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RESEARCH ARTICLE

Usnic Acid Exerts Antiproliferative and Apoptotic Effects by Suppressing NF-κB p50 in DU145 Cells

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ABSTRACT

Objective: Nuclear factor kappa B (NF-κB) is one pathway that controls the expression of genes involved in many cancer events such as proliferation, apoptosis, metastasis, and invasion. Usnic acid is a molecule with many biological effects such as being anti-cholinergic, gastroprotective, anti-inflammatory, anti-cancerous, and especially antioxidant. This study aims to mechanistically examine the apoptotic behaviors of usnic acid in DU145 prostate cancer cells and the molecules it acts on in the NF-κB pathway.

Materials and Methods: This study investigates the apoptotic changes in DU145 cells after usnic acid administration through JC-1 staining and caspase-3 activity measurements. In addition, it tests the effects of usnic acid on subunit p50 and p65 protein and gene expressions in the NF-κB pathway through the respective Western blot and qPCR measurements.

Results: The IC_{50} values of usnic acid at 24 and 48 h in DU145 cells were calculated as 167.06±12.35 µM and 42.15±3.76 µM, respectively. In addition, JC-1 staining showed usnic acid-treated DU145 cells to trigger apoptosis by increasing the membrane permeability of their mitochondria. NF-κB p50 protein expression was also found to be suppressed after usnic acid administration.

Conclusion: The results of this study show usnic acid administration to suppress proliferation and to induce mitochondrial apoptosis by suppressing the NF-κB pathway in DU145 cells. This effect of usnic acid indicates it to be combinable with chemotherapeutic agents and evaluable as an alternative in cancer treatment.

Keywords: Apoptosis, NF-κB pathway, prostate cancer, usnic acid

INTRODUCTION

Prostate cancer is the second most widespread type of carcinoma in males following lung cancer. According to the latest Globocan data, 375,608 deaths due to prostate cancer have been reported worldwide.[1] The most important risk factors for prostate cancer are genetic predisposition, smoking, excessive red meat diet, and excessive hormonal expression.[2] In particular, men with high serum amounts of insulin-like growth hormone-1 are reported to be 1.7 to 3.4 times more likely to develop prostate cancer than men with low serum levels.[3] Docetaxel, cabazitaxel, mitoxatrone and estramustine are the chemotherapeutics currently used in the treatment of prostate cancer.[4] These types of chemotherapeutics also cause side effects such as nausea, vomiting, and hair loss, which make routine life difficult. Therefore, the discovery of new agents in the treatment of cancer is an urgent necessity.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor is a pathway that has demonstrated a primary role in the inflammation and initiation of immune response. NF-κB has been emphasized to arrange the expression of genes apoptosis migration and to control cell proliferation in cancer development.[5,6] Due to NF-κB being overexpressed in cancer cells, studies aimed at elucidating this signaling pathway and developing NF-κB-targeted therapy strategies have gained momentum with regard to cancer treatment. Usnic acid is a dibenzofuran-origin compound found abundantly in lichen species.[7] Usnic acid has been reported to have many analgesic, antibiotic, antiviral, antiprotozoal, anti-inflammatory, and cytotoxic pharmacological effects.[8] In addition to these effects, usnic acid has also been reported to have similar activity with existing antioxidant molecules such as trolox and alpha tocopherol in tests regarding antioxidant capacity measurement such as Fe^{3+} and Cu^{2+} reduction, DPPH, $ABTS^{+}$, $DMPD^{+}$, and the $O_2.^{-}$ superoxide anion radical.[9] The phenolic groups present in the structure of usnic acid strengthen its radical scavenging effect, with usnic acid showing greater effects in aqueous media due to being a natural antioxidant.[10]

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Usnic acid has also been shown to have a high capacity to scavenge superoxide radicals in methanol extracts of usnic acid isolated from lichen species.[11] Investigating the anti-cancer effects of natural substances with antioxidant properties and high bioactivity and determining their effects on cellular processes are important. This study investigates the proliferative and apoptotic effects of usnic acid application in DU145 cells and additionally examines the possible effects of usnic acid applications on p50 and p65 expression in the NF-κB pathway.

MATERIALS AND METHODS

Chemicals and Cell Culture Equipment

Usnic acid was purchased from the Sigma company (Sigma 329967, Darmstadt, Germany). All equipment and supplements for cell culturation such as Trypsine-EDTA, penicillin-streptomycin, fetal bovine serum, and DMEM medium were bought from Gibco (Billings, MO, USA).

