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The Effects of Cordycepin on Benign Prostatic Hyperplasia through Autophagy Activation in BPH-1 Cell Line

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Abstract—Benign prostatic hyperplasia (BPH) is an age-related disease and characterized by nonmalignant enlargement of the prostate gland. Currently, it's etiology remains unclear. A previous report showed that autophagy is decreased in the human BPH cell line (BPH-1) compared with the normal prostate cells (RWPE-1). The purpose of this study was to investigate whether cordycepin (3'-deoxyadenosine) could alleviate BPH through activating autophagy in BPH-1 cells. BPH-1 cells were treated 100 μM cordycepin for 48-h and BPH-related markers, such as androgen receptor (AR), 5-alpha reductase 2 (5AR-2), prostate-specific antigen (PSA), and apoptosis-related markers (Bax and BCL-2) were examined via RT-qPCR, cell proliferation rates were measured via MTT assay, cell cycle-related proteins (Cdk1 and Cyclin B1), pro-inflammatory cytokines (TNF-α and IL-1β), and autophagy flux were determined via immunoblot. Treatment with 100 μM of cordycepin significantly decreased on AR (~63%), 5AR-2 (~46%), and PSA (~68%) mRNA expression levels in BPH-1 cells ($p < 0.05$). Cordycepin significantly decreased cell proliferation rates (~29%), Cdk1 (~65%), Cyclin B1 (~43%), and both TNF-α (~55%) and IL-1β (~60%) protein levels in BPH-1 cells ($p < 0.05$). Cordycepin (100 μM) significantly enhanced autophagy flux in accordance with increased AMPK and decreased ribosomal protein S6 (mTOR substrate) phosphorylation. These positive effects of cordycepin appear to occur via AMPK-mTOR-dependent autophagy signaling mechanism. This study suggests that cordycepin is a potent autophagy activator and a promising agent for the treatment of BPH and other autophagy-impaired diseases.

Keywords: autophagy, benign prostatic hyperplasia, cordycepin, prostate

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INTRODUCTION

BPH is a non-cancerous enlargement or growth of the prostate gland characterized by unregulated proliferation of epithelial and stromal cells in the periurethral region and the transition zone. Its prevalence increases with age, and over half of the male population will have BPH by the time they reach middle age (Thorpe and Neal, 2003). The pathogenesis of BPH is still largely unknown. Several theories have been proposed, which include genetic predisposition, androgen/androgen receptor signaling, chronic inflammation, imbalance between androgen/estrogen signaling, etc. (McVary, 2006; Parsons, 2010; Izumi et al., 2013). Recent studies have suggested that autophagy may also be involved in the development of BPH (Liu et al., 2013; Li et al., 2014; Lesovaya et al., 2015), and autophagy has been proposed as a novel therapeutic strategy for BPH treatment (Oh et al., 2020). However, there are not sufficient documents to support the benefits for the treatment of BPH via autophagy modulation.

Autophagy is an intracellular degradation system that delivers cytoplasmic contents to the lysosome via autophagosomes. This process plays a critical role in maintaining cellular quality control and regulating cellular metabolism and homeostasis. Autophagy is stimulated by a variety of stress stimuli, such as starvation, reactive oxygen species, protein aggregates, damaged organelles, and drugs (e.g. rapamycin), etc. (Feng et al., 2014). The activation of autophagy by these stimuli involves multiple signaling pathways. For example, the mechanistic target of rapamycin (mTOR) negatively regulates the activation of autophagy. Autophagy is regulated by autophagy-related (Atg) family of proteins, which participate in all stages of the autophagy process: induction, autophagosome formation, lysosomal fusion and autolysosome formation, and degradation (Dereticm and Levine, 2009). Autophagy is linked to some human pathophysiolo-

Abbreviations: AR—androgen receptor, 5AR-2—5-alpha reductase 2, PSA—prostate-specific antigen.

gies including cancer, myopathies, diabetes and neurodegenerative disease (Feng et al., 2014).

Cordycepin is a natural adenosine analogue found and extracted from various species of fungi, mainly those from *Cordyceps militaris* and *Ophiocordyceps sinensis*. Cordycepin has various intracellular potential targets, including RNA, DNA, protein, ROS, mTOR, AMPK, etc., and exhibits significant potential to regulate immune response, apoptosis, cell cycle, antimetastasis, antioxidant, anti-proliferation and promote autophagy (Huang et al., 2018; Rabie, 2022). The bioactivities of cordycepin have been tested in many human cancer cell lines and some disease models such as obesity, hypertension, type 2 diabetes, and hyperlipidemia, however, the therapeutic effects and efficacy for BPH treatment through autophagy activation by cordycepin have not been studies. We hypothesized that cordycepin was sufficiently able to increase autophagy and improve the indicators of BPH in an in vitro system using BPH-1 cell line. Therefore, the purpose of this study was to investigate whether cordycepin could alleviate BPH through activating autophagy using the BPH epithelial cell line (BPH-1).

MATERIALS AND METHODS

Chemicals and Antibodies

Bafilomycin A1 (B1793), Colchicine (C9754), anti-LC3B (L7543), and anti-actin (A2066) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cordycepin was purchased from TCI chemicals. Anti-phospho-S6 (ser235/236) (2211) and anti-S6 ribosomal protein (2217) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Atg5 (sc-133158), anti-AR (sc-7305), anti-Cdk1 (sc-53219), anti-cyclin B1 (sc-245), TNF-α (sc-130349), and anti-IL-1β (sc-32294) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

RWPE-1 cells (non-tumoral, wild-type AR^+), a normal human prostate cell line, were purchased from ATCC (www.atcc.org). BPH-1 cells, the benign prostate hyperplastic epithelial cell line, were purchased from the Leibniz-Institut DSMZ (ACC-143; www.dsmz.de). Each cell line was cultivated using its own medium at 37°C containing 5% CO_2 until the experiments were performed. RWPE-1 cells were cultivated using Keratinocyte Serum Free Medium supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL recombinant human EGF and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, USA). BPH-1 cells were maintained in RPMI 1640 medium supplemented with testosterone 20 ng/mL, transferrin 5 μg/mL, sodium selenite 5 ng/mL, insulin 5 μg/mL, 20% fetal bovine serum (www.pan-biotech.de).

siRNA Transfection

Atg5 siRNA (Cell Signaling Technology, #6345) or non-specific scrambled siRNA (Cell Signaling Technology, #6568, Beverly, MA, USA) was diluted in Opti-MEM reduced serum medium (Invitrogen, 31985-070, Carlsbad, CA, USA). The diluted siRNA and Lipofectamine 2000 transfection reagent (Invitrogen, USA; 11668–019) was mixed, and incubated at room temperature for 15 min. The siRNA mixture was added to each well of 6-well plates at a final siRNA concentration of 25 nM. RWPE-1 cells were harvested after being cultured in normal medium for 24 or 48 h.

