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Original Research

Crocin treatment improves testosterone induced benign prostatic hyperplasia in rats

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Abstract

Background and objective: Benign prostatic hyperplasia (BPH) is a typical nonmalignant growth of the prostate in the elderly. Crocin, a bioactive component of *Crocus sativus L*., commonly known as saffron, is known to have an anti-proliferative activity against numerous types of cancer, including prostate cancer. This study investigated the effects of crocin on testosterone-induced BPH development in rats.

Materials and methods: The study sample included three groups of adult male rats (3 months old, weighed 250 g): the control group received corn oil only, the second and the third groups were injected with testosterone (3 mg/kg dissolved in corn oil) subcutaneously. The second group was considered as testosterone-induced BPH (untreated) while the third groups were assigned as testosterone-induced BPH-crocin treated group (100 mg/kg orally for 14 days).

Results: After animal sacrifice, histopathological analysis of the prostate tissues was performed in parallel with gene expression of proliferation (PCNA), inflammation (IL-6), and vascularization (VEGF-A) markers, analyzed by qRT-PCR. Crocin treatment significantly reduced prostate index and the thickness of the epithelial layer in rats with BPH. Additionally, the mRNA expression levels of PCNA, a marker of cell proliferation; IL-6, an inflammatory cytokine; and VEGF-A, an angiogenesis marker, were significantly down-regulated in the BPH group that were treated with crocin.

Conclusions: The present study indicates that crocin can effectively prevent the development of experimentally induced BPH through inhibition of prostatic cellular proliferation, inflammation, and angiogenesis.

Keywords

Crocin; BPH; RT-PCR; Testosterone; Rats

1. Introduction

Benign prostatic hyperplasia or BPH is described as a nonmalignant prostate gland growth observed frequently in aging men [1]. The histological prevalence of BPH age-related disorder could reach more than 80% in men in their 80s [1]. Clinically, BPH causes symptoms known as lower urinary tract symptoms (LUTS) that include slow urinary flux and insufficient emptying, which may ultimately lead to acute urinary retention, chronic infections of the urinary tract, haematuria, bladder calculi, and renal failure [2]. For *in vivo* studies, the available model of BPH is generated by subcutaneous testosterone injection in rats. Different studies showed the similarity between induced BPH in rats and human BPH.

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TABLE 1. Sequence of primers used for qRT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	ATGGTGAAGGTCGGTGTG	GAACTTGCCGTGGGTAGA
PCNA	AGGCCCTCAAAGACCTCATC	CGGTATGTGTCGAAGCCTTC
IL-6	TCAACTCCATCTGCCCTTCA	CTGTGAAGTCTCCTCTCCGG
VEGF-A	CATCAGCCAGGGAGTCT	AGTGAAGGAGCAACCTCT

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PCNA, Proliferating cell nuclear antigen; IL-6, Interleukin-6; VEGF-A, Vascular Endothelial Growth Factor-A.

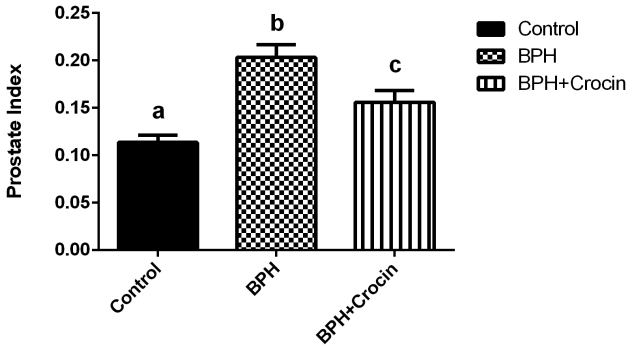

FIG. 1. Prostate index in the experimental rats showing the effect of crocin treatment. The results show the mean ± SEM. The significant difference was expressed as a different superscript letter in each group ($P < 0.05$). Abbreviations: BPH, benign prostatic hyperplasia.