Cell Culture

The DU145 human prostate cancer cell lines were bought from the American Type Culture Collection (ATCC HTB-81, Rockville, MD, USA). Cells were grown in a DMEM medium supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, 10% fetal bovine serum, and 2 mM L-Glutamine at 37°C and 5% CO_2 ambient conditions. The cell medium was renewed every two days. Once the cells reached 90% occupancy, the stocks were subcultured.

Cytotoxicity Test (MTT Assay)

The DU145 cells were added at a density of $5x10^3$ to 96-well plates in 100 µL of DMEM medium. Cells were incubated for 24 h at 37°C in the CO_2 incubator. Cells were incubated for 24 and 48 h after adding the 5, 10, 25, 50, 100, 250, and 500 µM usnic acid concentrations. At the end of the incubation, 5 mg/mL of MTT (ODC Research and Development Inc., Turkey) were added to the cells, and the cells were incubated for an additional 4 h. After discarding the medium from the cells, 100 µL of DMSO was suffixed to all wells to dissolve the formazan dye that had formed. The intensity of the color that formed was measured spectrophotometrically at 570 nm.[12] The percentage of viable cells was calculated utilizing the following formula:

Cell Viability (%)= (OD test sample/OD control) X 100 (1)

LumiTracker Mito JC-1 Staining

JC-1 is a fluorescent dye used to evaluate mitochondrial membrane potential in apoptotic studies. The DU145 cells were added at a density of $1x10^6$ to 6-well plates. After being incubated for 24 h, 40 µM usnic acid was applied to the cells for another 48 h. The JC-1 dye (ThermoFisher T3168, Waltham, Massachusetts, USA) was added to all wells at a concentration of 10µg/mL, and the cells were incubated at 37°C for 10 min before taking images under a fluorescent microscope.[13]

Caspase-3 Activity Measurement

The DU145 cells were seeded at a density of $1x10^6$ to 6-well plates and then incubated for 24 h. Cells were exposed to 40 µM usnic acid for 48 h, after which the cells were lysed. Caspase-3 activity was measured with the aid of a colorimetric assay kit (Abcam, ab39401, Cambridge, UK).

Protein Expression Analysis (Western Blotting)

The DU145 cells were added at a density of $1x10^6$ to 6-well plates. The cells were incubated for 24 h in a CO_2 oven. Next, the DU145 cells were incubated with 40 µM of usnic acid for 48 h. After the incubation, the cells were collected, and 350 µL of the sample loading buffer (2X) were added to each samples, with the cell proteins underwent denaturation by heating at 95°C.

The stacking and separation gel were prepared utilizing the solutions of pH 6.8 0.5 M Tris, pH 8.8 1.5 M Tris, 10% sodium dodecyl sulphate (SDS), N,N,N,N-tetramethylethylenediamine (TEMED), 30% (w/w) acrylamide-bis-acrylamide, and 10% ammonium persulfate. The samples were then filled onto a gel containing 5-10 µg of protein. The samples underwent electrophoresis with the running buffer (38.4 mM glycine, 1% SDS, 5 mM Tris) for 1-2 h at 100V Following the electrophoresis procedure, immunoblotting was carried out by transferring the gels to the polyvinylidene difluoride membrane. The membrane was respectively washed 3 times using the Tris-buffered saline with 0.1% Tween (TBST) and distilled water. The membrane was blocked with 2.5% bovine serum albumin for 2 h. After washing the membrane 3 times with TBST, it was incubated with the primary antibodies of NF-κB p50 (Santa Cruz SC8414), NF-κB p65 (Santa Cruz SC8008), and β-Actin (Santa Cruz SC47778) overnight at +4°C. The membrane washing process was performed 3 times with TBST for five min each time. After the washings, the membrane was treated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The membrane washing process was performed again 3 times with TBST for 5 min each time. The chemiluminescence reagent (ECL, Santa Cruz) was suffixed to the membrane for 1 min under dark conditions. Band imaging was performed with the help of an imaging system. Densitometric analysis of the protein blots was carried out with the program ImageJ.[14]

Gene Expression Analysis (qRT-PCR)

