Thermal cycling-hyperthermia in combination with polyphenols, epigallocatechin gallate and chlorogenic acid, exerts synergistic anticancer effect against human pancreatic cancer PANC-1 cells

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Abstract

Hyperthermia (HT) has shown feasibility and potency as an anticancer therapy. Administration of HT in the chemotherapy has previously enhanced the cytotoxicity of drugs against pancreatic cancer. However, the drugs used when conducting these studies are substantially conventional chemotherapeutic agents that may cause unwanted side effects. Additionally, the thermal dosage in the treatment of cancer cells could also probably harm the healthy cells. The purpose of this work was to investigate the potential of the two natural polyphenolic compounds, epigallocatechin gallate (EGCG) and chlorogenic acid (CGA), as heat synergizers in the thermal treatment of the PANC-1 cells. Furthermore, we have introduced a unique strategy entitled the thermal cycling-hyperthermia (TC-HT) that is capable of providing a maximum synergy and minimal side effect with the anticancer compounds. Our results demonstrate that the combination of the TC-HT and the CGA or EGCG markedly exerts the anticancer effect against the PANC-1 cells, while none of the single treatment induced such changes. The synergistic activity was attributed to the cell cycle arrest at the G2/M phase and the induction of the ROS-dependent mitochondria-mediated apoptosis. These findings not only represent the first *in vitro*

thermal synergistic study of natural compounds in the treatment of pancreatic cancer, but also highlight the potential of the TC-HT as an alternative strategy in thermal treatment.

Introduction

Pancreatic cancer is one of the leading causes in cancer death and remains one of the deadliest solid human malignancies worldwide [1]. Patients with pancreatic cancer are commonly diagnosed at the unresectable stage, and in most cases, patients with advanced pancreatic cancer have a poor response to chemotherapy or radiotherapy. In spite of the fact that therapeutic methods have been improved, the prognosis for pancreatic cancer patients still remains poor with a low five-year survival rate [2]. Therefore, there is a need for continued research in novel agents or alternative therapeutic strategies for treating pancreatic cancers, thereby making an improvement for the patients' quality of life.

Hyperthermia (HT) has emerged as a promising method for treating cancer over the past decades [3]. It is a procedure exposing the tumor tissue to high temperatures that cause cancer cell damage and death. Researches have shown that HT exhibits

66 therapeutic potential against cancer cells through multiple cellular changes, such as
67 protein denaturation and aggregation, inhibition of DNA synthesis, cytoskeleton
68 disruption, and alteration in the calcium homeostasis [4-6]. In addition, HT can directly
69 activate the immune response against the tumors, increase the tumor oxygenation, and
70 improve the drug delivery [7-9]. Although these encouraging results have expanded our
71 understanding of the cytotoxic effects of HT on the cancer cells, in the case of HT as
72 single treatment, it has been shown not to be sufficient to kill cancer cells [10]. To
73 strengthen the effectiveness of HT, several investigations have explored combinations
74 of HT and other cancer therapies, such as radiotherapy and chemotherapy [11]. It has
75 been demonstrated to be effective against various types of cancer, including pancreatic
76 cancer, in that HT enhanced the cytotoxicity of gemcitabine through the inhibition of
77 nuclear factor kappa B (NF-κB) [12-14]. There have also been reports of gemcitabine
78 and other drugs, such as cisplatin and carbonplatin, combined with HT, that
79 demonstrated the clinical efficacy in patients with pancreatic cancer [15, 16]. These
80 data suggest that HT could modify the cytotoxicity of the anticancer drugs, thereby
81 yielding better outcomes in treating pancreatic cancer. However, the drugs used in

these combined treatments are conventional chemotherapeutic drugs, which have been known to cause unpleasant and even dangerous side effects.

Nowadays, there has been an increasing interest in natural compounds research due to their lower toxicity and diverse biological properties. Phenolic compounds are among the most studied in cancer prevention and cure, and also the largest group of phytochemicals, as well as being widely distributed in our diet. Particularly, regular intakes of dietary polyphenols have been linked to lower risks of many cancers [17]. Tea and coffee are two of the most consumed beverages worldwide, and the natural phenolic compounds, epigallocatechin gallate (EGCG) and chlorogenic acid (CGA), are the major components in both drinks, respectively. It has been shown that EGCG and CGA have healthy benefits such as antioxidative, anti-inflammatory, and anticancer activities [18-20]. Recently, published findings from animal experimental [21, 22] and clinical studies [23, 24] have demonstrated the ability of EGCG and CGA to suppress tumor cell growth such as breast, lung and bladder cancer. On the other hand, there are a number of studies indicating that natural compounds, including polyphenols, can act as heat synergizers, and thereby improve the anticancer effect [25-29]. Therefore, this has prompted us to conduct the first combined experiment of

HT and natural phenolic compounds in pancreatic cancer. Furthermore, we propose a novel approach named thermal cycling-hyperthermia (TC-HT), which allows cells to receive the equivalent thermal dosage through the repeated heat-and-cold cycle. As a matter of fact, there is some evidence indicating that HT lacks tumor selectivity and could also cause harm to normal cells [30, 31]. In this thermal cycled strategy, the cells could avoid prolonged continuous heating under thermal treatment, thereby reducing the toxicity of HT to highlight the synergistic anticancer efficiency of the combined therapy. Therefore, the aim of this study was to investigate the synergistic anti-pancreatic cancer effect of the TC-HT with the CGA and the TC-HT with the EGCG.

In this paper, we examined the effects of the EGCG or CGA combined with the TC-HT on the growth inhibition of PANC-1 cells and evaluated the cell cycle regulation, apoptosis, and the expression of associated proteins to elucidate their underlying mechanisms. Our results demonstrate that the co-administration with the TC-HT and the EGCG or CGA significantly inhibited the cell proliferation and increased the cell death by inducing the cell cycle arrest and mitochondrial apoptotic pathway. These findings first indicate that both the EGCG and CGA might be effective

heat synergizers and that the maximum synergistic effects on the PANC-1 cells could be obtained when the TC-HT was administered. Furthermore, the TC-HT as a thermal treatment could be much gentler and feasible, mainly because of the view that the TC-HT itself is relatively harmless to the cells. We believe that this study provides the interesting concept of cyclic thermal application in treatment of cancer *in vitro*, and highlights the potential of the TC-HT as an alternative thermal treatment.

Materials and methods

Cell culture

Human pancreatic cancer cell line PANC-1 was obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated in 75 cm^3 cell culture flasks and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin (Gibco) in a humidified 5% CO_2 incubator at 37℃.

Drug treatment and TC-HT exposure

The EGCG was dissolved in distilled water at a concentration of 20 mM, and the CGA was dissolved in dimethyl sulfoxide (DMSO)(Sigma). Subsequently, the two samples were stored at -20℃. The stocks were diluted with a culture medium to the indicated concentration for treatment before usage, and the final concentration of DMSO in each well was 0.05% (v/v). After overnight incubation at 37^{o}C, cells were treated with various concentrations of EGCG or CGA, as well as the solvent vehicle (0.05% DMSO) for CGA. For the TC-HT, the cells were subject to a high and low temperature water bath using a temperature controller modified by a PCR machine (**Fig 1A**). The cells were exposed to HT (1-cycle TC-HT) at 46℃ for 30 min without a break or treated with 46-30℃ TC-HT through 3, 6, and 10 cycles of 10, 5, and 3 min, respectively, to receive the equivalent thermal dosages (**Fig 1B**). In the combined treatment, cells in a medium containing the EGCG or CGA were exposed to the TC-HT. The temperatures of the cancer cells actually sensed were monitored by placing a needle thermocouple at the bottom of the well (**Fig 1C**). During heat exposure experiment, the non-heating groups (non-treated and drug-treated groups) were exposed to a similar ambient environment as the heating groups over the experimental period of time. After the

148 treatment was completed, cells were stored in a cell culture incubator until the time of
149 analysis.
150
151 **Fig 1. Exposure to the TC-HT via a temperature controller.** (A)(B) Schematic of
152 experiment setup and the TC-HT programs setting. (C) The actual temperature
153 recorded every 20 sec in the PANC-1 cells throughout the exposure period.
154

155 ## MTT assay

156 Cell viability was accessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
157 bromide (MTT) (Sigma) assay. Cells were seeded in 24-well plates and incubated
158 overnight at 37℃. After a single-agent treatment or combined treatment of the TC-HT
159 with the 0.05% DMSO (vehicle control), EGCG or CGA for 24 h, the medium was
160 removed and the cells were washed with phosphate buffered saline (PBS). Cells were
161 then incubated in DMEM containing 0.5 mg/ml MTT for an additional 4 h at 37℃.
162 Then the medium was removed and DMSO was added to dissolve the formazan
163 crystals. The supernatant from each sample was transferred into 96-well plates, and the
164 absorbance was read at 570 nm using an ELISA microplate reader. The calculation of

synergism quotient (SQ) was dividing the combined effect by the sum of individual effects. The given treatment shows a synergy when SQ is greater than 1.0.

