

Epigallocatechin gallate and thermal cycling-stimulation synergistically promote apoptosis in A549 cells via endoplasmic reticulum stress-induced calcium ion dysregulation and oxidative stress

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Abstract. Non-small cell lung cancer (NSCLC), as the predominant subtype of lung cancer, presents a considerable

clinical challenge due to its high rates of recurrence and the significant adverse effects associated with conventional therapeutic modalities. In response to these challenges, the present study has explored the combined anticancer effects of epigallocatechin gallate (EGCG) together with thermal cycling-stimulation (TCS). The findings obtained demonstrated that the combination of EGCG and TCS led to a synergistic decrease in the viability of A549 and NCI-H460 NSCLC cells, whereas IMR-90 normal lung fibroblasts exhibited minimal cytotoxic effects. Further investigations revealed that EGCG could mitigate the TCS-induced upregulation of the heat shock proteins (HSPs) HSP70 and HSP105, whereas the expression levels of proteasome subunits were concurrently decreased. These alterations were accompanied by the disruption of proteostasis, and also by the activation of endoplasmic reticulum (ER) stress-associated pathways. Furthermore, pharmacological inhibition studies suggested a mutual interplay between inositol 1,4,5-trisphosphate receptor-mediated ER Ca²⁺ release and an excessive production of reactive oxygen species, ultimately causing the cells to undergo mitochondrial apoptosis. In summary, the present study has shown that the combined treatment of EGCG and TCS reduces the required dosage of EGCG, while also attenuating TCS-induced heat shock responses. Consequently, the present study offers a novel and promising *in vitro* proof-of-concept strategy for treating NSCLC.

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Abbreviations: NSCLC, non-small cell lung cancer; ER, endoplasmic reticulum; ROS, reactive oxygen species; TCS, thermal cycling-stimulation; HSP, heat shock protein; UPS, ubiquitin-proteasome system; EGCG, epigallocatechin gallate; IP₃R, inositol 1,4,5-trisphosphate receptor; BCRC, Bioresource Collection and Research Center; 2-APB, 2-aminoethoxydiphenyl borate; NAC, N-acetylcysteine; PERK, protein kinase R-like endoplasmic reticulum kinase; TRPM2, transient receptor potential cation channel subfamily M member 2; TRPV1, transient receptor potential cation channel subfamily V member 1; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TBST, Tris-buffered saline with Tween-20; PSMA3, proteasome subunit alpha type-3; PSMC3, 26S proteasome regulatory subunit 6A; p-JNK, phosphorylated c-Jun N-terminal kinase; p-eIF2 α , phosphorylated eukaryotic initiation factor 2 subunit α ; t-eIF2 α , total-eIF2 α ; t-JNK, total-JNK; PARP, poly(ADP-ribose) polymerase; XBP1, X-box binding protein 1; RT, reverse transcription; IRE1 α , inositol-requiring enzyme 1 α ; XBP1u, unspliced XBP1; XBP1h, heteroduplex XBP1; XBP1s, spliced XBP1; HBSS, Hanks' balanced salt solution; FSC, forward scatter; SSC, side scatter; DHE, dihydroethidium; MMP, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; PI, propidium iodide; ANOVA, analysis of variance; UPR, unfolded protein response; mPTP, mitochondrial permeability transition pore

Key words: EGCG, hyperthermia, NSCLC, combination treatment

Introduction

Lung cancer is the leading cause of cancer-associated deaths worldwide, with non-small cell lung cancer (NSCLC) representing ~85% of all cases (1,2). Although improvements have been made in terms of surgery, chemotherapy and targeted therapies, the prognosis for patients with advanced NSCLC remains poor, primarily due to the development of drug resistance associated with current treatment modalities (3). Therefore, there is an urgent clinical need to develop novel therapeutic approaches that demonstrate both high efficacy and minimal adverse effects.

Hyperthermia, employed as an adjuvant anticancer treatment, works by disrupting intracellular proteostasis through the elevation of temperature within tumor tissues (4). This thermal stress consequently leads to the accumulation of unfolded proteins, thereby inducing endoplasmic reticulum (ER) stress (4). When ER stress is severe or sustained, it can initiate apoptotic pathways via various mechanisms, including the disturbance of intracellular calcium ion (Ca^{2+}) homeostasis and the excessive generation of reactive oxygen species (ROS) (4,5). However, conventional hyperthermia techniques are often limited by the risk of thermal damage to adjacent normal tissues. To overcome this issue, our research group has developed a novel hyperthermic approach that has been termed 'thermal cycling-stimulation' (TCS). Unlike continuous heating, TCS involves alternating cycles of high and low temperatures, which has been demonstrated to minimize damage to normal cells, while preserving substantial inhibitory effects on cancer cell viability (6-10). Moreover, our previous studies (6-10) have shown that TCS works synergistically with various anticancer drugs, including herbal compounds and targeted therapies. This synergy both facilitates a reduction in the required drug dosages and enhances selective anticancer efficacy.

Although hyperthermia holds therapeutic potential, its clinical effectiveness is constrained by the activation of cellular protective mechanisms in response to heat stress. Exposure to heat stress has been shown to quickly trigger the upregulation of heat shock proteins (HSPs) within cancer cells (11). These molecular chaperones assist in the refolding of denatured proteins, thereby preserving proteostasis, which may lead to thermotolerance, also diminishing the susceptibility of cancer cells to hyperthermia-induced cytotoxicity (11,12). In addition to protein folding, the ubiquitin-proteasome system (UPS) contributes to proteostasis through degrading abnormal proteins, thereby helping cells to withstand stressful conditions (13). Hence, molecular inhibitors that target HSPs or the UPS have been proposed as promising approaches to potentiate the anticancer efficacy of hyperthermia (11,14,15).

On the other hand, epigallocatechin gallate (EGCG), the most prevalent and biologically active polyphenol found in green tea, has been recognized for its therapeutic potential against NSCLC (16). Importantly, previous studies have indicated that EGCG can suppress the expression of HSP70 and inhibit proteasome activity (17,18). Additionally, EGCG has been shown to induce the production of ROS, thereby leading to mitochondrial dysfunction in cancer cells (19). Despite these promising effects, however, the clinical application of EGCG is limited due to its poor chemical stability and low bioavailability, which restricts its accumulation in tumor tissues at therapeutically effective levels (20). Consequently, strategies to enhance the efficacy of EGCG are essential for its successful translation into clinical use.

In the present study, the synergistic anticancer effects of EGCG in combination with TCS on A549 NSCLC cells have been investigated. Our previous study demonstrated synergistic anticancer effects of EGCG combined with TCS in PANC-1 pancreatic cancer cells (8). However, the molecular basis underlying this interaction was not fully investigated. Consistent with published studies reporting that EGCG inhibits HSPs and the UPS (17,18), the findings of the present

study demonstrated that the combination of EGCG and TCS causes a substantial accumulation of ubiquitinated proteins, also exacerbating ER stress. Further mechanistic analysis suggested that this combined treatment may be associated with a reciprocal crosstalk between Ca^{2+} release from the ER via inositol 1,4,5-trisphosphate receptor (IP_3R) channels and ROS generation, ultimately inducing mitochondrial apoptosis. Importantly, the combination of EGCG and TCS was shown to exhibit no cytotoxic effects on IMR-90 normal lung fibroblast cells. Overall, the present study aimed to elucidate the synergistic anticancer effects of EGCG and TCS in combination, and the associated underlying molecular mechanisms, in an attempt to offer a promising *in vitro* proof-of-concept strategy to attenuate heat-induced cytoprotective responses, while lowering the required dosage of EGCG. Collectively, the findings of the present study have provided a valuable foundation for the potential development of combination therapies against NSCLC.

Materials and methods

Cell culture. The human NSCLC cell lines A549 (cat. no. 60074) and NCI-H460 (cat. no. 60373), as well as the human normal lung fibroblast cell line IMR-90 (cat. no. 60204), were obtained from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute. All cell lines were authenticated using short tandem repeat profiling and certified as mycoplasma-free by the BCRC prior to distribution. To maintain cell integrity, all experiments were performed using cells within 10 passages after resuscitation. Both A549 and NCI-H460 cells were cultured in Dulbecco's Modified Eagle Medium (HyClone™; Cytiva), whereas IMR-90 cells were maintained in Eagle's Minimum Essential Medium (Corning, Inc.). All culture media were supplemented with 10% fetal bovine serum (HyClone™; Cytiva) and 1% (v/v) penicillin-streptomycin (Gibco®; Thermo Fisher Scientific, Inc.). All cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2 . Cells were subcultured or harvested for subsequent experiments in 0.05% trypsin-0.5 mM EDTA solution (Gibco®; Thermo Fisher Scientific, Inc.).

TCS exposure. TCS was performed using a modified PCR system equipped with waterproof capabilities in the heating area, as described in previous studies (6-8). A minimal volume of water was employed as a thermal conduction medium, enabling the direct placement of cell culture dishes onto the heating area. The modified PCR apparatus controlled both heating and cooling phases. The TCS exposure comprised 15 cycles, each consisting of a 3-min high-temperature phase followed by a 30-sec passive cooling interval (Fig. 1A). This heating-cooling period was established based on prior investigations involving various cancer cell lines (6,7). The low-temperature setting was maintained at 37°C to simulate physiological human body temperature. Actual temperatures experienced by cells at the well bottom were monitored using a needle thermocouple. Temperature cycling reached a steady state after the third cycle, with the recorded temperature cycling between 41.4 and 43.8°C, as shown in Fig. 1B.

