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Original Article / 원저

AGS 인체 위암세포에서 육계 에탄올 추출물(CcEE)과 온열치료의 항암 시너지 효과

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Synergistic Anticancer Effect of the Cinnamomi Cortex Ethanol Extract (CcEE) and Hyperthermia in AGS Human Gastric Cancer Cells

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ABSTRACT

Objectives : In this study, we investigated the combination effects of Cinnamomi cortex Ethanol Extract (CcEE) and hyperthermia in the human AGS gastric cancer cell line.

Methods : AGS cells were treated with the indicated concentrations of CcEE (0, 50 or 60 μg/mL) for 1h prior to hyperthermia. And then incubated for a further 30 min at the indicated temperatures (37, 42 or 43℃) in a humidified incubator containing 5% CO_2 or a thermostatically controlled water bath for hyperthermia. The cell viability was measured by MTT assay, Morphology assay and Trypan blue assay. To investigate the possible molecular signaling pathways, the activation of mitogen-activated protein kinase (MAPK) proteins (ERK, p38 and JNK) and expression of various anti-apoptotic proteins such as Caspase-3, Caspase-9, p53, Cyclin D1 and MMP-2 were assessed by Western blot analysis. In addition, Annexin V and 7-amino-actinomycin D (7-AAD) staining was performed to examine the apoptotic mechanism.

Results : Combination of CcEE with hyperthermia effectively suppressed the cell viability and changed cellmorphology compared with CcEE or hyperthermia treatment alone. Combined treatment also abated the expression of Caspase-3, Caspase-9, Cyclin D1 and MMP-2. Whereas, the expression level of p53 was up-regulated by co-treatment. Moreover, combination treatment enhanced phosphorylation of ERK, p38 and JNK. In addition, this combination increased anti-cancer effect by inducing cell death through the apoptosis.

Conclusions : Taken together, all these findings suggest that the combination treatment with CcEE and hyperthermia may have therapeutic potential as a promising approach to patients with stomach cancer.

Key words : Gastric cancer (GC), AGS, Cinnamomi cortex, Hyperthermia, Apoptosis, Combination, Synergistic effect, Anti-cancer effect, Mitogen-activated protein kinase (MAPK).

Ⅰ. Introduction

Stomach cancer, also known as gastric cancer (GC), is one of the most common malignant tumor in the gastrointestinal (GI) system. GC ranks as the fifth most common cancer and is the third leading cause of cancer-related mortality worldwide[1]. The overall 5-year relative survival rate of patients with GC is low[2]. Although multifarious clinical anti-cancer treatments such as surgical techniques, chemotherapy, radiotherapy, hyperthermia and neoadjuvant therapy are improved, most GC patients are diagnosed with advanced-stage disease at diagnosis. Thus, the main treatment for patients with advanced gastric cancer is the combination treatments.

Natural products have been considered as one of the potential materials for developing the pharmacological agents. Because they are more suitable for prolonged use without side effects than chemical agents. *Cinnamomum cassia* (*C. cassia*), also named Chinese cassia or Chinese cinnamon, belongs to a member of Lauraceae family found in southern China, Vietnam, Myanmar and Laos[3]. Especially, the bark of *C. cassia* (Cinnamomi cortex) has been proven to have neuro-protective effect, anti-inflammatory effect and anti-tumor activity[4].

Heat therapy was used as a remedy for cancer in ancient Egypt over 5000 years ago and it was utilized to treat various diseases[5]. It has been reported that a temperature between 41℃ and 44℃ does not affect normal cells whereas significantly inhibiting cancer cell growth[6]. Commonly, hyperthermia usually induces cancer cell death *via* apoptosis or necrosis, but mainly through apoptosis. Hyperthermia induces various physiological modifications in cells, such as alteration in membrane permeability, modification of cytoskeletal system, changes in macromolecule synthesis, intracellular signal transduction, and inhibition of DNA repair[5,6]. Earlier literatures have turned out that combination of hyperthermia with chemotherapy proved better effectiveness than the alone because of thermotolerance which is its major obstacle[7,8].