Clonogenic survival assay

PANC-1 cells were seeded at 1000 cells/dish in 35 mm Petri dishes for 24 h and treated with 0.05% DMSO (vehicle control), CGA, EGCG and TC-HT alone or in combination. Cell medium was replaced after the treatment, and the dishes were cultured in a humidified 5% CO_2 incubator at 37℃ for additional 14 days. At last, the cells were fixed with 4% paraformaldehyde (PFA) (Sigma) for 10 min and stained with 0.1% crystal violet (Sigma). The colonies containing more than 50 cells were counted, and the number of colonies in each treatment group was normalized to control group.

Cell cycle analysis

Cells were seeded in 35 mm Petri dishes and incubated overnight at 37℃. After 24 h treatment with DMSO (vehicle control), CGA, EGCG and TC-HT alone or in combination, the cells were harvested, washed with PBS, and fixed with 70% ethanol at 4℃ for 30 min. The cells were then stained with propidium iodide (PI) (BD Bioscience) and RNase A (Thermal Scientific) for 30 min in the dark. The stained cells were

subject to the cell cycle analysis by using a flow cytometer (FACSCanto II; BD Biosciences), and data were analyzed with ModFit LT software.

DAPI staining assay

DAPI staining was used to detect morphological characteristics of the nucleus. Cells were cultured on glass coverslips in 35 mm Petri dishes. At the end of each 24 h treatment, cells were washed twice with PBS and fixed with 4% PFA for 10 min at room temperature. After washing twice with PBS, the glass coverslips with attached cells were mounted using Fluoroshield mounting medium with DAPI (Abcam) and examined with a fluorescence microscope (Axio Imager A1; ZEISS).

Annexin-V/PI double staining assay

Apoptosis was determined by using the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences). Briefly, PANC-1 cells were treated with the TC-HT combined with the DMSO (vehicle control), CGA or EGCG, and then the cells were harvested with trypsin-EDTA (Gibco) and collected by centrifugation at 2,000 × g for 5 min, washed twice with cold PBS, and resuspended in binding buffer containing Annexin V-FITC and PI. The cell suspensions were incubated for 15 min at room temperature in the dark and analyzed by a FACS Calibur flow cytometer.

Mitochondria membrane potential (MMP) measurement

The cells treated with 0.05% DMSO (vehicle control), CGA, EGCG and TC-HT alone or in combination for 24h were harvested, and resuspended with PBS followed by staining with 20 nM $DiOC_6(3)$ (Enzo Life Sciences International Inc.) for 30 min at 37℃ in the dark. The fraction of cells showing low MMP was then measured by a flow cytometer.

ROS detection

Cellular Reactive Oxygen Species (ROS) levels of superoxide anion ($O_2^{\bullet-}$) were detected using the fluorescent dye dihydroethidium (DHE) (Sigma). Cells were treated with the indicated treatments, washed with PBS, and then incubated with 5 μM DHE for 30 min at 37℃ in the dark. The fluorescence intensities were measured by flow cytometry and ROS levels were expressed as mean fluorescence intensity (MFI).

Western blot analysis

After treatment with CGA, EGCG, and TC-HT for 24 h alone or in combination, cells were harvested, washed with cold PBS, and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1.0% Triton X-100, 0.1% SDS, 1 mM EDTA, 1% phosphate and protease inhibitor cocktail)

(Millipore). Cell lysates were clarified by centrifugation at 23,000 × g for 30 min at 4℃, and the protein concentration in the supernatant fraction was quantified using the Bradford protein assay (Bioshop). Proteins were resolved by 10% SDS-PAGE and electrotransferred onto polyvinylidene fluoride membrane (PVDF) (Millipore) in transfer buffer (10 mM CAPS, pH 11.0, 10% methanol). The membranes were blocked with 5% nonfat dry milk/TBST (blocking buffer) for 1 h at room temperature and then incubated overnight at 4℃ with diluted primary antibodies in blocking buffer. The specific primary antibodies against Bcl-2, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3 (Cell Signaling), Bax (Santa Cruz), Cdc2, cyclin B1, cleaved PARP and β-actin (GeneTex) were used. After washing with TBST, the membranes were incubated with HRP-conjugated anti-goat (GeneTex) or anti-rabbit (Jackson Immunoresearch) secondary antibody. Chemiluminescence was detected using WesternBright ECL western blotting reagent (Advansta).

Statistical analysis

The results were presented as mean ± standard deviation (SD). Statistical analysis using one-way analysis of variance (ANOVA) performed with SigmaPlot software. The

231 results were considered to be statistically significant when *p*-values were less than 0.05.

232 Each experiment was done in triplicate.

233

234 Results

235 TC-HT in combination with polyphenols synergistically

236 inhibits proliferation of PANC-1 cells

237 The TC-HT was performed by a modified PCR machine to raise the temperature to a

238 desired level followed by a rapid return to normothermic temperature. The actual

239 temperature in the cells measured by a needle thermocouple could be elevated from

240 36^{o}C to 43.5, 44.2 and 44.9^{o}C within 3, 5 and 10 min, respectively, and rapidly returned

241 to the physiological temperature (**Fig 1C**). The effect of the TC-HT and the CGA or

242 EGCG on cell growth was first explored using MTT assay. As shown in **Figs 2A** and

243 **2B**, cells were treated with various concentrations of the CGA or EGCG for 24 h, and

244 both of the compounds only slightly affected the viability of PANC-1 cells. DMSO

245 (0.05%) treated cells with or without the TC-HT did not show a significant difference

246 in viability compared to untreated control cells. Moreover, the viability of PANC-1

247 cells in response to treatment with the TC-HT decreased in a cycle-dependent manner.

When the TC-HT was combined with either of the two compounds, a significant dose-dependent decrease in viability was observed at all four cycling parameters. Although the 1-cycle TC-HT and either the compound cooperatively reduced the proliferation of PANC-1 cells, the heat alone resulted in significant cytotoxicity to the cells. Notably, there was no difference in the cell viability under the condition of the 10-cycles TC-HT when compared to that in the control group, while it still was capable of working synergistically with either the CGA or the EGCG to exert the anti-proliferative activity. The SQ values for the corresponding treatments are as shown in **Table 1**. This suggests that both the CGA and the EGCG could exhibit a synergistic cytotoxic effect when co-administered with the TC-HT, particularly in the treatment of 10 cycles. We next performed a clonogenic survival assay to confirm the effect of the TC-HT combined with CGA or EGCG on cell proliferation. As shown in **Figs 2C** and **2D**, colony formation in PANC-1 cells was dramatically reduced following both combined treatments. Based on these data, the 10-cycles TC-HT (43.5-36 ℃) in combination with the concentration of 200 μM CGA, or the concentration of 20 μM EGCG, was used for all the following experiments.

Fig 2. Effects of CGA or EGCG combined with the TC-HT on proliferation in PANC-1 cells. (A) The cells were treated with different cycle numbers of the TC-HT, various concentrations of CGA and 0.05% DMSO (vehicle control) alone or in combination for 24 h. (B) The cells were treated with different cycle numbers of the TC-HT and various concentrations of EGCG alone or in combination for 24 h. (C) Representative images of clonogenic survival assay. (D) Analysis of colony formation rate. Data are presented as mean ± S.D. in triplicate.

Table 1. Synergy quotient for CGA or EGCG in combination with the TC-HT.

		TC-HT			
		1-cycle	**3-cycles**	**6-cycles**	**10-cycles**
CGA (μM)	**100**	0.84	0.86	1.08	1.51
	200	1.10	1.32	1.51	2.82
	300	1.11	1.34	1.38	2.40
EGCG (μM)	**10**	1.18	1.11	1.27	1.95
	20	1.28	1.35	1.76	3.09
	30	1.23	1.40	1.70	2.69

The combination of CGA or EGCG with TC-HT caused G2/M cell cycle arrest in PANC-1 cells

To evaluate the mode of the anti-proliferative effects of the TC-HT, CGA and EGCG, the cell cycle progression in the PANC-1 cells was examined by flow cytometric analysis. As shown in **Figs 3A** and **3B**, treatment with the CGA, EGCG, and TC-HT had no obvious effect on the cell progression when compared with the group of untreated cells. Also, 0.05% DMSO used as the solvent vehicle of CGA either alone or in combination with TC-HT had no effect on the cell progression. Interestingly, the TC-HT combined with either of the two polyphenols resulted in a significant accumulation of cells in the G2/M phase with a concomitant reduction of cells in the G1 phase. To investigate the molecular mechanism of the results of the combined effect on the cell cycle distribution, we examined the relevant proteins involved in the G2/M progression. As shown in **Fig 3C**, the expression of Cdc2 and cyclin B1 were markedly reduced in response to the combined treatment with TC-HT and CGA or the combined treatment with TC-HT and EGCG. These results indicate that either the CGA or EGCG combined with the TC-HT synergistically induced cell cycle arrest in the G2/M phase

by decreasing cyclin B1 and Cdc2 in the cells, and eventually inhibited the cell growth of PANC-1 cells.