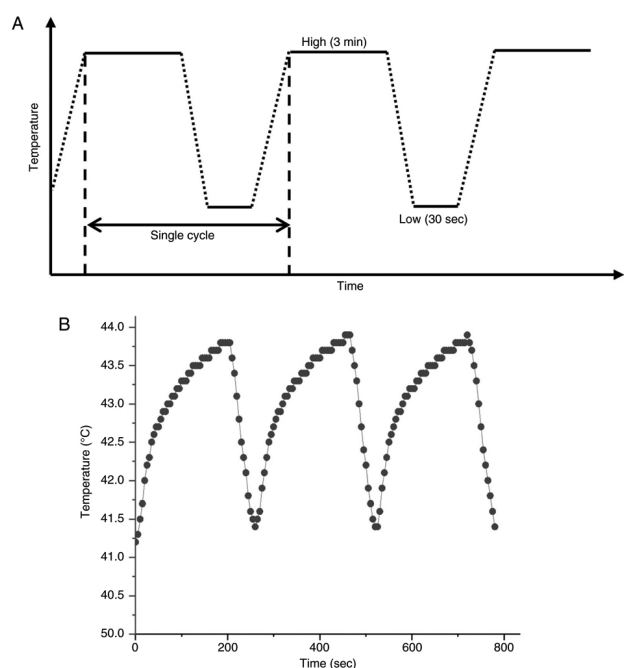


Figure 1. *In vitro* application of TCS. (A) A schematic diagram of the TCS parameter settings in the modified PCR system is shown. (B) Real-time temperature variation at the bottom of the well, measured with a needle thermocouple, is shown. Temperature readings were taken every 5 sec during the heating applications. TCS, thermal cycling-stimulation.

Cell treatments. EGCG (MedChemExpress; cat. no. HY-13653), 2-aminoethoxydiphenyl borate (2-APB) (MedChemExpress; cat. no. HY-W009724) and N-acetylcysteine (NAC; Sigma-Aldrich; Merck KGaA; cat. no. A9165) were dissolved in sterile distilled water to prepare stock solutions at concentrations of 20, 5 and 500 mM, respectively. By contrast, GSK2606414 [MedChemExpress; cat. no. HY-18072; an inhibitor of the protein kinase R-like ER kinase (PERK)], JNJ-28583113 [MedChemExpress; cat. no. HY-149143; an antagonist of transient receptor potential cation channel subfamily M member 2 (TRPM2)], and SB-705498 [MedChemExpress; cat. no. HY-10633; an antagonist of transient receptor potential cation channel subfamily V member 1 (TRPV1)] were solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) at concentrations of 1, 5 and 5 mM, respectively, to make stock solutions. All stock solutions were stored at -20°C until further use. Cells were seeded in 96-well plates at 3,000 cells/well, or in 6-well plates at 100,000 cells/well, and incubated overnight at 37°C to facilitate cell adherence. For the combined treatment of EGCG and TCS, EGCG was administered immediately prior to TCS exposure. Cells were pretreated with NAC or the molecular inhibitors 1 h prior to the administration of EGCG. Unless specifically indicated, cells were maintained at 37°C in a humidified incubator for an additional 24 h after treatment for further analysis.

Cell viability assay. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich; Merck KGaA) assay. MTT powder was dissolved in distilled water to make a 5 mg/ml stock solution, which was subsequently stored at 4°C . Immediately

before use, the MTT stock solution was diluted with culture medium to a final concentration of 0.5 mg/ml. At 24 h after the treatment, the culture medium in the 96-well plates was replaced with 100 μl of the MTT working solution. The cells were then incubated at 37°C in the dark for 4 h, during which time mitochondrial dehydrogenases in viable cells reduced MTT to insoluble formazan crystals. To dissolve the formazan crystals, 100 μl 10% sodium dodecyl sulfate (SDS) (Bioshop Canada, Inc.) solution in 0.01 M hydrochloric acid (HCl) (Echo Chemical Co., Ltd.) was added to each well. After an overnight incubation, the absorbance was measured at 570 nm with background subtraction at 690 nm using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Inc.). Finally, cell viability was expressed as a ratio based on the relative quantity of formazan compared with the untreated control.

Assessment of synergy using the Bliss independence model. To evaluate the synergistic interaction between EGCG and TCS, the Bliss independence model was applied. The expected fractional inhibition on cell viability for the combination treatment (E_{exp}) was calculated using the formula: $E_{\text{exp}} = E_{\text{EGCG}} + E_{\text{TCS}} - (E_{\text{EGCG}} \times E_{\text{TCS}})$, where E_{EGCG} and E_{TCS} represent the fractional inhibitions induced by individual treatments of EGCG and TCS, respectively. The Bliss excess value (ΔE) was defined as the difference between the observed fractional inhibition (E_{obs}) and E_{exp} (namely, $\Delta E = E_{\text{obs}} - E_{\text{exp}}$). A positive Bliss excess value ($\Delta E > 0$) was indicative of synergy (21).

Colony formation assay. To assess their colony-forming capabilities, A549 cells were seeded into 35-mm culture dishes at a density of 300 cells per dish and allowed to adhere overnight. On the following day, cells were treated with EGCG, TCS, or their combination. The culture medium was replaced after 24 h, followed by an additional 10-day incubation period. Subsequently, colonies were fixed using 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. Aggregates containing 50 or more cells were identified as viable colonies. Colony numbers were quantified manually using an optical microscope, and the results were expressed relative to the untreated control group.

Western blot analysis. Protein levels in A549 cells were assessed using western blotting. After treatment, cells were washed with phosphate-buffered saline (PBS) and subsequently lysed on ice for 1 h using RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1% Triton X-100, 0.1% SDS, 1 mM EDTA) (MilliporeSigma) supplemented with protease and phosphatase inhibitor cocktails (MilliporeSigma). Lysates were centrifuged at $10,000 \times g$ for 30 min at 4°C , and the supernatants were then collected. Protein concentrations were determined using the Bradford protein assay (Bioshop Canada Inc.). For the detection of total ubiquitinated proteins, cells were directly harvested and lysed in pre-heated sample loading buffer at 95°C for 10 min to rapidly deactivate endogenous deubiquitinating enzymes and preserve polyubiquitinated species. Equal amounts of protein samples (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide gels), and then transferred onto polyvinylidene fluoride (PVDF) membranes

(MilliporeSigma). To facilitate the detection of total ubiquitinated proteins, PVDF membranes were soaked in PBS and subsequently subjected to autoclaving at 121°C for 30 min prior to the blocking step, thereby improving epitope accessibility. Membranes were blocked with 5% bovine serum albumin (BioShop Canada Inc.) in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature, followed by an overnight incubation at 4°C with primary antibodies targeting proteasome subunit alpha type-3 (PSMA3; cat. no. 12446; 1:1,000), 26S proteasome regulatory subunit 6A (PSMC3; cat. no. 13923; 1:1,000), HSP70 (cat. no. 4872; 1:1,000) and phosphorylated c-Jun N-terminal kinase (p-JNK; Thr183/Tyr185; cat. no. 4668; 1:1,000) (all from Cell Signaling Technology, Inc.), and HSP105 (cat. no. GTX03248; 1:1,000), ubiquitin (cat. no. GTX128826; 1:5,000), phosphorylated eukaryotic initiation factor 2 subunit α (p-eIF2 α ; Ser51; cat. no. GTX130006; 1:1,000), total-eIF2 α (t-eIF2 α ; cat. no. GTX101241; 1:2,000), total-JNK (t-JNK; cat. no. GTX133806; 1:1,000), poly(ADP-ribose) polymerase (PARP; cat. no. GTX132329; 1:1,000) and GAPDH (cat. no. GTX100118; 1:10,000) (all from GeneTex, Inc.). After washing with TBST, the membranes were incubated overnight at 4°C with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.; 1:10,000). Finally, the immunoreactive signals were visualized using an enhanced chemiluminescence substrate (Advansta Inc.) and detected in an Amersham Imager 600 imaging system [Cytiva (formerly GE Healthcare Life Sciences)]. Finally, protein bands were quantified using Image Lab software (Bio-Rad Laboratories, Inc.).

RNA extraction, reverse transcription, and X-box binding protein 1 (XBP1) mRNA splicing analysis. Total RNA was extracted from A549 cells using the miTotal RNA Extraction Miniprep System (Viogene), following the manufacturer's instructions. Reverse transcription (RT) and PCR amplification were performed using a HiScript II One-Step RT-PCR Kit (Vazyme Biotech Co., Ltd.) with gene-specific primers. The primer sequences for human XBP1 were as follows: Forward, 5'-TTACGAGAGAAAACATCATGGCC-3' and reverse, 5'-GGGTCCAAGTTGTCCAGAATGC-3'; note that the primers flanked the 26-nucleotide intron that was excised by inositol-requiring enzyme 1 α (IRE1 α)-mediated splicing. GAPDH was amplified as an internal control using the following primers: Forward, 5'-GGAGCGAGATCCCTCCAAAT-3', and reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'. To achieve optimal resolution among the unspliced (XBP1u), heteroduplex (XBP1h) and spliced (XBP1s) isoforms, PCR products were separated on a 2.5% agarose gel prepared in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) and electrophoresed at 70 V for 1.5 h. Subsequently, the gel was stained for 45 min using GelRed Nucleic Acid Stain (Biotium, Inc.) diluted at a ratio of 3:10,000 in a 0.1 M NaCl aqueous solution. DNA bands were visualized using the Keta S Standalone Gel Documentation System (Wealtec Bioscience Co., Ltd.). Quantification of band intensities was performed using ImageJ software (version 1.53k; National Institutes of Health), and the ratio of XBP1 mRNA splicing was calculated according to the formula: Splicing ratio = (Intensity of XBP1s + 0.5x Intensity

of XBP1h)/(Intensity of XBP1s + Intensity of XBP1h + Intensity of XBP1u) (22).