Apoptosis is one of the cellular responses to anticancer agents, induced by herbs or hyperthermia[6]. Thus, targeted inhibition of the anti-apoptotic pathway is a tempted approach for combination tumor treatment therapies.

Here, we investigated the effects of combination with Cinnamomi cortex Ethanol Extract (CcEE) and hyperthermia-induced apoptosis and its associated molecular mechanisms in human AGS gastric cancer cell line.

Ⅱ. Materials and Methods

1. Preparation of Cinnamomi cortex Ethanol Extract

Cinnamomi cortex was obtained from Kwangmyungdang Medicinal herbs co., Ltd. (Ulsan, South Korea). To prepare Ethanol Extract, the material was mixed with 70% Ethanol at

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room temperature for 24h. The extract was filtered and concentrated in a boiling water bath. Finally, the concentrated extracts were dried using a vacuum drier. The resulted Cinnamomi cortex Ethanol Extract (CcEE) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4℃ until the experiment was performed.

2. Reagents

Tris base and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from AMRESCO (Solon, OH, USA). RPMI 1640 medium, 0.05% trypsin-EDTA, fetal bovine serum (FBS), penicillin-streptomycin (Pen-Strep, 10,000 units/mL-10,000 μg/mL) and phosphae-buffered saline (PBS) tablets were obtained from Gibco (Grand Island, NY, USA). Muse Annexin V and Dead cell kit was supplied from Merck Millipore (Billerica, MA, USA). Glycine was acquired from Gendepot (Houston, TX, USA). Bovine serum albumin (BSA) was supplied from Biosesang (Seongnam, Korea). Sodium dodecylsulfate (SDS), trypan blue solution and tween 20 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-p-ERK (Thr202/Tyr204), anti-ERK, anti-p-p38 (Thr180/Tyr182), anti-p38, anti-p-JNK (Thr183/Tyr185), anti-JNK, anti-Caspase-3, anti-Caspase-9, anti-p53 and cell lysis buffer (10X) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Cyclin D1 (A-12), anti-Matrix metalloproteinase-2 (MMP-2) (8B4), anti-β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bicinchoninic acid (BCA) protein assay reagent was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). RPMI 1640 and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Dimethyl sulfoxide (DMSO), ethanol (EtOH), methanol (MeOH) and hydrochloric acid (HCl) were obtained from Samchun Chem. (Seoul, Korea). Sodium chloride (NaCl) was acquired from Daejung chemicals and metals (Siheung, Korea).

3. Cell line and Cell culture

The human AGS gastric cancer cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured with RPMI 1640 medium containing 10% heat-inactivated FBS and 1% of Pen-Strep (10,000 U/mL) in a humidified incubator containing 5% CO_2 at 37℃.

4. Drug and Hyperthermia treatments

For drug and hyperthermia treatments, AGS cells were seeded at a density of 0.18 x 10^6 cells/well in 6-well plates. Indicated concentrations of CcEE were added to the culture media 1h prior to heating. After 1h, 6-well plates containing cells were sealed and incubated in a thermostatically controlled water bath for 30 min at 42 or 43℃. The AGS cells in the 37℃ served as the control. Following heat treatment, the AGS cells were returned to a standard culture conditions at 37℃ in a 5% CO_2 atmosphere for indicated duration.

5. Microculture tetrazolium (MTT) assay

The cell viability was examined by Microculture tetrazolium (MTT) assay. AGS cells were plated at a density of 1 x 10^4 cells/well in 96-well plate and allowed to adhere overnight. After added the indicated concentrations of CcEE for 1h prior to hyperthermia treatment, the AGS cells were incubated at 42 or 43℃ in a constant temperature water bath. And then they were further cultured in a humidified incubator with 5% CO_2 at 37℃. After 48h, 20 μL of MTT solution (2 mg/mL) was directly added to each well and then incubated for 1h 30 min to allow MTT to metabolize to formazan. The culture media were removed and the cells were lysed in 100 μL of DMSO. Absorbance was measured with an automated spectrophotometric plate

reader at a wavelength of 570 nm. The cell viability was normalized as relative percentages in comparison with untreated controls. The synergistic effect between CcEE and Hyperthermia was additionally determined by the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

