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The Role of NF-κB p65 in White Tea Aqueous Extract-Induced Cancer Cell Apoptosis

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Abstract

White tea encompasses a number of teas unique to eastern Fujian in China. Although white tea extracts have been reported to result in cancer cell apoptosis, to date, few studies have evaluated the mechanism of such apoptotic induction. A transcription factor that plays a critical role in cell apoptosis, NF-κB p65, is also likely critical by which white tea extracts induce cancer cell apoptosis. In this study, white tea aqueous extract (WTAE) was added to BEL-7402 and Hela cell media and NF-κB p65 activation was evaluated using western blotting and immunofluorescence. Results revealed that the phosphorylation of IKBα and p65 decreased in both cell lines after WTAE treatment. And the nuclear translocation of NF-κB p65 in both cell lines was also reduced with the WTAE treatment. NF-κB p65 inhibition was noted to accelerate apoptosis. Our findings suggest that NF-κB p65 was an important modulator in WTAE-induced apoptotic signal transduction and it acted as a negative regulator of apoptotic induction in BEL-7402 and Hela cancer cell lines.

Keywords

WTAE, NF-κB p65, Apoptosis, Cancer Cells

1. Introduction

Increasing studies are focusing on the potential benefits of foods and beverages in cancer prevention and treatment. Tea contains high levels of flavonoids, such as catechins and other polyphenols, and has recently garnered significant inter-

est in pharmaceutical research [1] [2]. Polyphenols are known for a wide spectrum of biological properties including exertion of anti-oxidant, -viral, -cancer, -bacterial, and -fungal effects [3] [4] [5] [6]. A number of clinical studies have reported green tea to inhibit tumor incidence and multiplicity in organs ranging from the mammary gland to the colon [7] [8] [9] [10]. Green tea has also been reported to induce the apoptosis of cancer cells and promote the arrest of cell growth by altering cell cycle regulatory protein expression, activating killer caspases and suppressing oncogenic transcription and pluripotency maintenance factors [11] [12] [13].

White tea, unique to China and mainly produced in Fujian province, has particularly high polyphenol concentrations [14]. The major pharmacological effects of white tea are mediated by (-)-epicatechin-3-gallate, (-)-epigallocatechin, and (-)-epicatechin [15] [16] [17]. Although the antineoplastic effects of green tea have been extensively studied, the beneficial health properties of white tea remained largely unrecognized until recently. As white tea has similar bioactive compounds to green tea, it is generally believed that these two kinds of tea have similar health function [2] [18].

Previous studies have reported inhibition of tumorigenesis by white tea extract via the induction of cancer cell apoptosis [14] [19] [20]. Compounds within white tea initiate a dynamic cascade of intracellular events, including the activation of caspase-3, modulation of genes related to cellular proliferation and apoptosis (e.g. p53, cyclin dependent kinase inhibitor 1α (CDK1α) and p21), and increased PPAR-γ activation [20] [21]. Our prior research revealed that white tea aqueous extract (WTAE) induces BEL-7402 and Hela cell apoptosis [14]. Here, we aim to elucidate the mechanism via which WTAE inhibits tumorigenesis.

NF-κB is a transcription factor vital in the regulation of a wide variety of biological responses [22] [23] [24]. It not only serves as a master switch for turning on certain immune and inflammatory responses [25] [26] [27] but also plays a key role in cellular proliferation, differentiation, migration and apoptosis by regulating genetic expression [28] [29] [30]. Under physiologic conditions, NF-κB activation is an inducible, transient process that can only be triggered by specific stimuli. In cancer cells, however, NF-κB is constitutively activated due to the failure of its regulatory mechanism. Although the activation of NF-κB is generally accepted to be responsible for resistance to apoptosis [31], this protein complex likely exerts a dual function, namely as either an inhibitor or activator of apoptotic cell death [32] [33] [34]. Teas or tea extracts have been reported to induce cancer cell apoptosis; green tea polyphenols were specifically reported to inhibit oxidized LDL-induced NF-κB activation in human umbilical vein endothelial cells [35].

In this study, WTAE was added to Hela and BEL-7402 cell media and a series of assays were performed to evaluate apoptotic activity. Assays performed included DAPI staining to assess cellular morphology, TUNEL staining to determine levels of oligonucleosomal DNA fragmentation, and MTT staining to assess cellular viability. The mRNA levels of p53, p21, and CD1 in Hela and BEL

7402 cells were subsequently measured to verify the effects of WTAE on apoptotic gene expression. Additionally, both nuclear translocation and phosphorylation of NF-κB p65 were studied to evaluate the influence of WTAE on NFκB signaling. Finally, PDTC, an inhibitor of NF-κB, was applied to investigate how NFκB activation affected WTAE-induced apoptosis.

2. Materials and Methods

2.1. Cell Culture and Treatment

Hela and liver cancer (BEL7402) cells were bought from ATCC (USA). Hela cells were cultured in Dulbecco's Minimum Essential Medium (DMEM; Gibco-BRL, NY, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells of the BEL7404 line were cultured in RPMI-1640 medium with the same aforementioned concentrations of FBS, penicillin and streptomycin. Medium was changed every other day.

Both cells, upon 70% - 90% confluence, were cultured in their respective media with corresponding IC_{50} concentrations of WTAE (0.1% or 0.05%) for 24h and 48 h as in our prior work [14]. Cells cultured in WTAE-free media were used as negative control. Cells were collected after culturing for further analyses.

2.2. Preparation of White Tea Aqueous Extract

White tea was purchased from commercially available sources. Tea infusion was prepared by heating 2 g of tea leaf powder in 100 ml of distilled water at 56˚C for 1 h. The infusion was then filtered through a 0.45 μm filter and aqueous extract was freeze-dried. The concentrated extract was diluted in culturing media and subsequently filtered again through a 0.22 μm filter to produce different concentrations of liquids containing WTAE for use in cell treatment.

