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*

49 thermal synergistic study of natural compounds in the treatment of pancreatic cancer,
50 but also highlight the potential of the TC-HT as an alternative strategy in thermal
51 treatment.
52

53 # Introduction

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

63 Hyperthermia (HT) has emerged as a promising method for treating cancer over
64 the past decades [3]. It is a procedure exposing the tumor tissue to high temperatures
65 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
69 directly activate the immune response against the tumors, increase the tumor
70 oxygenation, and improve the drug delivery [7-9]. Although these encouraging results
71 have expanded our understanding of the cytotoxic effects of HT on the cancer cells, in
72 the case of HT as single treatment, it has been shown not to be sufficient to kill cancer
73 cells [10]. To strengthen the effectiveness of HT, several investigations have explored
74 combinations of HT and other cancer therapies, such as radiotherapy and
75 chemotherapy [11]. It has been demonstrated to be effective against various types of
76 cancer, including pancreatic cancer, in that HT enhanced the cytotoxicity of
77 gemcitabine through the inhibition of nuclear factor kappa B (NF-κB) [12-14]. There
78 have also been reports of gemcitabine and other drugs, such as cisplatin and
79 carbonplatin, combined with HT, that demonstrated the clinical efficacy in patients
80 with pancreatic cancer [15, 16]. These data suggest that HT could modify the
81 cytotoxicity of the anticancer drugs, thereby yielding better outcomes in treating
82 pancreatic cancer. However, the drugs used in these combined treatments are

83 conventional chemotherapeutic drugs, which have been known to cause unpleasant
84 and even dangerous side effects.

85 Nowadays, there has been an increasing interest in natural compounds research
86 due to their lower toxicity and diverse biological properties. Phenolic compounds are
87 among the most studied in cancer prevention and cure, and also the largest group of
88 phytochemicals, as well as being widely distributed in our diet. Particularly, regular
89 intakes of dietary polyphenols have been linked to lower risks of many cancers [17].
90 Tea and coffee are two of the most consumed beverages worldwide, and the natural
91 phenolic compounds, epigallocatechin gallate (EGCG) and chlorogenic acid (CGA),
92 are the major components in both drinks, respectively. It has been shown that EGCG
93 and CGA have healthy benefits such as antioxidative, anti-inflammatory, and
94 anticancer activities [18-20]. Recently, published findings from animal experimental
95 [21, 22] and clinical studies [23, 24] have demonstrated the ability of EGCG and
96 CGA to suppress tumor cell growth such as breast, lung and bladder cancer. On the
97 other hand, there are a number of studies indicating that natural compounds, including
98 polyphenols, can act as heat synergizers, and thereby improve the anticancer effect
99 [25-29]. Therefore, this has prompted us to conduct the first combined experiment of

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

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

117 effective heat synergizers and that the maximum synergistic effects on the PANC-1
118 cells could be obtained when the TC-HT was administered. Furthermore, the TC-HT
119 as a thermal treatment could be much gentler and feasible, mainly because of the view
120 that the TC-HT itself is relatively harmless to the cells. We believe that this study
121 provides the interesting concept of cyclic thermal application in treatment of cancer *in*
122 *vitro*, and highlights the potential of the TC-HT as an alternative thermal treatment.
123

124 Materials and methods

125 Cell culture

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

132 Drug treatment and TC-HT exposure

133 The EGCG was dissolved in distilled water at a concentration of 20 mM, and the
134 CGA was dissolved in dimethyl sulfoxide (DMSO)(Sigma). Subsequently, the two
135 samples were stored at -20°C. The stocks were diluted with a culture medium to the
136 indicated concentration for treatment before usage, and the final concentration of
137 DMSO in each well was 0.05% (v/v). After overnight incubation at 37°C, cells were
138 treated with various concentrations of EGCG or CGA, as well as the solvent vehicle
139 (0.05% DMSO) for CGA. For the TC-HT, the cells were subject to a high and low
140 temperature water bath using a temperature controller modified by a PCR machine
141 (**Fig 1A**). The cells were exposed to HT (1-cycle TC-HT) at 46°C for 30 min without
142 a break or treated with 46-30°C TC-HT through 3, 6, and 10 cycles of 10, 5, and 3
143 min, respectively, to receive the equivalent thermal dosages (**Fig 1B**). In the
144 combined treatment, cells in a medium containing the EGCG or CGA were exposed
145 to the TC-HT. The temperatures of the cancer cells actually sensed were monitored by
146 placing a needle thermocouple at the bottom of the well (**Fig 1C**). During heat
147 exposure experiment, the non-heating groups (non-treated and drug-treated groups)
148 were exposed to a similar ambient environment as the heating groups over the

149 experimental period of time. After the treatment was completed, cells were stored in a

150 cell culture incubator until the time of analysis.

151

152 **Fig 1. Exposure to the TC-HT via a temperature controller.** (A)(B) Schematic of

153 experiment setup and the TC-HT programs setting. (C) The actual temperature

154 recorded every 20 sec in the PANC-1 cells throughout the exposure period.