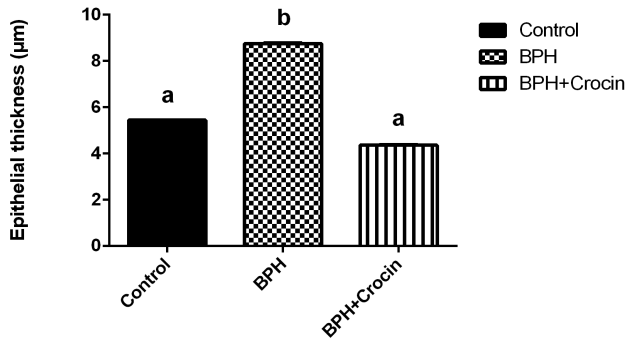

FIG. 2. Prostate epithelial thickness in the experimental rats showing the effect of crocin treatment. The results show the mean ± SEM. The significant difference was expressed as a different superscript letter in each group ($P < 0.05$). Abbreviations: BPH, benign prostatic hyperplasia.

Specifically, both pathologies present a distinctive expression of the major inflammatory, angiogenesis, and proliferative markers such as IL-6, VEGF, and PCNA, respectively [3, 4].

The mature prostate contains epithelial and stromal cells, both of which express androgen receptors [5]. BPH is a hyperplastic process resulting in a histologic alteration within the transitional zone and periurethral areas of the prostatic tissue with nodular overgrowth of the glandular-epithelial and fibromuscular tissue [1, 6]. Stromal-epithelial interactions under the influence of androgens engage in essential functions in the pathophysiology of BPH [5]. This interaction is mainly mediated by several stromal and epithelial factors and cytokines that work together to produce BPH by altering the balance of the prostatic cellular proliferation versus cell death [7, 8]. These factors include the vascular endothelial growth factor (VEGF), the primary factor for angiogenesis, and interleukin-6 (IL-6), a pleiotropic inflammatory cytokine.

Angiogenesis, the growth of new capillary vessels out of preexisting ones, provides expanded BPH nodules with oxygen and nutrients. High vessel densities were found in hyperplastic nodules in BPH where various proangiogenic factors regulate this process [9]. It was proposed that VEGF-A, a potent mitogen for endothelial cells, plays an effective role in the pathogenesis of BPH [7, 10]. Pieces of evidence have incriminated the role of inflammatory cytokines in the development of BPH [2, 7]. An increase in the inflammatory cytokine production by prostate epithelial/stromal cells and inflammatory cells such as IL-1β, IL-6,IL-8, IL-17, and TNF-α will create a local inflammatory microenvironment and may lead to the initiation and progression of BPH [2, 7, 11].

Currently, natural products are a promising source for new anti-BPH drug discovery and development. Crocin, a bioactive component of *Crocus sativus L.* (saffron) is known to have anti-inflammatory [12], antioxidant [13], and neuroprotective effects [14]. In addition, crocin's anticancer effect has been well-documented in several *in vitro* and *in vivo* studies [15, 16]. The anticancer activity of crocin is mainly attributed to the inhibition of cancer cell proliferation, metastasis, angiogenesis and by provoking cancer cell apoptosis [16–18]. To date, however, there have been no studies on possible crocin protective effects against BPH.

2. Materials and methods

The animal subjects were treated according to the guidelines of the Guide to the Care and Use of Laboratory Animals, and the Ethics Committee at Yarmouk University accepted the methodology (permission number: YU-12/6/2020). Thirty male Sprague Dawley rats three months old and weighing 220–250 g were supplied by the animal house unit at the Department of Biological Sciences at Yarmouk University. One week of adaptation to the laboratory environment in the animal house (25 °C, 40–60% humidity) was adopted for all experimental animals, then the rats were divided into three equal groups (n = 10/group): the normal control group received corn oil only, the second group (testosterone-induced BPH) received testosterone injection subcutaneously (3 mg/kg dissolved in corn oil for two weeks (testosterone enanthate 250 mg, Primoteston-Depot©, Schering, Germany). Finally, the third group was injected with testosterone for the BPH induction and treated with