The total RNA isolation was drawn out from $5x10^6$ DU145 cells as previously described.[15] In accordance with the cDNA Reverse Transcription Kit protocol (Applied Biosytems, Foster City, CA, USA), 1 µg total RNA was utilized for the reverse transcription. Real-time PCR reaction was performed using the *BAX, BCL2, NFKB1*, and *GAPDH* primers with a primer sequence of *BAX*: Forward 5'-GCCCTTTTGCTTCAGGGTTT-3', Reverse 5'-TCCAATGTCCAGCCCATGAT-3'; BCL2: Forward 5'-GACAGAAGATCATGCCGTCC-3', Reverse 5'-GGTACCAATGGCACTTCAAG-3'; *NFKB1*: Forward 5'-GGAGCACTACTTCTTGACCACC-3', Reverse 5'-TCTGTCCTGAGCATTGACGTC-3'; and *GAPDH*: Forward 5'-AGGGCTGCTTTTAACTCTGGT-3', Reverse 5'-CCCCACTTGATTTTGGAGGGA-3'. 100 ng of cDNA were copied using SYBR Green dye with the help of the ABI StepOne Plus detection system. The program characteristics for the amplification reaction involved heating at 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 59°C for 1 min, and 72°C for 30 sec. The qPCR value was calculated utilizing the StepOne Software v2.3 (Applied Biosystems, Foster City, CA, USA), with the the *BAX, BCL2*, and *NFKB1* gene results being normalized to the *GAPDH* gene results.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism (ver. 7.0) software. The statistical analysis performed data entry for all experiments in triplicate and offered the results as mean ± Standard Deviation (SD) . Alterations between groups were specified by one-way analysis of variance (ANOVA) testing.

RESULTS

Cytotoxic Effects of Usnic Acid on DU145 Cells

The cytotoxic effects of the 5, 10, 25, 50, 100, 250 and 500 µM usnic acid applications on DU145 cells for 24 and 48 h were studied using the MTT method. DU145 cell viability was found to decrease in a dosage-dependent manner at both the 24- and 48-h measurements (Figure 1a; $p<0.05$ and $p<0.001$, respectively). The IC_{50} value of usnic acid regarding the DU145 cells was calculated as 167.06 ± 12.35 µM at 24 h and 42.15 ±3.76 µM at 48 h. The morphologies of the DU145 cells treated with 40 µM (≈ IC_{50}) usnic acid were specified to change compared to the control group, with a decrease in intercellular contact (Figure 1b).

The Effects of Usnic Acids on Apoptosis

The intrinsic apoptotic pathway is known to be triggered by cytochrome c, a protein released as a result of deformation of the mitochondrial membrane. The mitochondrial membrane potentials of DU145 cells treated with 40 µM usnic acid were evaluated through JC-1 staining (Figure 2a). After JC-1 staining, the permeability of the mitochondrial membrane was determined to increase in DU145 cells that had been treated with usnic acid (Figure 2b). In addition, the study also measured caspase-3 activity, which is one of the intrinsic apoptotic molecules affected by cytochrome c released by increasing mitochondrial membrane permeability, and found the caspase-3 activity of DU145 cells treated with 40 µM usnic acid to have increased compared to the control group (Figure 2c).

The study tested the effects of usnic acid applications on *BAX* and *BCL2* gene expressions in the apoptotic pathway of DU145 cells using the qPCR method and found the DU145 cells treated with 40 µM usnic acid to have an increase in proapoptotic *BAX* gene expression and a decrease in anti-apoptotic *BCL2* gene expression compared to the control group (Figures 3a and 3b). In addition, the *BAX /BCL2* expression ratio of DU145 cells treated with 40 µM usnic acid was calculated to have increased by approximately eight compared to the control group (Figure 3c).

The Effects of Usnic Acid on NF-κB Pathways

The study evaluated the effect of usnic acid treatment on p50 and p65 protein expressions in the NF-κB pathway of DU145 cells using the Western blot method (Figure 4a) and found NF-κB p50 protein expression of DU145 cells treated with 40 µM usnic acid to decrease compared to the control group. However, no major change was found regarding p65 expression (Figures 4b and 4c). In addition, the *NFKB1* gene expressions of DU145 cells applied 40 µM usnic acid were found to decrease (Figure 4d).