155

156 MTT assay

157 Cell viability was accessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

158 bromide (MTT) (Sigma) assay. Cells were seeded in 24-well plates and incubated

159 overnight at 37°C. After a single-agent treatment or combined treatment of the

160 TC-HT with the 0.05% DMSO (vehicle control), EGCG or CGA for 24 h, the

161 medium was removed and the cells were washed with phosphate buffered saline

162 (PBS). Cells were then incubated in DMEM containing 0.5 mg/ml MTT for an

163 additional 4 h at 37°C. Then the medium was removed and DMSO was added to

164 dissolve the formazan crystals. The supernatant from each sample was transferred into

165 96-well plates, and the absorbance was read at 570 nm using an ELISA microplate

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

169 Clonogenic survival assay

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

178 Cell cycle analysis

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

183 Bioscience) and RNase A (Thermal Scientific) for 30 min in the dark. The stained
184 cells were subject to the cell cycle analysis by using a flow cytometer (FACSCanto II;
185 BD Biosciences), and data were analyzed with ModFit LT software.

186 DAPI staining assay

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

193 Annexin-V/PI double staining assay

194 Apoptosis was determined by using the Annexin V-FITC/PI apoptosis detection kit
195 (BD Biosciences). Briefly, PANC-1 cells were treated with the TC-HT combined with
196 the DMSO (vehicle control), CGA or EGCG, and then the cells were harvested with
197 trypsin-EDTA (Gibco) and collected by centrifugation at 2,000 × g for 5 min, washed
198 twice with cold PBS, and resuspended in binding buffer containing Annexin V-FITC

199 and PI. The cell suspensions were incubated for 15 min at room temperature in the

200 dark and analyzed by a FACS Calibur flow cytometer.

201 Mitochondria membrane potential (MMP) measurement

202 The cells treated with 0.05% DMSO (vehicle control), CGA, EGCG and TC-HT

203 alone or in combination for 24h were harvested, and resuspended with PBS followed

204 by staining with 20 nM $DiOC_6(3)$ (Enzo Life Sciences International Inc.) for 30 min

205 at 37°C in the dark. The fraction of cells showing low MMP was then measured by a

206 flow cytometer.

207 ROS detection

208 Cellular Reactive Oxygen Species (ROS) levels of superoxide anion ($O_2^{\bullet-}$) were

209 detected using the fluorescent dye dihydroethidium (DHE) (Sigma). Cells were

210 treated with the indicated treatments, washed with PBS, and then incubated with 5

211 μM DHE for 30 min at 37°C in the dark. The fluorescence intensities were measured

212 by flow cytometry and ROS levels were expressed as mean fluorescence intensity

213 (MFI).

214 Western blot analysis

215 After treatment with CGA, EGCG, and TC-HT for 24 h alone or in combination, cells
216 were harvested, washed with cold PBS, and lysed on ice for 30 min in lysis buffer (50
217 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1.0%
218 Triton X-100, 0.1% SDS, 1 mM EDTA, 1% phosphate and protease inhibitor cocktail)
219 (Millipore). Cell lysates were clarified by centrifugation at 23,000 × g for 30 min at
220 4°C, and the protein concentration in the supernatant fraction was quantified using the
221 Bradford protein assay (Bioshop). Proteins were resolved by 10% SDS-PAGE and
222 electrotransferred onto polyvinylidene fluoride membrane (PVDF) (Millipore) in
223 transfer buffer (10 mM CAPS, pH 11.0, 10% methanol). The membranes were
224 blocked with 5% nonfat dry milk/TBST (blocking buffer) for 1 h at room temperature
225 and then incubated overnight at 4°C with diluted primary antibodies in blocking
226 buffer. The specific primary antibodies against Bcl-2, cleaved caspase-8, cleaved
227 caspase-9, cleaved caspase-3 (Cell Signaling), Bax (Santa Cruz), Cdc2, cyclin B1,
228 cleaved PARP and β-actin (GeneTex) were used. After washing with TBST, the
229 membranes were incubated with HRP-conjugated anti-goat (GeneTex) or anti-rabbit
230 (Jackson Immunoresearch) secondary antibody. Chemiluminescence was detected
231 using WesternBright ECL western blotting reagent (Advansta).

232 ## Statistical analysis

233 The results were presented as mean ± standard deviation (SD). Statistical analysis
234 using one-way analysis of variance (ANOVA) performed with SigmaPlot software.
235 The results were considered to be statistically significant when *p*-values were less
236 than 0.05. Each experiment was done in triplicate.

237

238 # Results

239 ## TC-HT in combination with polyphenols synergistically
240 ## inhibits proliferation of PANC-1 cells

241 The TC-HT was performed by a modified PCR machine to raise the temperature to a
242 desired level followed by a rapid return to normothermic temperature. The actual
243 temperature in the cells measured by a needle thermocouple could be elevated from
244 36°C to 43.5, 44.2 and 44.9^{o}C within 3, 5 and 10 min, respectively, and rapidly
245 returned to the physiological temperature (**Fig 1C**). The effect of the TC-HT and the
246 CGA or EGCG on cell growth was first explored using MTT assay. As shown in **Figs**
247 **2A** and **2B**, cells were treated with various concentrations of the CGA or EGCG for
248 24 h, and both of the compounds only slightly affected the viability of PANC-1 cells.

