells, an effect which was prevented by 1–10 μM neferine (Fig. 9). Neferine ameliorated BPH through the regulation of oxidative stress.

## 4. Discussion

Neferine has been shown to inhibit the growth of human prostate cancer stem cells or prostate cancer cells by promoting apoptosis and autophagy through the p38 MAPK/JNK and JNK signaling pathway (Erdogan and Turkekul, 2020). Indeed, neferine inhibited prostate cancer cell growth by inhibiting 5α-reductase and androgen receptor expression via the PI3K/AKT signaling pathway in our

previous study (Liu et al., 2021). Neferine also reduced oxidative stress by activating the Nrf2-ARE pathway in a BPH epithelial cell line (Jahan et al., 2021). Proliferation of prostate stroma cells *in vitro* and inhibition of prostate enlargement *in vivo* in TP-induced BPH mice has been shown during the present work.

Androgen, age and metabolic syndrome may all affect the progress of BPH. The androgen /AR signaling pathway has been shown to promote the proliferation of prostate epithelial cells (Silva et al., 2001) and such cells support the growth of stromal cells during BPH development (Bayne et al., 1998). The nuclear androgen receptor binds testosterone and dihydrotestosterone and regulates the expression of genes, including PSA, which regulate prostate growth. Thus, abnormal activation of the androgen /AR signaling pathway may result in BPH or prostate cancer. The current study found that neferine reduced prostate enlargement and stromal cell growth by inhibiting expression of the androgen receptor, type Ⅱ 5α-reductase and PSA *in vivo* and *in vitro*. Prostatic inflammation contributes to the pathogenesis of BPH and has been observed in BPH patients and testosterone propionate-induced BPH animals (Fang et al., 2021; Gandaglia et al., 2013; Zang et al., 2021). Testosterone increased ROS levels in WPMY-1 cells, an effect which was reduced by neferine, indicating its anti-oxidative stress activity.

Impairment of the balance between apoptosis and proliferation results in the abnormal cell proliferation which characterizes BPH (Shah et al., 2021). The cysteine protease, caspase-3, is a key effector in apoptosis and poly (ADP-ribose) polymerase (PARP) is activated by the stress or DNA damage that accompanies apoptosis. The conversion of pro-capase-3 to cleaved caspase-3 is considered as activating apoptosis. Previous studies have shown that down-regulation of pro-caspase-3 levels implies upregulation of cleaved caspase-3 after drug treatment (Lee et al., 2009; Ye et al., 2012). Pro-caspase-3 and uncleaved PARP were upregulated in TP-induced BPH mice but neferine and finasteride treatment decreased expression. Cleaved PARP and cleaved caspase-3 were induced after neferine treatment. Thus, neferine treatment induced apoptosis in BPH mice.

The EMT is a physiological process related to cell migration, invasion, resistance to apoptosis and metastasis in cancer cells (Gundamaraju et al., 2022; Mashouri et al., 2019). BPH is thought to be composed of 88.4% stromal cells, which contribute to tissue remodeling and progression of BPH, and 9.0% epithelial cells (Svindland et al., 1996). Indeed, abnormal growth of prostatic stromal cells due to androgen /AR signaling and expression of growth factors and cytokines drives BPH (Sáez et al., 1999). Stromal cells consist of fibroblasts and myofibroblasts, the latter of which secrete growth factors to stimulate epithelial and stromal cell growth. The human prostatic stromal myofibroblast cell-line, WPMY-1, showed increased expression of EMT-associated mesenchymal markers, N-cadherin and vimentin, and decreased E-cadherin on testosterone treatment, as did the TP-induced BPH mouse model, consistent with a previous study (Kim et al., 2021). Thus, the androgen /AR signaling pathway was shown to regulate the EMT during prostate growth and neferine and finasteride treatments to downregulate the EMT *in vivo* and *in vitro*.