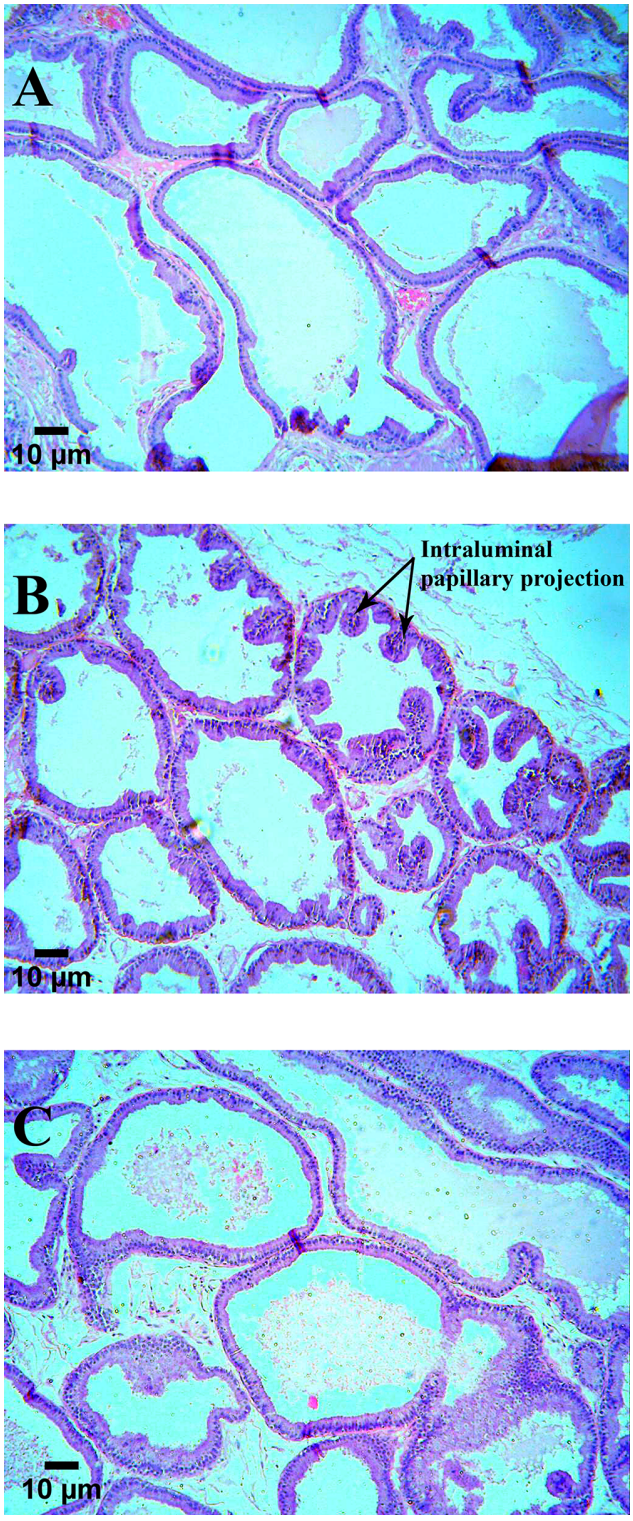

FIG. 3. The histomorphological changes of prostate sections thickness in the experimental rats showing the effect of crocin treatment (hematoxylin staining; magnification, 100×). (A) Control group show normal cellular architecture with normal glandular epithelial cells and fibromuscular stromal tissue. (B) BPH group displayed irregular morphology, including a greater level of proliferation in the glandular epithelial and in the fibromuscular stroma with multiple intraluminal papillary projections. (C) Crocin treated group showed that thecorcin treatment ameliorated histological morphology in the BPH treated rats compared to BPH-untreated group.