Fig 3. Effects of CGA or EGCG combined with the TC-HT on the G2/M cell cycle arrest in PANC-1 cells. Cells were treated with the TC-HT (10 cycles), 0.05% DMSO (vehicle control), CGA (200 μM), and EGCG (20 μM) alone or in combination (TC-HT + DMSO, TC-HT + CGA, TC-HT + EGCG) for 24 h and stained with propidium iodide (PI) for cell cycle analysis. (A) Representative DNA content profiles. (B) The percentage of cell population in each phase of the cell cycle. (C) Western bolt analysis of the expression of cell cycle regulator proteins cyclin B1 and Cdc2. β-actin was used as an internal control. Data are presented as mean ± S.D. in triplicate. ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated control)

TC-HT combined with CGA or EGCG induces apoptosis in PANC-1 cells

We then investigated as to whether the synergistic cytotoxic effect of the TC-HT with the CGA or EGCG was associated with the induction of apoptosis, and the flow

cytometric analysis was performed using Annexin V-FITC/PI staining. As shown in **Figs 4A** and **4B**, the fraction of apoptotic cells after the treatment of PANC-1 cells with either the single agent CGA, or the EGCG or TC-HT were barely increased as compared with untreated control cells. There was no significant difference in apoptosis between the 0.05% DMSO (vehicle control) combined with or without TC-HT and the untreated control. Interestingly, the TC-HT in combination with the CGA significantly increased the early apoptotic cell death in PANC-1 cells (18.6±5.2%). A similar result was also obtained in the combination of the TC-HT and the EGCG, but to a greater extent (24.3±0.8%). These results also showed that the late apoptotic cell death was increased following the exposure of TC-HT with CGA (15.7±4.6%) or EGCG (18.4±4.6%). Furthermore, in contrast to control cells, the DAPI staining demonstrated that the cells treated with either of the two combined treatments had more condensed and fragmented nuclei, which are typical morphological alterations for the apoptosis (**Fig 4C**). These results suggest that the TC-HT has a potential synergistic effect with the CGA or EGCG on the apoptosis in the PANC-1 cells.

Fig 4. Combination of the TC-HT and the CGA or EGCG induces apoptosis in PANC-1 cells. The apoptosis analysis for the cells following the treatment with the TC-HT (10 cycles), 0.05% DMSO (vehicle control), CGA (200 μM), and EGCG (20 μM) alone or in combination (TC-HT + DMSO, TC-HT + CGA, TC-HT + EGCG) for 24 h. (A) Flow cytometric detection of the apoptosis with Annexin V-FITC/PI double staining. (B) Histogram quantifying the percentage of PANC-1 cells in early and late apoptosis. (C) The nuclei morphology alterations (arrow) were examined using DAPI staining. Data are presented as mean ± S.D. in triplicate. ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated control)

Combination of the TC-HT with CGA or EGCG triggers significant loss in the mitochondrial membrane potential in PANC-1 cells

Collapse of the mitochondrial integrity is a critical event in the cells undergoing apoptosis. To examine whether the combination of the TC-HT and CGA- or EGCG-induced apoptosis involved mitochondrial disruption, the mitochondrial membrane potential (MMP) was assessed using $DiOC_6(3)$ fluorescence staining by

341 flow cytometric analysis [32]. As shown in **Figs 5A** and **5B**, the treatment with the
342 EGCG, the CGA and its corresponding vehicle control (0.05% DMSO) did not change
343 the MMP level in comparison with the untreated control. In response to treatment with
344 the TC-HT alone or together with vehicle control, there was no significant change in
345 MMP within cells; however, the cells treated with TC-HT showed a stronger effect on
346 the MMP depolarization after the administration of CGA or EGCG (44±6.4% and
347 75.7±7.6%), which was consistent with the results of the apoptosis analysis as
348 described in **Fig 4**. These results indicate that the TC-HT combined with the CGA- or
349 EGCG-induced apoptosis in the PANC-1 cells is mediated by mitochondrial
350 dysfunction.
351
352 **Fig 5. Combination of the TC-HT and the CGA or EGCG induces mitochondrial**
353 **dysfunction.** (A) Flow cytometric analysis of MMP using $DiOC_6(3)$ after treatment
354 with the TC-HT (10 cycles), 0.05% DMSO (vehicle control), CGA (200 μM), and
355 EGCG (20 μM) alone or in combination (TC-HT + DMSO, TC-HT + CGA, TC-HT +
356 EGCG) for 24 h. The M1 regions indicate the cells with the loss of MMP. (B)
357 Histogram represents the percentage of cells in the M1 region. Data are presented as

358 mean ± S.D. in triplicate. ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated

359 control)

360

361 Combination of the TC-HT with CGA or EGCG induces

362 apoptosis through the mitochondrial pathway

363 It is well known that Bcl-2 family proteins and caspases, along with PARP, play

364 important roles in the mitochondria-mediated apoptosis. To further explore the

365 mechanism by which the TC-HT combined with the CGA or EGCG triggered apoptosis

366 in the PANC-1 cells, we evaluated the expression of the apoptosis-related proteins

367 using western blot analysis. As shown in **Fig 6**, when compared with control cells, the

368 cleaved caspase-9, -3, and the cleaved PARP were markedly increased after the

369 co-administration with the TC-HT and either the CGA or EGCG. In addition, both of

370 the combined treatments down-regulated the expression of Bcl-2 and up-regulated the

371 expression of Bax, and thus decreased the ratio of Bcl-2 to Bax. These results reveal

372 that the TC-HT combined with the CGA or EGCG promotes apoptosis in the PANC-1

373 cells via activation of the mitochondrial pathway.

374

375 **Fig 6. Effects of the TC-HT, CGA and EGCG on the expression of the**
376 **apoptosis-related proteins in PANC-1 cells.** (A)(B) The protein levels of cleaved
377 caspase-9, -3, cleaved PARP, Bcl-2 and Bax of the PANC-1 cells treated with the
378 TC-HT (10 cycles), CGA (200 μM) and EGCG (20 μM) alone or in combination
379 treatment (TC-HT + CGA, TC-HT + EGCG) for 24 h were examined by western blot
380 analysis. GAPDH and β-actin were used as internal controls.

381

382 ## The role of ROS in the TC-HT combined with the CGA- or
383 ## EGCG-induced apoptosis in PANC-1 cells

384 As ROS generation is a critical event in the induction of the apoptosis, we next
385 examined the role of ROS in apoptosis induced by treatment with the TC-HT in
386 combination with either the CGA or EGCG. The intracellular ROS was measured by
387 flow cytometry using a fluorescence probe, DHE, which reacts with $O_2^{\bullet-}$ [33]. As
388 shown in **Fig 7A**, the treatment of PANC-1 cells with 0.05% DMSO (vehicle control),
389 CGA or EGCG alone did not alter the level of ROS when compared with the untreated
390 control cells. Also, the level of ROS induced by TC-HT and its combination with
391 DMSO were found to be comparable to the untreated control in the MFI. It is worth

mentioning that the TC-HT combined with CGA and the TC-HT combined with EGCG significantly increased the level of ROS in MFI by approximately 2.3- and 3.6-fold, respectively (**Fig 7B**). These results suggest that the TC-HT combined with the CGA or EGCG may induce apoptosis through the excessive ROS production in the PANC-1 cells.

Fig 7. Combined effects of the TC-HT and the CGA or EGCG on ROS generation in PANC-1 cells. (A) The DHE ($O_2^{\bullet-}$) levels were measured by flow cytometry after treatment with the TC-HT (10 cycles), 0.05% DMSO (vehicle control), CGA (200 μM), and EGCG (20 μM) alone or in combination (TC-HT + DMSO, TC-HT + CGA, TC-HT + EGCG) for 24 h. (B) Graph shows the fold change in MFI of DHE relative to control. Data are presented as mean ± S.D. in triplicate. ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated control)

Discussion

Despite previous studies having shown that HT or polyphenols, the EGCG and CGA, possessed anticancer activities against pancreatic cancer cells [34-37], the effect

409 of these two agents as a combined therapy on pancreatic cancer has not been reported.
410 In this study, we have explored as to whether the herbal compound, CGA or EGCG,
411 could cooperate with heat against the pancreatic cancer cells, and elucidated the cellular
412 mechanism underlying the biological effects. Our results have demonstrated that the
413 exposure to heat using the TC-HT in combination with the CGA or EGCG
414 synergistically inhibited the growth and induced the apoptosis in the PANC-1 cells.
415 Previous studies have shown the benefits of HT as an adjuvant in combination with
416 chemotherapy against many various cancers, including pancreatic cancer [13, 38, 39].
417 However, there is a major concern that HT can cause unavoidable thermal injury to
418 normal cells. Therefore, it is important to refine the method of heat administration to
419 achieve the desired thermal dosage with the minimal cytotoxicity. To the best of our
420 knowledge, this is the first report indicating a novel approach TC-HT synergizes with
421 the CGA or EGCG on pancreatic cancer PANC-1 cells.