Measurement of Autophagic Flux and Western Blot

RWPE-1 and BPH-1 cells, grown on 6-cm dishes were treated with DMSO, cordycepin (100 μM), EtOH, and rapamycin (10 μg/mL) for 48 h. To measure in vitro autophagic flux, cells were treated with and without 200 nM bafilomycin A1 (an autophagy inhibitor) or DMSO for 8 h. Cells were harvested, autophagy flux was quantified using LC3-II protein (an autophagosome marker) turnover measured via Western blot. The detailed method has been described previously (Ju et al., 2016; Oh et al., 2020). Since endogenous LC3-II levels can increase, decrease or remain unchanged in the setting of autophagic induction, it is necessary to measure LC3-II protein levels in the presence and absence of lysomotropic agents, such as bafilomycin A1 that block the degradation of LC3-II (Rubinsztein et al., 2009), which is also called LC3-II turnover assay. Bafilomycin A1 is a known inhibitor of the latter stages of autophagy, inhibiting fusion between autophagosomes and lysosomes by inhibiting vacuolar H^+ ATPase (Yamamoto et al., 1998). Extracted protein (20–60 μg) was subjected to SDS-PAGE (10–15% resolving gel) and transferred onto nitrocellulose membranes. After blocking in a solution of TBS-T containing 5% BSA for 2 h at room temperature, the proteins were identified using a primary antibody in 1% BSA and TBS-T at 4°C overnight. After incubation with the appropriate secondary antibody, bands were visualized by ECL solution (Thermo Fisher Scientific, Madison, WI, USA). Densitometry analysis was conducted using ImageJ software (NIH).

Cell Viability and Proliferation Assay

Cell viability and proliferation was assessed by the MTT assays. Briefly, 20 μL of MTT solution (5 mg/mL) was added to each well of the 96-well assay plate containing the samples in 100 μL of culture medium, the plate was incubated at 37°C for 3 h, then 150 μL of MTT solvent was added, and the absorbance

Table 1. Primer sequences used for RT-qPCR

Gene	Annealing temperature	Primers sequences (5' → 3')	Length
gapdh (human)	59°C	F: TGACCTTTCTGTAGCTGGGG	20
	59°C	R: CAAGCCCACCCCTTCTCTAA	20
lc3b (human)	59°C	F: TACATCCCTTTTCTGCCGGT	20
	59°C	R: ACCGGTGTCTGGAACTTAGG	20
bax (human)	60°C	F: TCACGGAGGAAGTCCAGTGT	20
	60°C	R: CCAAGAAGCTGAGCGAGTGT	20
bcl2 (human)	60°C	F: CACCTGTGGTCCACCTGAC	19
	60°C	R: AATCCCCATTTACGCTGATGATC	20
AR (human)	62°C	F: ATGTCCTGGAAGCATTGAGCCA	22
	62°C	R: CAGAAAGGATCTTGGGCACTTGC	23
PSA (human)	60°C	F: AGTTCATGCTGTGTGCTGGA	20
	60°C	R: TCCACTTCCGGTAATGCACC	20

F— forward primer; R—reverse primer

was measured at 570 nm. The experiments were performed in triplicate.

RT-qPCR Analysis

RT-qPCR was performed on the LightCycler® 96 system (Roche, Mannheim, Germany) with AccuPower® GreenStar™ RT-qPCR Master Mix (Bioneer, Dae-jeon, South Korea) in accordance with the manufacturer's instructions. The relative mRNA expression was normalized by GAPDH, and the primer pairs used for the detection are listed in Table 1.

Statistical Analysis

Data analyses were conducted with SPSS 22.0. Data are presented as means ± SE and were analyzed by a univariate analysis of variance (ANOVA) followed by Fisher's LSD post hoc comparisons at $p < 0.05$.

RESULTS

Comparisons of Autophagy Flux, BPH-Related mRNA Expression, and Cell Proliferation between RWPE-1 and BPH-1 Cells

The autophagy flux assay showed that treatment with bafilomycin A1 significantly increased the levels of LC3-II compared with the DMSO-treated control groups in both RWPE-1 and BPH-1 cells. When both cells were treated with bafilomycin A1, LC3-II protein levels of the BPH-1 cells were significantly less increased (~35%) than those of RWPE-1 cells ($p < 0.05$, Figs. 1a, 1b). Western blot showed a significant increase in P-S6 (ser235/236) of BPH-1 cells when compared with RWPE-1 cells with or without bafilomycin A1 treatment ($p < 0.05$, Figs. 1a, 1c). These data suggest that autophagy is decreased/impaired in BPH-1 cells.

RT-qPCR was used to determine mRNA expression levels of AR, as well as androgen-associated proteins such as 5AR-2, and PSA. The mRNA expression levels of AR (~200%), 5AR-2 (~150%), and PSA (~80%) of BPH-1 cells were significantly higher than those of RWPE-1 cells ($p < 0.05$, Fig. 1d). Cell proliferation was analyzed using an MTT assay, and the cell proliferation rate was significantly increased in BPH-1 cells (~87%) compared with RWPE-1 cells ($p < 0.05$, Fig. 1e). These data suggest that BPH-1 cell line has the pathological conditions of BPH.