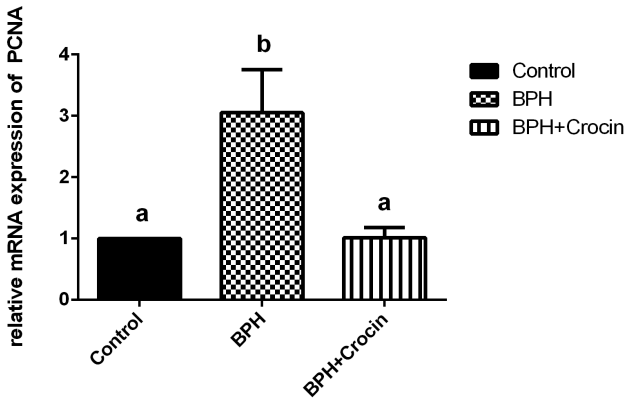

FIG. 4. PCNA mRNA expression in the experimental rats showing the effect of crocin treatment. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH, benign prostatic hyperplasia; PCNA, proliferating cell nuclear antigen.

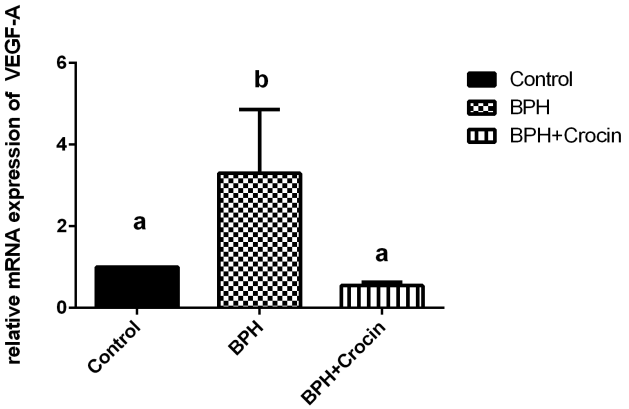

FIG. 5. VEGF-A mRNA expression in the experimental rats showing the effect of crocin treatment. The data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH, benign prostatic hyperplasia; VEGF-A, vascular endothelial growth factor-A.

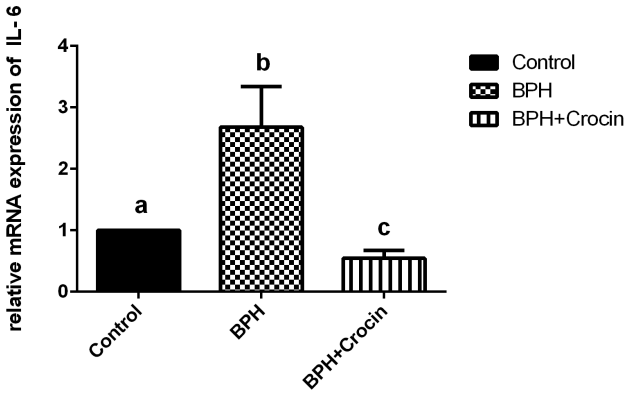

FIG. 6. IL-6 mRNA expression in the experimental rats showing the effect of crocin treatment. The data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH, benign prostatic hyperplasia; IL-6, interleukin-6.

crocin (CAS-No 42553-65-1, Sigma, MO, USA) (100 mg/kg dissolved in de-ionized water and administrated orally) for 14 days [19, 20]. After that, animals were anesthetized using ether and their prostates were removed immediately for further analysis. The prostate glands in all groups were divided into two symmetrical sections; the first segment was kept in liquid nitrogen for RNA analysis. Simultaneously, the second section was cleaned with saline solution, remedied in 4% buffered formaldehyde for 24 hours, followed by routine tissue processing and paraffin embedding.

2.1 The prostate index and histological analysis

The prostate index (prostate weight/body weight (PW/BW) percentage) of the experimental rats was determined by dividing the prostate weight by the bodyweight (mg/g) and multiplied by 100. Fixed prostate tissue processing, alcohol dehydration, and xylene clearing were performed on an automated tissue processor (Spin Tissue Processor STP 120-22050853, Thermo Fisher Scientific, Waltham, MA, USA). The paraffin-embedded tissue from each group was sectioned at 5 μm thickness sections and subjected to deparaffinization, rehydration in xylene, and alcohol serial concentrations. Finally, hematoxylin-eosin staining was performed for histological examination [21]. ImageJ software (64 bit Java 1.8.0_172, NIH, Bethesda, MD, USA) was used for the calculation of the prostate epithelial thickness.