249 DMSO (0.05%) treated cells with or without the TC-HT did not show a significant
250 difference in viability compared to untreated control cells. Moreover, the viability of
251 PANC-1 cells in response to treatment with the TC-HT decreased in a
252 cycle-dependent manner. When the TC-HT was combined with either of the two
253 compounds, a significant dose-dependent decrease in viability was observed at all
254 four cycling parameters. Although the 1-cycle TC-HT and either the compound
255 cooperatively reduced the proliferation of PANC-1 cells, the heat alone resulted in
256 significant cytotoxicity to the cells. Notably, there was no difference in the cell
257 viability under the condition of the 10-cycles TC-HT when compared to that in the
258 control group, while it still was capable of working synergistically with either the
259 CGA or the EGCG to exert the anti-proliferative activity. The SQ values for the
260 corresponding treatments are as shown in **Table 1**. This suggests that both the CGA
261 and the EGCG could exhibit a synergistic cytotoxic effect when co-administered with
262 the TC-HT, particularly in the treatment of 10 cycles. We next performed a
263 clonogenic survival assay to confirm the effect of the TC-HT combined with CGA or
264 EGCG on cell proliferation. As shown in **Figs 2C** and **2D**, colony formation in
265 PANC-1 cells was dramatically reduced following both combined treatments. Based

266 on these data, the 10-cycles TC-HT (43.5-36 °C) in combination with the

267 concentration of 200 μM CGA, or the concentration of 20 μM EGCG, was used for

268 all the following experiments.

269

270 **Fig 2. Effects of CGA or EGCG combined with the TC-HT on proliferation in**

271 **PANC-1 cells.** (A) The cells were treated with different cycle numbers of the

272 TC-HT, various concentrations of CGA and 0.05% DMSO (vehicle control) alone or

273 in combination for 24 h. (B) The cells were treated with different cycle numbers of

274 the TC-HT and various concentrations of EGCG alone or in combination for 24 h. (C)

275 Representative images of clonogenic survival assay. (D) Analysis of colony formation

276 rate. Data are presented as mean ± S.D. in triplicate.

277

278 **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
	10	1.18	1.11	1.27	1.95
EGCG (μM)	**20**	1.28	1.35	1.76	3.09
	30	1.23	1.40	1.70	2.69

279

280 **The combination of CGA or EGCG with TC-HT caused**

281 **G2/M cell cycle arrest in PANC-1 cells**

282 To evaluate the mode of the anti-proliferative effects of the TC-HT, CGA and

283 EGCG, the cell cycle progression in the PANC-1 cells was examined by flow

284 cytometric analysis. As shown in **Figs 3A** and **3B**, treatment with the CGA, EGCG,

285 and TC-HT had no obvious effect on the cell progression when compared with the

286 group of untreated cells. Also, 0.05% DMSO used as the solvent vehicle of CGA

287 either alone or in combination with TC-HT had no effect on the cell progression.

288 Interestingly, the TC-HT combined with either of the two polyphenols resulted in a

289 significant accumulation of cells in the G2/M phase with a concomitant reduction of

290 cells in the G1 phase. To investigate the molecular mechanism of the results of the

291 combined effect on the cell cycle distribution, we examined the relevant proteins

292 involved in the G2/M progression. As shown in **Fig 3C**, the expression of Cdc2 and

293 cyclin B1 were markedly reduced in response to the combined treatment with TC-HT

294 and CGA or the combined treatment with TC-HT and EGCG. These results indicate

295 that either the CGA or EGCG combined with the TC-HT synergistically induced cell

296 cycle arrest in the G2/M phase by decreasing cyclin B1 and Cdc2 in the cells, and

297 eventually inhibited the cell growth of PANC-1 cells.

298

299 **Fig 3. Effects of CGA or EGCG combined with the TC-HT on the G2/M cell**

300 **cycle arrest in PANC-1 cells.** Cells were treated with the TC-HT (10 cycles), 0.05%

301 DMSO (vehicle control), CGA (200 μM), and EGCG (20 μM) alone or in

302 combination (TC-HT + DMSO, TC-HT + CGA, TC-HT + EGCG) for 24 h and

303 stained with propidium iodide (PI) for cell cycle analysis. (A) Representative DNA

304 content profiles. (B) The percentage of cell population in each phase of the cell cycle.

305 (C) Western bolt analysis of the expression of cell cycle regulator proteins cyclin B1

306 and Cdc2. β-actin was used as an internal control. Data are presented as mean ± S.D.

307 in triplicate. ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated control)

308

309 **TC-HT combined with CGA or EGCG induces apoptosis in**

310 **PANC-1 cells**

311 We then investigated as to whether the synergistic cytotoxic effect of the TC-HT

312 with the CGA or EGCG was associated with the induction of apoptosis, and the flow

313 cytometric analysis was performed using Annexin V-FITC/PI staining. As shown in

314 **Figs 4A** and **4B**, the fraction of apoptotic cells after the treatment of PANC-1 cells

315 with either the single agent CGA, or the EGCG or TC-HT were barely increased as

316 compared with untreated control cells. There was no significant difference in

317 apoptosis between the 0.05% DMSO (vehicle control) combined with or without

318 TC-HT and the untreated control. Interestingly, the TC-HT in combination with the

319 CGA significantly increased the early apoptotic cell death in PANC-1 cells

320 (18.6±5.2%). A similar result was also obtained in the combination of the TC-HT and

321 the EGCG, but to a greater extent (24.3±0.8%). These results also showed that the late

322 apoptotic cell death was increased following the exposure of TC-HT with CGA

323 (15.7±4.6%) or EGCG (18.4±4.6%). Furthermore, in contrast to control cells, the