Measurement of cytosolic Ca²⁺ levels. Cytosolic Ca²⁺ levels were measured using the fluorescent dye Fluo-4 AM (MedChemExpress) to quantify cytosolic Ca²⁺ levels. A 1 mM stock solution of Fluo-4 AM was prepared by dissolving the dye in DMSO, after which it was stored at -20°C until further use. At 24 h after treatments, cells were harvested and washed with Hanks' Balanced Salt Solution (HBSS) and subsequently incubated with 1 μ M Fluo-4 AM in HBSS for 1 h at 37°C in the dark to load the dye. Following the incubation, cells were washed with HBSS and finally resuspended in fresh HBSS. Fluorescence intensity of Fluo-4 AM was measured using a FACSLytic flow cytometer (BD Biosciences) in the FITC channel. For each sample, at minimum of 9,000 events were acquired. Cellular debris was excluded based on the forward scatter (FSC) and side scatter (SSC) characteristics. Finally, cytosolic Ca²⁺ levels were quantified by calculating the mean fluorescence intensity and expressed as fold changes relative to the untreated control group.

Measurement of intracellular ROS levels. The dihydroethidium (DHE) (Sigma-Aldrich; Merck KGaA) fluorescent dye was employed to measure ROS levels in A549 cells. At 24 h after treatments, cells were harvested and washed with PBS prior to staining. Cells were subsequently incubated with a 5 μ M DHE working solution for 30 min at 37°C in the dark. Fluorescence intensity was measured using a FACSLytic flow cytometer (BD Biosciences) in the PE channel. For each sample, at minimum of 9,000 events were acquired. Cellular debris was excluded based on FSC and SSC characteristics. Finally, ROS levels were determined by calculating the mean fluorescence intensity and expressed as fold changes compared with the control group.

Mitochondrial membrane potential (MMP) analysis. The MMP integrity was assessed using the JC-1 (MedChemExpress) fluorescent dye. At 24 h after treatments, cells were harvested and rinsed with PBS. Subsequently, cells were incubated with 5 μ g/ml JC-1 in PBS for 30 min at 37°C in the dark. Fluorescence intensities of JC-1 were measured using the FACSLytic system (BD Biosciences) in both the PE (red fluorescence) and FITC (green fluorescence) channels. For each sample, at minimum of 9,000 events were acquired. Cellular debris was excluded based on the FSC and SSC characteristics, and MMP disruption was quantified through assessing the proportion of cells exhibiting a fluorescence shift from red (JC-1 aggregates) to green (JC-1 monomers).

Apoptosis analysis. Apoptotic rates were quantified using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining kit (BD Biosciences). At 24 h after treatments, cells were collected, washed with PBS, and subsequently incubated in a binding buffer containing annexin V-FITC and PI for 30 min at 37°C in the dark, following the manufacturer's instructions. Fluorescence intensities were measured using the FACSLytic system (BD Biosciences) in both the PE and FITC channels. Early and late apoptotic cells were quantified as the percentages of cells identified in the (PE⁺/FITC⁺) and (PE⁺/FITC⁺) quadrants, respectively. For each sample, a minimum of 9,000 events were acquired. Cellular debris was excluded based on

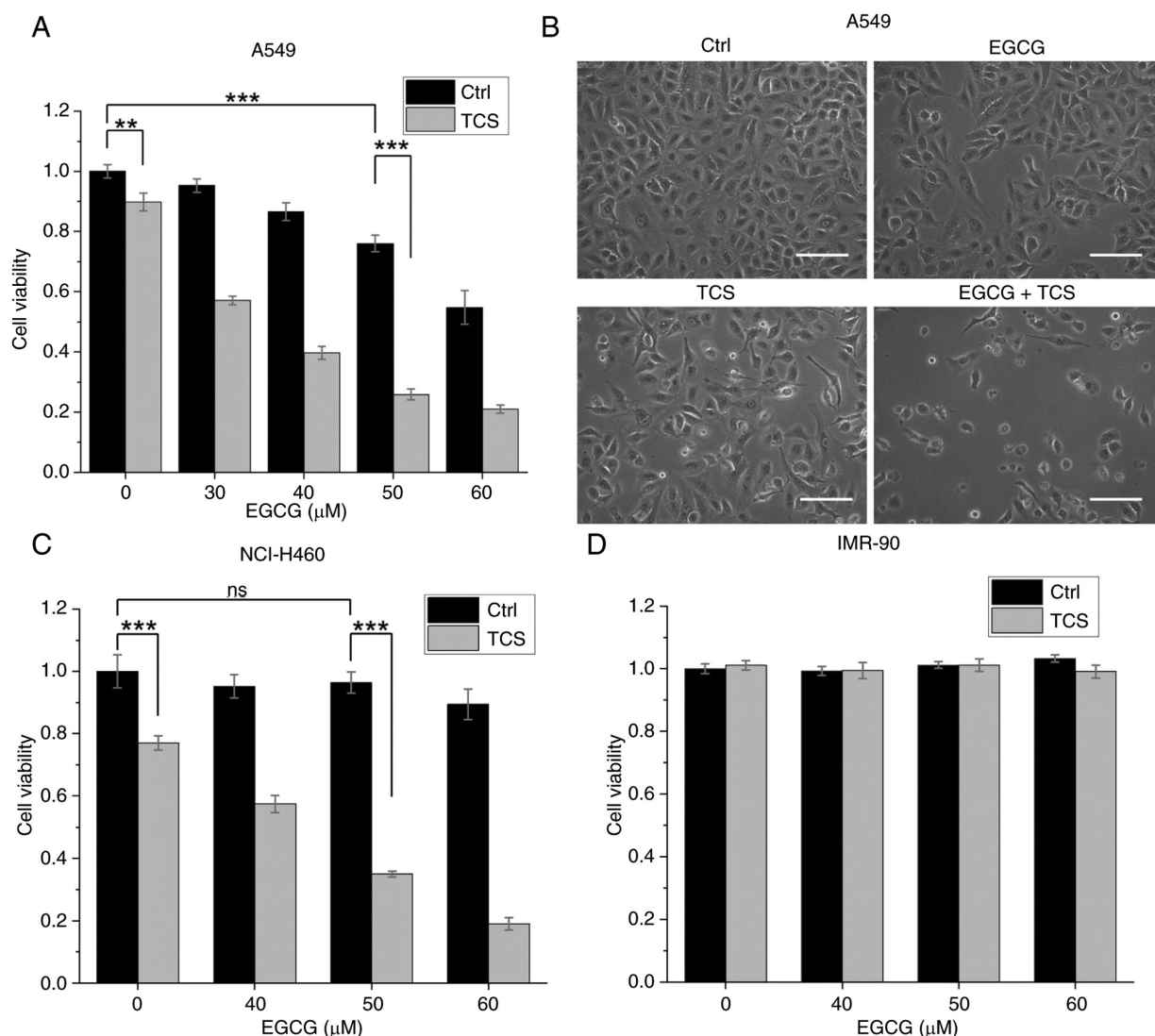


Figure 2. Effects of TCS and EGCG alone, or their combination treatment on the viability and morphology of A549, NCI-H460 and IMR-90 cells. (A) The viability of A549 cells was evaluated using the MTT assay 24 h after treatment with various concentrations of EGCG alone, or in combination with TCS. (B) Cellular morphology of A549 cells was examined using light microscopy 24 h after treatment with 50 μ M EGCG and/or TCS. Scale bar, 100 μ m. (C) The viability of NCI-H460 cells was measured 24 h after treatment with different concentrations of EGCG alone or combined with TCS. (D) The viability of IMR-90 cells was similarly assessed 24 h after treatment with various concentrations of EGCG alone or in combination with TCS. ** $P < 0.01$ and *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; ns, non-significant.

the FSC and SSC characteristics, and the percentages of these populations were calculated accordingly.

Statistical analysis. Three independent biological experiments were performed for each experimental condition using separately prepared cell cultures. Data are presented as the mean $\pm$ standard deviation. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, using the OriginPro 2015 software (version 92E; OriginLab Corporation). A P -value < 0.05 was considered to indicate a statistically significant difference. For clarity, only selected pairwise comparisons are shown in the figures.

Results

Synergistic inhibitory effects of EGCG and TCS on NSCLC cell viability. To assess the effect of EGCG and TCS on the viability of A549 cells, MTT assays were performed 24 h after

treatment. As shown in Fig. 2A, EGCG induced a significant and dose-dependent reduction in A549 cell viability. By contrast, TCS alone caused only a slight decrease in A549 cell viability, as $\sim 90\%$ of the control levels were maintained. Notably, however, the combined administration of EGCG and TCS produced a more substantial inhibition effect on A549 cell viability. The Bliss excess value (ΔE), as calculated in Table IA, confirmed a synergistic cytotoxic effect of EGCG and TCS, with $\Delta E > 0$ at EGCG concentrations ranging from 30 to 60 μ M. Consistent with the MTT results, morphological examination of the cells under a light microscope at 24 h post-treatment (Fig. 2B) demonstrated that the combined treatment of EGCG and TCS induced pronounced morphological alterations in A549 cells, including reduced cell density, cellular shrinkage, and an increased proportion of non-adherent cells. To further validate that these effects were more widely applicable, the MTT assay was subsequently performed on a different NSCLC cell line, NCI-H460. The results obtained

Table I. Bliss excess for EGCG in combination with TCS in A549 and NCI-H460 cells.