6. Trypan blue assay

The viability of AGS cells which treated with CcEE and hyperthermia was evaluated by cell counting. The AGS cells were seeded in 6-well plates at a density of 0.18 x 10^6 cells/well. The cells were treated with the indicated concentrations of CcEE for 1h and then continued for a further 30 min in order to expose the hyperthermia. After incubation at standard culture conditions for 48h, the cells were harvested from 6-well plate by trypsinization with 0.05% Trypsin-EDTA and centrifuged at 3500 rpm for 3 min at 4℃. The pallet was resuspended in 1 mL of medium and then 100 μL of this suspension was diluted with 400 μL of 0.4% trypan blue solution. The viable and non-viable cells on hematocytometer were counted under a light microscope. This test was done in triplicate. Survival rates were calculated according to the following equation; Survival rate (%) = Number of colorless cells / Total number of cells x 100.

7. Morphology assay

The cell growth was measured by Morphology assay. The AGS cells were grown in 6-well plates at a density of 0.18 x 10^6 cells/well. Briefly, the indicated concentrations of CcEE were added to the cell culture prior to the hyperthermia treatment. After 1h, all these cells were incubated in a temperature controlled water bath at 42 or 43℃ for 30 min. And then they were further cultured for 48h at standard culture conditions. The cells were visualized on a phase contrast microscopy and the pictures were captured with a phase contrast microscopy (Olympus CX-40, Olympus, Tokyo, Japan).

8. Western blot analysis

The AGS cells were harvested and lysed in ice-cold lysis buffer. The total protein concentrations were measured using a BCA protein assay. Equal amounts of protein resolved on sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in 1XTBS containing 0.1% Tween 20 for 1h at room temperature. After the blocking, the membranes were incubated overnight at 4℃ with the respective primary antibodies (1:3000). The membranes were washed three times and incubated with diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 1h at room temperature. After three times washing, the membranes were detected using an enhanced chemiluminescence (ECL) kit (Merck Millipore, Bedford, MA, USA).

9. Apoptosis assay by flow cytometry

To measure the ratio of the apoptosis, an Annexin V and Dead cell kit was used. AGS cells were seeded in 6-well plates at a density of 1 x 10^5 cells/well. At 16h after all treatments, AGS cells were collected and pallets were subjected to an Annexin V and 7-amino-actinomycin D (7-AAD) staining according to the manufaturer's instructions. After incubation, the cells were analyzed by using the Muse® Cell Analyzer (EMD Merck Millipore, Billerica, MA, USA).

10. Statistical analysis

All numeric values are represented as the mean ± SD. Statistical significance of the data compared with the untreated control was determined using the Student unpaired *t*-test. Significance was set at $p < 0.05$.

Ⅲ. Results

1. Combination with CcEE and hyperthermia activates ERK, p38 and JNK in AGS cells

First, to examine the possible role of MAPK signal pathways in the treatment of combination with CcEE and hyperthermia, the phosphorylation of ERK (p-ERK), p38 (p-p38) and JNK (p-JNK) were evaluated by western blotting. The results showed that the combined CcEE-hyperthermia treatment can substantially induce phosphorylation of MAPK family proteins such as ERK, p38 and JNK in AGS cells (Fig. 1A). Expression of p-JNK was increased after hyperthermia treatment with or without CcEE. p-ERK expression and p-p38 expression were dramatically up-regulated in the combined CcEE-hyperthermia treatment compared to the absence of CcEE. Total expressions of ERK, p38 and JNK were not significantly changed.