324 DAPI staining demonstrated that the cells treated with either of the two combined

325 treatments had more condensed and fragmented nuclei, which are typical

326 morphological alterations for the apoptosis (**Fig 4C**). These results suggest that the
327 TC-HT has a potential synergistic effect with the CGA or EGCG on the apoptosis in
328 the PANC-1 cells.
329
330 **Fig 4. Combination of the TC-HT and the CGA or EGCG induces apoptosis in**
331 **PANC-1 cells.** The apoptosis analysis for the cells following the treatment with the
332 TC-HT (10 cycles), 0.05% DMSO (vehicle control), CGA (200 μM), and EGCG (20
333 μM) alone or in combination (TC-HT + DMSO, TC-HT + CGA, TC-HT + EGCG)
334 for 24 h. (A) Flow cytometric detection of the apoptosis with Annexin V-FITC/PI
335 double staining. (B) Histogram quantifying the percentage of PANC-1 cells in early
336 and late apoptosis. (C) The nuclei morphology alterations (arrow) were examined
337 using DAPI staining. Data are presented as mean ± S.D. in triplicate. ($^{**}p < 0.01$ and
338 $^{***}p < 0.001$ compared with the untreated control)
339

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

343 Collapse of the mitochondrial integrity is a critical event in the cells undergoing
344 apoptosis. To examine whether the combination of the TC-HT and CGA- or
345 EGCG-induced apoptosis involved mitochondrial disruption, the mitochondrial
346 membrane potential (MMP) was assessed using $DiOC_6(3)$ fluorescence staining by
347 flow cytometric analysis [32]. As shown in **Figs 5A** and **5B**, the treatment with the
348 EGCG, the CGA and its corresponding vehicle control (0.05% DMSO) did not
349 change the MMP level in comparison with the untreated control. In response to
350 treatment with the TC-HT alone or together with vehicle control, there was no
351 significant change in MMP within cells; however, the cells treated with TC-HT
352 showed a stronger effect on the MMP depolarization after the administration of CGA
353 or EGCG (44±6.4% and 75.7±7.6%), which was consistent with the results of the
354 apoptosis analysis as described in **Fig 4**. These results indicate that the TC-HT
355 combined with the CGA- or EGCG-induced apoptosis in the PANC-1 cells is
356 mediated by mitochondrial dysfunction.
357
358 **Fig 5. Combination of the TC-HT and the CGA or EGCG induces mitochondrial**
359 **dysfunction.** (A) Flow cytometric analysis of MMP using $DiOC_6(3)$ after treatment

360 with the TC-HT (10 cycles), 0.05% DMSO (vehicle control), CGA (200 μM), and
361 EGCG (20 μM) alone or in combination (TC-HT + DMSO, TC-HT + CGA, TC-HT +
362 EGCG) for 24 h. The M1 regions indicate the cells with the loss of MMP. (B)
363 Histogram represents the percentage of cells in the M1 region. Data are presented as
364 mean ± S.D. in triplicate. ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated
365 control)
366

367 Combination of the TC-HT with CGA or EGCG induces
368 apoptosis through the mitochondrial pathway

369 It is well known that Bcl-2 family proteins and caspases, along with PARP, play
370 important roles in the mitochondria-mediated apoptosis. To further explore the
371 mechanism by which the TC-HT combined with the CGA or EGCG triggered
372 apoptosis in the PANC-1 cells, we evaluated the expression of the apoptosis-related
373 proteins using western blot analysis. As shown in **Fig 6**, when compared with control
374 cells, the cleaved caspase-9, -3, and the cleaved PARP were markedly increased after
375 the co-administration with the TC-HT and either the CGA or EGCG. In addition, both
376 of the combined treatments down-regulated the expression of Bcl-2 and up-regulated

377 the expression of Bax, and thus decreased the ratio of Bcl-2 to Bax. These results
378 reveal that the TC-HT combined with the CGA or EGCG promotes apoptosis in the
379 PANC-1 cells via activation of the mitochondrial pathway.
380
381 **Fig 6. Effects of the TC-HT, CGA and EGCG on the expression of the**
382 **apoptosis-related proteins in PANC-1 cells.** (A)(B) The protein levels of cleaved
383 caspase-9, -3, cleaved PARP, Bcl-2 and Bax of the PANC-1 cells treated with the
384 TC-HT (10 cycles), CGA (200 μM) and EGCG (20 μM) alone or in combination
385 treatment (TC-HT + CGA, TC-HT + EGCG) for 24 h were examined by western blot
386 analysis. GAPDH and β-actin were used as internal controls.
387

388 The role of ROS in the TC-HT combined with the CGA- or
389 EGCG-induced apoptosis in PANC-1 cells

390 As ROS generation is a critical event in the induction of the apoptosis, we next
391 examined the role of ROS in apoptosis induced by treatment with the TC-HT in
392 combination with either the CGA or EGCG. The intracellular ROS was measured by
393 flow cytometry using a fluorescence probe, DHE, which reacts with $O_2^{\bullet-}$ [33]. As

394 shown in **Fig 7A**, the treatment of PANC-1 cells with 0.05% DMSO (vehicle control),
395 CGA or EGCG alone did not alter the level of ROS when compared with the
396 untreated control cells. Also, the level of ROS induced by TC-HT and its combination
397 with DMSO were found to be comparable to the untreated control in the MFI. It is
398 worth mentioning that the TC-HT combined with CGA and the TC-HT combined
399 with EGCG significantly increased the level of ROS in MFI by approximately 2.3-
400 and 3.6-fold, respectively (**Fig 7B**). These results suggest that the TC-HT combined
401 with the CGA or EGCG may induce apoptosis through the excessive ROS production
402 in the PANC-1 cells.
403
404 **Fig 7. Combined effects of the TC-HT and the CGA or EGCG on ROS**
405 **generation in PANC-1 cells.** (A) The DHE ($O_2^{\bullet-}$) levels were measured by flow
406 cytometry after treatment with the TC-HT (10 cycles), 0.05% DMSO (vehicle
407 control), CGA (200 μM), and EGCG (20 μM) alone or in combination (TC-HT +
408 DMSO, TC-HT + CGA, TC-HT + EGCG) for 24 h. (B) Graph shows the fold change
409 in MFI of DHE relative to control. Data are presented as mean ± S.D. in triplicate.
410 ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the untreated control)