422 Previous research has reported the response of PANC-1 cells to mild HT at
423 different temperatures [40]. Particularly, the temperature in a range of about 42 to 46℃
424 has been shown capable of inducing the cell death of the pancreatic cell line PANC-1.
425 Our survival analysis revealed a consistent result that the cell growth of PANC-1 in the

426 1-cycle group was significantly inhibited. Although HT has a cooperative cytotoxicity
427 with the CGA or EGCG, its severe thermal toxicity to a cell is what we want to avoid.
428 The usage of the repeated cycles of heat exposure could provide a means to synergize
429 with the anticancer compounds in the heating process, and avoid the thermal damage
430 accumulation in the following non-heating process. Earlier studies have shown the
431 time-dependent modification of cancer cells during exposure to HT for which the
432 analysis of cellular growth indicated that the survival rate decreased with increasing HT
433 exposure time [41, 42]. Namely, the short exposure of cancer cells to HT may induce
434 cellular stress without affecting their proliferation, while the prolonged exposure may
435 lead to cytotoxicity. It has also been reported that short HT treatment of PANC-1 cells
436 at the temperature 46℃ for 5 min had no effect on cell viability [43]. Therefore, we
437 suggest that the 10-cycles TC-HT induced stress rather than damage in cells during
438 each heating cycle. As expected, the cells treated with the TC-HT of different cycling
439 parameters exhibited a decreased cytotoxicity with the increased cycles. It is worth
440 noting that 10 cycles of the TC-HT (43.5-36 ℃), or either compound, did not cause a
441 significant alteration in the viability, however, the combination of 10 cycles of the
442 TC-HT with the CGA or EGCG strongly resulted in a dose-dependent decline of the

443 cell viability. Similar results from colony formation were also obtained with the cells
444 treated with the combination of TC-HT and CGA or the combination of TC-HT and
445 EGCG. These results suggested that the either CGA or EGCG may act as heat
446 synergizer and co-administered with the use of the TC-HT could have significantly
447 combined effects against pancreatic cancer PANC-1 cells. Furthermore, we have also
448 tested one liver cancer (HepG2) and one non-cancerous (HEK293) cell lines to
449 examine the combined action. Results shown in **S1 Fig** revealed that the 10-cycles
450 TC-HT did not cause cytotoxicity in both cancer and non-cancerous cells.
451 Interestingly, such substantial cell death in the PANC-1 cells treated with the TC-HT
452 in combination with CGA or EGCG was not observed in the HepG2 and HEK293
453 cells, which indicated cell specificity of the combination treatments.

454 HT has been demonstrated to inhibit the growth of human cancer cells through
455 interfering with the cell cycle progression [27, 29, 44]. In our results, we found that the
456 TC-HT did not cause an obvious accumulation of cells in the G2/M phase. When cells
457 undergoing the TC-HT were given the CGA or EGCG concurrently, the proportion of
458 cells in the G2/M phase was significantly increased. Additionally, this observation was
459 supported by the marked downregulation of Cdc2 and cyclin B1 proteins in the

460 combination of the TC-HT and the CGA or EGCG. The Cdc2 (cell division cycle 2),
461 also known as the CDK1 (cyclin-dependent kinase 1), is a core regulator that drives the
462 cells through G2 and into mitosis. Several studies have reported that the binding of
463 Cdc2 to cyclin B1 complex plays an important role in the G2/M progression [45, 46].
464 Collectively, these results indicated that the synergistic cytotoxicity of CGA or EGCG
465 under the exposure of TC-HT, at least in part, was associated with the inhibition of the
466 Cdc2/cyclin B1 kinase activity.

467 Apoptosis, also the best known form of programmed cell death, plays a pivotal
468 role in defending against cancer. The conventional HT has been demonstrated to
469 affect apoptotic pathways in various types of cancer cells [47]. It has also been
470 reported that polyphenols exhibit anticancer activities against different human cancer
471 cells through activating the apoptotic pathway [48, 49]. However, in our study, the
472 results of apoptosis analysis performed using FITC Annexin V and PI double staining
473 indicated that neither the TC-HT nor these two compounds induced significant
474 apoptotic cells (**Fig 4**). Only when the TC-HT in combination with the CGA or
475 EGCG was conducted, both of the combined treatments significantly elevated the
476 percentage of the early and late apoptotic cells. This finding was further confirmed by

477 the nuclear morphological alterations of the apoptosis in the TC-HT-treated cells in
478 combination with the CGA or EGCG. The apoptotic process can be divided into the
479 death receptor and mitochondrial pathways, and the mitochondrial pathway of cell
480 death is thought to be the major mechanism of apoptosis in mammals. The Bcl-2
481 family proteins are key regulators of the mitochondrial apoptotic pathway, which
482 comprise of both the anti-apoptotic and pro-apoptotic members. Unbalanced
483 Bcl-2/Bax ratio within the cells induces the disruption of the mitochondrial membrane,
484 release of cytochrome *c*, activation of caspases, and the subsequent cleavage of PARP
485 [50, 51]. Results from **Figs 5** and **6** indicated that the combined treatment with the
486 TC-HT and the CGA or EGCG markedly decreased the level of MMP and Bcl-2
487 expression, and increased Bax expression. We also found that the activation of
488 caspase-9, -3, and the cleavage of PARP were promoted with both of the combined
489 treatments **(Fig 6**). Collectively, these data indicated that the mitochondria-dependent
490 pathway is involved in the synergistic apoptosis following the combined treatments.

491 Mitochondria are widely believed to be the main cellular source of ROS. The
492 excessive production of ROS could result in mitochondrial dysfunction, which in turn
493 triggers the apoptosis [52]. We then confirmed if the observation of mitochondrial

494 dysfunction in cells treated with the TC-HT in combination with the CGA or EGCG
495 was promoted by ROS generation. Our result showed that the cellular ROS generation
496 was elevated significantly following the administration of combination of the TC-HT
497 with the CGA or EGCG, indicating that the high levels of ROS production played an
498 important role in the apoptosis induced by the combination of the TC-HT with CGA
499 and the TC-HT with EGCG. HT has been known to act as an adjuvant treatment
500 modality to improve the cytotoxicity of several anticancer agents such as
501 chemotherapeutic drugs [53-55] and herbal compounds [56, 57]. It is thought that HT
502 can enhance the sensitivity of cancer cells to treatment with drugs [58], thereby
503 presenting synergistic anticancer activity. Therefore, these observations of this study
504 tempted us to suggest that the heat stress during temperature cycles may contribute to
505 the potentiation of the CGA or EGCG-triggered cytotoxic responses. Most
506 importantly, the TC-HT could serve as a mild approach to synergize with the natural
507 compound, CGA or EGCG. Furthermore, we also investigate the feasibility of the
508 TC-HT by using standard clinical compound gemcitabine shown to exhibit thermal
509 enhancement of cytotoxicity when combined with standard HT [53, 59]. Similar result
510 as the combination of 1-cycle TC-HT and gemcitabine was observed from analysis of

511 cell viability in the combined treatment with the 10-cycles TC-HT and gemcitabine,
512 whereas the 10-cycles TC-HT alone had no effect on cell viability (**S2 Fig**). This
513 finding suggests that this approach could be potentially extended to other anticancer
514 agent with heat synergy in cancer research. In practices, the type of thermal therapy is
515 usually associated with the location of tumor. At present time, the TC-HT
516 methodology might have difficulty in applying rapidly cycling heat on centrally
517 located malignancies *in vivo*. Further researches need to be conducted to overcome
518 this problem.

519 In conclusion, a novel method for producing a desired thermal dosage is
520 proposed in which it applies repeated thermal treatments of short exposure to prevent
521 the toxic effects from prolonged exposure. Our findings have demonstrated the
522 TC-HT shows capability of synergizing with the natural compound, CGA or EGCG,
523 and minimizing the thermal damage resulting from HT. The synergistic activity
524 against the PANC-1 cells was performed primarily via the G2/M arrest and the
525 apoptosis induced through the ROS-mediated mitochondrial pathway leading to an
526 imbalance between Bcl-2 and Bax, activation of caspase-9 and -3, and the cleavage of
527 PARP. This study represents an interesting concept of cyclic thermal application in

528 treatment of cancer *in vitro*, which may potentially demonstrate an alternative to heat
529 treatment. Gaining an optimal combined effect for a variety of cancers via the
530 examination of the relation between the TC-HT parameters and other anticancer drugs
531 is an attractive subject that warrants further investigation.