2.3. Caspase-3 Activity Assay

Initially, Hela and BEL-7402 cells were cultured to 80% - 90% confluence. Media were subsequently replaced by those containing WTAE in either 0.1 mg/ml or 0.05 mg/ml concentrations. Cells cultured in media without WTAE were used as controls. Cells were assayed for caspase-3 activity after treatment for 8 h, 16 h, 24 h, 36 h and 48 h intervals with a caspase-3 activity kit, according to manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, cells were digested with trypsin and washed with cold PBS, re-suspended in lysis buffer and left on ice for 15 min. Lysate was centrifuged at 16,000 × g at 4˚C for 15 min, mixed with reaction buffer (Beyotime, China), and incubated for 2 h at 37˚C. Absorbance of pNA was measured with a microplate reader (iMark™, Bio-Rad, USA) at 405 nm; caspase-3 activity was expressed as nmol pNA/min/mg protein.

2.4. DAPI Staining

Morphological assessment of apoptotic cells was conducted utilizing a DAPI

staining kit. Cells were seeded in a 6-well plate with sterilized 24 × 24 mm slides at the bottom of each well; 3 ml of media containing WTAE in either 0.1 mg/ml or 0.05 mg/ml concentrations was added to each well after cells adhered to plate walls. After treatment for either 24 h or 48 h, cells were fixed for 10 min, washed twice with PBS and stained with 0.5 ml of DAPI staining solution for 5 min at room temperature. Cells were mounted with an antifading agent and observed under UV light on an inverted fluorescent microscope (Leica, Germany) at 400 × magnification. Apoptotic cells were identified as those exhibiting chromatin condensation and nuclear fragmentation.

2.5. TUNEL Staining

Cells were seeded in a 6-well plate with sterilized 24 × 24 mm slides at the bottom of each well. After treatment with WTAE, cells were fixed in PBS containing 4% paraformaldehyde. Oligonucleosomal DNA fragmentation was evaluated by labeling 3-OH DNA ends generated during the apoptotic process by enzymatic addition of digoxygenin-labeled d-UTP via terminal deoxynucleotidyl transferase (*i.e.*, employing the TUNEL technique). The Boehringer TUNEL Staining Kit was used according to the manufacturer's instructions and results were observed under 550 nm UV light on an inverted fluorescent microscope (Leica, Germany) at 400 × magnification.

2.6. Real-Time PCR

RNA was extracted from cells using TRIzol (TaKaRa, Dalian, China) according to the manufacturer's instructions. RNA samples were treated with DNaseI (Fermentas, Shanghai, China) to remove residual genomic DNA. First-strand cDNA synthesis was performed using the RevertAid First Strand cDNA synthesis Kit (Fermentas) with oligo deoxynucleotidyl transferase. The mRNA of tested genes was quantitated by real-time quantitative PCR performed in a Bio-rad I-cycler 3.0 (Biorad) using QIAGEN Green Master Mix. Primer sequences were derived from genetic sequences available through GenBank (Table 1). Total mRNA was normalized to GAPDH.

2.7. Western Blotting

Cells were homogenized by sonication in RIPA lysis buffer (Beytime Institute of

Table 1. Primers for RT-PCR.

	GeneBank Accession Nos	primer sequence	Annealing temperature
p53	BC000474	Forword: AACTGCCTGGCTCTTGATGGT Reverse: CCTGGATTTCGGTCACTGGGTA	58˚C
p21	BC001935.1	Forword: TTAGCAGCGGAACAAGGAGTCA Reverse: AGAAACGGGAACCAGGACACAT	58˚C
GAPDH	NM_002046	Forword: 5'-GAAGGTCGGAGTCAACGG-3' Reverse: 5'-GGAAGATGGTGATGGGATT-3'	57˚C

Biotechnology, Jiangsu, China) with 1mM PMSF (Sigma-Aldrich, USA) and 0.1% phosphatase inhibitor cocktail (Sigma-Aldrich). Cell homogenate was centrifuged at 12,000 g for 5 min at 4˚C; supernatant was subsequently collected for western blotting. A 15 μl volume of lysate was applied to 1% SDS-PAGE for gel electrophoresis. Proteins in the gel were transferred to a PVDF membrane (Millipore, USA). After blocking with 5% skimmed milk, the membrane was incubated at 4˚C overnight in primary antibody against NF-*κ*B p65, p-NF-*κ*B p65, IKB*α*, p-IKB*α* or GAPDH (1:1000, Cell Signaling Technology). The membrane was subsequently incubated at room temperature for 1 h in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000, BOSTER, Wuhan, China) after washing with PBST (PBS supplemented 0.1% tween-20) three times. Immunoreactive bands were detected using enhanced chemiluminescent reagent (Tiangen biotech. Beijing, China) and quantitatively analyzed in triplicate by normalizing band intensities to control on scanned films using Image J software.

2.8. Immunofluorescence Staining

Both cancer cell lines were seeded in a 24-well plate. Sections were cultured in either control, WTAE-containing, PDTC-containing or WTAE- and PDTC-containing media for 2 h. Cells were subsequently collected for immunofluorescence labeling according to the manufacturer's instruction using a Cellular NF-*κ*Bp65 Translocation Kit (Beyotime Biotech). Results were observed using an inverted fluorescence microscope (Nikon, Japan) at 400 × magnification.

2.9. Selective Inhibition of Signal Transduction

To assess the mechanistic role of NF-*κ*B in WTAE-induced apoptosis, we selectively applied PDTC (Sigma, 10 μM) to inhibit NF-*κ*B activation; the effective dose of this inhibitor was obtained from the reagent specification. Briefly, cells were seeded in a 24-well plate and deprived of FBS overnight. Cells were then pretreated for 2 h with PDTC prior to exposure to WTAE-containing medium. The inhibitor was present during treatment.

2.10. Statistical Analyses

All experiments were repeated at least twice; representative data are presented as means ± standard deviation. Statistical analyses were performed using factorial analysis of variance (two-way ANOVA). A p value of less than 0.05 was considered statistically significant: *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).