2. Combination of CcEE and hyperthermia on changes expression of apoptosis associated proteins in AGS cells

To further investigate the molecular mechanisms of CcEE in response to hyperthermia, we examined expressions of various proteins involved in apoptosis, proliferation, metastasis and angiogenesis by western blot analysis. Caspases are considered as an important executioners in the apoptotic signaling pathway. As shown in Fig. 2A, only CcEE in combination with hyperthermia markedly reduced the expression of Caspase-3 and Caspase-9 in a concentration-dependent manner. The p53 is known as nuclear transcription factor which regulates cell cycle arrest and pro-apoptotic protein. Expression of activated p53 was observed in CcEE pre-treated cells and in cells after hyperthermia with or without CcEE (Fig. 2B). Western blot analysis also showed that the combined CcEE-hyperthermia treatment slightly down-regulated expression of Cyclin D1 which is a critical regulator of cell cycle progression (Fig. 2B). As shown in Fig. 2B, the results showed that CcEE led to a dose-dependent reduction in the protein level of MMP-2. In addition, CcEE with hyperthermia slightly more decreased expression of MMP-2 than CcEE alone. β-actin served as an internal reference.

3. Combination with CcEE and hyperthermia inhibits proliferation of AGS cells

To study anti-tumor activity of combination with CcEE and hyperthermia in AGS cells, cell viability was detected by MTT assay (Fig. 3A). The results showed that treatment of CcEE diminished the proliferation of AGS cells by 64% or 70% respectively, in a dose-dependent manner. Furthermore, dramatically inhibition of cell proliferation was observed after the cells were underwent with hyperthermia at 42 or 43℃ in a dose-dependent manner in comparison with CcEE alone. To confirm that the combination treatment with CcEE and hyperthermia has synergistic cytotoxicity on AGS cells, we used CompuSyn software. The synergy of combined treatment was assessed based on calculated combination index (CI) values, where CI values of > 1.0 implies antagonism, 1.0 implies additivity and < 1.0 implies synergistic type relationships between CcEE and hyperthermia. The results demonstrated that the combined treatment could synergistically inhibit the proliferation of AGS cells (Fig. 3B). And also cell morphological changes in response to the co-treatment with CcEE and hyperthermia were observed using phase contrast microscopy (Fig. 3C). To further examine the anti-cancer activity, we next investigated whether combination treatment of CcEE and hyperthermia induces cell death in AGS cells using Trypan blue assay (Fig. 3D). As shown in Fig. 3D, the proliferation-inhibitory effect was more significant in the combined treatment of CcEE and hyperthermia.

4. CcEE, with or without hyperthermia, elevates the ratios of apoptosis in AGS cells

In order to determine whether the treatment of CcEE with or without hyperthermia induced cell death through apoptotic mechanism, a double staining of Annexin V and 7-AAD staining was performed. As shown in Fig. 4A, apoptosis (early and late) was indicated by Annexin V (+) and 7-ADD (- and +). The proportions of apoptotic cells treated with CcEE alone as well as co-treated with CcEE and hyperthermia were dramatically increased.

Ⅳ. Discussion

Cinnamomum cassia (*C. cassia*) has been extensively studied in a various fields to reveal its pharmacological effects[3,4]. Moreover, previous reports have indicated that *C. cassia* includes vital oils and other derivatives including cinnamaldehyde, cinnamic acid, cinnamyl alcohol, cinnamate and coumarin related to variety of bio-activities[9,10]. However, the study of the effect of *C. cassia* as well as combination with another therapy (especially hyperthermia) on gastric cancer cells and the underlying mechanisms has not been elucidated unclearly.

In the present study, we demonstrated that combined treatment with Cinnamomi cortex Ethanol Extract (CcEE) and hyperthermia induced cell death significantly on AGS human gastric cancer cells. Upregulated phosphorylation of MAPK proteins (ERK, p38 and JNK) were involved in the underlying mechanisms. And also combined treatment increased p53 and diminished Caspase-3, Caspase-9, Cyclin D1 and MMP-2 which contribute to apoptotic signaling pathway.