532

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539

540 Competing Interests

541 The authors have declared that no competing interests exist.

542

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780 Supporting information

781 **S1 Fig. (A) HepG2 and (B) HEK293 cell viability results from MTT assay after**

782 **the treatments of CGA, EGCG, and TC-HT (10 cycles) alone or in combination**

783 **(TC-HT + CGA, TC-HT + EGCG) for 24 h.**

784 **S2 Fig. PANC-1 cell viability results from MTT assay after the treatments of**

785 **gemcitabine (5 μM) and TC-HT (1 cycle and 10 cycles) alone or in combination**

786 **for 24 h.**

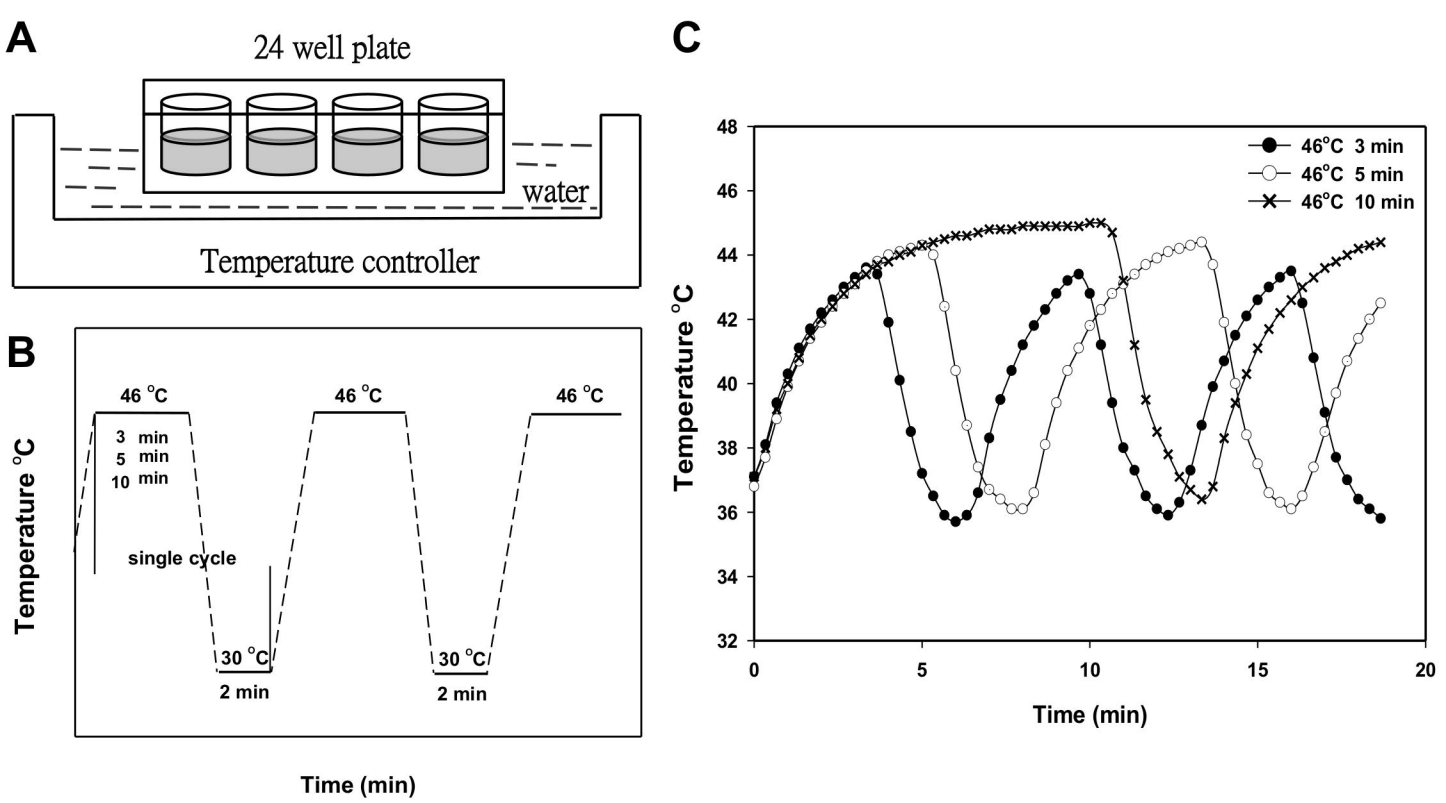
787 **S3 Fig. Raw figures of western blot analysis.**