3. Results

3.1. Morphological Nuclear Changes

Hela and BEL-7402 cells were respectively cultured in four different kinds of media as detailed above for either 24 h or 48 h; media used were either 1640 or DMEM medium, medium containing IC_{50} WTAE in either 0.05 mg/ml or 0.1

mg/ml quantities, and medium containing PDTC or both PDTC and WTAE. Cells were collected and examined for morphological nuclear changes using DAPI staining. The nuclei of BEL-7402 cells were found to be concentrated after culturing in WTAE-containing medium for 48 h (**Figure 1(A)**). Moreover, the nuclei of BEL-7402 cells treated with PDDTC only also became concentrated after being treated for 48 h. However, the nuclei of BEL-7402 cells treated with both PDTC and WTAE appeared to be more concentrated as compared to cells treated with WTAE only; similar findings were observed in Hela cells (**Figure 1(B)**) although morphological nuclear changes were different (*i.e.* numerous apoptotic bodies instead of nuclear concentration were observed in Hela cells after 24 h or 48 h treatment periods). Media containing both WTAE and PDTC were noted to result in greater apoptotic body formation as compared to WTAE alone or PDTC alone. Thus, inhibition of NF-κB by WTAE was found to trigger cancer cell apoptosis.

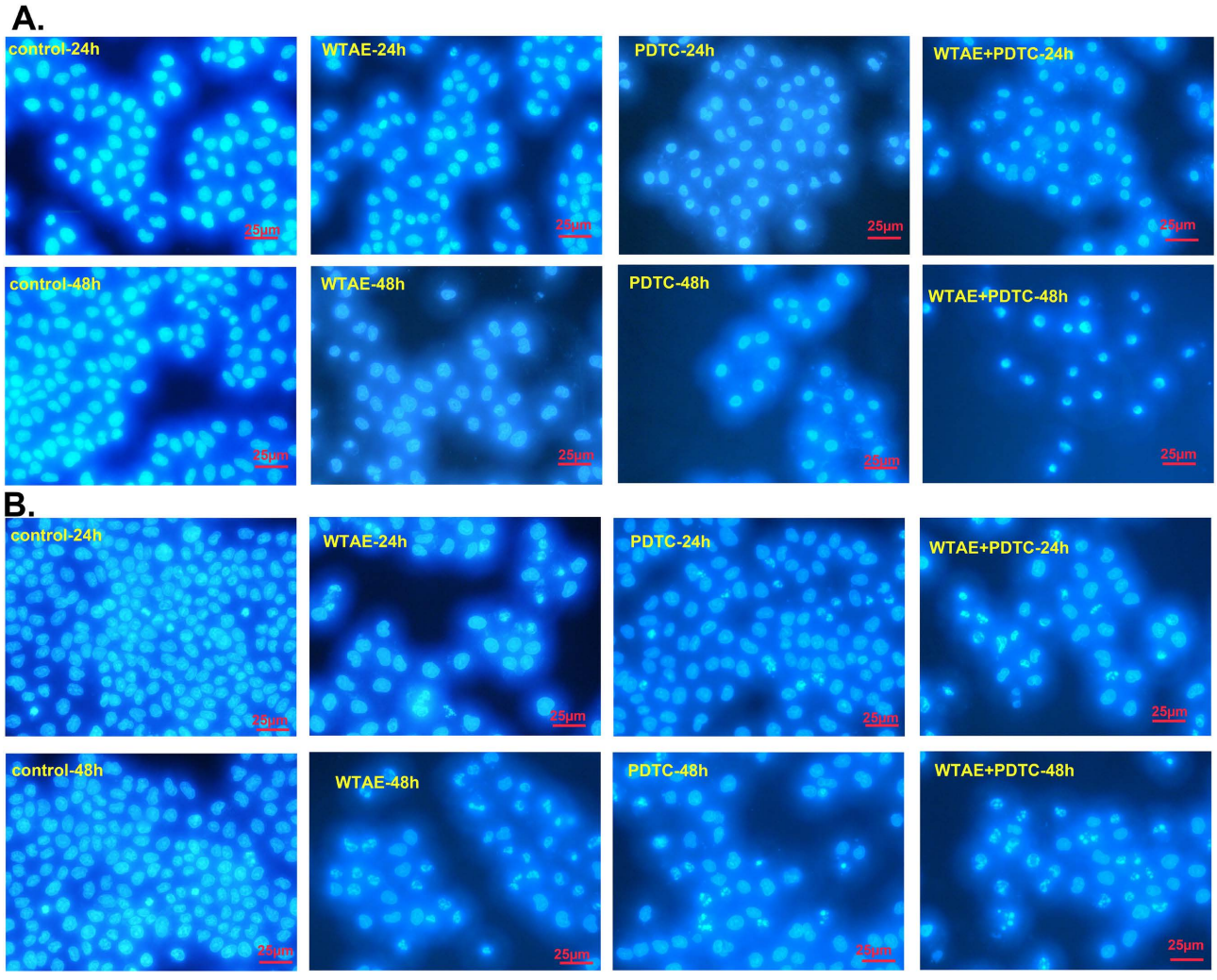

Figure 1. Morphological nuclear changes. The morphological nuclear changes of two kinds of cells were examined using DAPI staining after being cultured under four different conditions for 24 h or 48 h. (A) Nuclear staining in BEL-7402 cells. (B) Nuclear staining in Hela cells. Results were observed under fluorescence microscopy (400×).

3.2. DNA Fragmentation

The fragmentation of DNA in both aforementioned cancer cell lines was evaluated via TUNEL assay; cells were treated with WTAE, PDTC or WTAE and PDTC after 24 h or 48 h. Red fluorescence of both Hela and BEL-7402 cells was observed in cells treated with Three kinds of medium (Figure 2). Nuclei of both cancer cell lines treated with WTAE and PDTC exhibited a greater degree of red fluorescence as compared to those of cells other two treated groups (respectively treated with WTAE or PDTC) (Figure 2). Control group nuclei revealed no fluorescence (Figure 2). Inhibition of NF-κB was thus found to lead to DNA fragmentation in cancer cells.

