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Synergistic Effect of Apigenin and Curcumin on Apoptosis, Paraptosis and Autophagy-related Cell Death in HeLa Cells

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Abstract. *Background/Aim: We aimed to investigate the synergistic effects of apigenin and curcumin on the cross-talk between apoptosis and autophagic cell death, as well as on paraptosis in HeLa cells. Materials and Methods: Cell viability was measured using the MTT assay. Synergistic effects were measured using the Bliss independence model. qRT-PCR was used to study the expression of genes related to apoptosis, autophagic cell death, and cross-talk. GRP78/BiP immunostaining was used to identify endoplasmic reticulum (ER) stress. Results: Treatment with a combination of apigenin and curcumin increased the expression levels of genes related to cell death in HeLa cells 1.29- to 27.6-fold. The combination of curcumin and apigenin showed a synergistic anti-tumor effect via cross-talk between processes leading to apoptosis and autophagic cell death, as well as ER stress-associated paraptosis. GRP78 expression was down-regulated, and massive cytoplasmic vacuolization was observed in HeLa cells. Conclusion: The combination of curcumin and apigenin is an effective potential therapeutic for cervical cancers.*

Cancer is a disease caused by the abnormal proliferation and differentiation of cells and is governed by tumorigenic factors (1). Cervical cancer is a major cause of death among young women. There have been significant advances in the diagnosis and treatment of cervical cancer (2). One of the most effective ways of treating cancer is chemotherapy. However, chemotherapy alone may not be adequate for effective treatment due to drug resistance and systemic toxicity. Thus, multiple cytotoxic drugs may need to be used in combination for effective treatment. The combined use of biological agents with cytotoxic features has yielded promising results while lowering their toxic effect on cells, showing the benefits of combination therapy (3, 4). In light of this information, new combination therapies have to be developed for cancer treatment.

Many anti-cancer drugs derived from natural products are widely used in cancer treatment due to their high efficacy and lower toxicity (5). Recent studies have revealed that flavonoids from plant sources can be used as anti-cancer agents. Phenolic compounds, including apigenin and curcumin, are characterized by their broad biological activities, and their benefits have been demonstrated in numerous mammalian systems *in vitro* and *in vivo*. Phenolic compounds act as free-radical scavengers and antioxidants, exhibiting anti-mutagenic, anti-inflammatory, antiviral, and anti-tumorigenic effects (6-9).

Apigenin has well-known antioxidant effects, and it can also be used as a therapeutic agent to fight diseases, including autoimmune and neurodegenerative diseases, inflammation, and several types of cancers (10, 11). Apigenin also targets signaling pathways regulating cell cycle and cellular migration. The different mechanisms underlying the potential therapeutic action of apigenin and its anti-cancer, anti-inflammatory, and antioxidant effects have been investigated (12, 13). Apigenin has nutraceutical potential in addition to its many interesting pharmacological activities. As a dietary supplement, apigenin is under investigation for its potential to prevent the recurrence of neoplasias in a phase 2 clinical study that includes patients with colorectal cancer (14). Apigenin's potential therapeutic action derives from the arrest of the cell cycle at different proliferation stages and induction of apoptosis (12). It has been shown that apigenin has a cytotoxic effect mainly on

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cancer cells rather than on normal cells and is less toxic compared to other flavonoids (15).

Curcumin, a naturally occurring compound, has been shown to have pleotropic pharmacological properties, including antioxidant, anti-inflammatory, antiviral, and antitumor effects (16-19). Various studies have shown the effective use of curcumin in several types of cancer (18, 19). Curcumin modulates several cellular signaling pathways involved in cell cycle, apoptosis, and cellular differentiation, thus suppressing the proliferation of cancer cells (20, 21). Curcumin may also sensitize tumor cells to targeted therapy agents and reverse resistance to chemotherapeutic drugs (16, 19). In addition to *in vitro* and *in vivo* models, the effectiveness of curcumin combination chemotherapy has been reported in several clinical trials (22). The combination of apigenin and curcumin has been shown to effectively reduce tumor activity in various cancers, either when used independently (23-27) or in combination with various chemotherapeutic drugs (28-31). Combined therapeutic strategies involving apigenin or curcumin and other chemotherapeutic drugs have been developed as adjuvant chemotherapy to enhance the anti-cancer effects of these natural compounds on various types of cancers and overcome cancer drug resistance or alleviate other adverse effects of chemotherapy. Apigenin has been shown to reduce the side effects of chemotherapeutic drugs (10, 11), and curcumin reverses resistance to chemotherapeutic drugs (22, 30). Choudhury et al. have shown that the synergistic effect of apigenin and curcumin can induce the death of A549 lung cancer cells (17). They reported that the anticancer activity of these two compounds inhibited microtubule polymerization and triggered apoptosis in cancer cells through this mechanism (17).

One of the first targeted strategies in cancer treatment is blocking cell proliferation and inducing apoptosis. After exposure to anti-tumor agents, cells may undergo apoptosis, autophagic cell death, or necroptosis; these pathways may be activated independently or after cross-talk between relevant signaling pathways, which can be initiated by a common stimulus but with different mechanisms of action (32, 33). Apoptosis can be initiated by either intrinsic or extrinsic cellular triggers. Bcl-2 family members, such as antiapoptotic (including Bcl-2, Bcl-xL, Mcl-1, and Bcl-W) and proapoptotic (including Bax, Bak, Bad, Bid, Bif, Bik, Bcl-xS, and NOXA) proteins, are responsible for the intrinsic pathway, while death receptors (including FAS, TRAILs, and TNFR) play important roles in the extrinsic pathway (33). Apoptosis is a caspase-dependent process that mainly involves Caspase-3, -8 and -9 (33, 34). Autophagy is an important process in balancing cellular survival. A breakdown or increase in autophagic activity may induce cell death. Depending on the conditions, autophagy-related proteins, such as Atg12, Atg5, and Beclin-1 can direct the pathways to cellular death (32, 35). A common stimulus within the same cell can activate apoptotic and autophagy-mediated cell death using different mechanisms (32, 33). Recent studies have shown that cells can choose one of two death pathways by a cross-talk mechanism that uses well-known regulatory genes, such as *Bcl-2, Bcl-xL, Atg5, p53, Beclin-1, Bif-1,* and *BNIP-3* (32, 36). Targeting autophagy-related cell death and/or apoptosis can increase the efficacy of cancer treatment (32).