A, A549 cells					
EGCG (μ M)	0	30	40	50	60
Bliss excess (ΔE)	0.00	0.28	0.38	0.42	0.28
B, NCI-H460 cells					
EGCG (μ M)	0	40	50	60	
Bliss excess (ΔE)	0.00	0.16	0.39	0.50	
TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate.					

(as shown in Fig. 2C) confirmed that a synergistic cytotoxic effect also occurred upon treatment with EGCG and TCS in NCI H460 cells, with the corresponding Bliss excess values presented in Table IB. According to the data from Table IA, the combination of 50 μ M EGCG and TCS produced the highest Bliss excess value in A549 cells, thereby justifying the selection of 50 μ M EGCG for subsequent experiments in the present study. Notably, as shown in Fig. 2D, the combination of EGCG and TCS did not lead to a reduction in the viability of the IMR-90 normal lung fibroblasts, demonstrating that this combined treatment is well tolerated by non-malignant lung fibroblasts. To further evaluate whether this combined treatment could impair long-term reproductive viability, a colony formation assay was performed using A549 cells. As shown in Fig. S1A and B, the combination of EGCG and TCS significantly suppressed the clonogenic capability of the A549 cells, resulting in a significantly lower colony count compared with single treatments. Taken together, these results showed that EGCG and TCS work synergistically to inhibit both the short-term metabolic viability, and the long-term clonogenic survival, of NSCLC cells, while exhibiting minimal cytotoxicity on IMR-90 normal lung fibroblasts *in vitro*.

Effects of EGCG and TCS on proteostatic impairment in A549 cells. To elucidate the mechanism underlying the synergistic effect of EGCG and TCS, it was first investigated whether these treatments could influence the regulation of HSPs in A549 cells. Western blot analysis was employed to quantify the expression levels of HSP70 and HSP105. As shown in Fig. 3A and B, administering TCS increased the expression levels of both HSP70 and HSP105 in A549 cells, whereas combined treatment with EGCG significantly reduced this induction of protein expression. In addition to HSPs, the expression levels of the proteasome subunits, PSMA3 and PSMC3, were also evaluated via western blot analysis. It was revealed that TCS alone exerted a negligible effect on PSMA3 expression, and induced only a slight reduction in PSMC3 levels (Fig. 3C and D). By contrast, EGCG was found to significantly decrease the expression of both PSMA3 and PSMC3 in A549 cells treated with TCS. To evaluate the overall status of cellular proteostasis under these conditions,

the levels of total ubiquitinated proteins were subsequently measured. As shown in Fig. S2A and B, co-treatment with EGCG and TCS resulted in a significant increase in the accumulation of ubiquitinated proteins compared with either treatment in isolation. Taken together, these findings suggested that EGCG suppresses the expression of critical components of the proteostatic machinery, including HSP70, HSP105, and the proteasome subunits. Moreover, this inhibition is associated with an increased accumulation of ubiquitinated proteins in the combination treatment group, reflecting heightened proteostatic dysfunction in A549 cells.

EGCG and TCS induce ER stress in A549 cells. The intracellular accumulation of unfolded proteins is known to provoke ER stress, which, if sustained or severe, may culminate in cellular damage (23). In the present study, the involvement of the PERK-eIF2 α signaling axis, a key ER stress pathway, was therefore explored in mediating cell death induced by EGCG and TCS. As shown in Fig. 4A, administration of the PERK inhibitor GSK2606414 partially mitigated the reduction in A549 cell viability caused by combined EGCG and TCS treatment in a dose-dependent manner. Additionally, western blot analysis of the downstream signaling components revealed that TCS significantly enhanced the phosphorylation of eIF2 α , with the combination of EGCG and TCS producing an even more pronounced effect (Fig. 4B). Concurrently, TCS treatment alone induced substantial XBP1 mRNA splicing (Fig. S3A), thereby confirming IRE1 α activation. Notably, co-treatment with EGCG and TCS led to a significant enhancement in JNK phosphorylation (Fig. S3B), a pro-apoptotic signaling event that can be triggered either downstream of IRE1 α or via ROS-apoptosis signal-regulating kinase 1 (ASK1)-dependent pathways. These findings suggest that the combination of EGCG and TCS activates both the PERK-eIF2 α and the IRE1 α -JNK pathways of ER stress signaling, which may partially contribute to the observed reduction in viability of A549 cells.

Induction of IP₃R-mediated ER Ca²⁺ release by the combined administration of EGCG and TCS in A549 cells. ER stress is frequently associated with intracellular Ca²⁺ dysregulation, in which Ca²⁺ stored in the ER can be released via IP₃R channels (24). Therefore, cytosolic Ca²⁺ levels were further quantitatively measured using the Fluo-4 AM fluorescent dye via flow cytometric analysis. As shown in Fig. 5A and B, treatment with EGCG alone did not significantly affect cytosolic Ca²⁺ levels in A549 cells, whereas TCS alone led to only a slight increase. By contrast, the combination of EGCG and TCS produced a substantial elevation in cytosolic Ca²⁺ levels. Notably, this increase was found to be greatly attenuated through administering the IP₃R inhibitor 2-APB at a concentration of 50 μ M, suggesting that the elevation in Ca²⁺ level might be mediated via the activation of IP₃R channels. To further elucidate the role of ER Ca²⁺ release in the synergistic cytotoxicity induced by EGCG and TCS in A549 cells, a rescue experiment employing 2-APB was performed using the MTT viability assay. As revealed in Fig. 5C, 2-APB significantly ameliorated the combined treatment's reduction of A549 cell viability in a dose-dependent manner. In addition to its effect on IP₃R channels, 2-APB is also known to

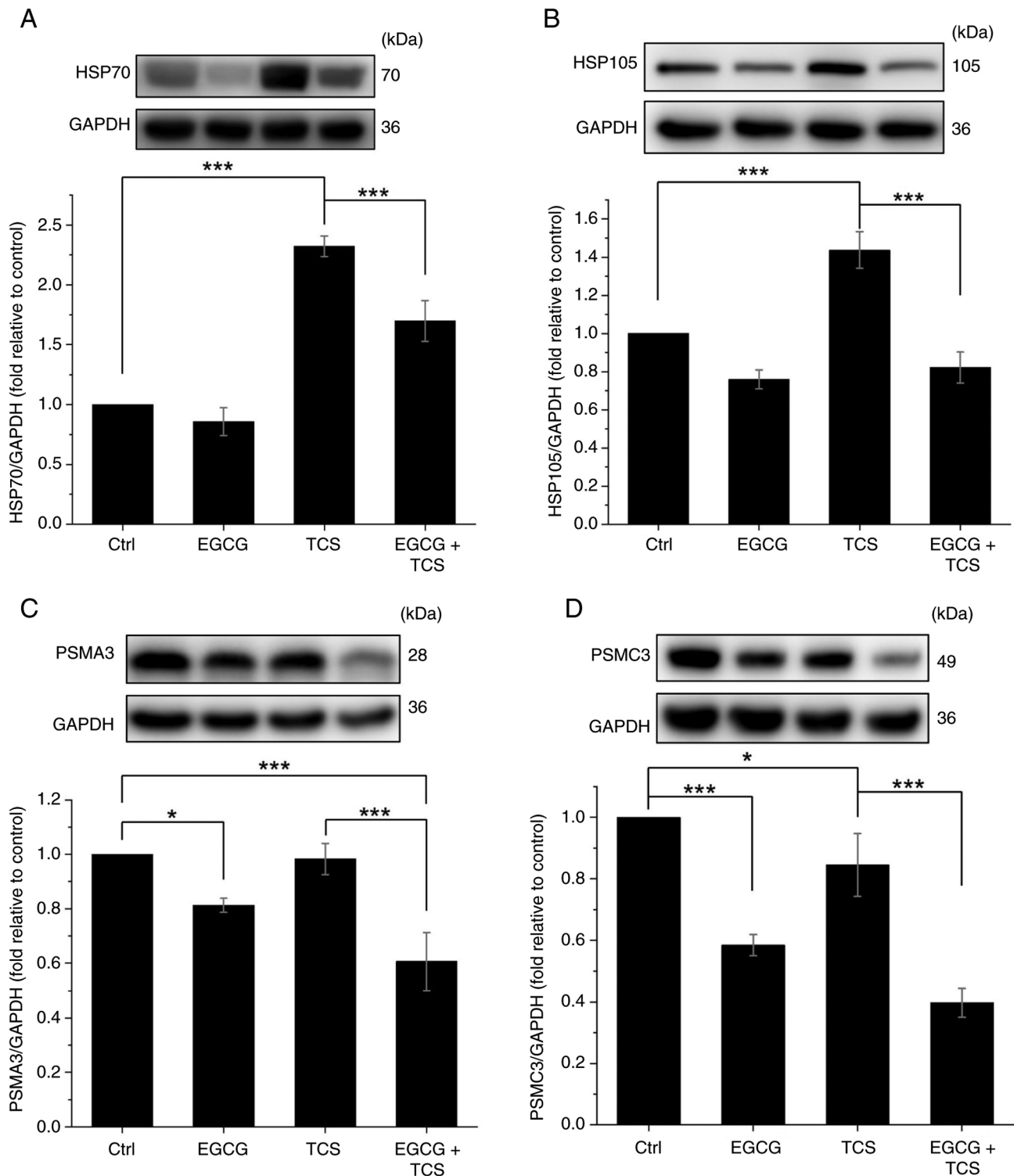


Figure 3. Effects of TCS, EGCG and their combined application on the expression levels of HSPs and proteasome subunits in A549 cells. (A-D) Western blot analysis was employed to measure the expression levels of (A) HSP70, (B) HSP105, (C) PSMA3 and (D) PSMC3. GAPDH was used as the internal loading control. * $P < 0.05$ and *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; HSP, heat-shock protein; PSMA3, proteasome subunit alpha type-3; PSMC3, 26S proteasome regulatory subunit 6A.