Autophagy Inhibition in RWPE-1 Cells Mimics the Characteristics of BPH-1 Cells

To demonstrate whether decreased autophagy is associated with the etiology of BPH, autophagy was inhibited through Atg5 siRNA transfection in RWPE-1 cells in the present study. Western blot analysis showed that Atg5 was significantly knocked down by ~75% compared with the control siRNA transfection ($p < 0.05$, Figs. 2a, 2b). The mRNA expression levels of AR, 5AR-2, and PSA in cells transfected with the Atg5-targeted siRNA were increased by ~85, ~75, ~8%, respectively, compared with the sham control ($p < 0.05$, Fig. 2c). In addition, compared with the sham control, Atg5 knockdown significantly elevated cell proliferation rates of RWPE-1 cells after 24- and 48-h siRNA transfection ($p < 0.05$, Fig. 2d). Together, these results indicate that impaired autophagy may be one of the causes of BPH.

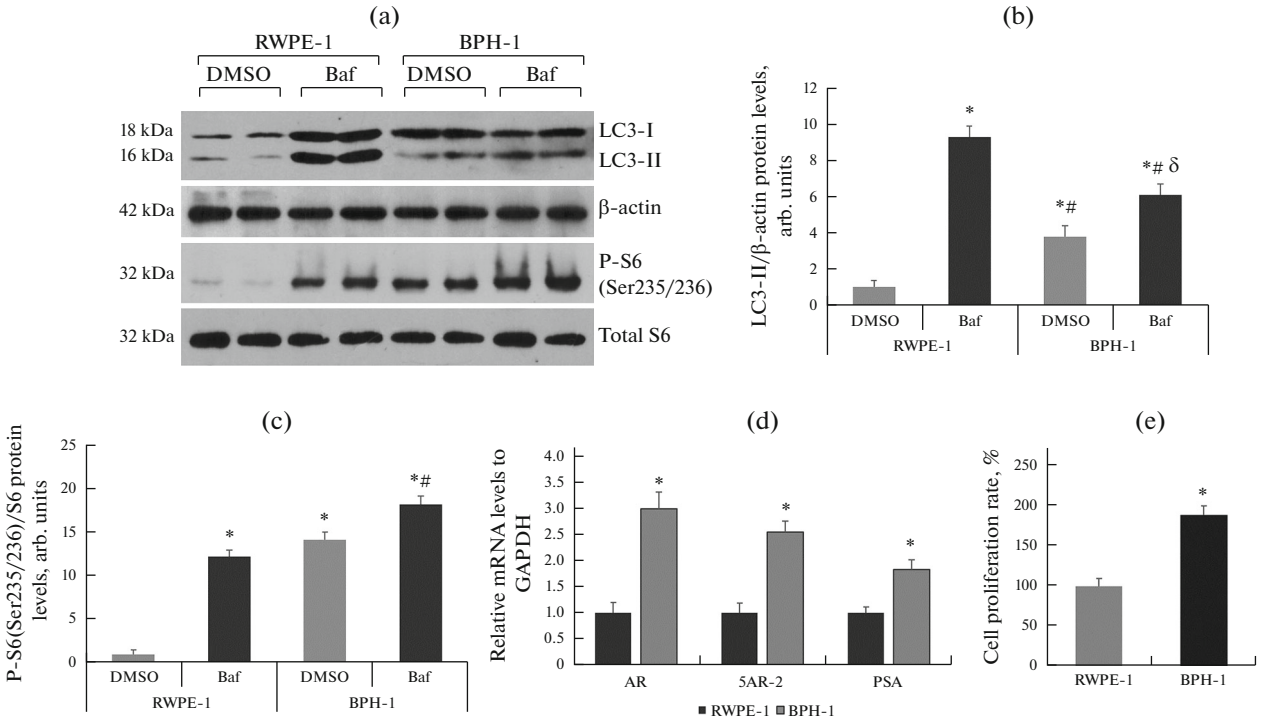


Fig. 1. Autophagy flux assay, an in vitro LC3 protein turnover assay via Western blot, showed that autophagy flux was decreased in BPH-1 cells compared with RWPE-1 cells. (a) Representative immunoblot images of LC3, β-actin, phosphor-S6 (ser235/236) or total S6. (b) LC3-II/β-actin and (c) P-S6/total S6 ratios were quantitated via densitometry from 8 samples per treatment conditions. $^{\#}$ $p < 0.05$ vs. RWPE-1 + DMSO, $^{\#}$ $p < 0.05$ vs. RWPE-1 + bafilomycin (Baf), $^{\delta}$ $p < 0.05$ vs. BPH-1 + DMSO. BPH-related mRNA expression and cell proliferation rates were elevated in BPH-1 cells compared with RWPE-1 cells. (d) The mRNA levels of AR, 5AR-2 and PSA were determined by RT-qPCR. (e) Cell proliferation rates of RWPE-1 and BPH-1 cells were measured using MTT assay. Values are means ± SE; * $p < 0.05$ vs. RWPE-1.

Effects of Cordycepin on Cell Viability and Autophagy Flux in RWPE-1 and BPH-1 Cells

BPH-1 cells were treated with different concentrations of cordycepin (0, 50, 100, 200, 300, 400 μM) for 24- and 48-h and the cell viability was assessed by an MTT assay. As shown in Fig. 3a, the OD values of BPH-1 cells treated with 50 and 100 μM of cordycepin for 48 h were not significantly different from those of the control (no cordycepin), but 200, 300, and 400 μM of cordycepin concentrations significantly decreased cell viability ($p < 0.05$, Fig. 3a). To circumvent cellular toxic effects of cordycepin, concentrations lower than 200 μM were used throughout the study. Cell proliferation was determined using MTT assay at two time point, 24 and 48 h after treatment with DMSO or cordycepin (100 μM) demonstrating that the cell proliferation rates were significantly decreased, and cordycepin exhibited an inhibitory effect on cell proliferation in BPH-1 cells ($p < 0.05$, Fig. 3b).

RWPE-1 cells were treated with two different concentrations of cordycepin (50 and 100 μM) for 48 h, and autophagy flux and AMPK/mTOR activation were assessed by western blot analyses. The protein levels of LC3-II were significantly higher in both 50 (~190%) and 100 μM (~225%) concentrations in the (cordycepin + bafilomycin) groups than those in the (DMSO + bafilomycin) group ($p < 0.05$, Figs. 3c, 3d). The levels of phosphorylation of AMPK (Thr172) were significantly higher in the 100 μM (~120%) than the DMSO control ($p < 0.05$, Figs. 3c, 3e). S6 (ser235/236) phosphorylation was significantly decreased in both 50 μM (~50%) and 100 μM (~88%) of the DMSO group ($p < 0.05$, Figs. 3c, 3f). These data suggested that treatment with 100 μM of cordycepin induced AMPK activation and downregulated mTOR signaling suggesting that cordycepin sufficiently induces autophagy flux in RWPE-1 cells.