2.2 RNA extraction and quantitative real time-PCR

Total RNA extraction from prostate tissues was performed using the TRI Reagentas recommended by the manufacturer (R2050-1-50, Zymo, Irvin, CA, USA). The amount of RNA was measured using the QuantiFluor RNA System (E3310, Promega, Madison, WI, USA) and the QuantusFluorometer (E6150, Promega, Madison, WI, USA). The mRNA was reverse-transcribed using an oligo-(DT) 15 primer in a total volume of 20 μL as instructed by the producer (Reverted First Strand cDNA Synthesis Kit, K1621, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The produced cDNA was stored at –20 °C until used for qRT-PCR.

qRT-PCR for IL-6, VEGF, and PCNA expressions was performed using Line-Gen 9600 thermal cycler (Bioer Technology, Hangzhou, China) under the following conditions: activation step at 95 °C for 3 minutes followed by 35 cycles at 95 °C for 5 seconds and 60 °C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as a housekeeping gene. Primers were designed using the Primer3 application and are designed by IDT (Integrated DNA Technologies, INC., Coralville, IA, USA) as shown in Table 1. Agarose gel was tested to ensure the primers' specificity, in which a single band of the required size was obtained. SYBR-PCR experiments have been performed using the TB Green Premix Ex TaqII PCR master mixture (RR420B, Takara, San Jose, CA, USA) in 20 μL of the final volume (10 μL SYBR green, 1 μL of uM forward primer, 1 μL of uM reverse primer, 6 μL nuclease-free water, and 2 μL CDNA). The relative gene expression rates were calculated by the $2^{-\Delta\Delta CT}$ method. Gene expression levels were expressed as the normalized gene expression ratio relative to the GAPDH mRNA level using one sample of the control group as a calibrator.

2.3 Statistical analysis

All statistical analyses were performed using the SPSS program (version 14.0) (SPSS Inc., Chicago, IL, USA). One-way variance analysis (ANOVA) was computed between groups, followed by the least significant difference (LSD) posthoc test analysis. When the P-value < 0.05, the difference was considered significant.

3. Results

The results of the current study demonstrated a higher prostate index and the thickness of the epithelial layer in the BPH group compared to the normal prostatic group,whereas treatment with crocin significantly decreased the prostate index and the epithelial thickness in the treated rats compared to the BPH group ($P < 0.05$; Figs. 1,2). The histological examination of the prostate tissues in the control rats showed typical cellular architecture with normal glandular epithelial cells and fibromuscular stromal tissue. On the other hand, the prostatic tissues in the BPH group displayed irregular morphology and showed a greater proliferation level in both the glandular epithelial and the fibromuscular stroma with multiple intraluminal papillary projections (Fig. 3). Compared to the BPH untreated group, the crocin treated BPH group showed a significant improvement in the histological morphology of the prostate tissue as shown in Fig. 3.

Moreover, the relative prostatic mRNA expression levels of PCNA, a marker of cell proliferation; VEGF-A, a biomarker of angiogenesis; and IL-6, an inflammatory cytokine, showed a significant increase in the BPH group compared to the control rats' group. However, this was significantly rescued in the crocin-treated group compared to the untreated BPH group, as the mRNA expression of PCNA, VEGF-A, and IL-6 were reduced comparable to the control group ($P < 0.05$; Figs. 4,5,6 respectively).

4. Discussion

Further research leading to the development of new effective and safe treatments for BPH with fewer side effects is needed. Crocin has shown a considerable pharmaceutical effect on the treatment of cancer [16]. In the current study,we aimed to assess the *in vivo* effects of crocin on the development of testosterone-induced BPH which might be the first study on such a model to the best of our knowledge. The results of this study found that crocin can effectively prevent the pathological progression of the BPH in the experimental rats by abrogating the proliferation of the prostatic tissues, inhibiting inflammatory signs, and prevention of angiogenesis-inducing process as shown by the qRT-PCR profile of the

PCNA, IL-6, and VEGF-A. Thus, we conclude that crocin could be used as a potentially effective treatment against the BPH disease.