3.3. Effects of WTAE Alone as Well as WTAE and PDTC on Cancer Cells' Caspase-3 Activity

To further verify whether NF-κB was related to WTAE-induced cancer cell apoptosis, we evaluated caspase-3 activity of BEL-7402 and Hela cells cultured in media containing WTAE, PDTC or both WTAE and PDTC. In BEL-7402 cells, caspase-3 activity was found to be increased after exposure to WTAE-containing medium or PDTC-containing medium for 8 h, peaking 24 h post-exposure (Figure 3(A)). However, Caspase-3 activity in cells cultured in medium containing both WTAE and PDTC was found to have peaked at 16 h post-treatment and was significantly greater when compared to that of cells treated with WTAE or PDTC alone (Figure 3(A); $p < 0.001$). In addition, caspase-3 activity in cells of three treatment groups was markedly higher than that of contemporaneous control group after being treated for 16 h, 24 h or 36 h (Figure 3(A)).

Hela cells caspase-3 activity of WTAE, PDTC, as well as WTAE plus PDTC treatment groups was also found to be increased and significantly higher than that of cells in control groups after being treated for 8 h, 16 h and 24 h and 36 h (Figure 3(B); $p < 0.001$), and reached the peak after 16 h. Although the caspase-3 activity of cells in the WTAE plus PDTC treatment group was found to be slightly higher than that of WTAE group and PDTC group cells, the difference between these groups was not significant (Figure 3(B); $p > 0.05$). Our findings suggest that inhibition of NF-κB by PDTC further improved caspase-3 activity in cancer cells.

3.4. Effects of WTAE, PDTC or WTAE plus PDTC on Expression of Genes Related to Apoptosis

The mRNA levels of p21 and p53 were studied using quantitative real-time PCR to evaluate what effects WTAE, PDTC and WAE plus PDTC exerted on the expression of genes related to cancer cell apoptosis. After treatment with WTAE, PDTC or WTAE plus PDTC for 24 h or 48 h, mRNA levels of p53 and p21 in both cancer cell lines were found to be markedly increased as compared with those of corresponding control group or PDTC treatment groups (Figure 4; $p < 0.001$). However, combination treatment of cells with WTAE and PDTC was

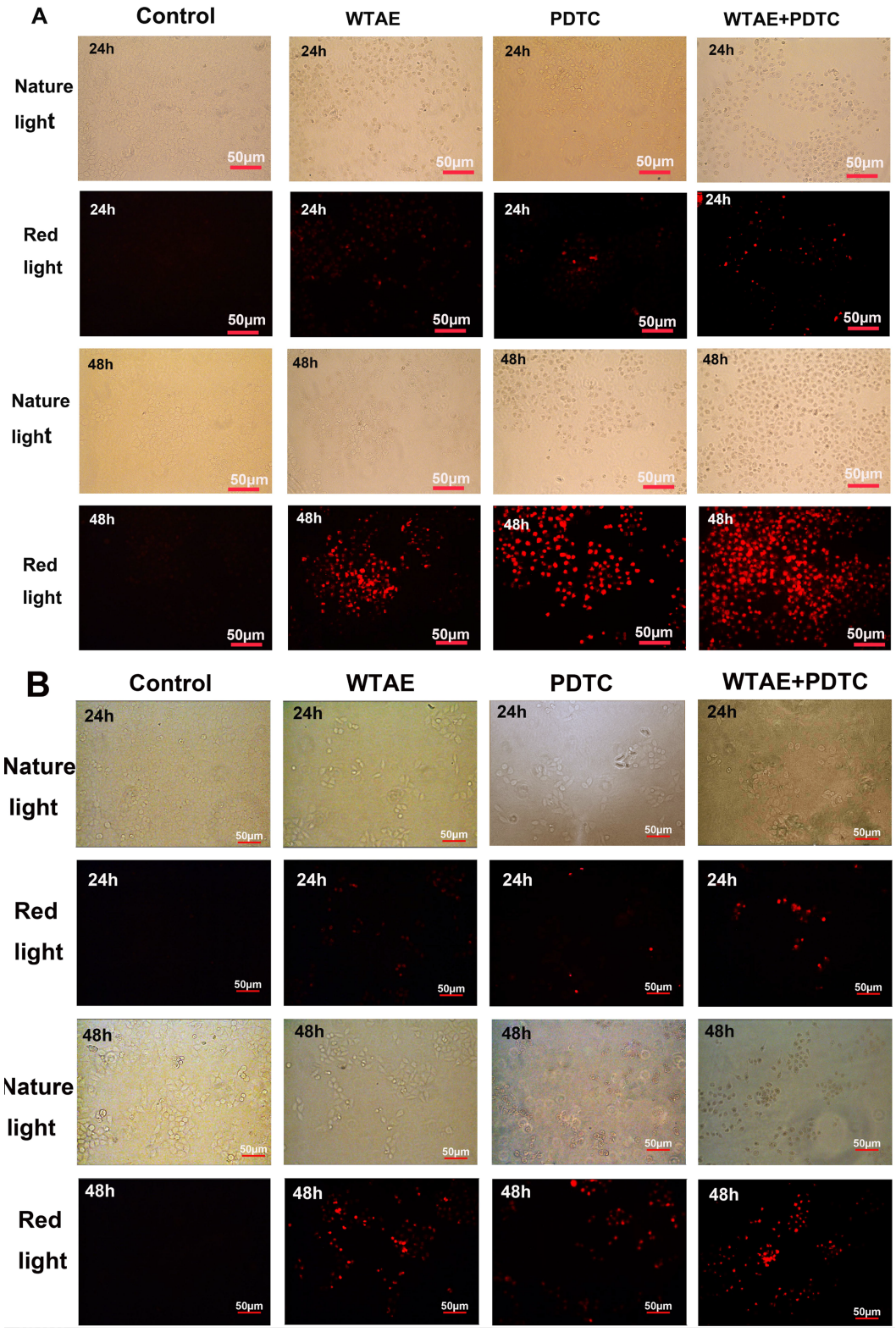

Figure 2. TUNEL assay detected the oligonucleosomal fragmentation of DNA. BEL-7402 and Hela cells were seeded on slides and then cultured respectively with four different kinds of medium for 24 h and 48 h. Oligonucleosomal fragmentation of DNA was detected using the TUNEL assay. Results were observed with fluorescence microscopy (200×). (A) TUNEL assay results of BEL-7402 cells. (B) TUNEL assay results of Hela cells.