affect thermosensitive transient receptor potential (TRP) channels, such as TRPV1 and TRPM2 (25,26). However, in the experiments of the present study, it was found that selective inhibitors for TRPM2 (JNJ-28583113) and TRPV1 (SB-705498) did not reverse the synergistic cytotoxic effects of EGCG and TCS (Fig. 5D), suggesting that these

TRP channels are unlikely to mediate a significant role in this process. Taken together, these pharmacological rescue experiments suggested the potential involvement of IP₃R channels and associated ER Ca²⁺ release in contributing to the synergistic anticancer effects induced by the combined EGCG and TCS treatment.

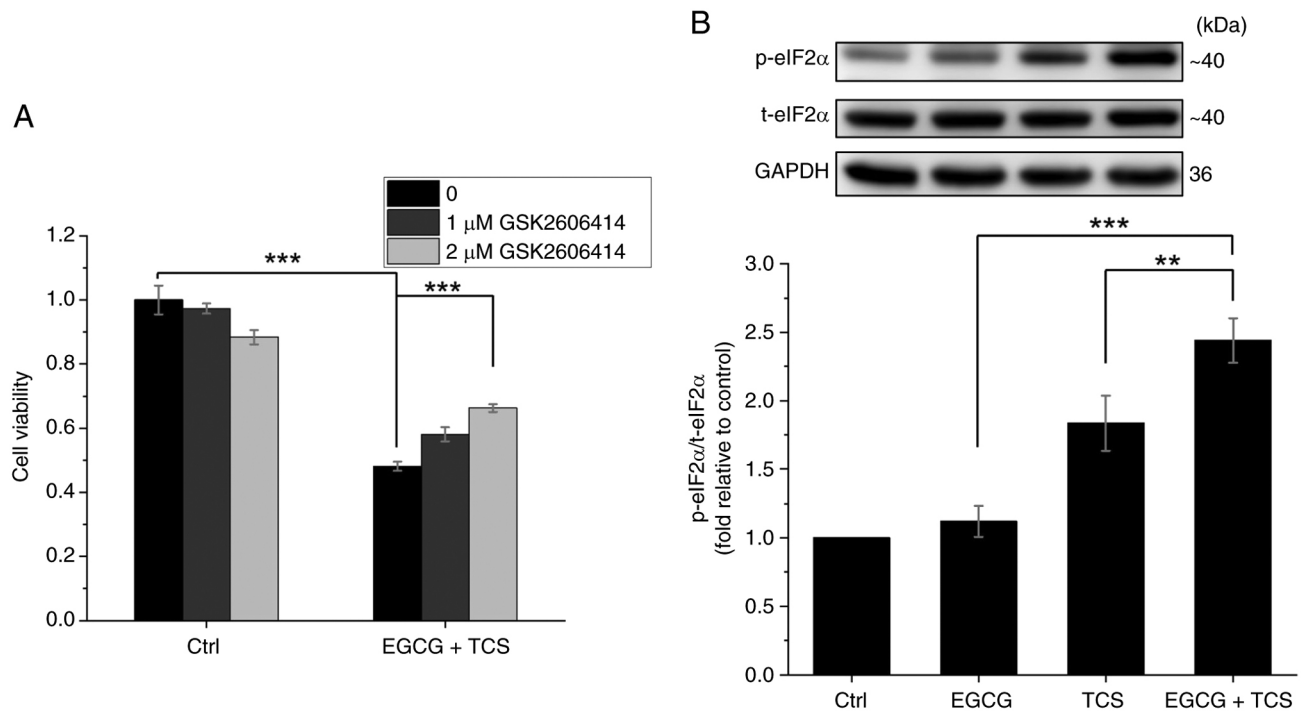


Figure 4. The effects of TCS and EGCG on the PERK-eIF2 α signaling pathway in A549 cells. (A) Cell viability of A549 cells was measured using the MTT assay 24 h following combined treatment with EGCG and TCS, in the presence or absence of the PERK inhibitor GSK2606414, which was administered 1 h prior to the combined treatment. (B) The protein levels of p-eIF2 α and t-eIF2 α were examined by western blot analysis 3 h after the treatment. GAPDH was utilized as the internal loading control. ** $P < 0.01$ and *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; PERK, protein kinase R-like endoplasmic reticulum kinase; eIF2 α , eukaryotic initiation factor 2 subunit α ; p-eIF2 α , phosphorylated eIF2 α ; t-eIF2 α , total eIF2 α .

The combination of EGCG and TCS induces ROS production in A549 cells. ER stress and intracellular Ca^{2+} dysregulation have previously been shown to coincide with excessive ROS generation (5). In the present study, intracellular ROS levels in A549 cells were measured using the fluorescent dye DHE via flow cytometric analysis. As demonstrated in Fig. 6A and the resultant quantification of the data in Fig. 6B, treatment with either EGCG or TCS alone resulted in only a slight, and statistically non-significant, increase in ROS levels. On the other hand, the combined administration of EGCG and TCS produced a pronounced and statistically significant elevation of ROS levels in A549 cells. To explore the role of ROS in the reduction of cell viability caused by this combination, a rescue experiment employing the antioxidant NAC was performed using the MTT assay. The results shown in Fig. 6C demonstrate that NAC could effectively mitigate the synergistic cytotoxicity induced by the combination of EGCG and TCS in A549 cells, suggesting that the excessive ROS production may contribute to the reduced viability in A549 cells.

Potential reciprocal interplay of ROS production and ER Ca^{2+} release under combined EGCG and TCS treatment in A549 cells. To explore the potential association between ER Ca^{2+} release and ROS generation, ROS levels in A549 cells were first assessed by DHE staining in the presence of 50 μ M 2-APB, an inhibitor of IP_3R -mediated Ca^{2+} release. As shown in Fig. 7A and B, 2-APB treatment led to a significant attenuation of the ROS elevation triggered by the combined EGCG and TCS treatment, suggesting that ER Ca^{2+} release through IP_3R channels may contribute to the substantial increase in

ROS levels. On the other hand, the cytosolic Ca^{2+} levels were measured using Fluo-4 AM staining in the presence of 2 mM NAC (Fig. 7C and D). These experiments revealed that NAC treatment effectively suppressed the rise in cytosolic Ca^{2+} levels that was induced by the combined EGCG and TCS treatment, suggesting that elevated ROS levels may facilitate ER Ca^{2+} release. Collectively, these convergent pharmacological findings support a working model of reciprocal interplay between the ER Ca^{2+} release and ROS generation events, wherein mutual amplification of these signals culminates in enhanced cell death.

The combination of EGCG and TCS induces mitochondrial apoptosis in A549 cells. Excessive ROS accumulation and mitochondrial Ca^{2+} overload are known to cause mitochondrial damage (27,28). Given that, in these experiments, the combined EGCG and TCS treatment effectively triggered ER Ca^{2+} release and an increase in ROS levels, it was sought to further evaluate changes in the MMP using the fluorescent dye JC-1 via flow cytometric analysis. As revealed in Fig. 8A, the combined EGCG and TCS treatment caused a significant shift in JC-1 fluorescence from red to green, indicative of MMP disruption. The quantitative analysis shown in Fig. 8C demonstrates that the combined treatment caused a more substantial loss of MMP compared with either treatment alone. Mitochondrial damage may eventually activate the intrinsic apoptotic pathway (27,28). Accordingly, the evaluation of apoptosis was performed using annexin V-FITC/PI double staining by flow cytometric analysis. As demonstrated in Fig. 8B and D, the combination of EGCG and TCS caused a significant increase in the proportion of apoptotic cells