On the other hand, an alternative, non-apoptotic cell death pathway, such as paraptosis, may be targeted for the development of new therapies. Paraptosis is characterized by extensive cytoplasmic vacuolization due to the dilation of the endoplasmic reticulum (ER) and/or mitochondria (37, 38). Cytoplasmic vacuolization may be preceded by induction of ER stress. The chaperone glucose-regulated protein (GRP78/BiP) is a major regulator of ER stress. GRP78/BiP has critical cytoprotective roles in oncogenesis, and it is often over-expressed in cancer cells (39). Increased expression of GRP78/BiP has been related to drug resistance, high pathological grades, and short survival times in various types of cancers (40).

In this study, our aim was to investigate the synergistic effects of the apigenin, which was isolated from the *C. nerimaniae* plant, a species endemic to Turkey, and curcumin, on gene expression specifically related to apoptosis and autophagy-related cell death and to determine the potential cross-talk between apoptotic and autophagic cell death mechanisms and ER stress-associated paraptosis in the HeLa cell line.

Materials and Methods

Cell culture. The human cervical cancer cell line (HeLa) was purchased from ATCC (ATCC-CCL-2, Manassas, VI, USA). HeLa cells were grown in Minimum Essential Medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) which contained 10% Fetal Bovine Serum (FBS, Gibco, Thermo Fisher, Waltham, MA, USA) and 100 U/ml penicillin and 50 mg/ml streptomycin. The cells were grown as monolayer in 25 cm^2 flasks in a 5% CO_2 incubator at 37˚C until 80-85% confluency (41). In the study, the cells were divided in in four groups: 1) curcumin alone, 2) apigenin alone, 3) both curcumin and apigenin (combination group) and 4) control group without curcumin and apigenin.

Drugs. Apigenin (4□, 5, 7-trihydroxyflavone) was isolated from *C. nerimaniae*, an endemic plant in Turkey and was characterized following the procedure published elsewhere (42). The extract of dried whole plant was first isolated by petroleum ether and then with 95% ethanol. The concentrated ethanol extract was diluted with distilled H_2O and extracted with benzene and chloroform. Apigenin was obtained from the chloroform extract. For the isolation and purification of the apigenin, column chromatography, preparative thin layer chromatography (TLC), and paper chromatography (PC) were performed. The structures of apigenin were determined by comparison with authentic samples (TLC, UV, IR) (42). The stock

solution for apigenin was prepared by adding and dissolving crystallized apigenin in 100% dimethyl sulfoxide (DMSO) and the molar extinction coefficient was used to determine the concentration. Curcumin was obtained from Sigma-Aldrich. Final DMSO concentrations were set to be lower than 0.1% (v/v).

MTT proliferation assay. The cell proliferation rates and cytotoxic effects of apigenin and curcumin on Hela cells were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich) and trypan blue. Ninety-six well plates (30×10^3 cells/well) were used to determine the viability of HeLa cells. MTT assay was performed as previously described (43, 44). The medium was removed as soon as the incubation finished and a total of 40 μl MTT solution which was dissolved in phosphate buffer solution (PBS) (5 mg/ml) was added into each well. After incubating at 37˚C for 4 h, 160 μl DMSO was added. After overnight incubation, optical densities of the samples were measured with μQuant Elisa plate reader (Bio-tek Inst, Vinoski, VT, USA) at 570 nm and 690 nm (45). All experiments were repeated three times and the median values were calculated.

Total RNA extraction. For each experimental group, 150×10^3 cells/ml were seeded in 12 well plates. Following a 24 h incubation at 37˚C, trypsin was added into the wells and the cells were re-suspended and centrifuged at 12.000 × *g* for 2 min and the supernatant was removed. Total RNA was extracted from HeLa cells as described by the manufacturer (Ambion PureLink RNA Mini Kit, Cat. No: 12183-018A, Thermo Fisher Scientific, Austin, TX, USA) and was measured using Qubit (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, CA, USA). The extracted total RNA was used to synthesize cDNA to determine the expression levels of *TRADD, FADD, Bid, Bcl-xL, Fas, TRAF2, TNFR1, TNF, Caspase-3, -8, -9, NOXA, Bad, Bif-1, Atg5, Atg12, DAPk, BNIP-3* and *Beclin-1* by quantitative RT-PCR (qRT-PCR) method. All cDNA products were stored at –20˚C until experiments.

qRT-PCR experiment. qRT-PCR was used to assess the gene expression profiles of apoptosis and autophagic cell death related genes (46, 47). Eight wells were used for each experimental group. Each reaction consisted of 200 ng total RNA, 10 μl buffer, 1μl enzyme mix, in a total volume of 20 μl (Applied Biosystems, High Capacity RNA-to-cDNA Kit, Cat. No: 4387406, Foster City, CA, USA). The reaction mix was incubated for 60 min at 37˚C followed by 5 min at 95˚C. The PCR reaction was run in 96 well plates (TaqMan Array 96-Well Fast Plate, Custom Format 48, Applied Biosystems) using a master mix (Applied Biosystem, TaqMan Gene, expression Master Mix, Cat. No: 4369016), nuclease free dH_2O, and cDNA with probes and primers in 7500 Fast Real-Time PCR Systems (Applied Biosystems), each well contained a total of 20 μl of reaction mix. Each experiment was repeated three times and the median values were calculated.