788 **S1 File. Raw data of cell viability and colony formation results.**

789 **S2 File. Raw data of various time points of temperatures.**

790 **S3 File. Raw data of flow cytometry result.**

791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Temperature °C
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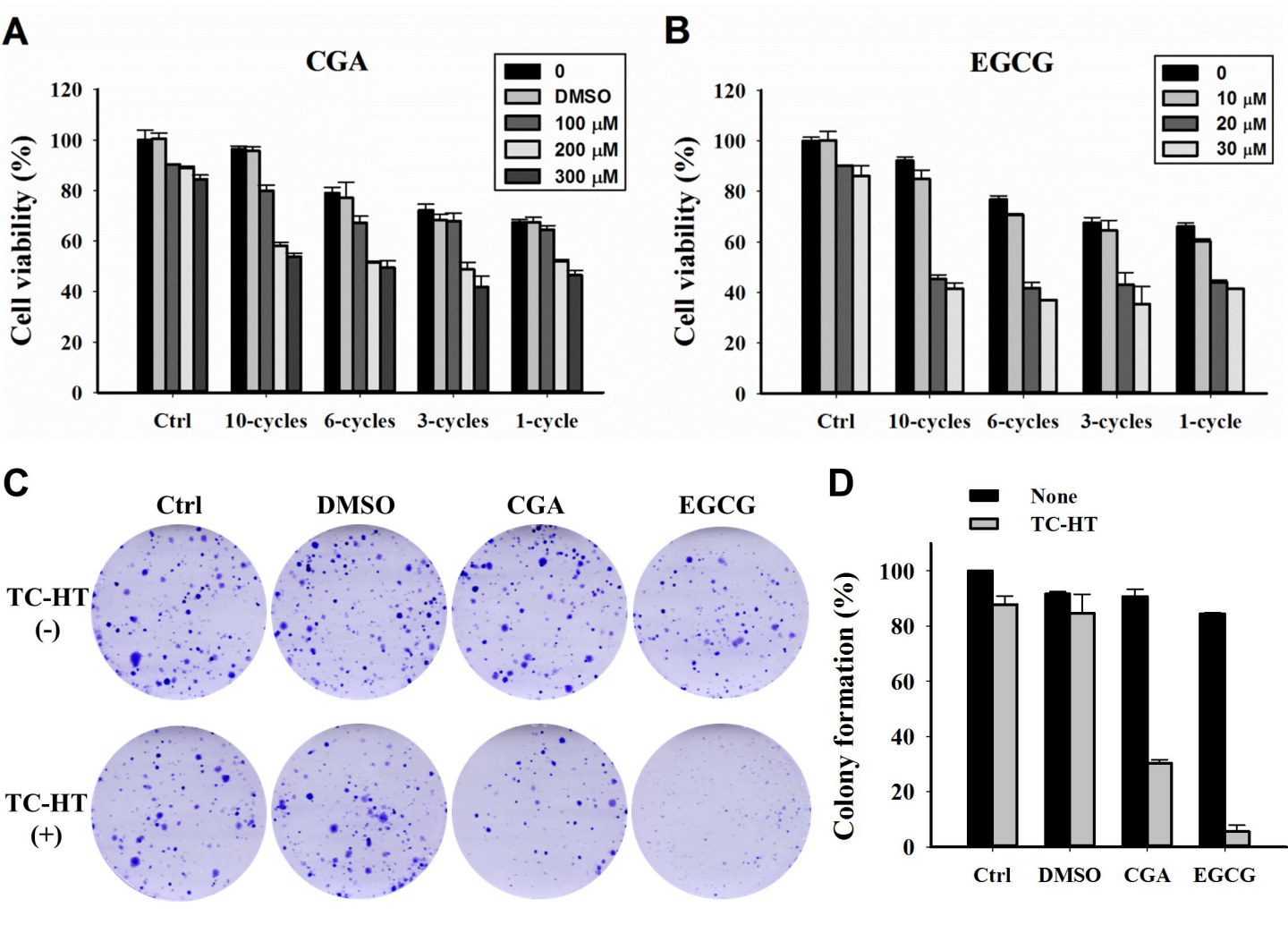
A
CGA
0
DMSO
100 μM
200 μM
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Cell viability (%)
120
100
80
60
40
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10-cycles
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1-cycle
B
EGCG
0
10 μM
20 μM
30 μM
Cell viability (%)
120
100
80
60
40
20
0
Ctrl
10-cycles
6-cycles
3-cycles
1-cycle
C
Ctrl
DMSO
CGA
EGCG
TC-HT
(-)
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D
None
TC-HT
Colony formation (%)
100
80
60
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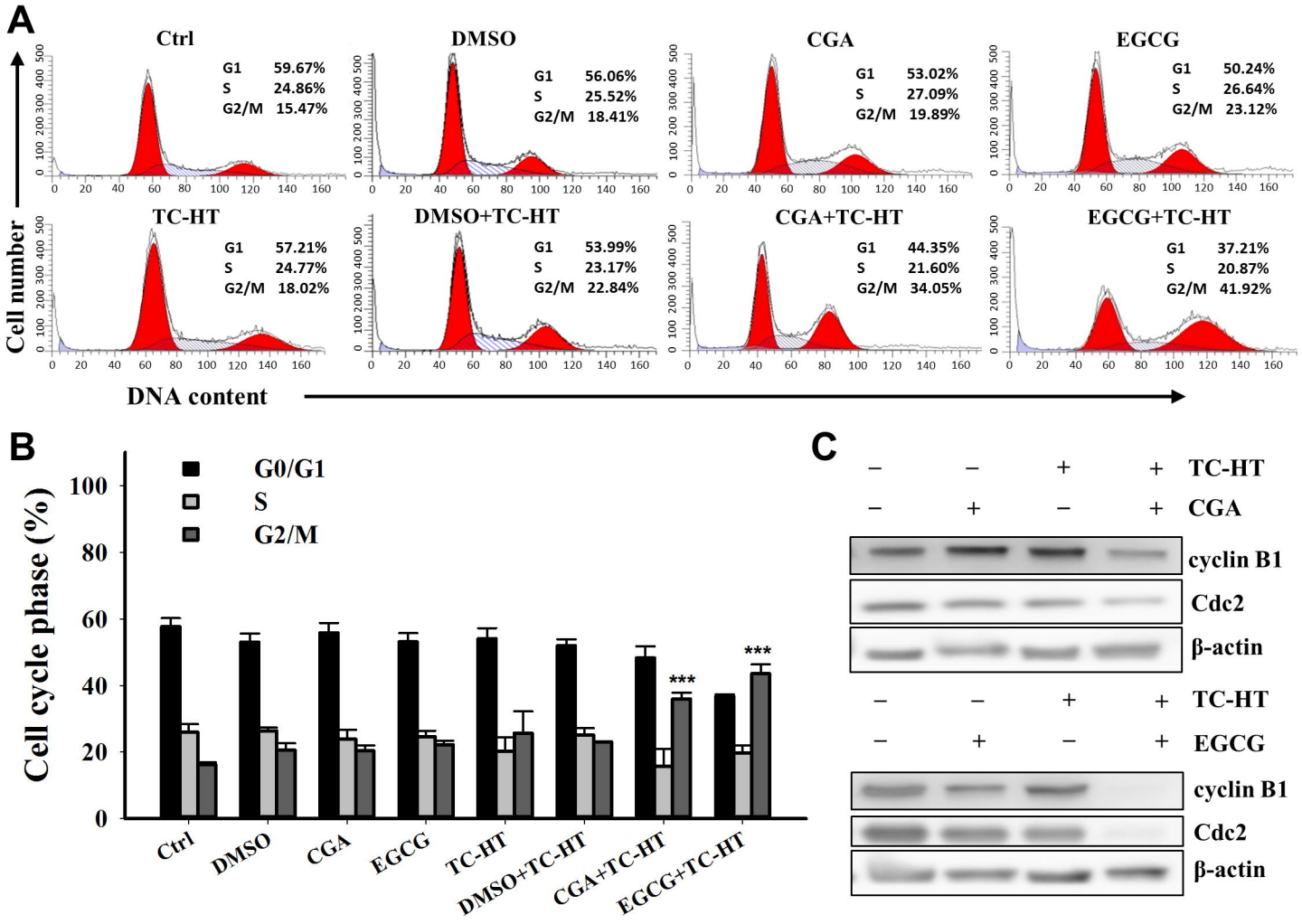
20
0
Ctrl
DMSO
CGA
EGCG