TGF-β has an impact on cell growth, differentiation and apoptosis and has been shown to induce the proliferation of WPMY-1 cells (Cao et al., 2022; Thongphichai et al., 2022; Webber et al., 1999). The relationship of the EMT to increased androgen /AR and TGF-β/Smad signaling, activated by LPS/TLR4 signaling in BPH progression has been previously demonstrated (He et al., 2016). TGFBR2 is predominant in BPH tissue (Royuela et al., 1998) and the TGF-β/Smad signaling pathway is activated by phosphorylated Smad2/3. Abnormal TGF-β/Smad activation may stimulate BPH progression. The current study found testosterone to induce the

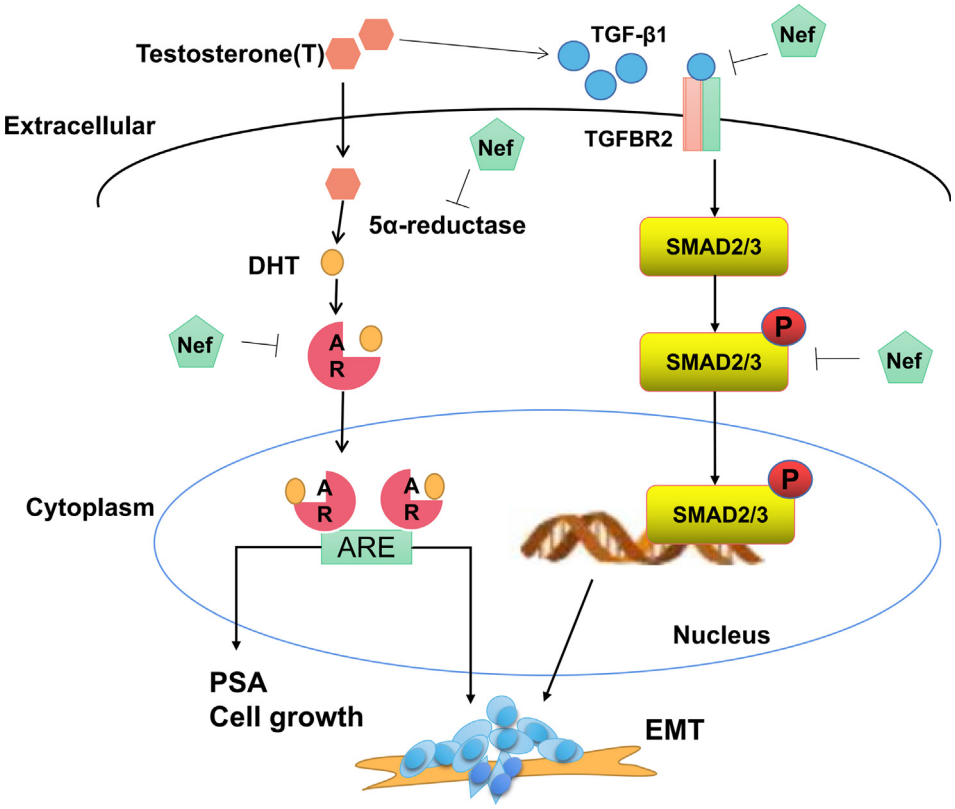


**Fig. 10.** Mechanism of neferine inhibition of prostate growth in testosterone propionate-induced BPH mouse model and in testosterone-stimulated WPMY-1 cells. In the present study, neferine blocked androgen /AR signaling pathway, TGF-β/Smad signaling pathway and reversed EMT in the prostate. EMT: epithelial mesenchymal transition; Nef: neferine; PSA: prostate specific antigen; AR: androgen receptor; DHT: dihydrotestosterone; ARE: androgen response elements; TGF-β: transforming growth factor-β.

EMT and to activate the TGF-β/Smad signaling pathway *in vivo* and *in vitro* while neferine decreased the expression of TGF-β1, TGFBR2 and p-Smad2/3. TGF-β1 also induced the EMT but neferine reversed the effect in WPMY-1 cells. In summary, Neferine inhibited TGF-β/Smad signaling and reversed the EMT in BPH.

## 5. Conclusion

In conclusion, neferine relieved BPH and reduced prostate enlargement by inhibiting the androgen/AR signaling pathway and inducing apoptosis (Fig. 10). Neferine prevented TGF-β-mediated EMT and had anti-oxidative stress activity. Neferine is suggested as a potential therapeutic agent for BPH.

## 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.

## Acknowledgements

The authors would like to express their gratitude to EditSprings (https://www.editsprings.cn) for the expert linguistic services provided.