DISCUSSION

Known as the plague of our age, cancer is the disease that causes the most death after cardiovascular system diseases. Intensive studies have been carried out to elucidate upon the formation mechanism of cancer and its treatment. The NF-κB pathway is known to be overexpressed in cancer cells.[16] The NF-κB transcription factor family includes five members: Rel A (p65), RelB, c-Rel, p105/p50, and p100/p52. Each of these proteins comprises an N-terminal Rel chain that enables nuclear localization, DNA binding, and dimerization. The NF-κB signaling pathway shows its molecular effects in two different ways: canonically and non-canonically. [17] Activation of the canonical pathway occurs with stimuli such as tumor necrosis factor (TNF) and interleukin 1 (IL1). Through pathway activation, IKKα, IKKβ, and IKKγ from the κB protein inhibitors trimerize to form the IκB complex. Under basal conditions, the IκB complex binds to the p65-p50 dimer, allowing the p65-p50 dimer to cross the nuclear membrane. The IκB complex, which

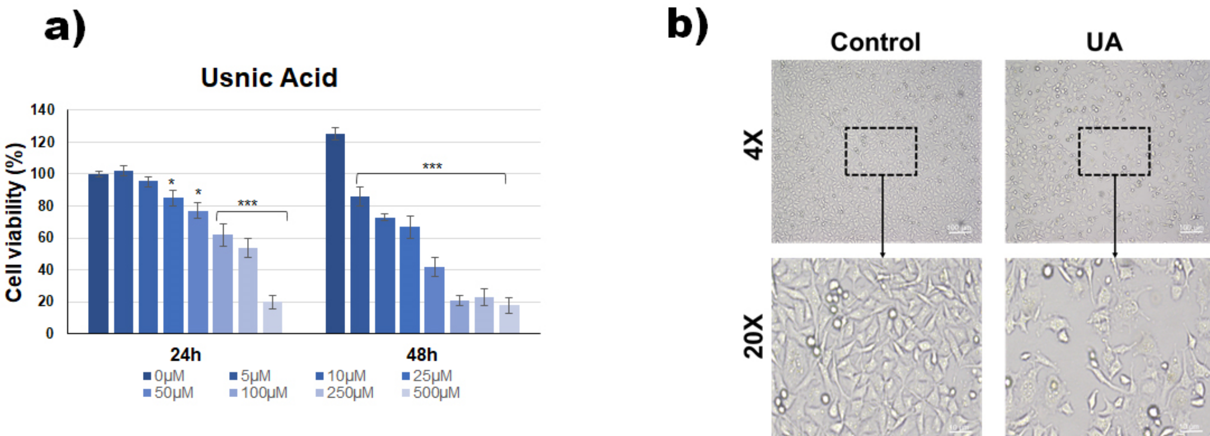

Figure 1. **(a)** Cell viability graph of DU145 cells treated with usnic acids for 24 and 48 h. **(b)** Inverted microscope images of DU145 cells treated with usnic acids (Scale bar= 100 µm) (*p<0.05; ***p<0.001).