To determine whether cordycepin would also be able to induce autophagy flux sufficiently in BPH-1 cells, the autophagy flux assay was performed 48 h after cordycepin treatment. Cordycepin treatment led to a further increase in the protein levels of LC3-II than bafilomycin alone (the DMSO + Bafilomycin group) in both cell lines, RWPE-1 (~88%) and BPH-1 (~50%) ($p < 0.05$). However, LC3-II protein levels of the BPH-1 cells were significantly less increased (~50%) than those of RWPE-1 cells when both cells were treated with cordycepin (100 μM) ($p < 0.05$, Figs. 3g, 3h). Cordycepin decreased p62 protein levels by ~60% in RWPE-1 cells but p62 protein levels were

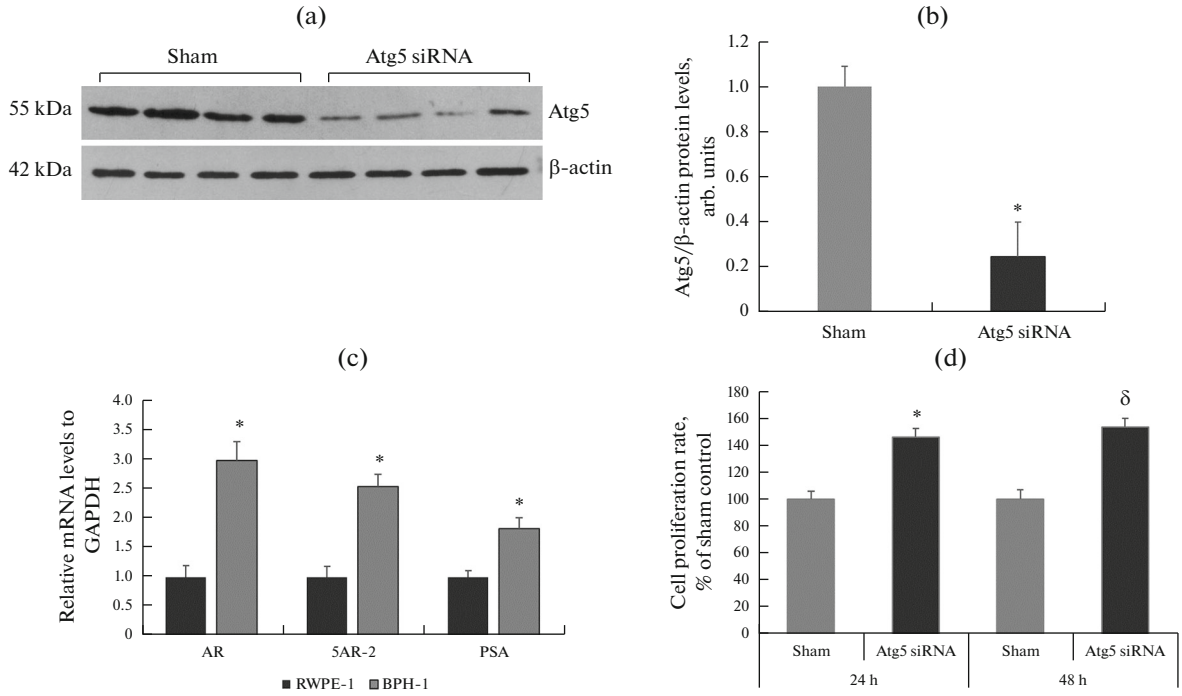


Fig. 2. Inhibition of autophagy using siRNA technique in RWPE-1 cells mimics the characteristics of BPH-1 cells. (a) Representative immunoblot images of Atg5 knockdown cells. (b) siRNA knockdown of Atg5 effectively suppressed Atg5 protein expression levels. (c) After knockdown of Atg5, the mRNA expression levels of AR, 5AR-2 and PSA were determined by RT-qPCR and (d) cell proliferation rates were measured using MTT assay. Each bar represents the mean ± SE for Atg5 siRNA transfected RWPE-1 cells. * $p < 0.05$ vs. 24 h sham control; $^{\delta}p < 0.05$ vs. 48 h sham control. AR—androgen receptor, 5AR-2—5-alpha reductase2, and PSA—prostate-specific antigen.

significantly less decreased (~30%) in BPH-1 cells ($p < 0.05$, Figs. 3g, 3i). It has been indicated that p62 decreases when autophagy is induced and accumulates when autophagy is inhibited. Together, these data suggested that cordycepin activated autophagy in both cell lines but activation of autophagy through cordycepin treatment was significantly attenuated in BPH-1 cells.