## Funding

This work was supported by Yichun University Local Development Research Center (grant no. DF2019002), the PhD Research Foundation of Yichun University (grant no. 2113360118006), Science and Technology Project, Jiangxi Provincial Department of Education (Grant No. GJJ190852).

## References

Afdal, A., Darwin, E., Yanwirasti, Y., Hamid, R., 2019. The Expression of Transforming Growth Factor Beta-1 and Interleukin-6 on Human Prostate: Prostate Hyperplasia and Prostate Cancer. Open Access Maced J Med Sci. 7 (12), 1905–1910. https://doi.org/10.3889/oamjms.2019.548.

Alonso-Magdalena, P., Brössner, C., Reiner, A., Cheng, G., Sugiyama, N., Warner, M., Gustafsson, J.A., 2009. A role for epithelial-mesenchymal transition in the etiology of benign prostatic hyperplasia. Proc Natl Acad Sci U S A. 106 (8), 2859–2863. https://doi.org/10.1073/pnas.0812666106.

Baek, E.B., Hwang, Y.H., Park, S., Hong, E.J., Won, Y.S., Kwun, H.J., 2022. Eriochloa villosa Alleviates Progression of Benign Prostatic Hyperplasia in vitro and in vivo. Res Rep Urol. 14, 313–326. https://doi.org/10.2147/RRU.S381713.

Bakir, B., Chiarella, A.M., Pitarresi, J.R., Rustgi, A.K., 2020. EMT, MET, Plasticity, and Tumor Metastasis. Trends Cell Biol. 30 (10), 764–776. https://doi.org/10.1016/j.tcb.2020.07.003.

Bayne, C.W., Donnelly, F., Chapman, K., Bollina, P., Buck, C., Habib, F., 1998. A novel coculture model for benign prostatic hyperplasia expressing both isoforms of 5 alpha-reductase. J Clin Endocrinol Metab. 83 (1), 206–213. https://doi.org/10.1210/jcem.83.1.4486.

Cao, Y., Tian, Y., Zhang, H., Luo, G.H., Sun, Z.L., Xia, S.J., 2022. Imbalance in the estrogen/androgen ratio may affect prostate fibrosis through the TGF-β/Smad signaling pathway. Int Urol Nephrol. 54 (3), 499–508. https://doi.org/10.1007/s11255-021-03079-z.

Chen, J., Rong, N., Liu, M., Xu, C., Xiong, Q., Lei, Y., 2021. The exosome-like vesicles derived from androgen exposed-prostate stromal cells promote epithelial cells proliferation and epithelial-mesenchymal transition. Toxicol Appl Pharmacol. 411, 115384. https://doi.org/10.1016/j.taap.2020.115384.

Chiu, K.M., Hung, Y.L., Wang, S.J., Tsai, Y.J., Wu, N.L., Liang, C.W., et al., 2021. Anti-Allergic and Anti-Inflammatory Effects of Neferine on RBL-2H3 Cells. Int J Mol Sci. 22 (20), 10994. https://doi.org/10.3390/ijms222010994.

Erdogan, S., Turkekul, K., 2020. Neferine inhibits proliferation and migration of human prostate cancer stem cells through p38 MAPK/JNK activation. J Food Biochem 44 (7), e13253. https://doi.org/10.1111/jfbc.13253.

Fang, C., Wu, L., Zhao, M.J., Deng, T., Gu, J.M., Guo, X.P., et al., 2021. Periodontitis Exacerbates Benign Prostatic Hyperplasia through Regulation of Oxidative Stress and Inflammation. Oxid Med Cell Longev. 2021, 2094665. https://doi.org/10.1155/2021/2094665.

Gandaglia, G., Briganti, A., Gontero, P., Mondaini, N., Novara, G., Salonia, A., et al., 2013. The role of chronic prostatic inflammation in the pathogenesis and progression of benign prostatic hyperplasia (BPH). BJU Int. 112 (4), 432–441. https://doi.org/10.1111/bju.12118.

Gu, M., Liu, C., Yang, T., Zhan, M., Cai, Z., Chen, Y., et al., 2021. High-Fat Diet Induced Gut Microbiota Alterations Associating With Ghrelin/Jak2/Stat3 Up-Regulation to Promote Benign Prostatic Hyperplasia Development. Front Cell Dev Biol. 9, 615928. https://doi.org/10.3389/fcell.2021.615928.