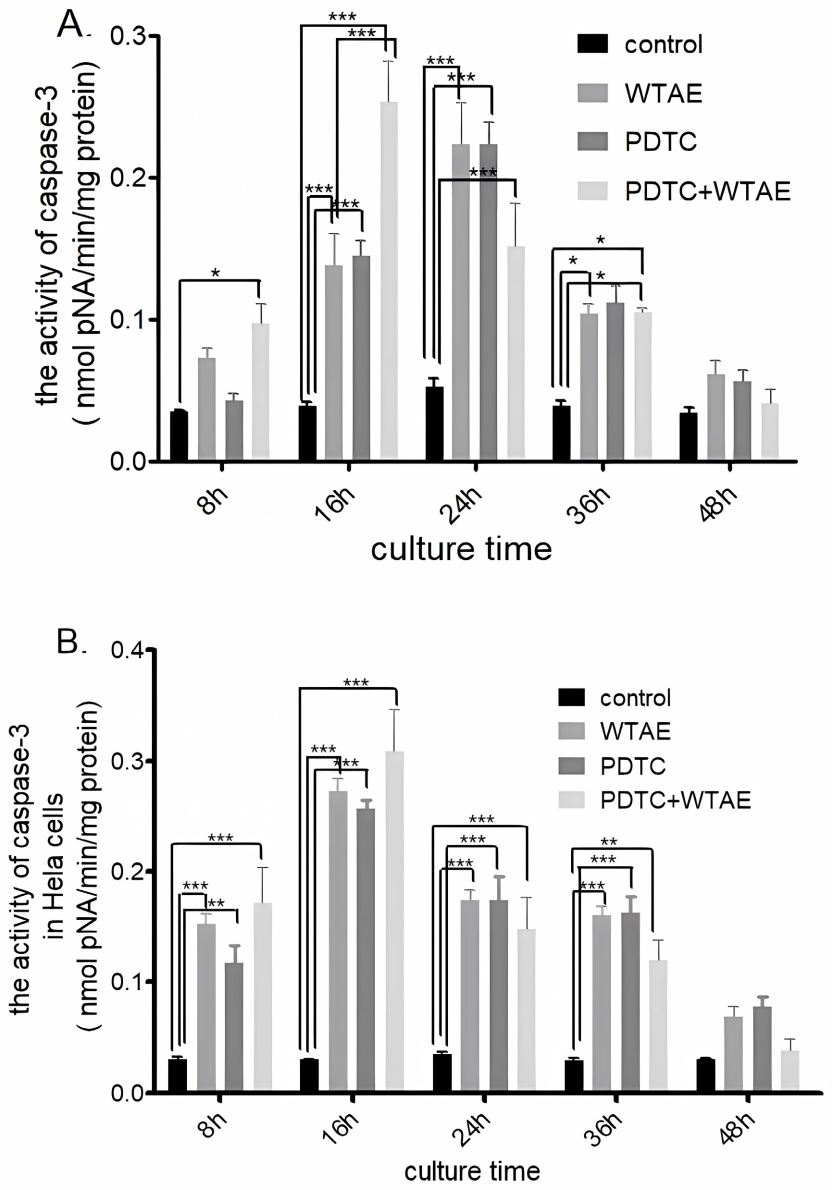

Figure 3. Activity of caspase-3 in Hela and BEL-7402 cells. Both cell types were collected to evaluate caspase-3 activity after cultural with medium, medium plus WTAE, medium plus PDTC or medium containing WTAE and PDTC for 8 h, 16 h, 24 h, 36 h and 48 h. Caspase-3 activity was expressed as nmol pNA/min/mg protein. (A) Caspase-3 activity in BEL-7402 cells. (B) Caspase-3 activity in Hela cells. Results were analyzed using two-way ANOVA followed by Dunnett's test for comparison of each treatment. *($p < 0.05$) indicated statistical significance, **indicated strong statistical significance ($p < 0.01$) and ***($p < 0.001$) was considered extremely significant.

found to lead to no higher levels of p21 and p53 expression in both cancer cell lines as compared to WTAE treatment alone (**Figure 4**; $p < 0.05$) at 24 h post-treatment. Moreover, PDTC didn't lead to significant effect on the expression of p21 and p53. These findings underscore that PDTC didn't intensify the effects exerted by WTAE on the expression of apoptosis-related genes in both cancer cell lines.