Reactive oxygen species (ROS) are derived from the metabolism of oxygen as the by-product of cell respiration. Oxidative stress occurs as a result of an imbalance between ROS production and the available neutralizing antioxidants[5,11]. Furthermore, Oxidative stress plays a crucial role in apoptosis by triggering a lot of cellular responses by the activation of stress-activated protein kinases. Thus, ROS generation leads to DNA damage and culminates in apoptosis[12]. Previous studies indicate that anti-tumor agents which induce ROS generation can also simultaneously activate MAPK pathways in multiple tumor cells[13,14]. Thus, we investigated whether combination treatment of CcEE and hyperthermia induces ERK, p38 and JNK MAPK activation in AGS cells. In our results, expression of p-ERK and p-p38 were significantly increased in the combined treatment comparing with hyperthermia treatment alone. And expression of p-JNK was increased after treatment hyperthermia regardless of the absence or presence of CcEE (Fig. 1A). On the basis of these results, our findings indicate that activation of the MAPK signaling pathway is one of the important signal transduction pathways involved in the induction of apoptosis by co-treatment with CcEE and hyperthermia.

Besides, we also noticed that the expression of diverse proteins involved in apoptosis, proliferation, metastasis and angiogenesis was altered upon combined treatment of CcEE and hyperthermia (Fig. 2A and 2B). Apoptosis is a thoroughly regulated cell suicide program[15]. Caspases are a family of protease enzymes and are crucial mediators in the apoptotic mechanism[5,16]. It is reported that there are three-pathways to the activation of caspases: the intrinsic or mitochondrial pathway, the extrinsic or death receptor pathway and the intrinsic endoplasmic reticulum (ER) pathway[5]. p53 is a nuclear transcription factor and transactivates numerous target genes in connection with the induction of cell cycle arrest and apoptosis[17,18]. Since p53 plays an important role in the regulation of cell fate in response to DNA damage, the therapeutic strategies which activate

p53-mediated pro-apoptotic pathway should be required. Cyclin D1 is important for the development and progression of several cancers[19,20]. Cyclin D1 overexpression is a common event in cancer. Numerous therapeutic agents have been observed to induce Cyclin D1 degradation. Finally, mitochondrion plays a pivotal role in regulating of intrinsic cell death. And also the loss of the MMP leads to cell death subsequent an apoptotic insult[21]. MMP-2, which degrades the extracellular matrix, has been related to lymph node metastasis of gastric cancer[22,23].

Taken together, our data demonstrate that the combination with CcEE and hyperthermia is a capable stimulator of ROS-mediated MAPKs signaling pathway and can also induce apoptosis by modulating various anti-apoptotic proteins in AGS human gastric cells.

Ⅴ. Conclusion

Our approach was to study the synergistic anticancer effect of CcEE and hyperthermia through the apoptotic mechanism on the AGS human gastric cancer cell line. In conclusion, our results demonstrated the apoptotic effect of CcEE, which was effectively elevated in co-treatment with hyperthermia. Although the possibility of other mechanisms should be studied, our finding suggests that combined therapy with CcEE and hyperthermia could be considered as an alternative strategy and might be beneficial especially for stomach cancer.

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Figure legends

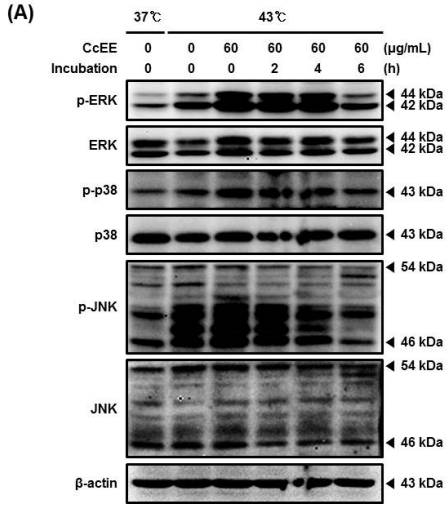

Fig. 1. Combination effects of CcEE and hyperthermia on the protein expressions of ERK, p38 and JNK MAPK activation. (A) Western blot analysis of ERK, p38 and JNK and their phosphorylation. AGS cells (0.35 x 10^6 cells/well) were treated at 37 or 43℃ for 30 min with or without CcEE pre-treatment at 60 μg/mL for 1h before hyperthermia. And then they were incubated at standard culture conditions during different indicated time. Whole-cell extracts were prepared, then equal amounts of lysates were examined by Western blot analysis using antibodies against p-ERK, ERK, p-p38, p38, p-JNK and JNK. β-actin was used as an internal control.