Gundamaraju, R., Lu, W., Paul, M.K., Jha, N.K., Gupta, P.K., Ojha, S., et al., 2022. Autophagy and EMT in cancer and metastasis: Who controls whom? Biochim Biophys Acta Mol Basis Dis. 1868 (9), 166431. https://doi.org/10.1016/j.bbadis.2022.166431.

He, Y., Ou, Z., Chen, X., Zu, X., Liu, L., Li, Y., et al., 2016. LPS/TLR4 Signaling Enhances TGF-β Response Through Downregulating BAMBI During Prostatic Hyperplasia. Sci Rep. 6, 27051. https://doi.org/10.1038/srep27051.

Heinlein, C.A., Chang, C., 2002. Androgen receptor (AR) coregulators: an overview. Endocr Rev. 23 (2), 175–200. https://doi.org/10.1210/edrv.23.2.0460.

Hong, G.L., Park, S.R., Jung, D.Y., Karunasagara, S., Lee, K.P., Koh, E.J., et al., 2020. The therapeutic effects of Stauntonia hexaphylla in benign prostate hyperplasia are mediated by the regulation of androgen receptors and 5alpha-reductase type 2. J Ethnopharmacol. 250, 112446. https://doi.org/10.1016/j.jep.2019.112446.

Huang, Y., Chen, H., Zhou, X., Wu, X., Hu, E., Jiang, Z., 2017. Inhibition effects of chlorogenic acid on benign prostatic hyperplasia in mice. Eur J Pharmacol. 809, 191–195. https://doi.org/10.1016/j.ejphar.2017.04.017.

Huang, X., Lee, C., 2003. Regulation of stromal proliferation, growth arrest, differentiation and apoptosis in benign prostatic hyperplasia by TGF-beta. Front Biosci. 8, s740–s749. https://doi.org/10.2741/1093.

Jahan, N., Chowdhury, A., Li, T., Xu, K., Wei, F., Wang, S., 2021. Neferine improves oxidative stress and apoptosis in benign prostate hyperplasia via Nrf2-ARE pathway. Redox Rep. 26 (1), 1–9. https://doi.org/10.1080/13510002.2021.1871814.

Karunasagara, S., Hong, G.L., Jung, D.Y., Kim, K.H., Cho, K., Jung, J.Y., 2020. Protective effects of combination of Stauntonia hexaphylla and Cornus officinalis on testosterone-induced benign prostatic hyperplasia through inhibition of 5α-reductase type 2 and induced cell apoptosis. PLoS One 15 (8), e0236879. https://doi.org/10.1371/journal.pone.0236879.

Kim, Y., Lee, D., Jo, H., Go, C., Yang, J., Kang, D., Kang, J.S., 2021. GV1001 interacts with androgen receptor to inhibit prostate cell proliferation in benign prostatic hyperplasia by regulating expression of molecules related to epithelial-mesenchymal transition. Aging (Albany NY). 13 (3), 3202–3217. https://doi.org/10.18632/aging.202242.

Larson, C., Oronsky, B., Carter, C.A., Oronsky, A., Knox, S.J., Sher, D., Reid, T.R., 2020. TGF-beta: a master immune regulator. Expert Opin Ther Targets. 24 (5), 427–438. https://doi.org/10.1080/14728222.2020.1744568.

Lee, D.S., Lee, M.K., Kim, J.H., 2009. Curcumin induces cell cycle arrest and apoptosis in human osteosarcoma (HOS) cells. Anticancer Res. 29 (12), 5039–5044.

Liu, C.M., Wu, Z., Pan, B., An, L., Zhu, C., Zhou, J., Jiang, Y., 2021. The antiandrogenic effect of neferine, liensinine, and isoliensinine by inhibiting 5-α-reductase and androgen receptor expression via PI3K/AKT signaling pathway in prostate cancer. Pharmazie. 76 (5), 225–231. https://doi.org/10.1691/ph.2021.1301.