The expression levels of apoptotic genes (*caspase-3, caspase-8, caspase-9*), proapoptotic Bcl-2 member genes of mitochondria related to apoptotic pathway (*Bcl-xL, Bid, Bad* and *NOXA*), receptor mediated apoptotic pathway genes (*TRADD, FADD, TRAF2, TNF, TNFR1* and *Fas*), autophagy related cell death genes (*Atg5, Atg12, DAPK* and *Beclin-1*), and crosstalk genes related to apoptosis and autophagy (*Bif-1* and *BNIP-3*) were measured by qRT-PCR. The 18S, *β-actin* and *GAPDH* were used as reference. Relative mRNA levels were calculated using $2^{-\Delta\Delta Ct}$ method (48).

Determination of synergistic effects. All drug-interaction experiments were performed by a 4×4 checkerboard assay. The cell viabilities in the presence of curcumin (Sigma-Aldrich Company, C1386) (0-100 μM) and apigenin (0-100 μM) were determined by MTT assay. Initial drug concentration was zero (no drug) and the drug doses were gradually increased with 12 different doses (from 0 to 100 μM). In experiments, doses of apigenin and curcumin were adjusted so that as the dose increased cell viability decreased. Cell viability was higher than 50% at lowest dose and lower than 50% at highest dose (49). All interactions and independent effects of the drugs were measured using the Bliss independence model utilizing probabilistic theory using MATLAB (50).

Immunocytochemistry. The Histostain Plus Bulk Kit (Cat no: 85-8943, Invitrogen) was used for immunoperoxidase staining. The HeLa cells were grown in 24-well plates on coverslips then fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. The cells were treated by 3% H_2O_2 prepared in methanol for 5 min, washed for 5 min in phosphate-buffered-saline (PBS) and then blocked by the blocking solution for 15 min. The coverslips were incubated with an anti-GRP78 (1:100; sc-376768, Santa Cruz, CA, USA) antibody at 4˚C overnight, washed with PBS. Specific staining was performed by biotinylated universal secondary antibody, horseradish peroxidase streptavidin complex. The enzyme activity was developed using AEC substrate and then the cells were counterstained with hematoxylin. All procedures except incubation with the primary antibody were applied on the negative control.

Cytological semi-quantitative analysis. Leica DM 2500 light microscope was used for the cytological score analysis, and the samples were photographed with a Leica DFC280 digital camera system (Leica Microsystems, Wetzlar, Germany). Cytological score analysis was performed at a magnification of 400×. Ten different areas from each slide were scored by two researchers in a blinded manner. The intensity of HeLa cell immunostaining was semiquantitatively evaluated using the following categories: 0 (no staining), 1+ (weak, but detectable staining), 2+ (moderate staining), 3+ (distinct staining), and 4+ (intense staining). A histologic score (H score) was derived for each slide by summing the percentage of stained cells at each intensity category and multiplying that value by the weighted intensity of the staining, using the following formula: H Score $\Sigma Pi\ (i + 1)$ (46).

Statistical analysis. One-way ANOVA test was used to determine the differences between the drug and control groups in terms of time and dose. The differences between drug and control groups in terms of cytotoxicity were determined by Dunnett's test ($p<0.05$) (GraphPad Prism v. 4, San Diego, CA, USA).

Results

Cytotoxicity and cell viability assays. Cell proliferation rates and cytotoxicity of apigenin and curcumin in HeLa cells were measured using the MTT assay. The viability graph (%) based on the results is shown in Figure 1. We observed significant differences between the treated and control groups after 24 h ($p<0.001$). While the cell viability rates for curcumin and apigenin alone were 50.98% and 50.64%, respectively, the combination treatment inhibited cell

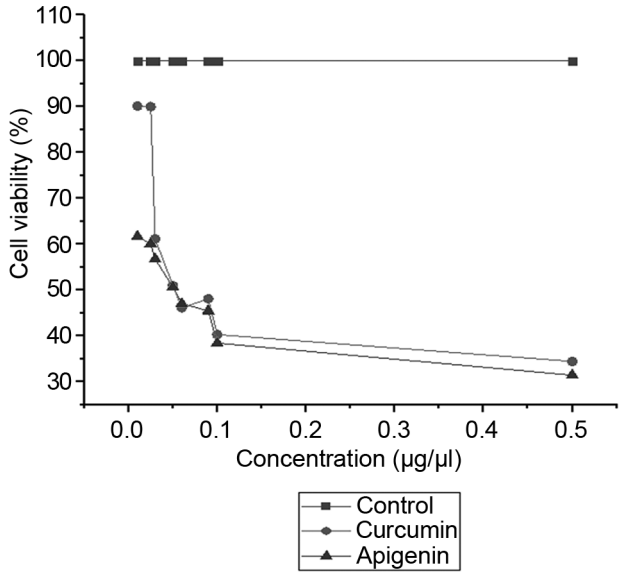

Figure 1. *Cell viability of HeLa cells based on drug (apigenin and curcumin) concentrations.*

viability at a rate of 89.26%. Our results showed that the combined use of these drugs induced significant cytotoxicity in the treatment group compared to the control group in a time- and dose-dependent manner. Cell proliferation was significantly inhibited even at the lowest concentrations.