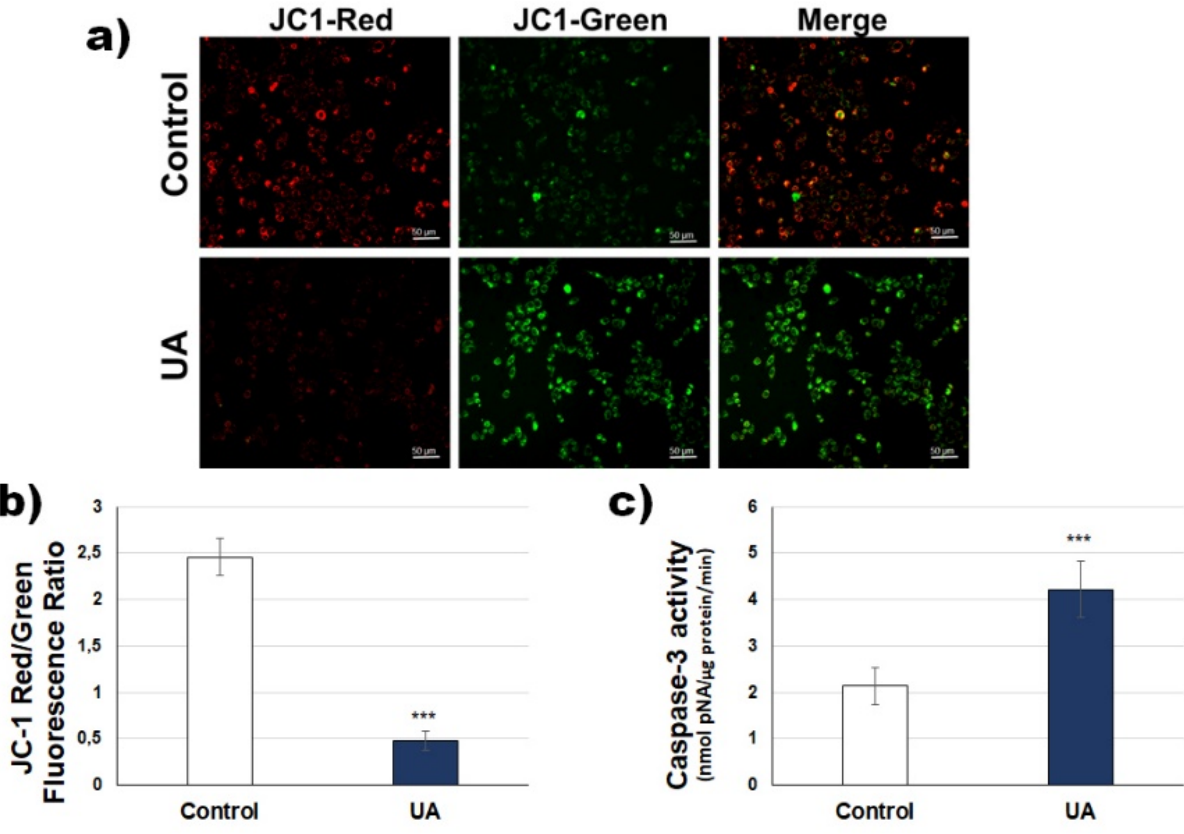

Figure 2. **(a)** JC-1 staining images of DU145 cells treated with usnic acid (Scale bar= 50 µm) **(b)** Graphical illustration of red/green ratio of DU145 cells treated with usnic acid **(c)** Caspase-3 activity graph of DU145 cells treated with usnic acid (***p<0.001).

returns to the cytoplasm, is degraded by ubiquitination. The p65-p50 dimer activates the transcription of target genes such as anti-apoptotic factors, cytokines (IL6), and proliferation factors (Cyclin D1) in the nucleus.[18,19] Studies are continuously increasing on the discovery of new molecules targeting the destruction of cancer cells by regulating this pathway. Various molecules have been discovered to inhibit the receptors that initiate the NF-κB pathway, such as adalimumab, etanercept, anakinra, brentuximab, and denosumab.[20] Cevik et al. stated a 1 nM cabazitaxel application reduced the expression of NF-κB 50 and led to apoptosis in PC3 cells.[21]