Lu, T., Lin, W.J., Izumi, K., Wang, X., Xu, D., Fang, L.Y., et al., 2012. Targeting androgen receptor to suppress macrophage-induced EMT and benign prostatic hyperplasia (BPH) development. Mol Endocrinol. 26 (10), 1707–1715. https://doi.org/10.1210/me.2012-1079.

Manogaran, P., Somasundaram, B., Viswanadha, V.P., 2022. Reversal of cisplatin resistance by neferine/isoliensinine and their combinatorial regimens with cisplatin-induced apoptosis in cisplatin-resistant colon cancer stem cells (CSCs). J Biochem Mol Toxicol 36 (3), e22967. https://doi.org/10.1002/jbt.22967.

Marconi, G.D., Fonticoli, L., Rajan, T.S., Pierdomenico, S.D., Trubiani, O., Pizzicannella, J., Diomede, F., 2021. Epithelial-Mesenchymal Transition (EMT): The Type-2 EMT in Wound Healing. Tissue Regeneration and Organ Fibrosis. Cells. 10 (7), 1587. https://doi.org/10.3390/cells10071587.

Marthandam Asokan, S., Mariappan, R., Muthusamy, S., Velmurugan, B.K., 2018. Pharmacological benefits of neferine - A comprehensive review. Life Sci. 199, 60–70. https://doi.org/10.1016/j.lfs.2018.02.032.

Mashouri, L., Yousefi, H., Aref, A.R., Ahadi, A.M., Molaei, F., Alahari, S.K., 2019. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. Mol Cancer. 18 (1), 75. https://doi.org/10.1186/s12943-019-0991-5.

Micalizzi, D.S., Farabaugh, S.M., Ford, H.L., 2010. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia. 15 (2), 117–134. https://doi.org/10.1007/s10911-010-9178-9.

Paniagua Cruz, A., Zhu, K.Y., Ellimoottil, C., Dauw, C.A., Sarma, A., Skolarus, T.A., 2020. Characterizing the Benign Prostatic Hyperplasia Literature: A Bibliometric Analysis. Urology. 136, 202–211. https://doi.org/10.1016/j.urology.2019.11.033.

Parsons, J.K., Dahm, P., Köhler, T.S., Lerner, L.B., Wilt, T.J., 2020. Surgical Management of Lower Urinary Tract Symptoms Attributed to Benign Prostatic Hyperplasia: AUA Guideline Amendment 2020. J Urol. 204 (4), 799–804. https://doi.org/10.1097/ju.0000000000001298.

Qi, Z., Wang, R., Liao, R., Xue, S., Wang, Y., 2021. Neferine Ameliorates Sepsis-Induced Myocardial Dysfunction Through Anti-Apoptotic and Antioxidative Effects by Regulating the PI3K/AKT/mTOR Signaling Pathway. Front Pharmacol. 12, 706251. https://doi.org/10.3389/fphar.2021.706251.

Rastrelli, G., Vignozzi, L., Corona, G., Maggi, M., 2019. Testosterone and Benign Prostatic Hyperplasia. Sex Med Rev. 7 (2), 259–271. https://doi.org/10.1016/j.sxmr.2018.10.006.

Roy, A.K., Chatterjee, B., 1995. Androgen action. Crit Rev Eukaryot Gene Expr. 5 (2), 157–176. https://doi.org/10.1615/critreveukargeneexpr.v5.i2.30.

Royuela, M., De Miguel, M.P., Bethencourt, F.R., Sanchez-Chapado, M., Fraile, B., Paniagua, R., 1998. Transforming growth factor beta 1 and its receptor types I and II. Comparison in human normal prostate, benign prostatic hyperplasia, and prostatic carcinoma. Growth Factors. 16 (2), 101–110. https://doi.org/10.3109/08977199809002121.

Sáez, C., González-Baena, A.C., Japón, M.A., Giráldez, J., Segura, D.I., Rodríguez-Vallejo, J.M., et al., 1999. Expression of basic fibroblast growth factor and its receptors FGFR1 and FGFR2 in human benign prostatic hyperplasia treated with finasteride. Prostate. 40 (2), 83–88. https://doi.org/10.1002/(sici)1097-0045(19990701)40:2<83::aid-pros3>3.0.co;2-n.