Synergistic effect of curcumin and apigenin on autophagic and apoptotic cell death. We investigated whether the combined use of apigenin and curcumin had a synergistic effect on apoptotic and autophagic cell death in the HeLa cell line. Curcumin or apigenin treatment alone at concentrations of 0.025-0.01 μg/μl did not have any significant effect on cell proliferation (Figure 1). Cell proliferation was reduced after a combination treatment with curcumin and apigenin at concentrations of 0.03, 0.05, and 0.09 μg/μl, and the inhibitory rates were 46.64%, 49.4%, and 38.17%, respectively. While some concentrations of curcumin (0.025 and 0.05 μg/μl) and apigenin (0.03-0.05 μg/μl) showed an antagonistic effect, the combined use of 0.09 μg/μl curcumin and 0.06 μg/μl apigenin showed a synergistic anti-tumor effect (Figure 2).

mRNA expression analysis. We studied the expression levels of genes related to receptor-mediated and mitochondria-related apoptotic pathways, autophagy, and the cross-talk between autophagy and apoptosis using qRT-PCR. Our data revealed that treatment with the combination of curcumin and apigenin increased the expression levels of the genes studied compared to the control group. The levels of transcriptional activation ranged from 1.29-fold to 27.6-fold. As shown in Figure 3, we detected an increase in the expression levels of *Caspase-8* (19.8-fold) and *Caspase-9* (7.38-fold), while the levels of *Caspase-3* expression decreased by 0.69 compared to the control. *Bad* (5.73-fold) and *Bid* (3.63-fold) gene expression was increased in the drug combination group, while the increase in the expression levels of *NOXA* was smaller (1.29-fold). We also observed significant differences in the expression levels of genes related to the *TRADD* receptor-mediated apoptotic pathway. As shown in Figure 3, the expression of *TNFR1* (11.45-fold), *FADD* (6.94-fold), and *TRADD* (4.34-fold) genes was increased in the drug combination group. *TNF* (27.6-fold), *FAS* (8.41-fold), and *TRAF2* (6.92-fold) showed the higher increases. Among genes related to autophagic cell death, *Atg12* (12.71-fold), *DAPK* (10.89-fold), *Atg5* (6.02-fold), and *Beclin-1* (4.81-fold) were highly expressed in the drug combination group compared to the control group. Expression levels of genes related to cross-talk, such as *Bif-1* (12.23-fold), *BNIP-3* (11.61-fold), and *Bcl-xL* (6.8-fold) were increased in HeLa cells in the drug combination group compared to the control group (Figure 3).

In the apigenin-only group, expression levels of *Caspase-9* (40.64-fold), *Bad* (17.44-fold), and *TRAF2* (1.12-fold) were significantly increased compared to the control group. On the other hand, expression levels of *Caspase-3* (0.13-fold), *Caspase-8* (0.17-fold), *Bid* (0.27-fold), *FADD* (0.28-fold), *FAS* (0.52-fold), *TNF* (0.26-fold), and *TRADD* (0.09-fold) were significantly lower than those of other apoptotic genes. Expression levels of autophagy-related cell death genes, such as *Atg12* (0.08-fold), *Atg5* (0.4-fold), *Beclin-1* (0.07-fold), *Bcl2-xL* (0.12-fold), and *DAPK1* (0.2-fold) were also significantly decreased. After apigenin-only treatment, *BNIP3* and *Bif1* gene expression was not detected.

In the curcumin-only group, the expression levels of genes related to apoptosis, such as *Caspase-9* (273.86-fold), *Caspase-3* (2.05-fold), *Caspase-8* (1.84-fold), and the *Bcl-2* family members, *Bad* (21.48-fold), *Bid* (2.27-fold) and *NOXA* (4.4-fold) were increased. The expression levels of apoptosis receptor-mediated pathway genes, such as *TNF* (4.28-fold), *FADD* (2.08-fold), *FAS* (4.85-fold), and *TRAF2* (1.74-fold) were also increased, while *TRADD* gene expression (0.53-fold) was lower compared to other genes. The expression levels of autophagy-related genes, such as *Atg12* (4.65-fold), *Atg5* (1.31-fold), *Beclin-1* (1.53-fold), and *DAPK1* (3.02-fold) were increased. The expression levels of cross-talk genes, including *BNIP3* (2.44-fold) and *Bif1* (4.6-fold) were increased.

Immunocytochemistry. The expression levels of GRP78/BiP were determined by immunostaining (Figure 4). The intensity of GRP78/BiP immunostaining in the curcumin- or apigenin-only groups was decreased in HeLa cells compared to the control group. In the drug combination group, the