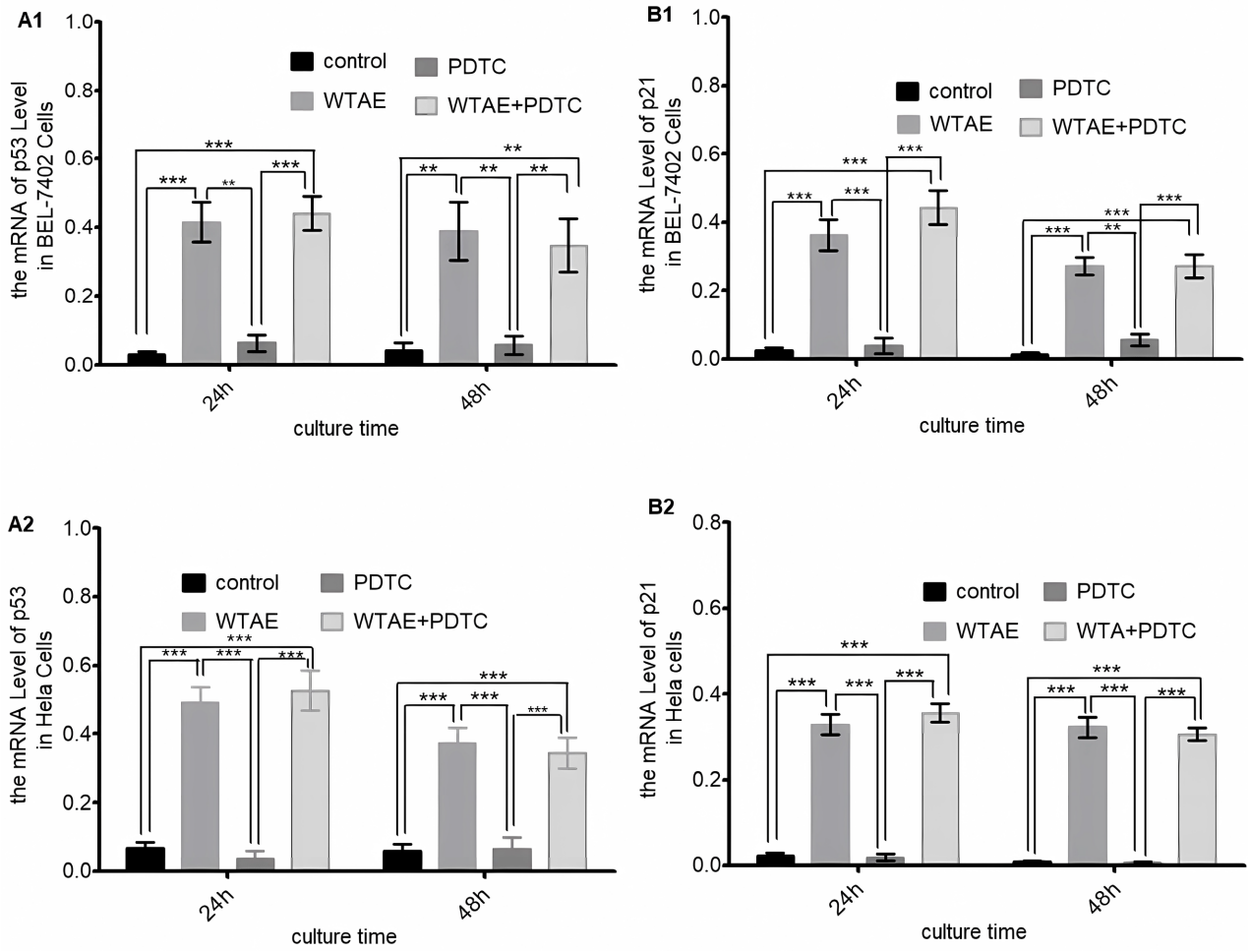

Figure 4. Expression levels of p53 and p21 in two kinds of cancer cells under four different conditions. Cancer cells were respectively treated with WTAE, PDTC or WTAE plus PDTC for 24 h and 48 h. Cells cultured in medium without WTAE or PDTC were used as controls. Gene expression level was evaluated by qRT-PCR. (A1 and A2) qRT-PCR analysis of p53 expression levels in BEL-7402 and Hela cells. (B1 and B2) qRT-PCR analysis of p21 expression levels in BEL-7402 and Hela cells. Results were analyzed using two-way ANOVA followed by Dunnett's test for comparing each treatment to controls. **indicated strong statistical significance ($p < 0.01$) and ***($p < 0.001$) was considered extremely significant.

3.5. Nuclear Translocation of NF-κBp65

Nuclear translocation of NF-κB p65 in both cancer cell lines was evaluated using immunofluorescence after culturing in all four types of media for 2 h. Red fluorescence could be seen in many 7402 cells of control groups. However, almost no red fluorescence was observed in cells of three treated groups: WTAE treated group, PDTC treated group or PDTC plus WTAE treated group (**Figure 5**). Similar findings were reported in experiments studying Hela cells. These data suggest that WTAE and PDTC could effectively inhibited NF-κB p65 nuclear translocation.

3.6. Phosphorylation of IKBα and NF-κB p65 in Cancer Cells

To further confirm findings noted during immunofluorescence assays, we evaluated IKBα and NF-κB p65 phosphorylation in both cancer cell lines among