Studies on the isolation and use of plant-derived secondary

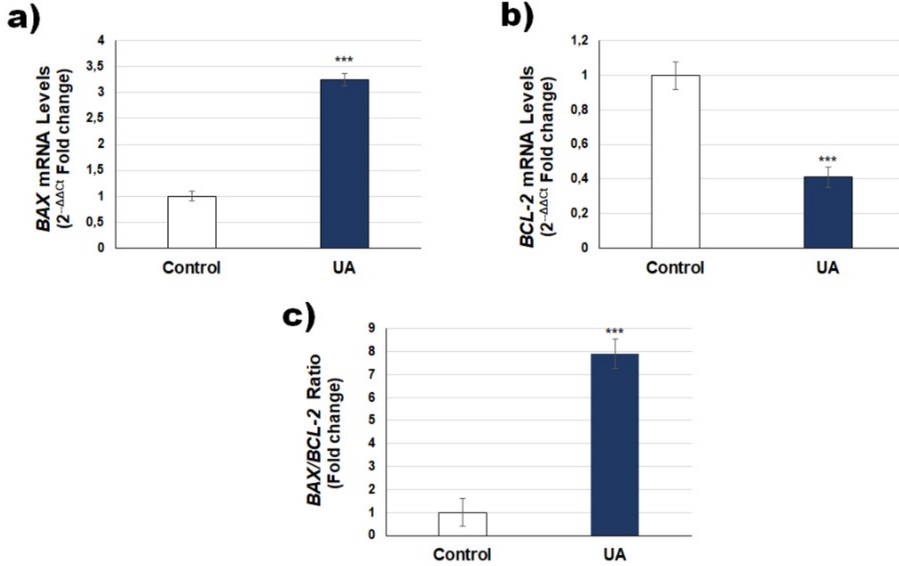

Figure 3. **(a)** Graphs of *BAX* gene expressions of DU145 cells after administration of usnic acids **(b)** Graphs of *BCL2* gene expressions of DU145 cells after administration of usnic acids **(c)** Graphical illustration of Bax/Bcl-2 ratio of DU145 cells treated with usnic acid (***p<0.001).

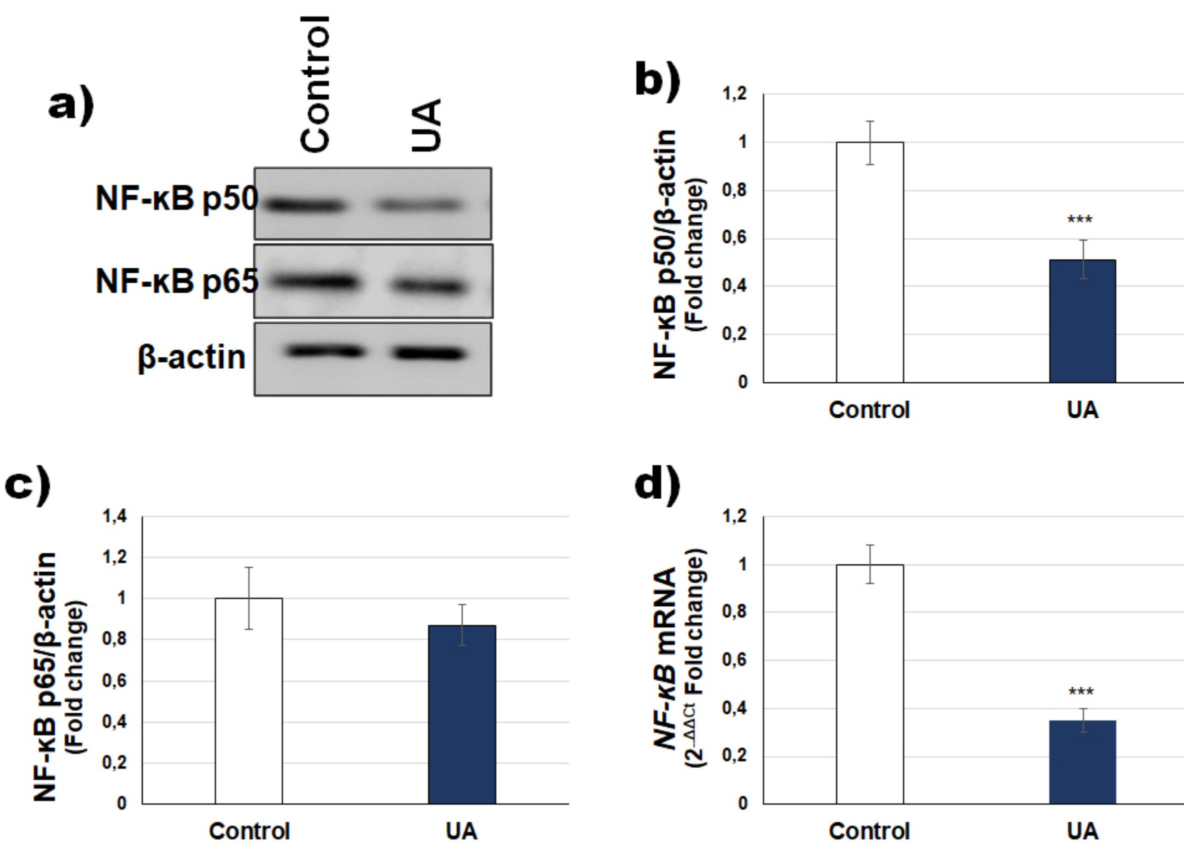

Figure 4. **(a)** NF-κB p50, NF-κB p65 and β-actin Western blot bands of DU145 cells treated with usnic acid **(b)** Graphs of NF-κB p50 protein expressions of DU145 cells after administration of usnic acid **(c)** Graphs of NF-κB p65 protein expressions of DU145 cells after administration of usnic acid **(d)** Graphs of *NFκB1* gene expressions of DU145 cells after administration of usnic acid (*** p<0.001).