411

412 # Discussion

413 Despite previous studies having shown that HT or polyphenols, the EGCG and
414 CGA, possessed anticancer activities against pancreatic cancer cells [34-37], the
415 effect of these two agents as a combined therapy on pancreatic cancer has not been
416 reported. In this study, we have explored as to whether the herbal compound, CGA or
417 EGCG, could cooperate with heat against the pancreatic cancer cells, and elucidated
418 the cellular mechanism underlying the biological effects. Our results have
419 demonstrated that the exposure to heat using the TC-HT in combination with the
420 CGA or EGCG synergistically inhibited the growth and induced the apoptosis in the
421 PANC-1 cells. Previous studies have shown the benefits of HT as an adjuvant in
422 combination with chemotherapy against many various cancers, including pancreatic
423 cancer [13, 38, 39]. However, there is a major concern that HT can cause unavoidable
424 thermal injury to normal cells. Therefore, it is important to refine the method of heat
425 administration to achieve the desired thermal dosage with the minimal cytotoxicity.
426 To the best of our knowledge, this is the first report indicating a novel approach
427 TC-HT synergizes with the CGA or EGCG on pancreatic cancer PANC-1 cells.

428 Previous research has reported the response of PANC-1 cells to mild HT at
429 different temperatures [40]. Particularly, the temperature in a range of about 42 to
430 46°C has been shown capable of inducing the cell death of the pancreatic cell line
431 PANC-1. Our survival analysis revealed a consistent result that the cell growth of
432 PANC-1 in the 1-cycle group was significantly inhibited. Although HT has a
433 cooperative cytotoxicity with the CGA or EGCG, its severe thermal toxicity to a cell
434 is what we want to avoid. The usage of the repeated cycles of heat exposure could
435 provide a means to synergize with the anticancer compounds in the heating process,
436 and avoid the thermal damage accumulation in the following non-heating process.
437 Earlier studies have shown the time-dependent modification of cancer cells during
438 exposure to HT for which the analysis of cellular growth indicated that the survival
439 rate decreased with increasing HT exposure time [41, 42]. Namely, the short exposure
440 of cancer cells to HT may induce cellular stress without affecting their proliferation,
441 while the prolonged exposure may lead to cytotoxicity. It has also been reported that
442 short HT treatment of PANC-1 cells at the temperature 46°C for 5 min had no effect
443 on cell viability [43]. Therefore, we suggest that the 10-cycles TC-HT induced stress
444 rather than damage in cells during each heating cycle. As expected, the cells treated

445 with the TC-HT of different cycling parameters exhibited a decreased cytotoxicity
446 with the increased cycles. It is worth noting that 10 cycles of the TC-HT (43.5-36 °C),
447 or either compound, did not cause a significant alteration in the viability, however, the
448 combination of 10 cycles of the TC-HT with the CGA or EGCG strongly resulted in a
449 dose-dependent decline of the cell viability. Similar results from colony formation
450 were also obtained with the cells treated with the combination of TC-HT and CGA or
451 the combination of TC-HT and EGCG. These results suggested that the either CGA or
452 EGCG may act as heat synergizer and co-administered with the use of the TC-HT
453 could have significantly combined effects against pancreatic cancer PANC-1 cells.
454 Furthermore, we have also tested one liver cancer (HepG2) and one non-cancerous
455 (HEK293) cell lines to examine the combined action. Results shown in **S1 Fig**
456 revealed that the 10-cycles TC-HT did not cause cytotoxicity in both cancer and
457 non-cancerous cells. Interestingly, such substantial cell death in the PANC-1 cells
458 treated with the TC-HT in combination with CGA or EGCG was not observed in the
459 HepG2 and HEK293 cells, which indicated cell specificity of the combination
460 treatments.

461 HT has been demonstrated to inhibit the growth of human cancer cells through
462 interfering with the cell cycle progression [27, 29, 44]. In our results, we found that
463 the TC-HT did not cause an obvious accumulation of cells in the G2/M phase. When
464 cells undergoing the TC-HT were given the CGA or EGCG concurrently, the
465 proportion of cells in the G2/M phase was significantly increased. Additionally, this
466 observation was supported by the marked downregulation of Cdc2 and cyclin B1
467 proteins in the combination of the TC-HT and the CGA or EGCG. The Cdc2 (cell
468 division cycle 2), also known as the CDK1 (cyclin-dependent kinase 1), is a core
469 regulator that drives the cells through G2 and into mitosis. Several studies have
470 reported that the binding of Cdc2 to cyclin B1 complex plays an important role in the
471 G2/M progression [45, 46]. Collectively, these results indicated that the synergistic
472 cytotoxicity of CGA or EGCG under the exposure of TC-HT, at least in part, was
473 associated with the inhibition of the Cdc2/cyclin B1 kinase activity.