Shah, A., Shah, A.A., Nandakumar, K., Lobo, R., 2021. Mechanistic targets for BPH and prostate cancer-a review. Rev Environ Health. 36 (2), 261–270. https://doi.org/10.1515/reveh-2020-0051.

Silva, I.S., Morsch, D.M., Urnauer, L., Spritzer, P.M., 2001. Androgen-induced cell growth and c-myc expression in human non-transformed epithelial prostatic cells in primary culture. Endocr Res. 27 (1–2), 153–169. https://doi.org/10.1081/erc-100107177.

Solanki, A., Patel, S., Solanki, N., Shah, U., 2021. Inhibitory Effect of Artemisinin on Testosterone Propionate Induced Benign Prostatic Hyperplasia. Curr Drug Discov Technol. 18 (4), 518–524. https://doi.org/10.2174/1570163817666200612151150.

Svindland, A., Eri, L.M., Tveter, K.J., 1996. Morphometry of benign prostatic hyperplasia during androgen suppressive therapy. Relationships among epithelial content, PSA density, and clinical outcome. Scand J Urol Nephrol Suppl. 179, 113–117.

Thongphichai, W., Uttarawichien, T., Chanvorachote, P., Pitiporn, S., Charoen-Ame, T., Kwankhao, P., et al., 2022. Standardization of the ethanolic extract of Crinum latifolium leaves by two bioactive markers with antiproliferative activity against TGF-β-promoted prostate stromal cells (WPMY-1). BMC Complement Med Ther. 22 (1), 139. https://doi.org/10.1186/s12906-022-03617-x.

Wang, Y.T., Liu, Y.T., Bai, X.Y., 2021a. The role of epithelial-mesenchymal transition in cancer metastasis: a context-dependent regulation process. Chemistry of life. 41 (3), 428–436. https://doi.org/10.13488/j.smhx.20200550.

Wang, Z., Zhang, Y., Zhao, C., Li, Y., Hu, X., Wu, L., et al., 2021b. The miR-223-3p/MAP1B axis aggravates TGF-β-induced proliferation and migration of BPH-1 cells. Cell Signal. 84, 110004. https://doi.org/10.1016/j.cellsig.2021.110004.

Webber, M.M., Trakul, N., Thraves, P.S., Bello-DeOcampo, D., Chu, W.W., Storto, P.D., et al., 1999. A human prostatic stromal myofibroblast cell line WPMY-1: a model for stromal-epithelial interactions in prostatic neoplasia. Carcinogenesis. 20 (7), 1185–1192. https://doi.org/10.1093/carcin/20.7.1185.

Wicha, P., Onsa-Ard, A., Chaichompoo, W., Suksamrarn, A., Tocharus, C., 2020. Vasorelaxant and Antihypertensive Effects of Neferine in Rats: An In Vitro and In Vivo Study. Planta Med. 86 (7), 496–504. https://doi.org/10.1055/a-1123-7852.

Ye, M.X., Zhao, Y.L., Li, Y., Miao, Q., Li, Z.K., Ren, X.L., et al., 2012. Curcumin reverses cis-platin resistance and promotes human lung adenocarcinoma A549/DDP cell apoptosis through HIF-1α and caspase-3 mechanisms. Phytomedicine. 19 (8–9), 779–787. https://doi.org/10.1016/j.phymed.2012.03.005.

Zang, L., Tian, F., Yao, Y., Chen, Y., Shen, Y., Han, M., et al., 2021. Qianliexin capsule exerts anti-inflammatory activity in chronic non-bacterial prostatitis and benign prostatic hyperplasia via NF-κB and inflammasome. J Cell Mol Med. 25 (12), 5753–5768. https://doi.org/10.1111/jcmm.16599.

Zhu, B., Kyprianou, N., 2005. Transforming growth factor beta and prostate cancer. Cancer Treat Res. 126, 157–173. https://doi.org/10.1007/0-387-24361-5_7.

Zou, Y., Aboshora, W., Li, J., Xiao, T., Zhang, L., 2017. Protective Effects of Lepidium meyenii (Maca) Aqueous Extract and Lycopene on Testosterone Propionate-Induced Prostatic Hyperplasia in Mice. Phytother Res. 31 (8), 1192–1198. https://doi.org/10.1002/ptr.5838.