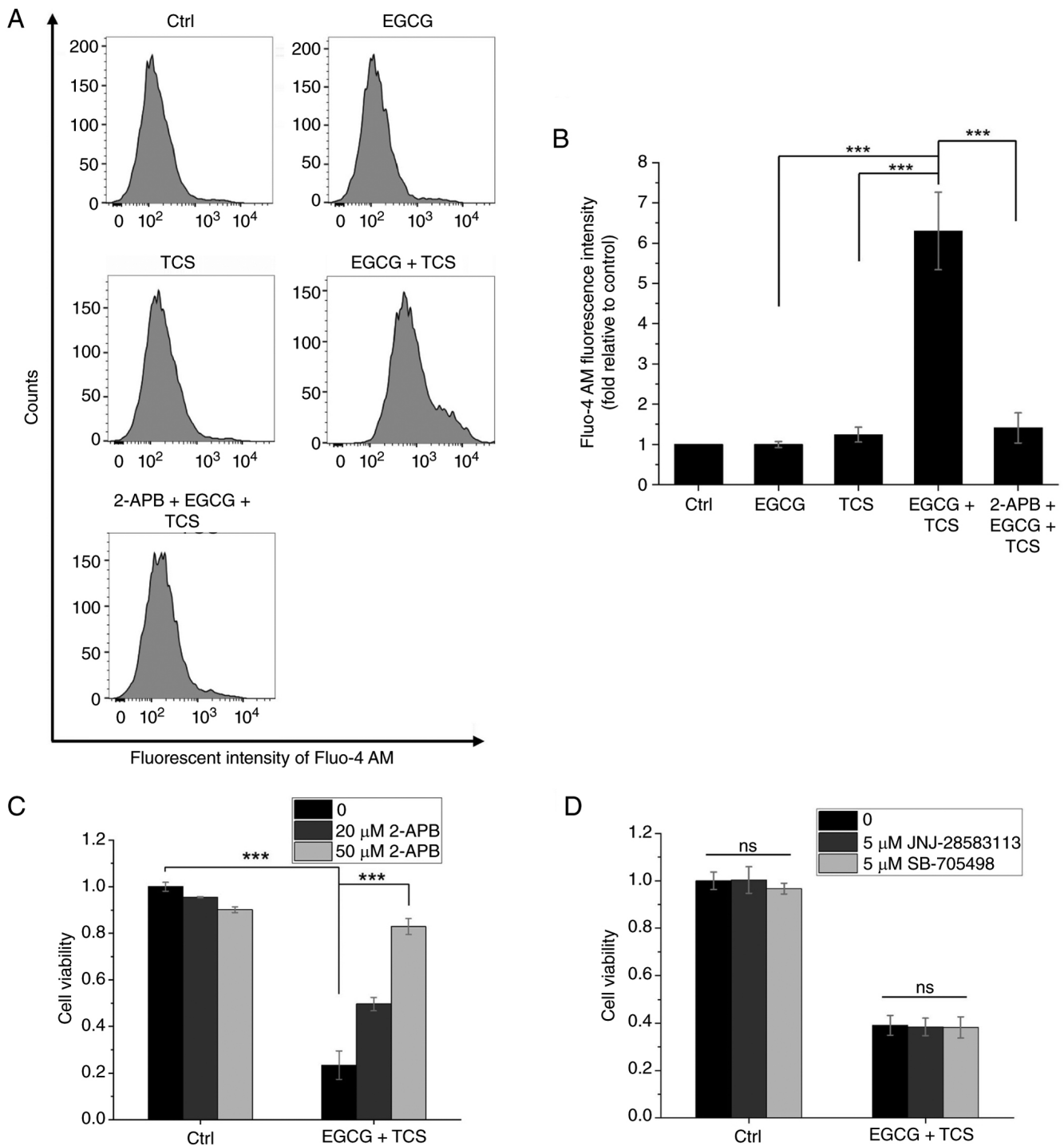


Figure 5. Investigation of the effects of TCS and EGCG on Ca^{2+} homeostasis in A549 cells. (A) Cytosolic Ca^{2+} levels in A549 cells were measured utilizing the fluorescent dye Fluo-4 AM via flow cytometry. (B) Quantitative analysis of the mean fluorescence intensities of Fluo-4 AM, as shown in panel (A). (C) Cell viability of A549 cells was evaluated using the MTT assay 24 h following combined EGCG and TCS treatment, in the presence or absence of the IP_3R inhibitor 2-APB. The 2-APB inhibitor was administered 1 h prior to the combined EGCG and TCS treatment. (D) Assessment of A549 cell viability was also performed using the MTT assay 24 h after combined EGCG and TCS treatment, with or without the TRPM2 inhibitor JNJ-28583113 and the TRPV1 inhibitor SB-705498. Similarly, these inhibitors were applied 1 h before the combined treatment with EGCG and TCS. *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; 2-APB, 2-aminoethoxydiphenyl borate; TRPM2, transient receptor potential cation channel, subfamily M, member 2; TRPV1, transient receptor potential cation channel subfamily V member 1; ns, non-significant.

compared with single treatments. To further confirm the occurrence of apoptosis at the molecular level, the cleavage of PARP, a well-established indicator of caspase activation, was examined. As shown in Fig. 8E, western blot analysis demonstrated that the combined treatment with EGCG and TCS led

to a significant enhancement in the cleavage of full-length PARP (116 kDa) into its characteristic 89-kDa inactive fragment. Collectively, these results confirmed that the combined treatment of EGCG with TCS effectively induced mitochondrial apoptosis in A549 cells.

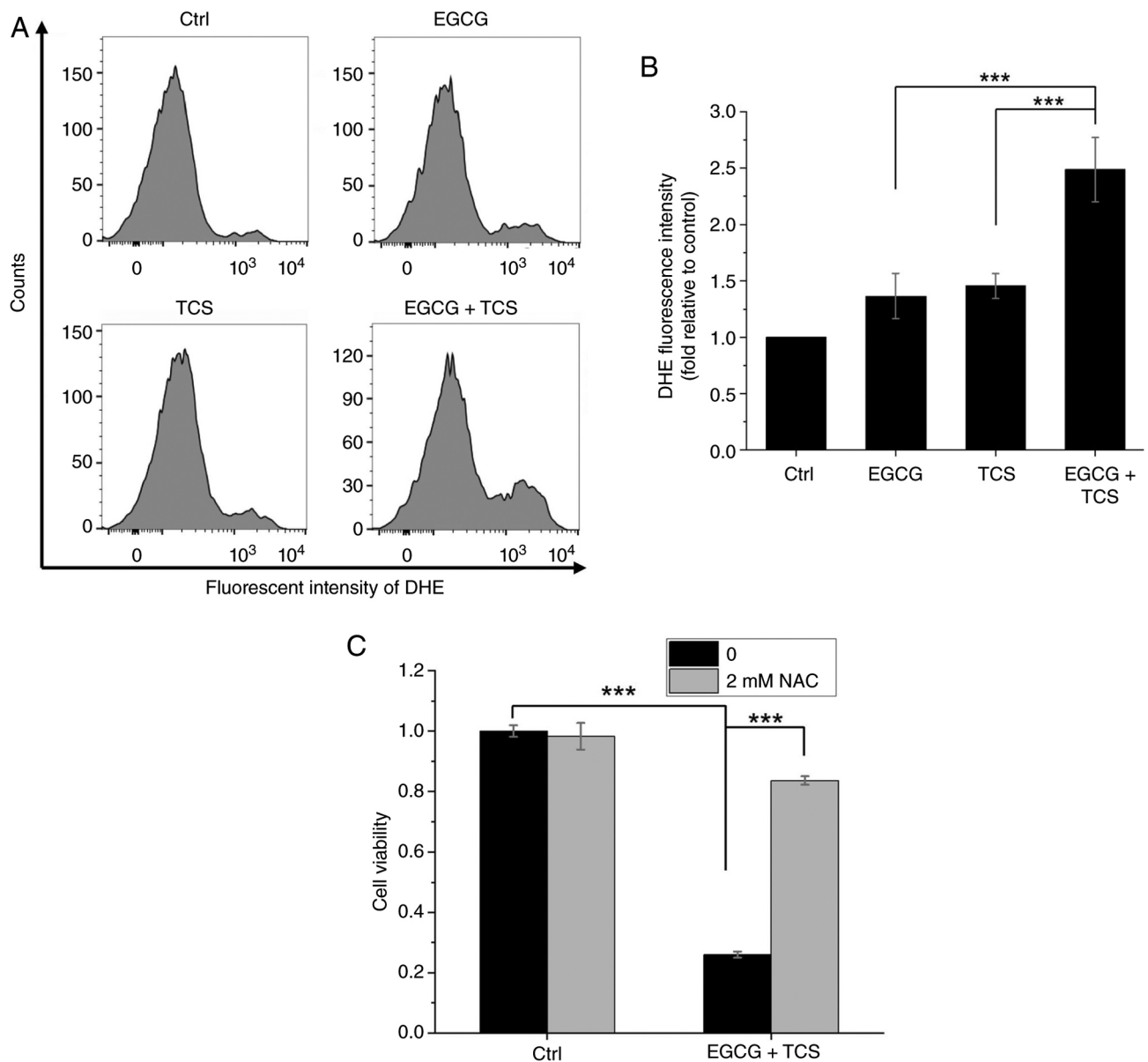


Figure 6. Evaluation of the effects of TCS and EGCG on ROS regulation in A549 cells. (A) Intracellular ROS levels in A549 cells were determined using the fluorescent dye DHE via flow cytometry. (B) Quantification of the mean fluorescent intensities of DHE, as shown in panel (A). (C) A549 cell viability was measured using the MTT assay 24 h following combined treatment with EGCG and TCS, with or without the ROS scavenger NAC. NAC was administered 1 h prior to the combined treatment. *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; ROS, reactive oxygen species; DHE, dihydroethidium; NAC, N-acetylcysteine.

Discussion

The considerable challenges associated with the treatment of NSCLC have prompted extensive investigation into more effective combination therapies (2,6). EGCG, a natural compound, exhibits strong anticancer properties, although its clinical application has been limited due to poor bioavailability, low stability and potential hepatotoxicity at elevated doses (20,29). The data presented in the present study, as shown in Fig. 2, demonstrate that the combined administration of 50 μ M EGCG and TCS synergistically suppresses the viability of the NSCLC cell lines A549 and NCI-H460, while exhibiting minimal cytotoxic effects on IMR-90 normal lung fibroblasts. These findings suggest that TCS is able to enhance the cytotoxic effect of EGCG *in vitro*, implying

that combination treatment may provide a potential strategy for partially improving the therapeutic efficacy of EGCG by lowering its required dosage.