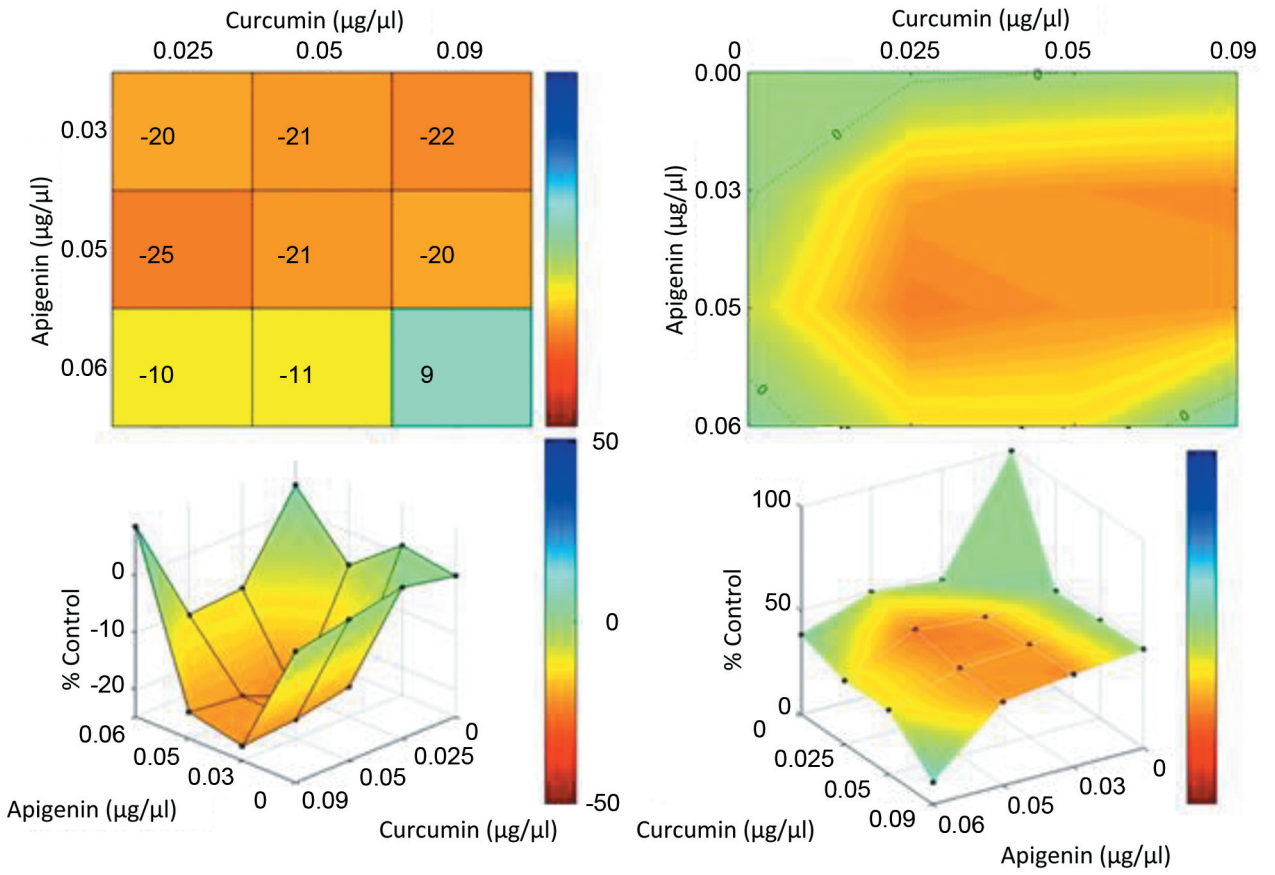

Figure 2. *The upper left shows the scores using the Bliss independence model of drug-drug interactions for the curcumin-apigenin combination used on the HeLa cell line (negative scores represent stimulated antagonism). The upper right demonstrates the 2D response surface. The lower left and right show the 3D response surfaces of the drug combination. The yellow and reddish areas of the surfaces demonstrate more antagonistic interactions (decreased/lower growth inhibition) compared to the green and blue areas, which represent more additive/synergistic interactions (increased/higher growth inhibition). Lower left and right: The X- and Y-axes show drug concentrations plotted against the percentage difference of observed and model-predicted growth inhibition of the HeLa cells at different concentrations of drug combinations.*

immunostaining density of GRP78/BiP was markedly lower than that in the other groups. The combination of apigenin and curcumin resulted in the down-regulation of GRP78/BiP expression. We observed cytoplasmic vacuolization in cells in all drug-treated groups. In the drug combination groups, the cytoplasm of the cells was almost completely occupied by vacuoles of various sizes (Figure 4).

Discussion

Drug combination therapeutic strategies enhance the anti-cancer effects of drugs on various cancers, overcoming cancer drug resistance or reducing the adverse effects of chemotherapy. The combined use of drugs generally reduces toxicity and increases treatment efficacy. It also enables physicians to use low doses of drugs (6, 28, 49). Apigenin and curcumin are natural phenolics with strong anti-cancer effects that use different molecular mechanisms and have low toxicity to normal tissues (11, 51). Previous studies have shown that the use of apigenin in combination with other drugs significantly increases the anti-cancer efficacy of chemotherapeutics in various cancer types by targeting multiple signaling pathways (10, 28, 52). Curcumin also has a potential cytotoxic effect on several cancer types through the induction of apoptosis and cell cycle arrest regulated by the p53 signaling pathway (18, 19, 53). Several clinical trials have reported the combined use of curcumin with docetaxel or doxorubicin during chemotherapy, in which curcumin increased the other drug's efficacy (22, 54, 55). Apigenin or curcumin have been used as anti-cancer agents with other chemotherapeutic drugs in various types of cancers (10, 19). However, there is only one study that has focused on the synergistic effect of apigenin and curcumin on lung cancer cells resulting in cell death (17). Choudhury *et al*. have