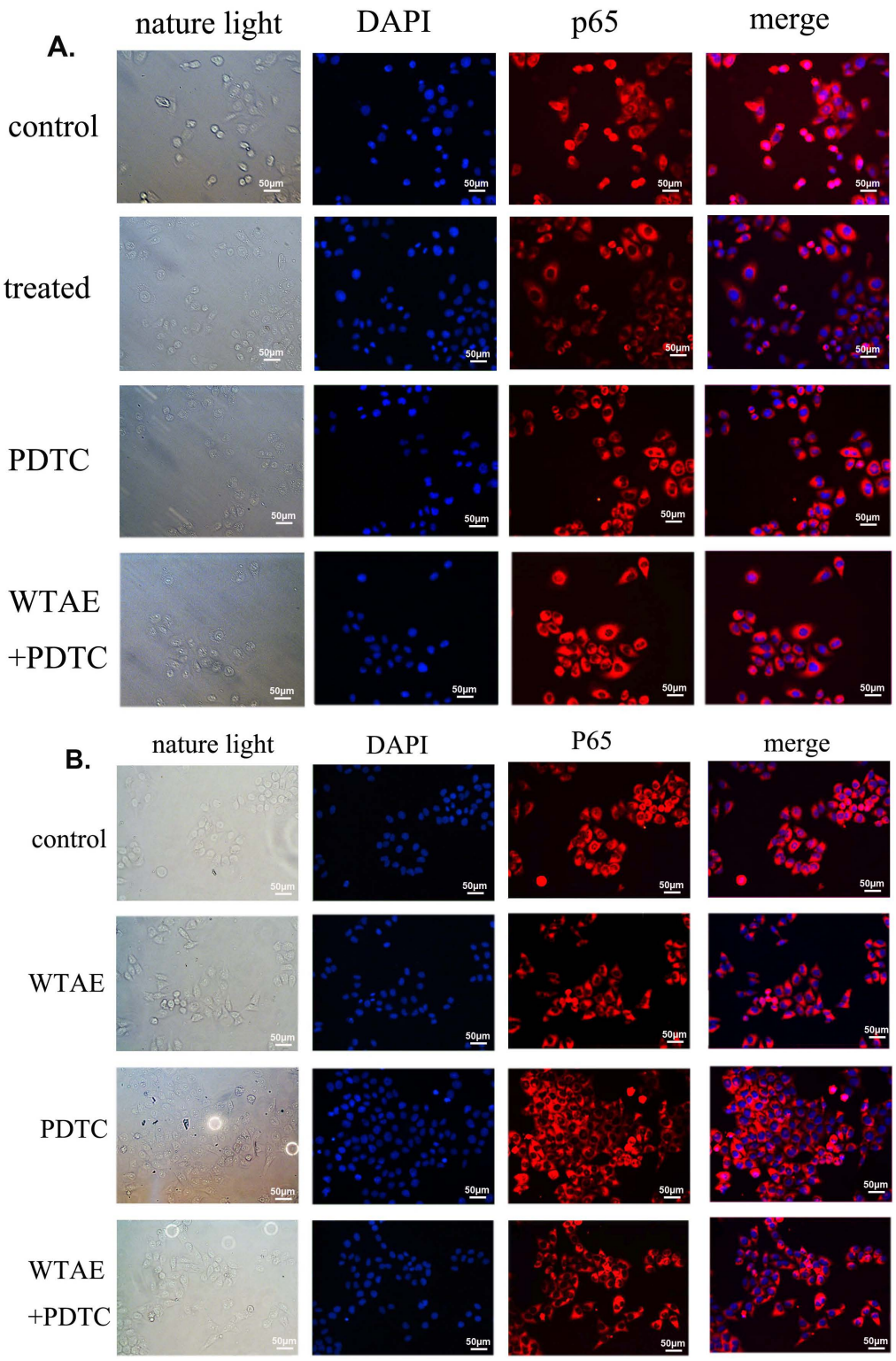

Figure 5. The nuclear translocation status of NF-κB in two kinds of cancer cells under four different conditions. Cells seeded on slides were cultured with four different kinds of medium for 2 h and then were assayed by Immunofluorescence staining. Fluorescence microscopy (200×) showed the location of NF-κB subunit, p65. The location of p65 (red fluorescence) was compared with nuclear (DAPI, blue). (A) The nuclear translocation status of NF-κB in BEL-7402 cells. (B) The nuclear translocation status of NF-κB in Hela cells.