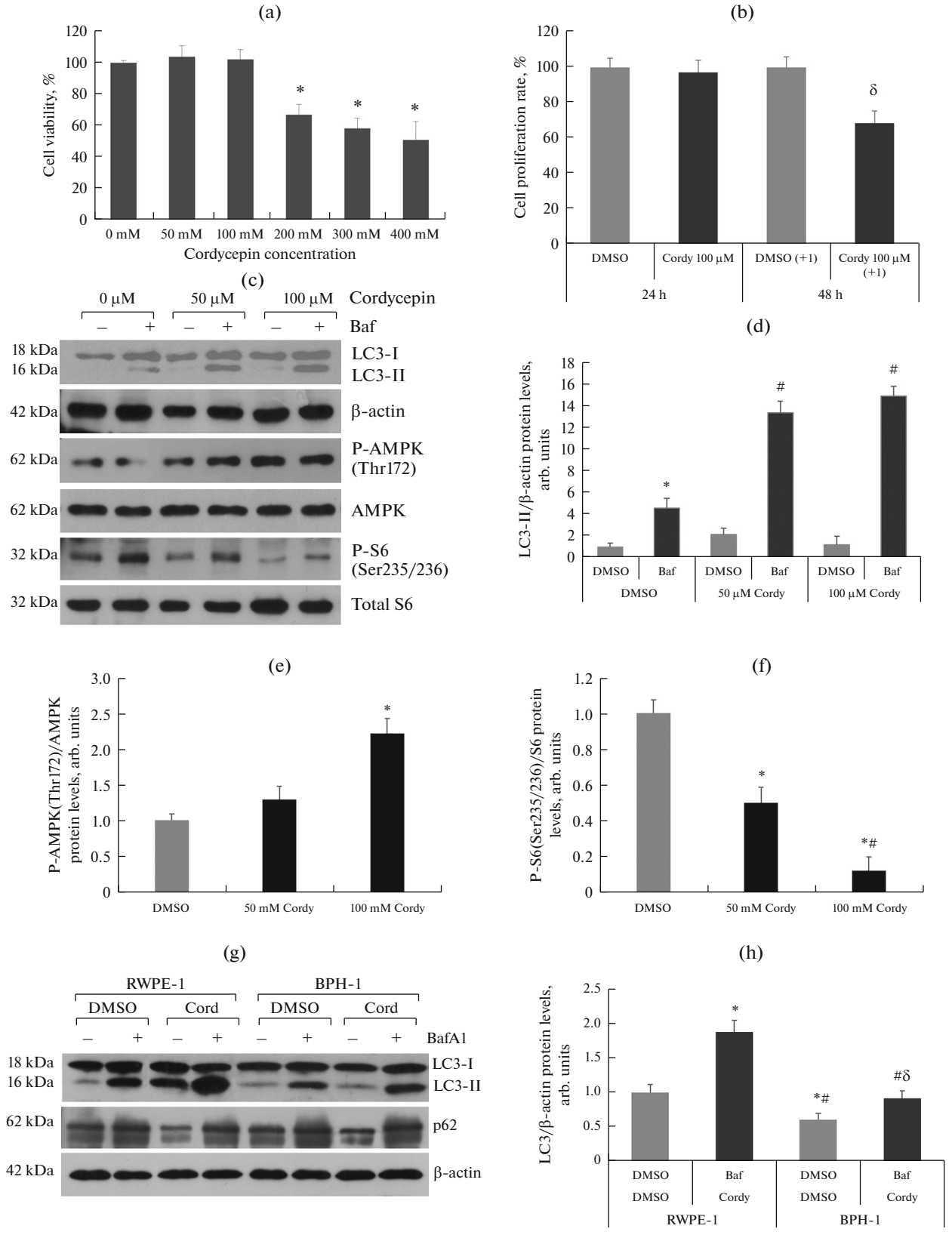
Effects of Cordycepin on BPH-Related mRNA Expression and Cell Proliferation in BPH-1 Cells

BPH-1 cells were treated with cordycepin (100 μM) or DMSO, and rapamycin (10 μg/mL) or EtOH for 48 h and the mRNA levels of AR, 5AR-2, and PSA, which are BPH-related markers, were quantified using RT-qPCR. Treatment with 100 μM of cordycepin significantly decreased AR (~63%), 5AR-2 (~46%), and PSA (~68%) mRNA expression levels in BPH-1 cells ($p < 0.05$, Fig. 4a). Consistent with these results, mRNA expression of these BPH-related genes was also significantly reduced by rapamycin (10 μg/mL), which is an mTOR inhibitor and well-known autophagy activator ($p < 0.05$, Fig. 4a). Cell proliferation rates were determined using MTT assay at 48 h after treatment with DMSO, cordycepin (100 μM), EtOH, and rapamycin (10 μg/mL). Both treatments of cordycepin and rapamycin reduced cellular proliferation rates in BPH-1 cells by ~29 and ~39%, respectively ($p < 0.05$, Fig. 4b). These effects of cordycepin on inhibition of BPH-related gene expression and cell proliferation appears to be elicited through autophagy activation

We investigated whether cordycepin could inhibit the expression of cyclin-dependent kinase 1 (Cdk1) and Cyclin B1, which are cell proliferation markers. Cordycepin significantly reduced Cdk1 (~65%), and Cyclin B1 (~43%) protein levels in BPH-1 cells ($p < 0.05$). Similarly, protein expression of these cell proliferation markers was also significantly decreased by rapamycin by ~83 and ~45%, respectively ($p < 0.05$, Figs. 4c–4e).

Effects of Cordycepin on Apoptosis-Related mRNA Expression and Inflammatory Marker Proteins in BPH-1 Cells

To investigate whether cordycepin could affect apoptosis in BPH-1 cells, cells were treated with cordycepin (100 μM) or DMSO, and rapamycin (10 μg/mL) or EtOH for 48 h, and mRNA expression levels of Bax and Bcl-2, which are apoptosis-related markers, were detected by RT-qPCR. Treatment with either cordycepin or rapamycin had no effect on mRNA expression of Bax and Bcl-2 ($p > 0.05$,


(a)
Cell viability, %
120
100
80
60
40
20
0
0 mM
50 mM
100 mM
200 mM
300 mM
400 mM
Cordycepin concentration
(b)
Cell proliferation rate, %
DMSO
Cordy 100 μM
DMSO (+1)
Cordy 100 μM (+1)
24 h
48 h
(c)
0 μM
50 μM
100 μM
Cordycepin
Baf
18 kDa
16 kDa
LC3-I
LC3-II
42 kDa
β-actin
62 kDa
P-AMPK (Thr172)
62 kDa
AMPK
32 kDa
P-S6 (Ser235/236)
32 kDa
Total S6
(d)
LC3-II/β-actin protein levels, arb. units
DMSO
Baf
50 μM Cordy
100 μM Cordy
(e)
P-AMPK(Thr172)/AMPK protein levels, arb. units
DMSO
50 mM Cordy
100 mM Cordy
(f)
P-S6(Ser235/236)/S6 protein levels, arb. units
DMSO
50 mM Cordy
100 mM Cordy
(g)
RWPE-1
BPH-1
DMSO
Cord
BafA1
18 kDa
16 kDa
LC3-I
LC3-II
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Cordy
RWPE-1
BPH-1