474 Apoptosis, also the best known form of programmed cell death, plays a pivotal
475 role in defending against cancer. The conventional HT has been demonstrated to
476 affect apoptotic pathways in various types of cancer cells [47]. It has also been
477 reported that polyphenols exhibit anticancer activities against different human cancer

478 cells through activating the apoptotic pathway [48, 49]. However, in our study, the
479 results of apoptosis analysis performed using FITC Annexin V and PI double staining
480 indicated that neither the TC-HT nor these two compounds induced significant
481 apoptotic cells (**Fig 4**). Only when the TC-HT in combination with the CGA or
482 EGCG was conducted, both of the combined treatments significantly elevated the
483 percentage of the early and late apoptotic cells. This finding was further confirmed by
484 the nuclear morphological alterations of the apoptosis in the TC-HT-treated cells in
485 combination with the CGA or EGCG. The apoptotic process can be divided into the
486 death receptor and mitochondrial pathways, and the mitochondrial pathway of cell
487 death is thought to be the major mechanism of apoptosis in mammals. The Bcl-2
488 family proteins are key regulators of the mitochondrial apoptotic pathway, which
489 comprise of both the anti-apoptotic and pro-apoptotic members. Unbalanced
490 Bcl-2/Bax ratio within the cells induces the disruption of the mitochondrial membrane,
491 release of cytochrome *c*, activation of caspases, and the subsequent cleavage of PARP
492 [50, 51]. Results from **Figs 5** and **6** indicated that the combined treatment with the
493 TC-HT and the CGA or EGCG markedly decreased the level of MMP and Bcl-2
494 expression, and increased Bax expression. We also found that the activation of

495 caspase-9, -3, and the cleavage of PARP were promoted with both of the combined
496 treatments **(Fig 6)**. Collectively, these data indicated that the mitochondria-dependent
497 pathway is involved in the synergistic apoptosis following the combined treatments.

498 Mitochondria are widely believed to be the main cellular source of ROS. The
499 excessive production of ROS could result in mitochondrial dysfunction, which in turn
500 triggers the apoptosis [52]. We then confirmed if the observation of mitochondrial
501 dysfunction in cells treated with the TC-HT in combination with the CGA or EGCG
502 was promoted by ROS generation. Our result showed that the cellular ROS generation
503 was elevated significantly following the administration of combination of the TC-HT
504 with the CGA or EGCG, indicating that the high levels of ROS production played an
505 important role in the apoptosis induced by the combination of the TC-HT with CGA
506 and the TC-HT with EGCG. HT has been known to act as an adjuvant treatment
507 modality to improve the cytotoxicity of several anticancer agents such as
508 chemotherapeutic drugs [53-55] and herbal compounds [56, 57]. It is thought that HT
509 can enhance the sensitivity of cancer cells to treatment with drugs [58], thereby
510 presenting synergistic anticancer activity. Therefore, these observations of this study
511 tempted us to suggest that the heat stress during temperature cycles may contribute to

512 the potentiation of the CGA or EGCG-triggered cytotoxic responses. Most
513 importantly, the TC-HT could serve as a mild approach to synergize with the natural
514 compound, CGA or EGCG. Furthermore, we also investigate the feasibility of the
515 TC-HT by using standard clinical compound gemcitabine shown to exhibit thermal
516 enhancement of cytotoxicity when combined with standard HT [53, 59]. Similar result
517 as the combination of 1-cycle TC-HT and gemcitabine was observed from analysis of
518 cell viability in the combined treatment with the 10-cycles TC-HT and gemcitabine,
519 whereas the 10-cycles TC-HT alone had no effect on cell viability (**S2 Fig**). This
520 finding suggests that this approach could be potentially extended to other anticancer
521 agent with heat synergy in cancer research.

522 In conclusion, a novel method for producing a desired thermal dosage is
523 proposed in which it applies repeated thermal treatments of short exposure to prevent
524 the toxic effects from prolonged exposure. Our findings have demonstrated the
525 TC-HT shows capability of synergizing with the natural compound, CGA or EGCG,
526 and minimizing the thermal damage resulting from HT. The synergistic activity
527 against the PANC-1 cells was performed primarily via the G2/M arrest and the
528 apoptosis induced through the ROS-mediated mitochondrial pathway leading to an

529 imbalance between Bcl-2 and Bax, activation of caspase-9 and -3, and the cleavage of
530 PARP. This study represents an interesting concept of cyclic thermal application in
531 treatment of cancer *in vitro*, which may potentially demonstrate an alternative to heat
532 treatment. Gaining an optimal combined effect for a variety of cancers via the
533 examination of the relation between the TC-HT parameters and other anticancer drugs
534 is an attractive subject that warrants further investigation.
535