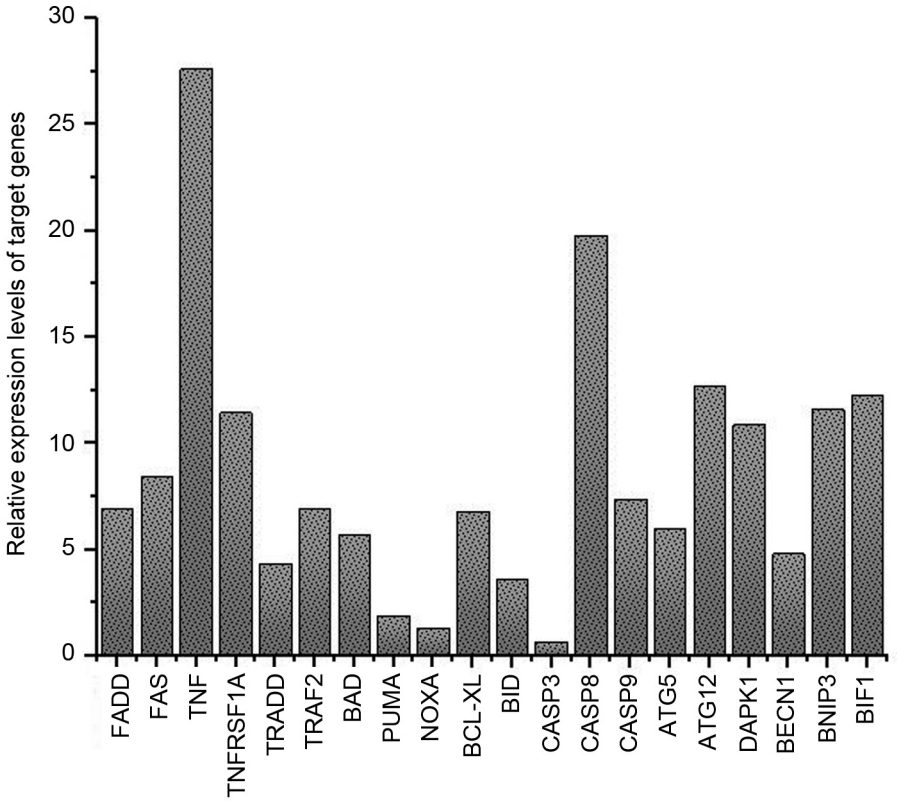

Figure 3. *Changes in the expression levels of apoptotic genes (Caspase-3, Caspase-8, Caspase-9, FAS, TNF, TNFR1, FADD, TRAF2, TRADD, NOXA, Bad, Bcl-xL, and Bid), autophagy-related cell death genes (Atg12, Atg5, Beclin-1, and DAPK) and genes related to the cross-talk between apoptotic and autophagy-related cell death (Bif-1 and BNIP-3) after the combined drug treatment.*

shown that a combination of apigenin and curcumin triggers apoptosis by inhibiting microtubule polymerization and by blocking cell cycle progression in A549 lung cancer cells (17). To our knowledge, no previous study has focused on the synergistic effects of the combined use of apigenin and curcumin on regulatory genes of apoptosis- and/or autophagy-related cell death, as well as on paraptosis-like type of cell death.

In our previous, preliminary study, we observed that treatment with either 0.05 μg/μl of curcumin or 0.05 μg/μl of apigenin induced apoptosis (*via Caspase-3, FAS,* and *TNF*) and autophagy-related cell death (*via Atg5* and *Beclin-1*) (56). In the present study, we aimed to evaluate the efficacy and synergistic effect of the combination of curcumin and apigenin on genes that regulate apoptotic and/or autophagy-related cell death in HeLa cells. In order to identify the molecular targets of curcumin and apigenin, the expression of the following genes was analyzed: *Bif*, *Beclin-1*, and *BNIP-3* (for cross-talk between apoptotic and autophagic cell death); *Atg12*, *Atg5*, and *DAPK* (for autophagic cell death); and *Bcl-xL*, *Bad*, *Bid*, *NOXA*, *FAS*, *TNFR1*, and the *Caspases 3, 8,* and *9* (for apoptotic cell death). In this study, curcumin (0.01-0.5 μg/μl) or apigenin (0.01-0.5 μg/μl) independently reduced the viability of HeLa cells in a dose-dependent manner, while the combined use of apigenin and curcumin showed a greater synergistic inhibitory effect on cell viability (0.09 μg/μl of curcumin and 0.06 μg/μl of apigenin). Our results showed that a combination of low doses triggered the expression of genes involved in both apoptosis and autophagy-related cell death.

Paraptosis, an alternative, caspase-independent, non-apoptotic cell death mechanism, has been discovered in recent years. It is characterized by massive cytoplasmic vacuolization due to swelling of the ER and mitochondria (37, 38). It has been shown that the ER stress marker, expression of the unfolded protein response regulator GRP78/Bip, is increased during paraptosis of cancer cells (39). GRP78/BiP expression is increased in many cancer types. GRP78/BiP expression can be related to increased tumor aggressiveness and poor prognosis (40). GRP78/BiP down-regulation results in sensitization of cancer cells to drug-induced apoptosis (57). In our study, the combination of