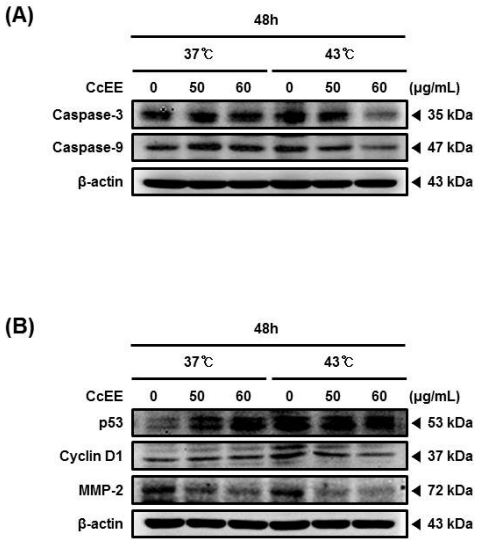

Fig. 2. Combination effects of CcEE and hyperthermia on the expressions of various proliferative, anti-apoptotic and metastatic proteins. (A and B) AGS cells (0.18 x 10^6 cells/well) were treated at 37 or 43℃ for 30 min with or without CcEE pre-treatment at 50 or 60 μg/mL for 1h before hyperthermia. And then they were incubated at standard culture conditions for 48h. Whole-cell extracts were prepared, then equal amounts of lysates were examined by

Western blot analysis using antibodies against Caspase-3, Caspase-9, p53, Cyclin D1 and MMP-2. β-actin was used as an internal control.