To understand the mechanisms underlying this synergistic effect, the influence of EGCG and TCS was first studied on HSPs and the UPS, two critical regulators of intracellular proteostasis. HSPs, especially HSP70, are often overexpressed in various types of malignancies, including NSCLC, where they facilitate cell proliferation, inhibit apoptosis, and contribute to drug resistance (30-32). Consequently, several molecular inhibitors targeting HSPs have been developed as anticancer agents (11,12). Nevertheless, hyperthermia-induced upregulation of HSP70 and HSP105 has been shown to be associated with the development of thermotolerance, which diminishes the clinical efficacy of thermal therapies (11,33).

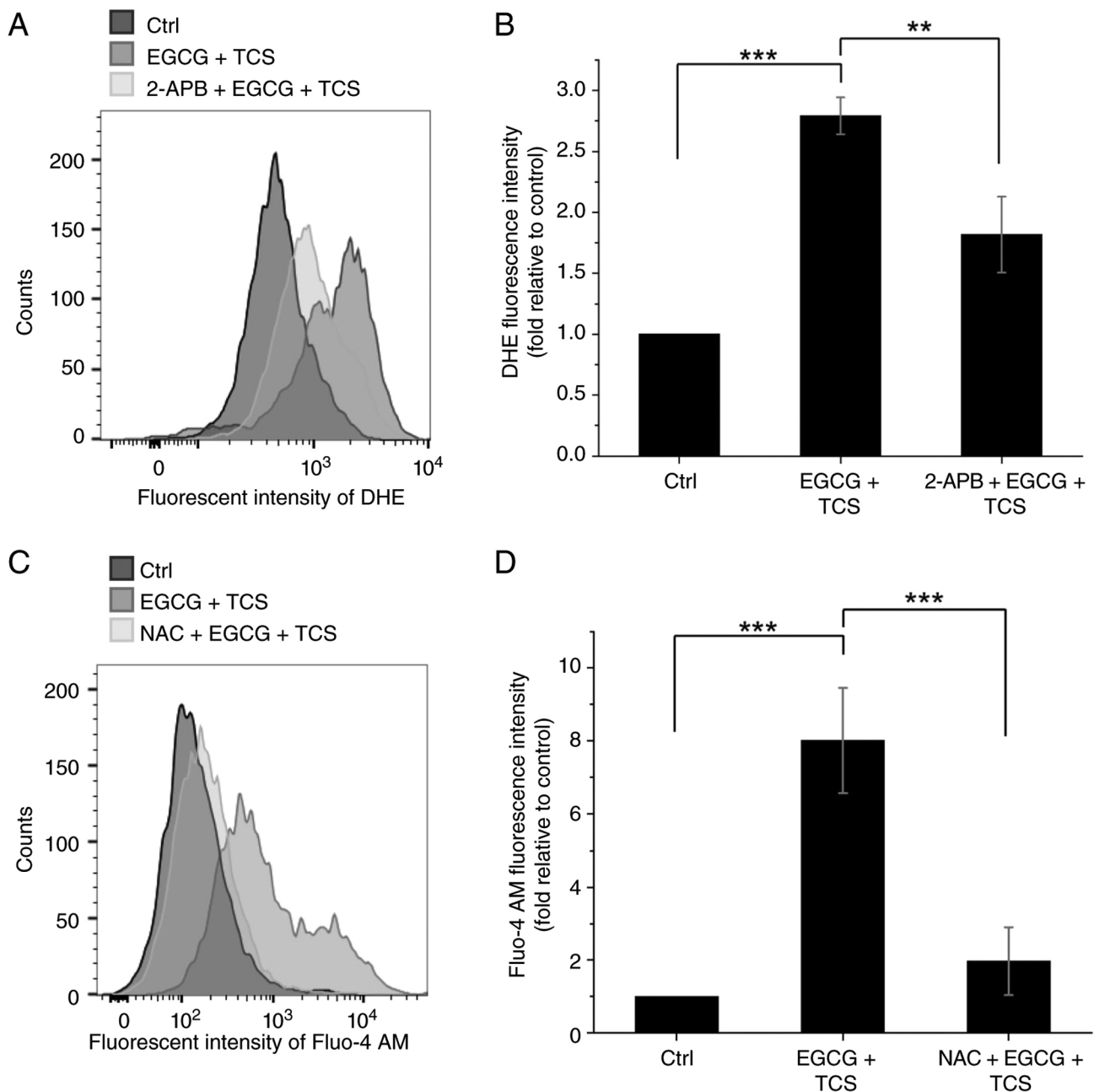


Figure 7. Potential effects of TCS and EGCG on the ROS- Ca^{2+} reciprocal interplay in A549 cells. (A) Intracellular ROS levels in A549 cells were measured using the fluorescent dye DHE via flow cytometry, following combined treatment with EGCG and TCS in the absence or presence of 2-APB. (B) Quantitative analysis of the mean fluorescent intensities of DHE, as shown in panel (A). (C) Cytosolic Ca^{2+} levels in A549 cells were assessed using the fluorescent dye Fluo-4 AM via flow cytometry following combined EGCG and TCS treatment, in the absence or presence of NAC. (D) Quantification of the mean fluorescent intensities of Fluo-4 AM, as shown in panel (C). ** $P < 0.01$ and *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; ROS, reactive oxygen species; DHE, dihydroethidium; 2-APB, 2-aminoethoxydiphenyl borate; NAC, N-acetylcysteine.

Notably, EGCG has been shown to suppress the expression of HSP70 and to competitively bind to its ATPase domain, thereby inhibiting its chaperone function (17). Consistent with these findings, the results obtained in the present study have demonstrated that EGCG effectively attenuates the TCS-induced elevation of HSP70 and HSP105 levels (Fig. 3A and B), suggesting that EGCG may both disrupt adaptive thermotolerance and sensitize A549 cells to TCS-induced proteotoxic stress. In addition to HSPs, the UPS has a crucial role in oncogenesis and progression, rendering it a viable therapeutic target in cancer treatments (15,34). A study by

Nam *et al* (35) demonstrated that EGCG acts as a blocker to the chymotrypsin-like function of the 20S proteasome. The findings of the present study have further revealed that the combination of EGCG and TCS caused a marked decrease in the protein expression of the proteasome subunits, PSMA3 and PSMC3 (Fig. 3C and D). Importantly, these alterations in the proteostatic machinery were accompanied by a pronounced accumulation of total ubiquitinated proteins in the combination treatment group (Fig. S2). Collectively, these results suggested that the combined administration of EGCG and TCS suppresses both chaperones and proteasome components,