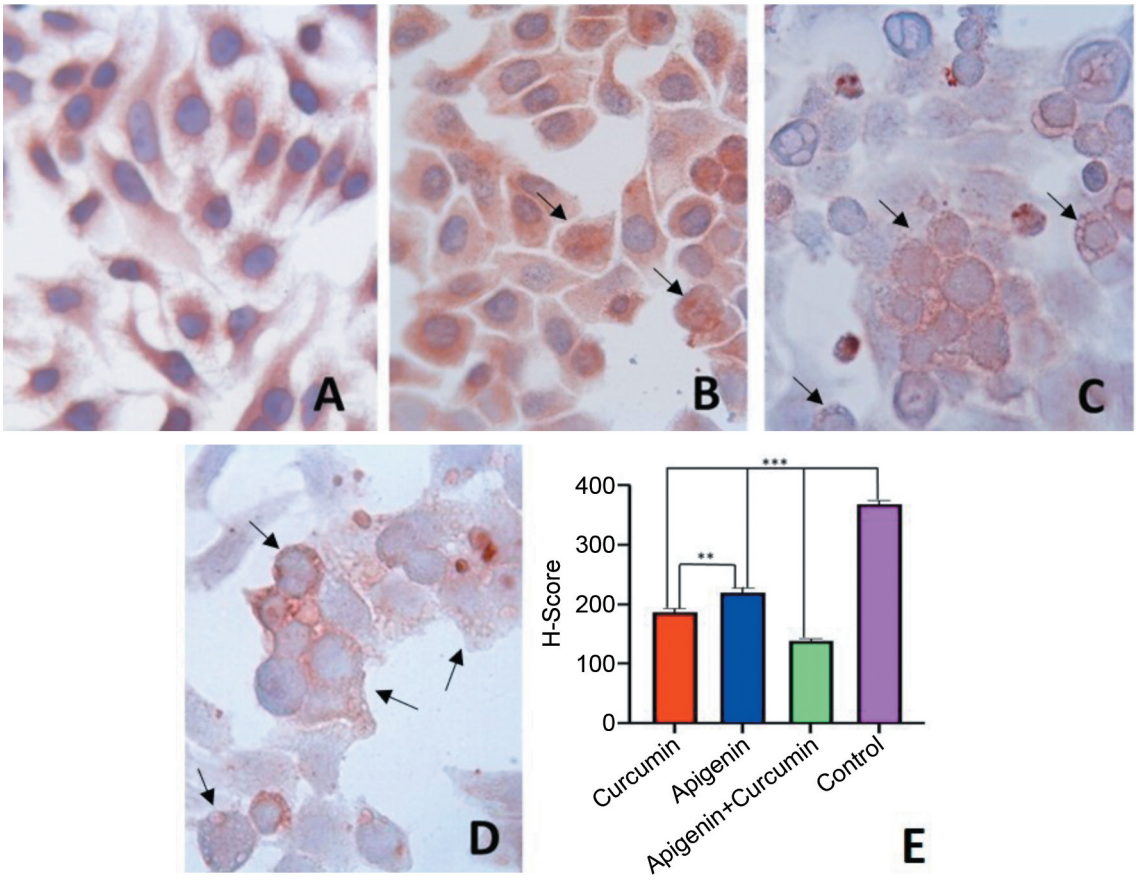

Figure 4. *Immunocytochemistry for GRP78/BiP. Expression levels of GRP78/BiP according to the H-score (E) were markedly decreased in the curcumin and apigenin combination group in contrast to the control group (A) and the other drug-treated groups (B, C) (**p<0.01, ***p<0.00). Extensive cytoplasmic vacuolization (arrows) was observed in HeLa cells in the combination group (D). (A: Control, B: Apigenin, C: Curcumin, D: Curcumin and Apigenin combination groups).*

curcumin and apigenin induced GRP78/BiP down-regulation and produced extensive cytoplasmic vacuolization, similar to paraptosis-like cell death.

Many studies have reported that targeting apoptosis and/or autophagy-related cell death yields successful results in cancer treatment. As a response to therapeutic agents, cancer cells may undergo apoptosis, necroptosis, or autophagy-related cell death. These pathways have a common initiation mechanism; however, the pathways themselves are different (32, 36). Caspases play a major role in triggering apoptosis, either *via* mitochondrial or intrinsic pathways, resulting in activation of *Caspase-3* and *Caspase-9* (33). In this study, we showed that the use of curcumin in combination with apigenin increased *Caspase-8* and *Caspase-9* gene expression; however, *Caspase-3* expression was lower than when either curcumin or apigenin alone were applied. Depending on the complex interactions between cellular proteins, apoptosis can be initiated by different pathways, which are either caspase-dependent or -independent (58, 59). Caspase-3 also has a role in tissue regeneration and cell proliferation in neighboring non-apoptotic cells (34, 60). Overexpression of Caspase-3 has also been reported in a variety of cancer types, which suggests possible crucial roles in oncogenic transformation (60, 61). Flanagan *et al*. have shown that low levels of Caspase-3 predict a favorable response to 5-fluorouracil (5-FU) based chemotherapy in advanced colorectal cancer cases; thus, they have suggested that Caspase-3 inhibition might be used as a therapeutic approach (62). In this study, apigenin from an endemic plant may have down-regulated *Caspase-3* expression by interacting with curcumin in an unknown way. This is the first study showing that an antioxidant, such as apigenin or curcumin, reduces *Caspase-3* gene expression. The cytoplasmic vacuolization in the absence of caspase activation is a characteristic of paraptosis. However, it has been suggested that Caspase-9 can induce non-apoptotic cell death, including paraptosis. Paraptosis requires Caspase-9 in the absence of cytochrome C and lacks apoptotic bodies

(63). Curcumin has been shown to induce cytoplasmic vacuolization-mediated cell death and paraptosis in malignant breast cancer (64) and glioblastoma (65) cell lines. We observed a higher expression of *Caspase-9* in the curcumin-only treatment group, while in the combination group expression was down-regulated. When apigenin was used together with curcumin, the toxicity of curcumin was reduced. We showed that the synergistic effects of these drugs affected different cell death modalities, such as apoptosis, autophagy, and paraptosis-like cell death.