The pathological development of BPH is described by histological changes and distinguished proliferation in the stromal and glandular epithelial cells due to the loss of homeostasis between cellular creation and apoptosis [22]. PCNA is a non-histone nuclear protein necessary for DNA synthesis and has been identified as a G1/S phase marker in the cell cycle [22–24]. This protein's expression has been used in several studies as a reliable diagnostic and prognostic marker for many diseases [22], including prostate cancer and BPH [24]. An increase in the PCNA expression in BPH relative to normal prostate tissues was reported in the current study, showing consistency with some previous findings [21, 24]. The results of the current study proven that crocin treatment significantly reduced the prostate index and attenuated the prostatic stromal and epithelial hyperplasia as indicated by the histological examination. In addition, crocin treatment down-regulated the mRNA expression of PCNA and reduced the prostate epithelial thickness in the BPH group, indicating that crocin has an anti-proliferative effect in experimentally induced BPH. It was reported that crocin inhibits the proliferation of several types of carcinoma cells, including breast, prostate, gastric, colon,and pancreatic cancer [20, 25–27]. Different mechanisms for the anti-proliferation effect of crocin have been proposed. Crocin was found to hinder the proliferation of different types of cancer cells through inhibition of cell cycle progression [20, 25], induction of apoptosis [16, 17, 20, 25], and by disrupting the microtubule network [27].

Prostatic inflammation may alter the balance between proliferation and apoptotic cell death, leading to BPH development [7]. The interaction between prostatic stromal cells and immune system cells (mainly T lymphocytes) within the prostatic tissue is responsible for prostate cell proliferation and BPH development [7, 28]. Prostatic epithelial and stromal cells respond to different cytokines released by T lymphocytes (such as IL-2 and IL-17) by increasing IL-8 and IL-6, which are the leading cause of the growth in the stromal BPH [28]. Crocin has been reported to have an anti-inflammatory effect in many inflammatory diseases and to significantly decrease the systemic and tissue level of IL-6 [29, 30]. In agreement with a previous study [21], the prostatic mRNA expression level of IL-6 level was significantly elevated in the BPH rats than those of normal rats. As was expected, treatment with crocin suppressed the prostatic mRNA IL-6 expression level in the BPH rats. This result indicates that crocin could be used to manage BPH development through its anti-inflammatory effects.

Vascular alterations are also linked to BPH development [9]. An increase in the proangiogenic factor secretions (fibroblast growth factor, VEGF) derived from BPH cells has been reported during BPH development [10, 17]. Crocin has an anti-angiogenic effect in breast cancer cells [31]. Furthermore, crocin inhibits angiogenesis and lung metastasis of melanoma cells by suppressing VEGF in the experimental melanoma lung metastasis mouse model [17]. The current findings showed that the VEGF-A's level was markedly increased in the BPH rats, which supports the previous reports [17, 21, 32]. In addition, we displayed that crocin could attenuate the development of BPH in rats by hindering the expression of VEGF-A (the angiogenesis marker). Therefore, the current outcome simply that preventing the angiogenesis induced by VEGF-A may play an crucial role in crocin's beneficial effect against experimentally induced BPH development. Based on our finding, more examinations are recommended to be conducted to evaluate the impact of crocin of the development of BPH by revealing the involved molecular pathways which will help in application of required phases of clinical trials.

5. Conclusions

In conclusion, this study showed that crocin administration to rats prevented testosterone-induced BPH progression by reducing prostate weight and prostatic hyperplasia. This effect can be attributed, at least in part, to the anti-proliferative, anti-inflammatory, and anti-angiogenic activities of crocin. These findings suggest that crocin has potential as a novel therapeutic agent for the treatment of BPH.

Author contributions

JQ, BT, and MZ conceived and planned the experiments. DF, AA, and SO carried out the experiments. JQ, BT, MZ and KB contributed to the interpretation of the results. BT, GE, and MZ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Ethics approval and consent to participate

The animal subjects were treated according to the guidelines of the Guide to the Care and Use of Laboratory Animals, and the Ethics Committee at Yarmouk University accepted the methodology (permission number: YU-12/6/2020).

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Conflict of interest

The authors declare no conflict of interest.

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