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543 **Competing Interests**

544 The authors have declared that no competing interests exist.
545

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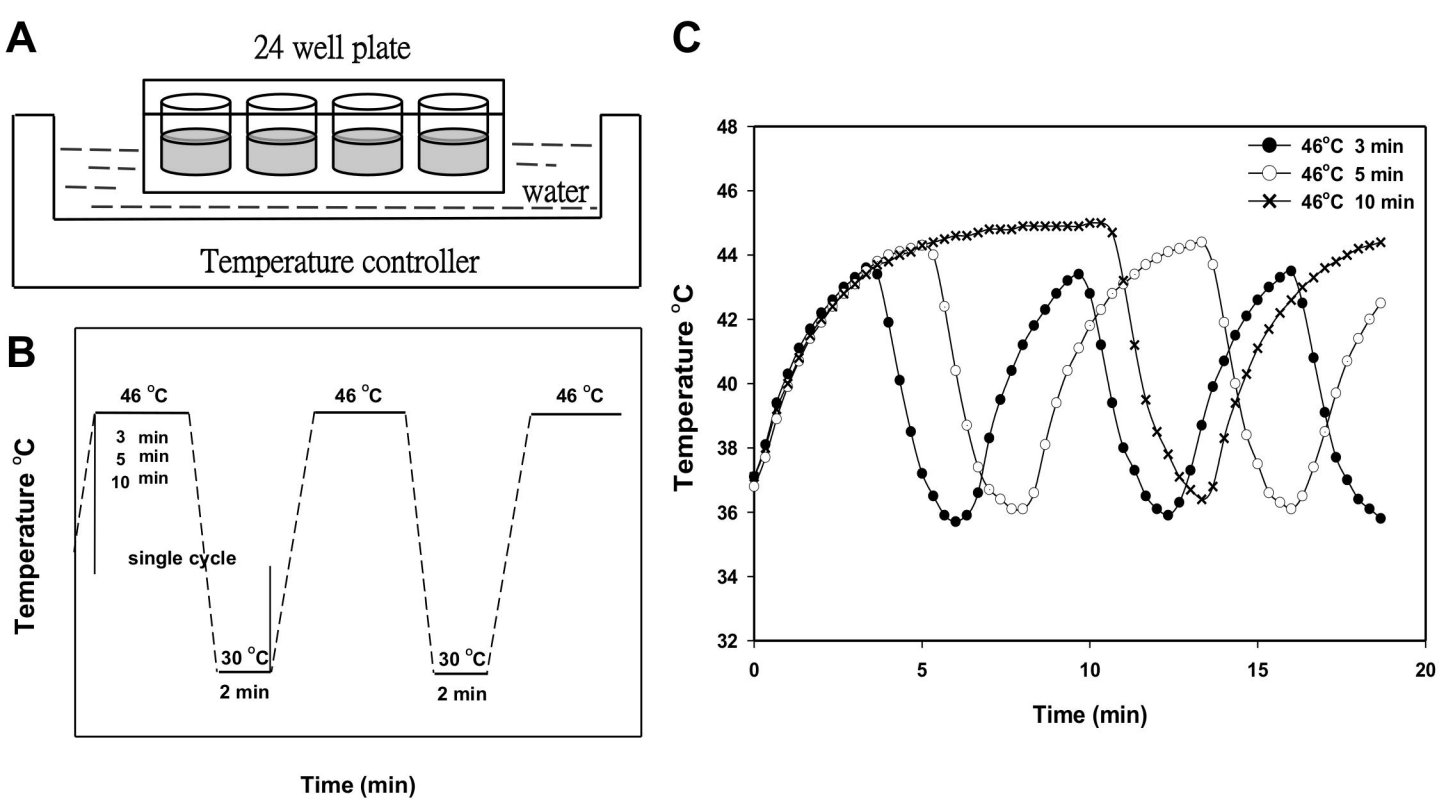
Supporting information

S1 Fig. (A) HepG2 and (B) HEK293 cell viability results from MTT assay after the treatments of CGA, EGCG, and TC-HT (10 cycles) alone or in combination (TC-HT + CGA, TC-HT + EGCG) for 24 h.

S2 Fig. PANC-1 cell viability results from MTT assay after the treatments of gemcitabine (5 μM) and TC-HT (1 cycle and 10 cycles) alone or in combination for 24 h.