A
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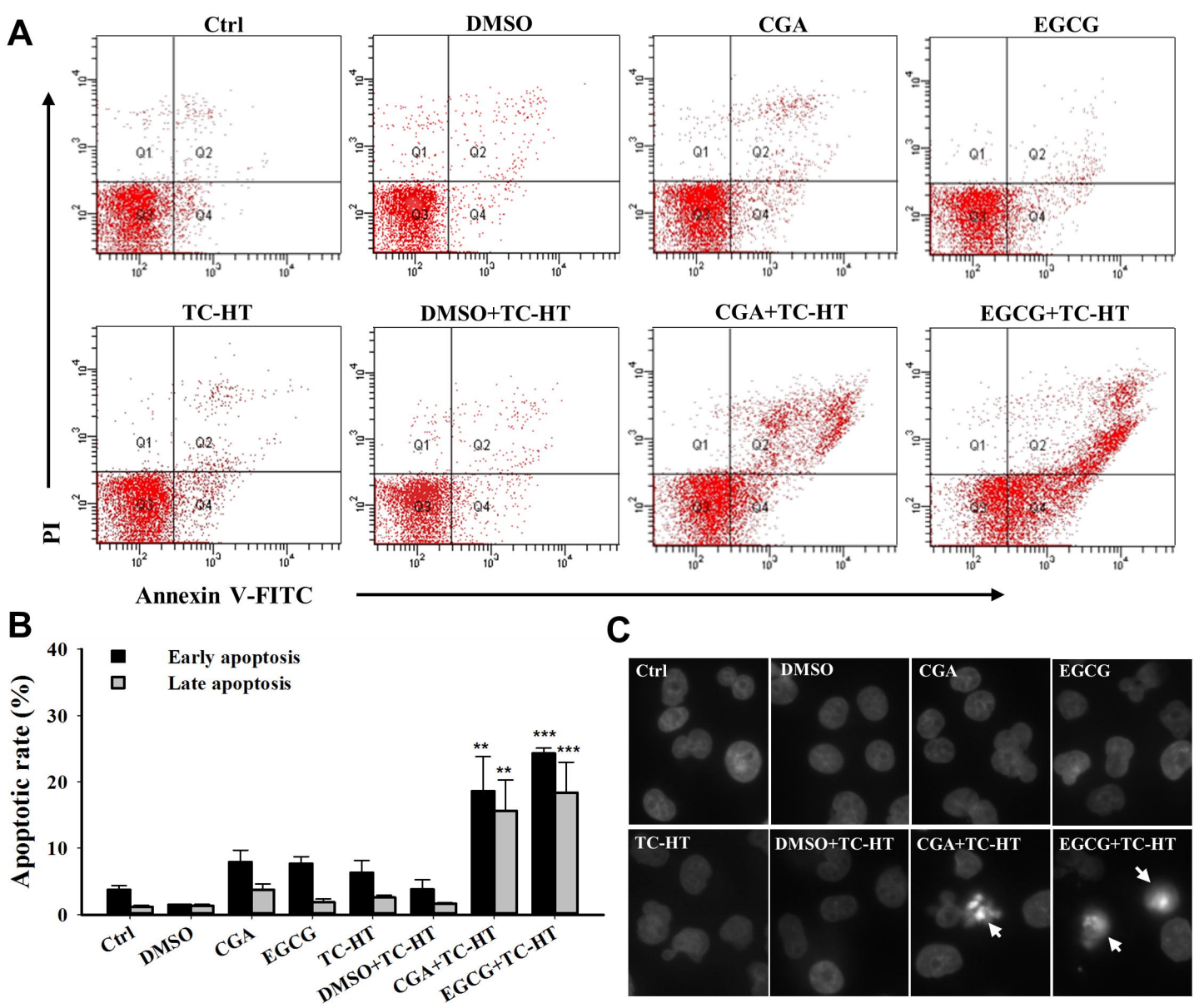
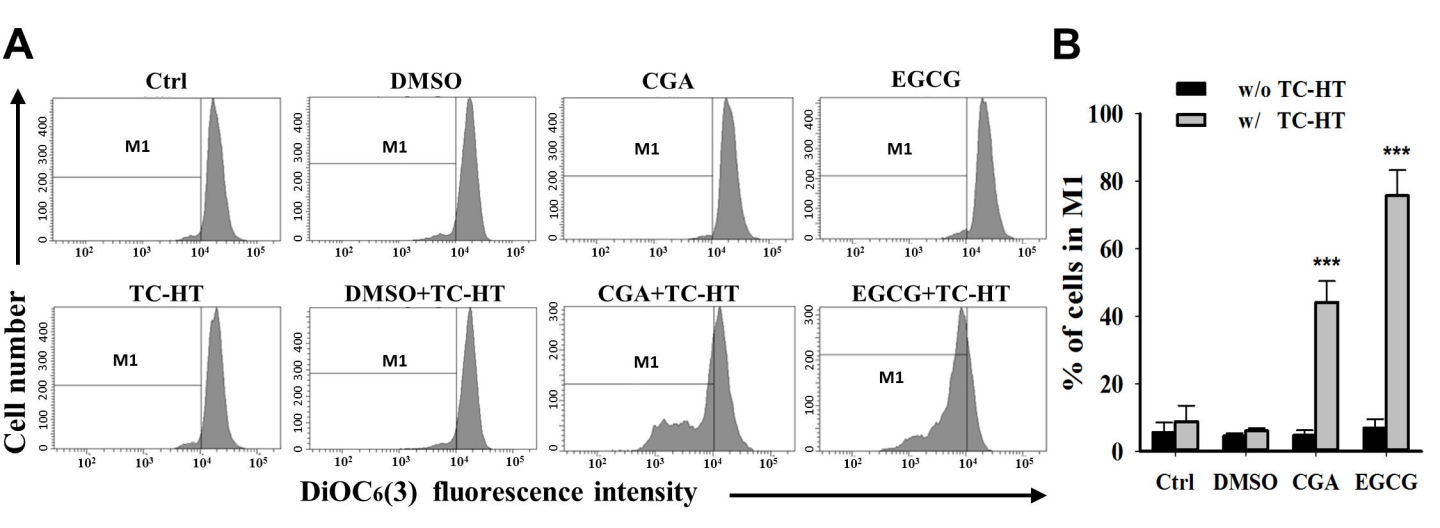
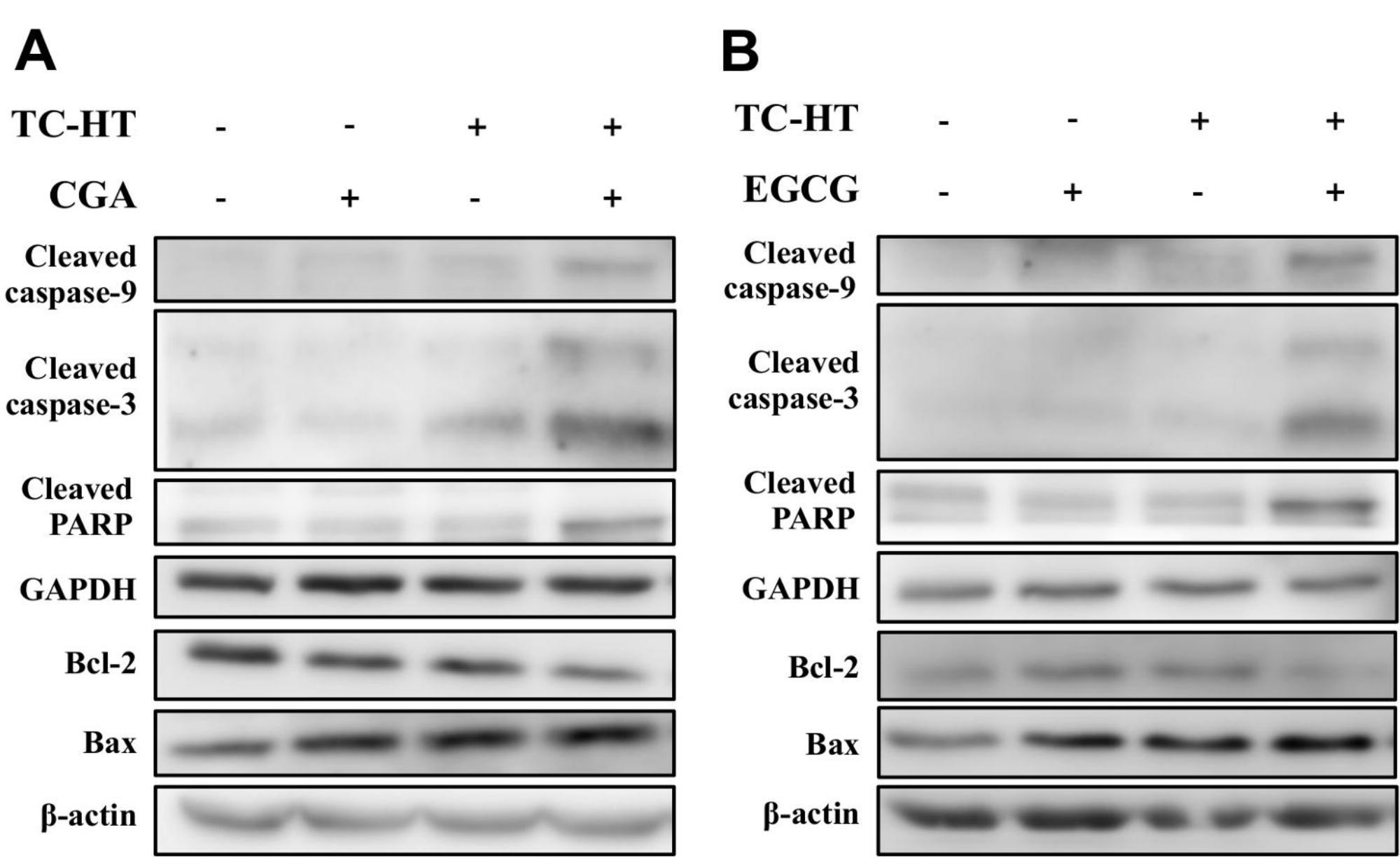
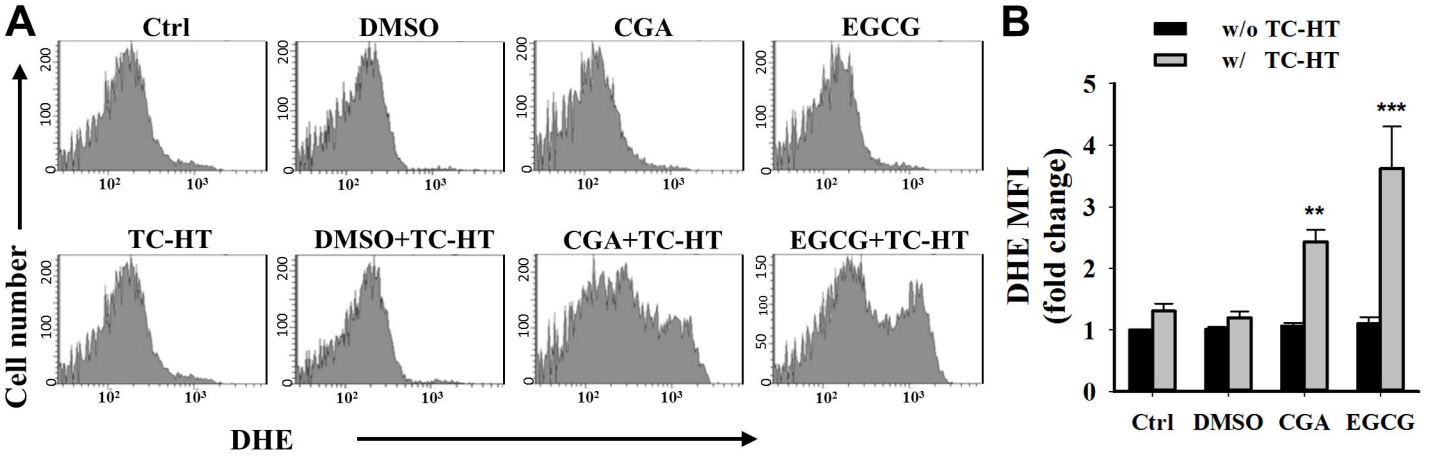
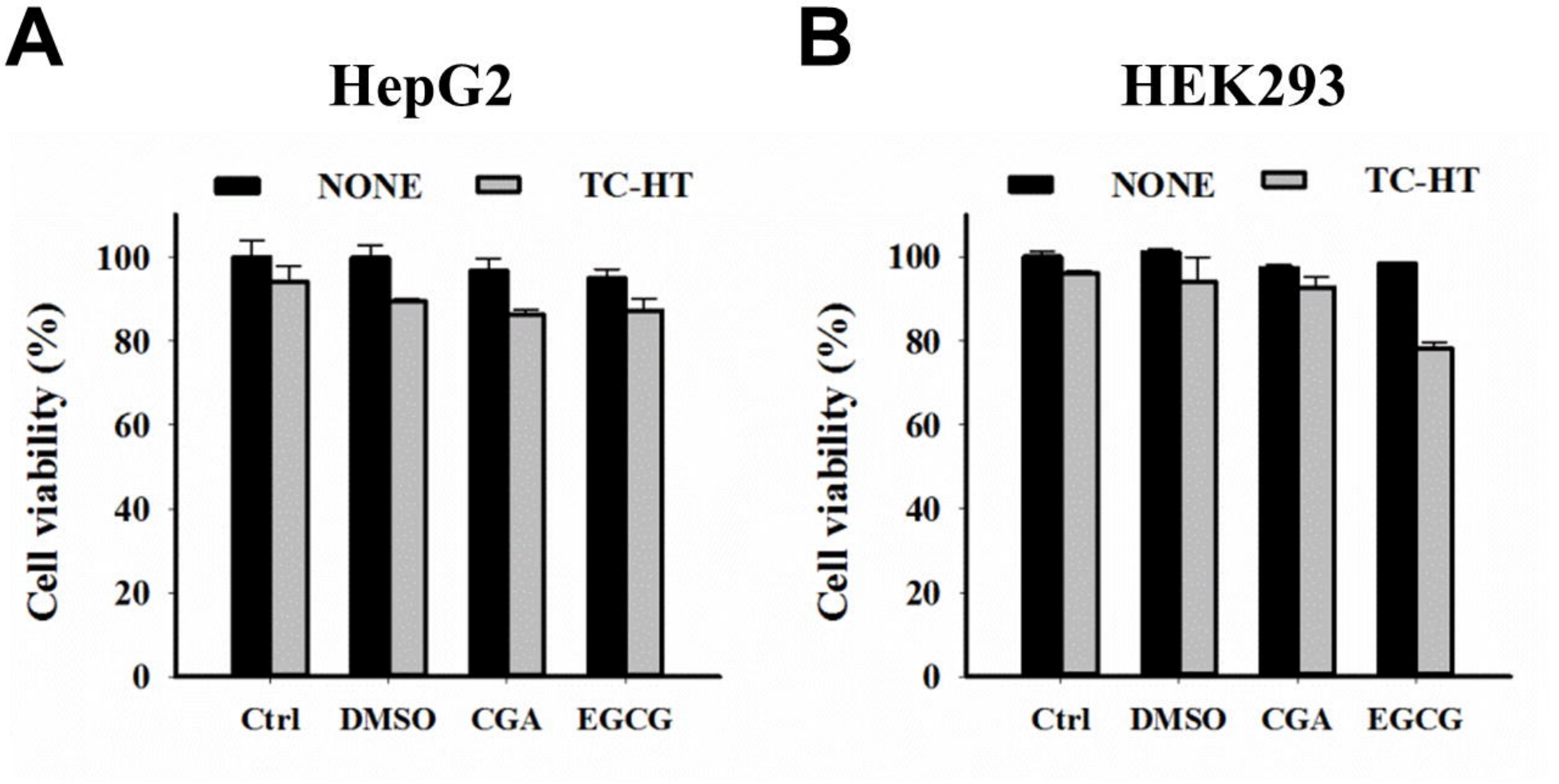
G1 59.67%
S 24.86%
G2/M 15.47%
DMSO
G1 56.06%
S 25.52%
G2/M 18.41%
CGA
G1 53.02%
S 27.09%
G2/M 19.89%
EGCG
G1 50.24%
S 26.64%
G2/M 23.12%
TC-HT
G1 57.21%
S 24.77%
G2/M 18.02%
DMSO+TC-HT
G1 53.99%
S 23.17%
G2/M 22.84%
CGA+TC-HT
G1 44.35%
S 21.60%
G2/M 34.05%
EGCG+TC-HT
G1 37.21%
S 20.87%
G2/M 41.92%
Cell number
DNA content
B
G0/G1
S
G2/M
Cell cycle phase (%)
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
***
C
TC-HT
CGA
cyclin B1
Cdc2
β-actin
TC-HT
EGCG
cyclin B1
Cdc2
β-actin