metabolites have increased due to their high compatibility with living systems. Intensive research continueon studying the effectiveness of usnic acid isolated from lichen species.[22] The scavenging effect of reactive oxygen species, especially those produced in large amounts in cancer, has made usnic acid an agent that should be focused on in cancer treatment.[23] Cakmak and Gulcin reported usnic acid to perhaps be a promising agent in pharmaceutical applications due to having antioxidant properties such as high reducing ability, metal chelating, and radical scavenging.[9] On the other hand, usnic acid has been reported to cause free radical production at high doses, as well as mitochondrial stress in healthy hepatocyte cells.[24] A study conducted on healthy hepatocytes reported that usnic acid to cause mitochondrial glutation (GSH) depletion in cells at concentrations of 5 μM or higher and to dysregulate oxidative phosphorylation through adenosine triphosphate (ATP) reduction.[25] In isolated rat hepatocytes, the administration of usnic acid has been shown to cause toxicity and to decrease GSH by increasing lipid peroxidation.[26] Furthermore, when examining this effect over human hepatocellular cancer cells (HepG2), usnic acid was reported to increase cytochrome p450 activity, to cause mitochondrial dysregulation, and to trigger the oxidative stress mechanism.[27] The current study has shown this effect from usnic acid on mitochondria through JC-1 staining in prostate cancer cells to disrupt the mitochondrial membrane potential and lead the cells to apoptosis. Usnic acid decreased the permeability of mitochondria in DU145 cells and disrupted the oxidative phosphorylation balance of the cell. Changes in mitochondrial permeability may also alter transcription factors in cells and increase cellular stress. In addition, NF-κB signaling can regulate the expression of genes involved in mitochondrial function, including those encoding the components of the electron transport chain and antioxidant enzymes. This suggests that NF-κB may affect mitochondrial function beyond permeability.[28] NF-κB, particularly its p50 subunit, can affect mitochondrial permeability and function through various mechanisms, ultimately influencing cellular responses and physiological processes.[29] The current study has found usnic acid administration to suppress the NF-κB's p50 subunit by reducing mitochondrial permeability.

The most studied molecules in the apoptotic pathway regarding cancer treatment are the B-cell lymphoma-2 (BCL-2) for suppressing apoptosis and the Bcl-2-associated X protein (BAX) for promoting apoptosis. Various studies are also found in the literature to have examined the cytotoxic and anti-apoptotic effects of metabolites obtained from lichen species.[8] Takai et al. found usnic acid to exhibit cytotoxic effects regarding the Lewis lung carcinoma test system.[30] Cardarelli et al. found a decrease in the proliferation of K-562, Ishikawa, and HEC50 cells that had been treated with 50 μg/mL usnic acid.[31] Backorova et al. reported A2780 and HT-29 cells that had been treated with usnic acid to increase the Bax protein expression and to decrease p53 and Bcl-2 expression, thus activating programmed cell death mechanisms.[32] Dincsoy and Duman emphasized usnic acid as a secondary metabolite of lichen to induce apoptosis by increasing the *BAX* expression and decreasing the *BCL2* expression of HEP2C, RD, and Wehi cells.[33] Similar to these studies, the current article also has found usnic acid to trigger cell death in DU145 cells by activating apoptotic pathways. Jin et al. reported usnic acid to exhibit anti-inflammatory behaviors by suppressing the NF-κB pathway in Raw 264.7 macrophage cells.[34] Although usnic acid is known to suppress the NF-κB pathway, its effects on NF-κB p50 and NF-κB p65 have yet to be clarified. This study has shown usnic acid administration to decrease proliferation by suppressing NF-κB p50 in DU145 human prostate cancer cells.

CONCLUSION

Prostate cancer remains one of the biggest health problems among men, and the need exists to develop new treatment options for prostate cancer. The current study's results have shown usnic acid regarding prostate cancer treatment to suppress NF-κB p50 by reducing mitochondrial permeability in the DU145 prostate cancer cells, thus triggering apoptosis in the cells. The results from this study may shed light on new molecular mechanisms for treating prostate cancer and may offer another treatment option for the use of usnic acid alone or in combination with existing chemotherapeutics used in treatment.

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