It has been shown that BNIP-3 activates caspase-independent apoptosis, known as the AIF-EndoG pathway (33). Activation of BNIP-3 induces the release of AIF and EndoG from mitochondria, and these molecules translocate to the nucleus to initiate apoptosis (59). We considered that a high level of expression of *BNIP-3* (an 11.61-fold increase) might have activated the caspase-independent pathway, resulting in the lower expression of *Caspase-3*. Meanwhile, apigenin alone decreased the expression levels of genes related to apoptosis, such as *Caspase-8*, *Caspase-3*, and other genes related to the apoptotic cell death pathway, indicating that the effect of these molecules on apoptosis was lower than that of the combination group. In the combination group, apigenin+curcumin may have the opposite effect on *Caspase-3* expression. It has been suggested that the efficacy of chemotherapeutic agents can be improved by inhibiting the activity of caspases in some cancer cells (59, 66). The limitation of the current study is that protein levels of the expressed genes were not examined; further study is required to examine the expression levels of the Caspase-3 protein.

In this study, we showed that combination treatment induced the expression of proapoptotic genes of the *Bcl-2* family, including *Bad* (5.73-fold), *Bid* (3.63-fold), and *NOXA* (1.29-fold) in mitochondrial-mediated pathways. Moreover, the expression of receptor-mediated apoptotic genes, such as *TNFR1, FAS, FADD, TRAF2,* and *TRADD* increased between 4.34- and 11.45-fold; the most remarkable increase in gene expression was observed for *TNF*, with a 27.6-fold increase after the combination treatment. These results suggest that the synergistic effect of curcumin and apigenin together triggered apoptosis by affecting the death receptor-mediated pathway. A number of studies have also emphasized that combined treatment with curcumin can induce apoptosis in cancer cell lines (54, 55). As previously reported, autophagy-related cell death may also be simultaneously induced by extrinsic apoptotic pathway components, including *FAS, FADD, TRADD*, and *TNF* (67). Autophagy or apoptosis can promote cell death by acting in sequence or in parallel (68, 69). These two processes share common signaling pathways; only a specific stimulus decides which pathway will be triggered. Recent studies have shown that Atg5, Atg12, Beclin-1, Bcl-xL, Bif-1, and BNIP-3 can mediate the cross-talk between apoptotic and autophagic cell death mechanisms (35, 70-74). Atg5 induces autophagy *via* cytoprotective and proapoptotic mechanisms, suggesting an effective role in the cross-talk between the two cell death mechanisms. Atg5 knockdown has been shown to inhibit autophagy, triggering apoptosis, and overexpression of Atg5 can sensitize cells to apoptotic stimuli (75). Beclin-1 is a major player in autophagosome formation and functions in the cross-talk with autophagy and apoptotic pathways (35). In addition, DAPK can interact with many proteins with death domains, including Beclin-1, ERK, and TNFR-1, suggesting that protein interaction may increase its expression levels (72).

In this study, we found that the expression of genes related to autophagic cell death – *Atg12* (12.71-fold), *DAPK* (10.89-fold), *Atg5* (6.02-fold), *Beclin-1* (4.81-fold), and *Bcl-xL* (6.8-fold) – significantly increased after combination treatment. Bcl-xL is an important anti-apoptotic factor, inhibiting Beclin-1-mediated autophagy. In addition, Beclin-1 and Bcl-xL can cooperate with Atg5 and regulate both autophagy and apoptosis. Thus, Bcl-xL represents a molecular link between autophagy and apoptosis (74, 76). Once Beclin-1 is cleaved by Caspase-8 and is inactivated, it induces autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria (77). In our study, the increase in *Beclin-1*, *Bcl-xL*, and *Caspase-8* expression after combination treatment supported a synergistic effect and the cross-talk between apoptosis and autophagic cell death.

Our study showed that the expression of *Bif-1* and *BNIP-3*, genes involved in the cross-talk between apoptosis and autophagy-related cell death, increased 12.23- and 11.61-fold, respectively. Bif-1 is important in the mitochondrial pathway of apoptosis, as it interacts with Bax and Bak. Bif-1 can regulate autophagy and suppress tumorigenesis by interacting with Beclin-1. Loss of Bif-1 suppresses autophagosome formation, which triggers tumor development in mice (70). A possible interaction with other genes functioning in autophagy and apoptosis increase the expression of *Bif-1* and *BNIP-3*, suggesting that apoptosis and autophagy are triggered simultaneously (78, 79). It has been reported that autophagy can be induced by proapoptotic BH3-only proteins, such as NOXA and BNIP-3 (79). Our data demonstrates that co-treatment with curcumin and apigenin increased the expression of the proapoptotic genes *BNIP-3* (11.61-fold) and *NOXA* (1.29-fold) in the HeLa cell line. This is the first study showing significantly increased levels of *BNIP-3* and *Bif-1* in the HeLa cell line, so we suggest that the synergistic effect of apigenin and curcumin induces the cross-talk between autophagy-related cell death and apoptosis. Indeed, the complex mechanisms regulating autophagy, apoptosis, and paraptosis have not been fully revealed. In order to clarify the mechanisms of the cross-talk between these cell death modalities, further studies must be conducted.

Conclusion

In conclusion, our data showed that co-treatment with apigenin and curcumin has significant and synergistic effects on the inhibition of cellular growth in HeLa cells by triggering autophagy-related cell death, apoptosis, and paraptosis-like cell death, and inducing cross-talk between these pathways. We suggest that curcumin may have a remarkable anti-cancer effect due to its low toxicity and has a therapeutic potential when used in combination with apigenin in cervical cancer.

Conflicts of Interest

The Authors report no conflicts of interest in relation to this study.

Authors' Contributions

SK, KY and MO designed the study and the concept; SK, KY and ASA performed experiments; SK, KY and MO performed data analysis and wrote the manuscript. SK, KY, ASA, FKD, GM, MO reviewed the data, made adjustments for the study protocols. All Authors read and approved the final version of the manuscript.

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