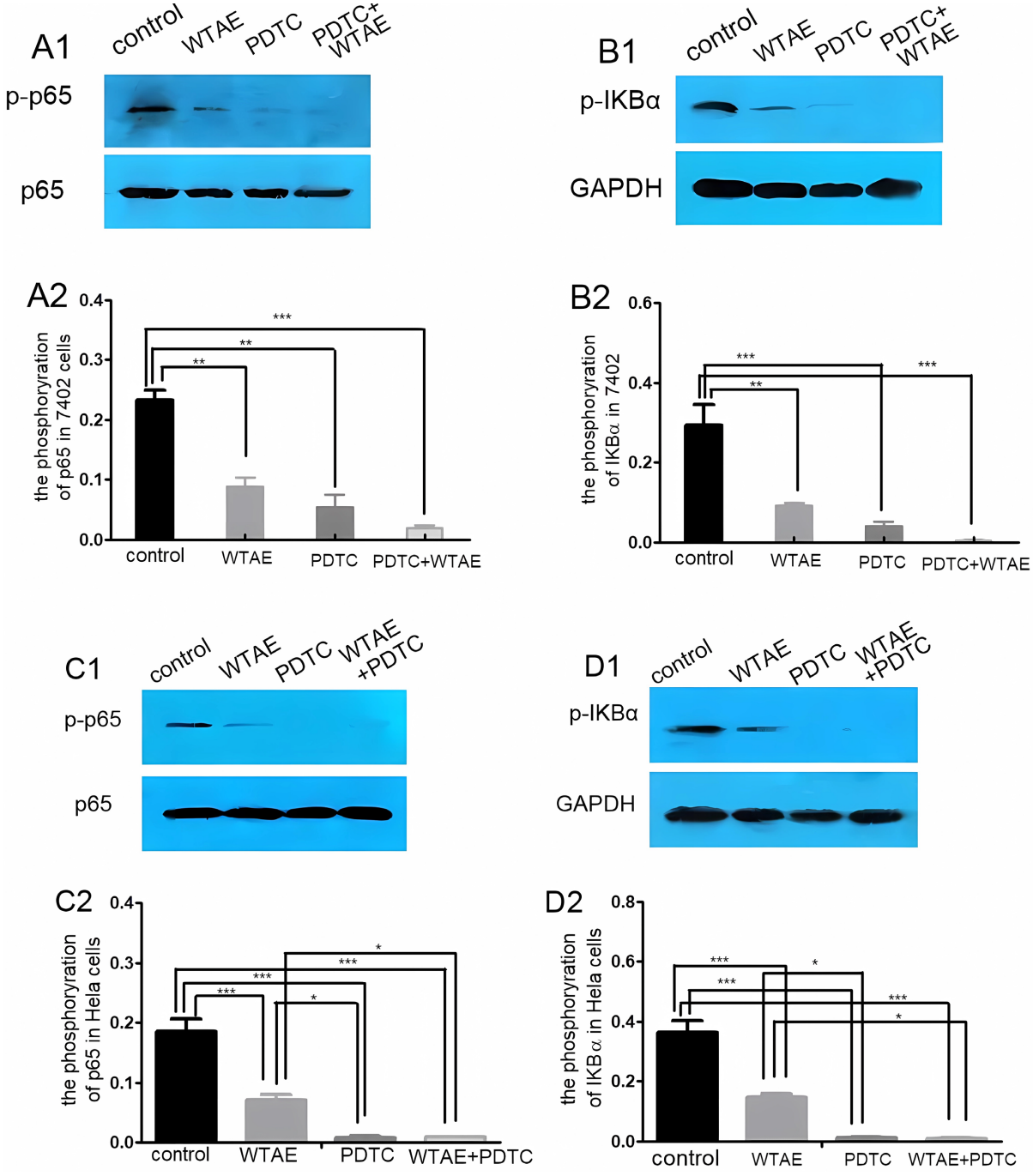

Figure 6. Activation level of p65 and IKB*α* in Hela cells and BEL-7402 culture under four different conditions. (A1) The phosphorylation of p65 in BEL-7402 cells. (A2) Densitometric measure of band intensity for p-p65 in BEL-7402 cells was analyzed by Image J software and normalized by corresponding total p65. $p < 0.05$ indicated statistically significant, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (B1) The phosphorylation of IKB*α* in BEL-7402 cells. (B2) Densitometric measure of band intensity for p-IKB*α* in BEL-7402 cells was analyzed by Image J software and normalized by the corresponding GAPDH. $p < 0.05$ indicated statistically significant, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (C1) The phosphorylation of p65 in Hela cells. (C2) Densitometric measure of band intensity for p-p65 in Hela cells was analyzed by Image J software and normalized by the corresponding total p65. $p < 0.05$ indicated statistically significant, *$p < 0.05$,**$p < 0.01$ and ***$p < 0.001$. (D1) The phosphorylation of IKB*α* in Hela cells. (D2) Densitometric measure of band intensity for p-IKB*α* in Hela cells was analyzed by Image J software and normalized by the corresponding GAPDH. $p < 0.05$ indicated statistically significant, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

control, WTAE-treated, WTAE- and PDTC-treated as well as PDTC-treated groups. Phosphorylation levels of both p65 and IKBα in the control groups of both cell lines were significantly higher than those in three corresponding treated groups. With the treatment of WTAE, the phosphorylation of p65 and IKB in two kinds of cancer cells decreased obviously. Furthermore, levels of both p65 and IKBα phosphorylation in both cell lines were inhibited by PDTC (**Figure 6(A)** and **Figure 6(B)**). Thus, WTAE was found to reduce the activation of NF-κB.

4. Discussion

Teas or tea extracts are known to induce cancer cell apoptosis [22] [36] [37]. Our previous study also reported that WTAE inhibits cancer cell proliferation via apoptotic induction, although the mechanism of this phenomenon remained unclear. As NF-κB is an important transcription factor related to cellular proliferation and apoptosis [38] [39] and tea or tea extracts have been reported to attenuate NF-κB activation [40], we considered whether NF-κB participates in WTAE-induced apoptotic signal transduction among cancer cells.

In this study, we evaluated the effects of white tea on BEL-7402 and Hela cells, specifically studying WTAE, mainly as traditional tea-drinking customs involve placement of either tea leaves or powder into boiling water. Our experiments revealed that WTAE decreased NF-κB p65 nuclear translocation in those two cancer cell lines. Thus, our findings suggest that WTAE could lessen the activation of NF-κB p65. Our findings were further confirmed by western blotting. We found that phosphorylation of IKBα and p65 weakened in both BEL-7402 and Hela cells cultured in WTAE-containing media. Moreover, we also found that inhibition of NF-κB p65 by PDTC increased manifestation of WTAE-induced apoptotic characteristics in both Hela and BEL-7402 cell lines, including morphological nuclear changes, oligonucleosomal DNA fragmentation, high levels of p53 and p21 expression, and elevated caspase-3 activity. Our findings suggest that the inhibition to NF-κB p65 activation was an important way for WTAE to induce apoptosis in cancer cells.

Our findings agreed with those reported in literature. Green tea extracts were previously reported to inhibit oxidized LDL-induced NF-κB activation in human umbilical vein endothelial cells [32]. The main bioactive compounds in white tea are similar to those in green tea. Accordingly, white tea extract could lead to a similar effect on cancer cells as green tea extracts.

Additionally, our findings also revealed that PDTC exacerbates WTAE-induced cancer cell apoptosis. Moreover, PDTC alone was also found to result in cancer cells apoptosis. Thus, PDTC could intensify WTAE-induced cancer cells apoptosis. Although NF-κB has been generally considered to play both inhibiting and activating roles in apoptosis, our findings suggest that it chiefly exerts an inhibitory function. Our data thus confirm that restraining NF-κB p65 activation is an important way for WTAE to induce apoptosis in cancer cells.

5. Conclusion

Here, we show that NF-κB p65 is not a key regulator of WTAE-induced apoptosis in cancer cells and further confirm that NF-κB p65 acts as an apoptotic inhibitor. However, this study did not detail how WTAE mechanistically led to cancer cell apoptosis. Further research should focus on elucidating the signal transduction mechanism of such apoptotic induction.

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Authors' Contributions

Liyue Liu designed the experiment and drafted the manuscript. Liyue Liu, Qin Ling, Bo Liu, and Shengyang Zhen carried out experiments. Liyue Liu and Zuyun Ye analyzed experimental results. Weirui Zhang assisted with data collection and figure construction.

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Practical Application

Here, we aim to detail how white tea aqueous extract induces cancer cell apoptosis. Our mechanistic findings will also be helpful in formulating novel anti-cancer therapeutics.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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