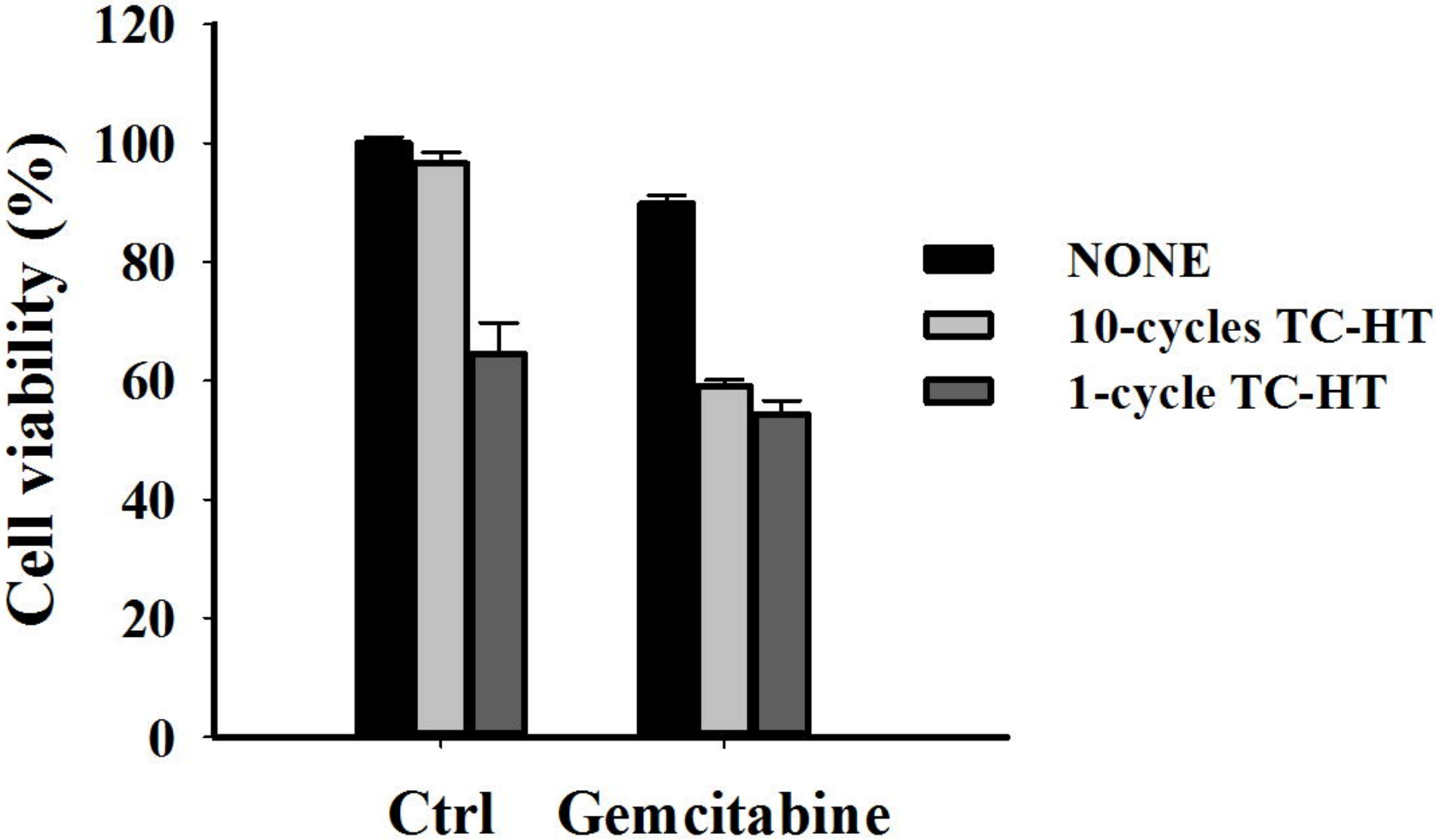

A
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
Q1
Q2
Q3
Q4
PI
Annexin V-FITC
B
Early apoptosis
Late apoptosis
Apoptotic rate (%)
40
30
20
10
0
**
**
***
***
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
C
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT


A
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
M1
Cell number
DiOC6(3) fluorescence intensity
B
w/o TC-HT
w/ TC-HT
% of cells in M1
***
Ctrl
DMSO
CGA
EGCG


A
TC-HT - - + +
CGA - + - +
Cleaved caspase-9
Cleaved caspase-3
Cleaved PARP
GAPDH
Bcl-2
Bax
β-actin
B
TC-HT - - + +
EGCG - + - +
Cleaved caspase-9
Cleaved caspase-3
Cleaved PARP
GAPDH
Bcl-2
Bax
β-actin


A
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
Cell number
DHE
B
w/o TC-HT
w/ TC-HT
DHE MFI
(fold change)
**
***
Ctrl
DMSO
CGA
EGCG


A
HepG2
NONE
TC-HT
Cell viability (%)
100
80
60
40
20
0
Ctrl
DMSO
CGA
EGCG
B
HEK293
NONE
TC-HT
Cell viability (%)
100
80
60
40
20
0
Ctrl
DMSO
CGA
EGCG


120
100
80
60
40
20
0
Cell viability (%)
Ctrl
Gemcitabine
NONE
10-cycles TC-HT
1-cycle TC-HT