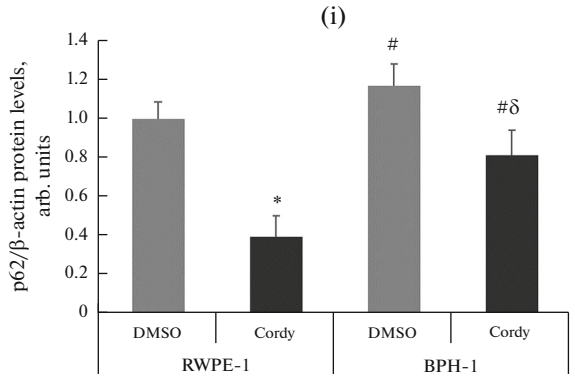

Fig. 3. (a) The cell viability of BPH-1 cells treated with different concentrations of cordycepin (Cordy: 0, 50, 100, 200, 300, 400 μM) was evaluated and (b) the cell proliferation was determined at two time points of 24 and 48 h after treatment with DMSO or Cordy (100 μM). * $p < 0.05$ vs. 0 μM, $^{\delta}$ $p < 0.05$ vs. DMSO control. An in vitro autophagy flux assay showed that cordycepin (50 and 100 μM) sufficiently induces autophagy flux in RWPE-1 cells. (c) Representative immunoblot images of LC3, β-actin, phosphor-AMPK (thr172), total AMPK, phosphor-S6 (ser235/236) or total S6. (d) LC3-II/actin, (e) P-AMPK/total AMPK, and (f) P-S6/total S6 ratios were quantitated via densitometry; values are means ± SE ($n = 8$). * $p < 0.05$ vs. DMSO; $^{\#}$ $p < 0.05$ vs. 50 μM Cordy. When autophagy flux was compared, Cordy induces autophagy less potently in BPH-1 cells compared RWPE-1 cells. (g) Representative immunoblot images of LC3 and p62 following 48 h Cordy treatment (100 μM) in RWPE-1 and BPH-1 cells. (h) LC3-II/β-actin and (i) p62//β-actin ratios were quantitated via densitometry; values are means ± SE; ($n = 8$). * $p <$ 0.05 vs. RWPE-1 + DMSO + DMSO; $^{\#}$ $p < 0.05$ vs. RWPE-1 + Cordy + Baf); $^{\delta}$ $p < 0.05$ vs. BPH-1 + DMSO + DMSO. Baf—bafilomycin A1.

Figs. 5a, 5b). In the present study, two pro-inflammatory markers were used to see if cordycepin could sufficiently reduce inflammation in BPH-1 cells. Cordycepin treatment significantly decreased both TNF-α and IL-1β protein levels in BPH-1 cells by ~55% and ~60%, respectively ($p < 0.05$, Figs. 5c–5e). In addition, rapamycin also significantly reduced IL-1β protein levels ($p < 0.05$) but failed to decrease TNF-α protein levels in BPH-1 cells ($p > 0.05$).

DISCUSSION

The present study demonstrated that cordycepin significantly reduced BPH-related gene expression, cellular proliferation rates, and cell cycle marker proteins in BPH-1 cells and these effects appeared to be mediated via autophagy activation by cordycepin treatment. When autophagy was inhibited by siRNA knockdown, these positive effects were abolished in BPH-1 cell line. Therefore, it is plausible that the etiology of BPH could be the result of impaired autophagy. This notion was previously proposed in a recent study (Oh et al., 2020). The current study has also shown that autophagy flux was significantly decreased in BPH-1 cells compared with RWPE-1 cells. This defective autophagy observed in BPH-1 cells seems to parallel with the pathological conditions of BPH. Autophagy-impaired BPH-1 cells showed that mRNA expression of AR and its associated proteins such as 5AR-2 and PSA was significantly increased compared with that of RWPE-1 cells. In addition, cell proliferation rates were shown to be a significant increase in BPH-1 cells compared with those of RWPE-1 cells (Fig. 1). Atg5 knockdown caused a significant increase in mRNA expression levels of AR, 5AR-2, PSA, and cell proliferation rates in RWPE-1 cells (Fig. 2). This suggests that decreased autophagy may not entirely but partly account for BPH disease.

Cordycepin has been shown to activate autophagy in various cell lines (Choi et al., 2011; Lee et al., 2014; Yu et al., 2017; Chaicharoenaudomrung et al., 2018). The current study has also shown that cordycepin was able to induce autophagy flux in both RWPE-1 and BPH-1 cell (Fig. 3). This raised the question of whether the increase in autophagy by cordycepin treatment still could reduce the pathological markers of BPH in BPH-1 cells. Cordycepin significantly decreased on AR, 5AR-2, and PSA mRNA expression levels in BPH-1 cells. Consistent with these results, mRNA expression of these BPH-related genes was also significantly reduced by rapamycin in the current study. Both cordycepin and rapamycin reduced cellular proliferation rates in BPH-1 cells. Therefore, the effects of cordycepin on inhibition of BPH-related gene expression and cell proliferation rates are assumed to occur via mTOR-dependent autophagy mechanism. A recent study demonstrated the mechanism of activation of AMPK by cordycepin (Hawley et al., 2020). Cordycepin enters cells via adenosine transporters and is converted by cellular metabolism into monophosphate (cordycepin monophosphate), which then acts as an AMP analog. Cordycepin monophosphate activates AMPK by mimicking multiple effects of its natural activator, AMP. In addition, 100 μM of cordycepin, which was the same concentration used in this study, activated AMPK in human cells