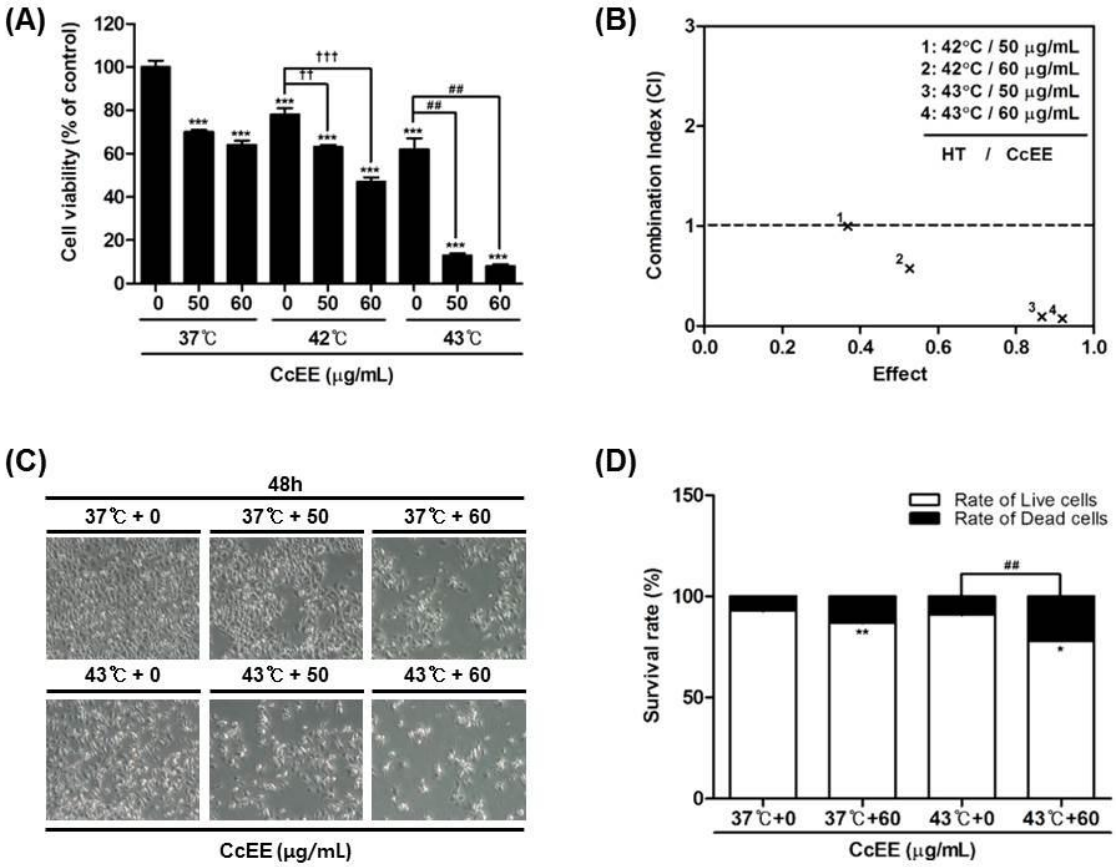

Fig. 3. Effects of combination with CcEE and hyperthermia on the viability in AGS cells. (A and B) AGS cells (1 x 10^4 cells/well) were treated at different temperatures (37, 42 and 43℃) for 30 min with the indicated concentrations (0, 50 and 60 μg/mL) for 1h before hyperthermia. And then they were incubated at standard culture conditions for 48h. (A) Percentage of cell viability was measured using MTT assay. (B) Combination index (CI) for combined effects under different conditions (CcEE and hyperthremia) was calculated by CompuSyn software. CI = 1, < 1 and > 1 indicates additive effect, synergism and antagonism, respectively. (C) AGS cells (0.18 x 10^6 cells/well) were treated at 37 or 43℃ for 30 min with or without CcEE pre-treatment at 60 μg/mL for 1h before hyperthermia. And then they were incubated at standard culture conditions for 48h. Representative microscopy images demonstrated cellular morphological comparisons between different treatment groups of AGS cells. (D) The viable cell was evaluated by trypan blue assay. Bar graph shows cell viability of different treatment groups of AGS cells. Data represents means ± S.D. for three separate measurements. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ vs. control group (CcEE 0 μg/mL and 37℃), †† indicates $p < 0.01$, ††† indicates $p < 0.001$ vs. group of CcEE 0 μg/mL and 42℃ and $^{\#\#}$ indicates $p < 0.01$ vs. group of CcEE 0 μg/mL and 43℃.

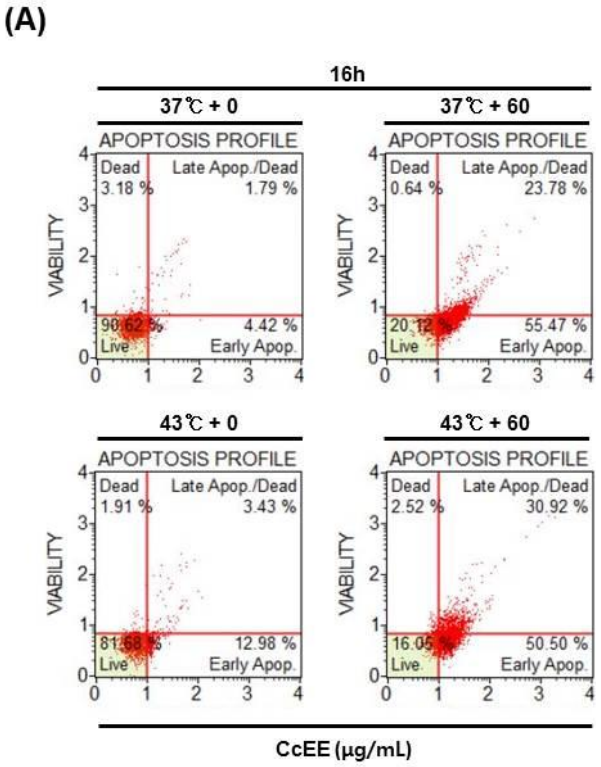

Fig. 4. Assessment of apoptosis by flow cytometry in AGS cells treated CcEE and with or without hyperthermia. (A) AGS cells (1 x 10^5 cells/well) were treated at 37 or 43℃ for 30 min with or without CcEE pre-treatment at 60 μg/mL for 1h before hyperthermia. And then they were incubated at standard culture conditions for 16h. Annexin V and 7-AAD staining was used to detect apoptosis and then analyzed by a flow cytometer.