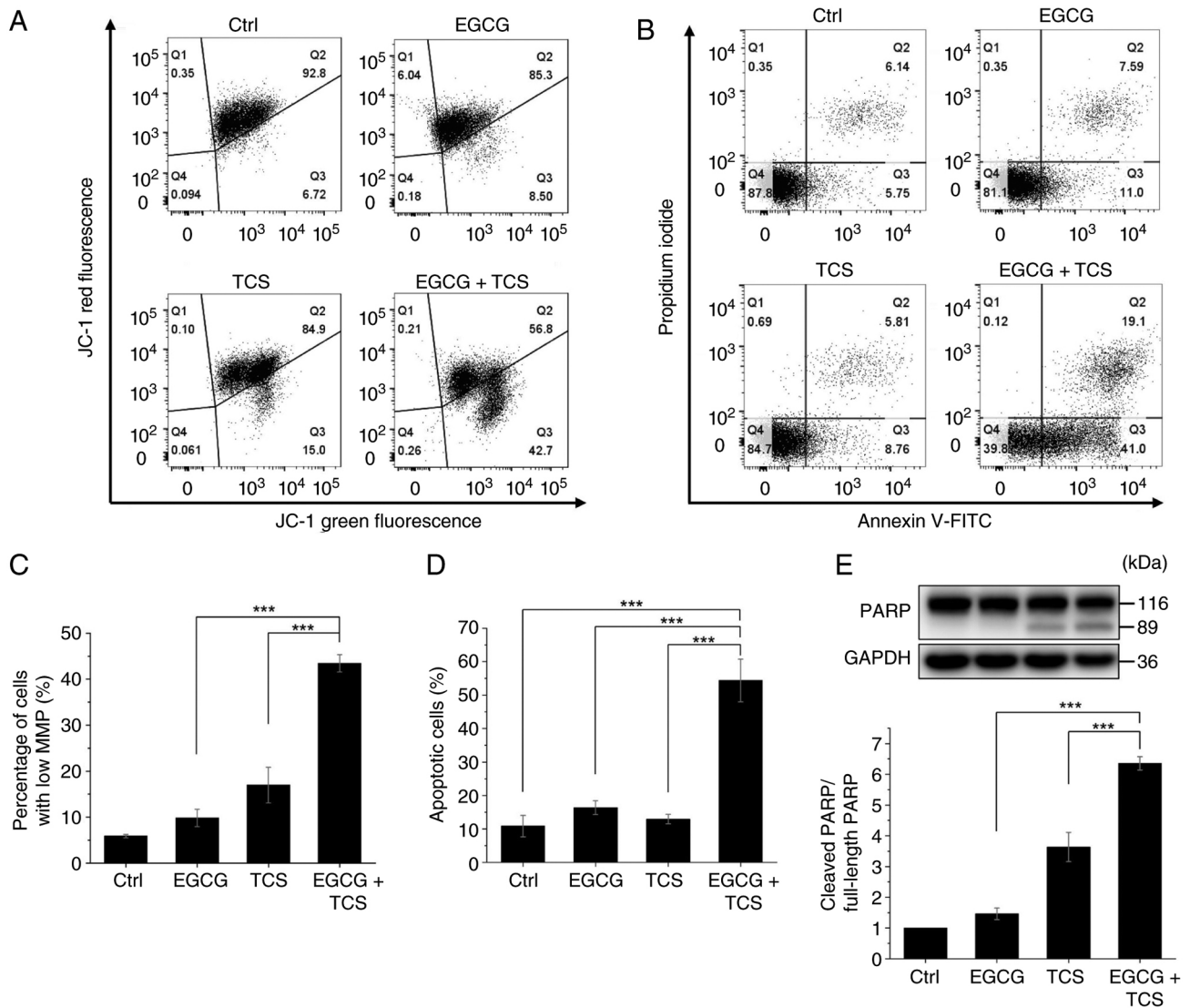


Figure 8. Effects of TCS and EGCG on mitochondrial apoptosis in A549 cells. (A) MMP in A549 cells was evaluated using the fluorescent dye JC-1 by flow cytometric analysis. (B) Apoptotic A549 cells were detected using the annexin V-FITC/PI double staining method and analyzed via flow cytometry. (C) Quantification of the percentages of cells with low MMP [Q3 regions in panel (A)] is shown. (D) Quantitative measurement of the percentages of apoptotic cells [Q2 + Q3 regions in panel (B)] is shown. (E) Representative western blot images, and corresponding quantitative analysis of full-length PARP (116 kDa) and cleaved PARP (89 kDa) levels, are shown. *** $P < 0.001$. TCS, thermal cycling-stimulation; EGCG, epigallocatechin gallate; MMP, mitochondrial membrane potential; PARP, poly(ADP-ribose) polymerase.

and that this was associated with disrupted proteostasis, therefore having a possible role in the induction of ER stress in NSCLC cells.

ER stress serves as a crucial cellular response to stress and has a dual effect on cancer cells. When cancer cells experience mild proteotoxic stress in the tumor microenvironment, they activate the unfolded protein response (UPR) to enhance survival. However, if ER stress becomes persistent or severe, it overwhelms this adaptive capacity, eventually leading to apoptosis (23,36). Consequently, manipulating this balance to shift the UPR signaling from a pro-survival to a pro-apoptotic mode represents a promising therapeutic strategy for treating NSCLC (36-38). In the present study, it has been demonstrated that pretreatment with the PERK inhibitor GSK2606414 partially restored the reduction in viability of A549 cells that was caused by the combined treatment of EGCG and TCS (Fig. 4A).

Additionally, phosphorylation of the downstream target eIF2 α was found to have been enhanced in the combination treatment group (Fig. 4B). In addition to the PERK pathway, ER stress induces activation of the IRE1 α sensor, which facilitates the splicing of XBP1 mRNA (39). The results shown in Fig. S3A demonstrated that administering TCS led to a significant increase in XBP1 mRNA splicing. In addition to XBP1 splicing, IRE1 α activation and stress signals such as ROS-ASK1 converge on the pro-apoptotic JNK signaling cascade (39,40). In the present study, it was shown that co-treatment with EGCG and TCS significantly increased JNK phosphorylation compared with either treatment in isolation (Fig. S3B). Collectively, these findings suggested that activation of both the PERK-eIF2 α signaling axis and pro-apoptotic JNK signaling may contribute to the reduced viability of NSCLC cells following combined EGCG and TCS treatment.

Severe ER stress is frequently associated with ER Ca^{2+} release, which can cause further cellular damage (5,24,41). Notably, activation of the PERK pathway has been shown to promote Ca^{2+} release from ER via the IP_3R (24,41). Consistent with these findings, the data shown in Fig. 5A and B revealed that the combination of EGCG and TCS significantly elevates cytosolic Ca^{2+} levels. Furthermore, treatment with the IP_3R inhibitor 2-APB led to a marked reduction in this Ca^{2+} increase, suggesting that the effect might be dependent on IP_3R activity. Concurrently, ER stress is often accompanied by excessive ROS production (5,42). The results shown in Fig. 6A and B confirmed that the combined treatment of EGCG and TCS effectively increased intracellular ROS levels in A549 cells. Importantly, both the release of Ca^{2+} from the ER and the production of ROS are widely recognized as inter-related processes. Previous studies have shown that oxidation of key thiol groups in the IP_3R protein enhances its channel activity (43,44). At the same time, increased cytosolic or mitochondrial Ca^{2+} levels activate ROS-generating enzymes, thereby facilitating free radical formation (45-47). In the present study, it was found that the increase in cytosolic Ca^{2+} caused by the combination treatment of EGCG and TCS was reduced by administering the antioxidant NAC, whereas ROS levels were lowered by 2-APB (Fig. 7). Although pharmacological agents inherently possess potential off-target effects, these reciprocal observations point toward a plausible bidirectional crosstalk between Ca^{2+} dysregulation and excessive ROS production, a well-documented pathophysiological amplification loop governing cellular stress responses (48). Moreover, treatments with either NAC or 2-APB alone significantly lessened the decrease in cell viability caused by the combined administration of EGCG and TCS in A549 cells (Figs. 5C and 6C), supporting a candidate working model wherein this crosstalk mediates the synergistic cytotoxic effects observed in A549 cells. Future investigations utilizing genetic silencing (for example, IP_3R knockdown) and organelle-targeted ROS probes are essential to definitively establish causality, to identify specific ROS species, and to delineate their primary sources.

Mitochondrial ROS and Ca^{2+} overload are potential triggers for the opening of the mitochondrial permeability transition pore (mPTP), which subsequently leads to the loss of MMP (24,27,28). Building upon the present findings that the combination of EGCG and TCS causes intracellular Ca^{2+} dysregulation and ROS generation, in subsequent experiments it was further demonstrated that this combination could effectively reduce the levels of MMP in A549 cells, as shown by JC-1 staining (Fig. 8A and C). Opening of the mPTP results in mitochondrial matrix swelling and rupture of the outer mitochondrial membrane, facilitating the release of cytochrome *c* from the intermembrane space into the cytosol, thereby initiating the intrinsic apoptotic pathway (49,50). Consistent with this mechanism, combined EGCG and TCS treatment was shown to cause a significant increase in the proportion of apoptotic cells (Fig. 8B and D), also significantly enhancing the cleavage of PARP (Fig. 8E), a signature downstream event in the caspase cascade. Considered altogether, these findings suggested that the combination of EGCG and TCS induces intrinsic apoptosis through promoting mitochondrial ROS accumulation and Ca^{2+} overload in A549 cells.

Although the present study has provided valuable mechanistic insights derived from *in vitro* experiments, the successful clinical translation of this combination therapy necessitates overcoming substantial practical challenges, especially in the areas of precise drug delivery and targeted hyperthermia techniques. The 50 μM concentration of EGCG employed herein surpassed plasma levels attainable through oral administration; however, strategies such as liposomal encapsulation combined with surface ligand modifications may enhance EGCG stability (51,52). Furthermore, TCS could be implemented using clinically applicable heating modalities, such as radiofrequency (53), focused ultrasound (54) or modulated electro-hyperthermia (55). Delivering heat in cycles may exploit tissue-specific hemodynamics, as heterogeneous tumor perfusion hinders heat dissipation during the passive cooling phase and promotes intratumoral heat accumulation (55). Therefore, TCS has the potential to augment tumor-specific heating, while minimizing thermal exposure to surrounding healthy tissues. Nevertheless, further *in vivo* studies are essential to optimize the kinetics of localized EGCG delivery, and also to refine TCS dosing protocols.

In conclusion, the present study has demonstrated the synergistic effects of EGCG and TCS in reducing the viability of NSCLC cells; furthermore, a potential underlying mechanism has been proposed. Specifically, TCS causes protein unfolding, whereas EGCG suppresses the activity and expression of HSPs (for example, HSP70 and HSP105) and proteasomes, accompanied by the accumulation of ubiquitinated proteins and the activation of ER stress-associated pathways. Furthermore, the results obtained in the present study have also suggested a mutual amplification between IP_3R -mediated ER Ca^{2+} release and excessive ROS production, which ultimately induces mitochondrial apoptosis. Notably, this combination treatment selectively damages NSCLC cells while sparing IMR-90 normal lung fibroblasts *in vitro*, and this effect is potentially attributable to the elevated basal ROS levels, and more vulnerable proteostasis regulation, in cancer cells (56,57). This combined approach potentially reduces the necessary dose of EGCG and also attenuates TCS-induced heat-shock responses, thereby offering a promising *in vitro* proof-of-concept strategy. Although the A549 cell line has served as the primary representative model for the present study, however, further validation using other NSCLC cell lines, and subsequent *in vivo* evaluations (such as assessing normal tissue toxicity, pharmacokinetics and therapeutic efficacy), are essential to both confirm these mechanistic insights and advance the clinical translation of this combination therapy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CYC conceived and supervised the study. FTH and CYC designed the study. FTH, HHL, YK and CJL participated in the acquisition of data. FTH, HHL, YK, CJL and CYC confirm the authenticity of all the raw data. FTH and CYC contributed to data analysis and wrote the manuscript. All authors performed the experiments. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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