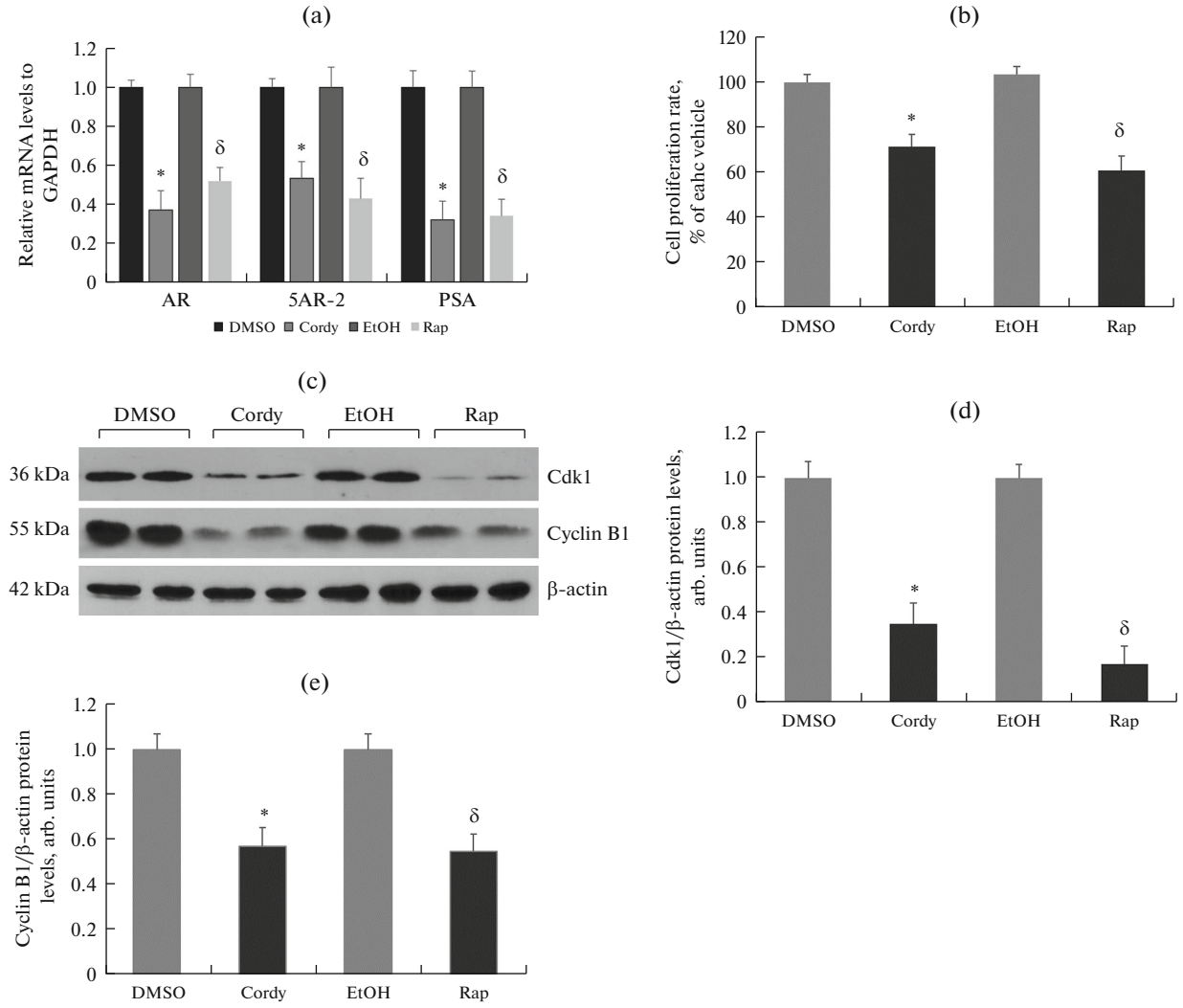

Fig. 4. Effects of cordycepin (Cordy) on BPH-related mRNA expression and cell proliferation in BPH-1 cells. (a) Cells were treated with DMSO, Cordy (100 μM), EtOH, and rapamycin (Rap, 10 μg/mL) for 48 h and the mRNA levels of androgen receptor (AR), 5-alpha reductase 2 (5AR-2), and prostate-specific antigen (PSA) were quantified using RT-qPCR. (b) Cell proliferation rates were determined using MTT assay at 48 h after treatment with DMSO, Cordy (100 μM), EtOH, and Rap (10 μg/mL). The expression levels of cell proliferation marker proteins were determined via Western blot following the same treatments were applied. (c) Representative immunoblot images of Cdk1, Cyclin B1 and β-actin. (d) Cdk1/β-actin and (e) Cyclin B1/β-actin ratios were quantitated via densitometry from 8 samples per treatment conditions. * $p < 0.05$ vs. DMSO; $^{\delta}$ $p < 0.05$ vs. EtOH.

and higher concentrations were shown to be toxic (Hawley et al., 2020), which similarly cell toxicity was observed above the concentration in the current study. These and the present research suggest that cordycepin increases autophagy via AMPK activation. This study has also shown that cordycepin activated autophagy via AMPK-mTOR-dependent signaling pathway (Fig. 3).

There was a significant decrease in the expression of Cdk1 and cyclin B1, both of which are key molecular regulators of G2/M progression (Williams and Stoeber, 2012), by cordycepin treatment (Fig. 4). These results indicate that cordycepin plays a role in antiproliferative function by downregulating the levels of cell cycle related proteins. It was found that cordycepin (100 μM) did not change the mRNA expression of apoptosis-related proteins, Bax and Bcl-2. Studies have shown that cordycepin inhibits cell cycle and induces apoptosis, especially cancer cell lines (Jung et al., 2012; Liao et al., 2015). However, in this study, cordycepin (100 μM) was sufficient to induce the suppression of cell proliferation, but 100 μM of cordycepin may not stimulate apoptosis in BPH-1 cells (Fig. 5). Cell viability for BPH-1 cells was not affected by treatment with cordycepin at a concentration of 100 μM for 48 h. Cell cycle arrest and apoptosis are both likely responses to certain events, such as cellular stress and DNA damage, etc. The two pathways could overlap somehow, but there might be no guarantee over sequential links from cycle arrest to cell death. In

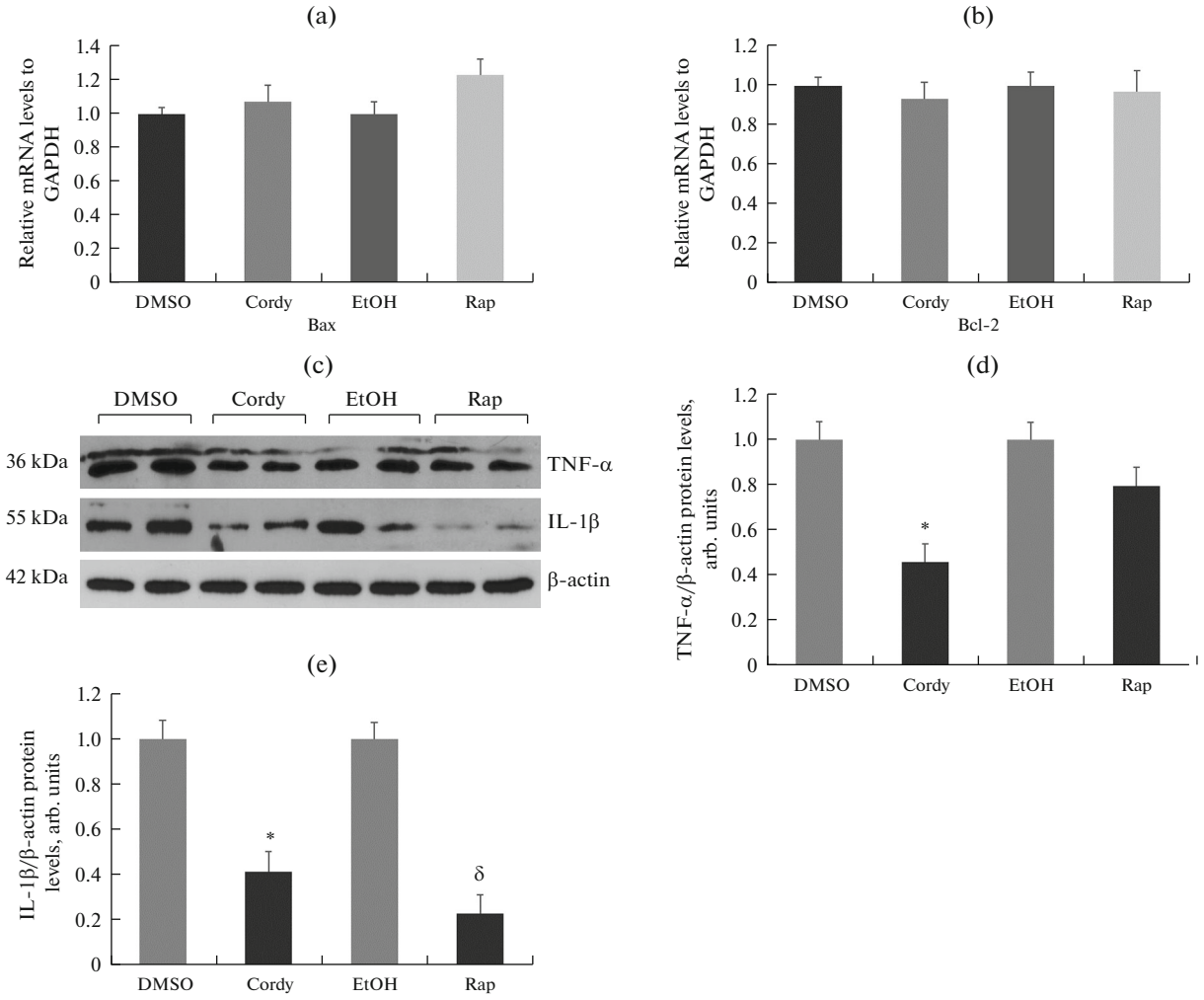

Fig. 5. Effects of cordycepin (Cordy) on expression levels of apoptosis-related mRNA and inflammatory marker proteins in the BPH-1 cells. The mRNA expression levels of (a) Bax and (b) Bcl-2 were determined by RT-qPCR. (c) Representative immunoblot images of TNF-α, IL-1β, β-actin. (d) TNF-α/β-actin and (e) IL-1β/β-actin ratios were quantitated via densitometry. Values are means ± SE; ($n = 8$). $^{\#}$ $p < 0.05$ vs. RWPE-1 + DMSO. * $p < 0.05$ vs. DMSO; $^{\delta}$ $p < 0.05$ vs. EtOH. Rap—rapamycin.

the present study, cordycepin (100 μM) sufficiently activated autophagy but it might not lead to autophagy-dependent cell death (type 2 programmed cell death) in BPH-1 cells. Indeed, in some cases the same proteins control both autophagy and apoptosis. Apoptotic signaling can regulate autophagy and conversely autophagy can regulate apoptosis (and most likely other cell death mechanisms). However, the molecular connections between autophagy and cell death are complicated and, in different contexts, autophagy may promote or inhibit cell death (Lane et al., 2013). Many researchers have used an apoptosis-inducing approach for the treatment of BPH and it is questionable that targeting apoptosis-based therapy would be an optimal strategy for BPH treatment. Targeting inhibition of cell cycle progression and cellular proliferation in the prostate with BPH without causing cellular toxicity and/or cell death through using higher concentrations of cordycepin or other drugs might be better and suitable therapeutic intervention tactics for treating and alleviating BPH.

As shown in Fig. 5, cordycepin significantly decreased both TNF-α and IL-1β protein levels in BPH-1 cells. Several investigations have indicated that cordycepin has an anti-inflammatory potential by suppressing the NF-κB signaling pathway, suggesting that cordycepin could be used as an anti-inflammatory agent in the treatment of inflammation-associated disorders. For example, cordycepin inhibits LPS-induced proinflammatory mediators and/or cytokines in RAW 264.7 macrophage (Kim et al., 2006) and BV2 microglial cell models (Jeong et al., 2010) by blocking NF-κB activation. Cordycepin also prevents LPS-induced airway neutrophilia in mice and effectively blocks LPS-induced expression of vascular adhesion molecule-1 in human lung epithelial cells (Kim et al., 2010). To date, there have been no studies of anti-

inflammatory effects of cordycepin on BPH. This is the first study to show that anti-inflammatory effects of cordycepin in BPH-1 cell line.

Taking advantage of the in vitro BPH model system, the present study demonstrated that autophagy is impaired/decreased in BPH-1 cells. The pathological conditions of BPH, such as high levels of BPH-related markers (AR, 5AR-2, and PSA), cell proliferation, cell cycle, and inflammation may be due in part to defective autophagy observed in BPH-1 cells. Treatment with 100 μM cordycepin sufficiently elevated autophagy flux in autophagy-impaired BPH-1 cells. Cordycepin significantly decreased AR, 5AR-2, and PSA mRNA expression levels, cell proliferation rates, and the markers proteins of both cell cycle and inflammation in BPH-1 cells. These positive effects of cordycepin appear to occur via AMPK-mTOR-dependent autophagy signaling mechanism. This study suggests that cordycepin is a potent autophagy activator and may be an excellent agent for the treatment of BPH and other autophagy-impaired diseases and activating autophagy flux in the prostate with BPH may be a promising potential strategy for BPH treatment and prevention of BPH development.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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