A
24 well plate
water
Temperature controller
B
46 °C
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46°C 5 min
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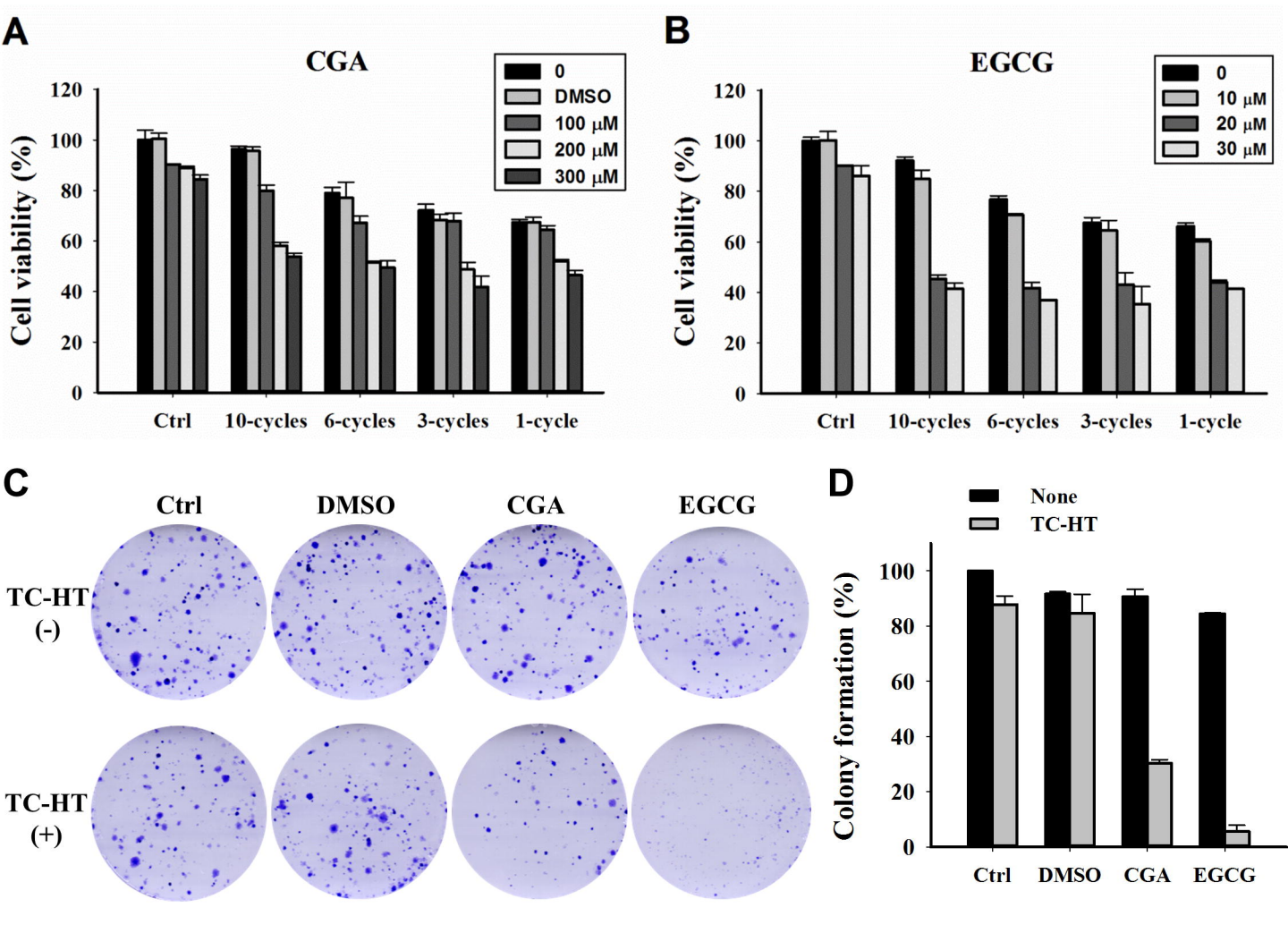
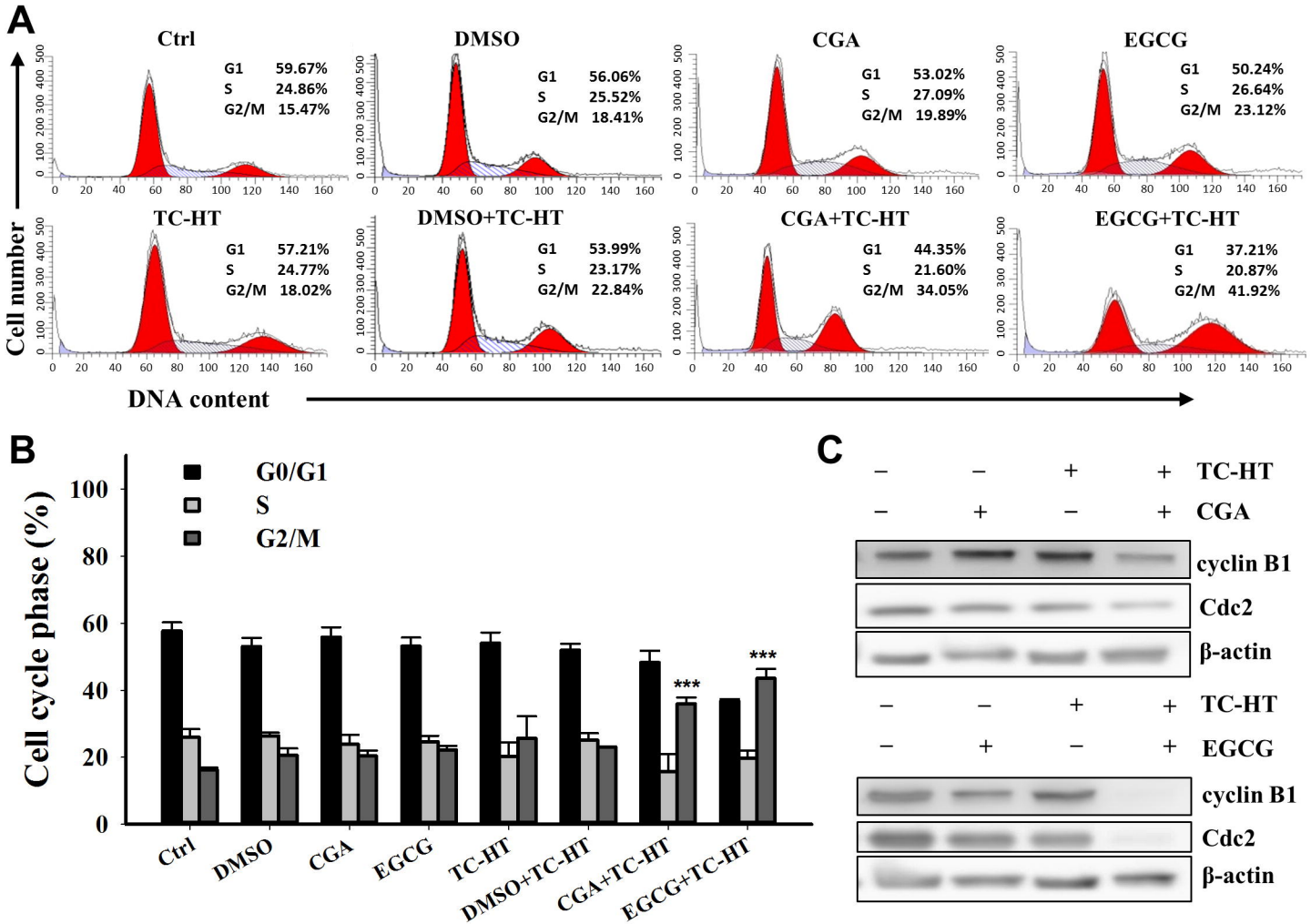
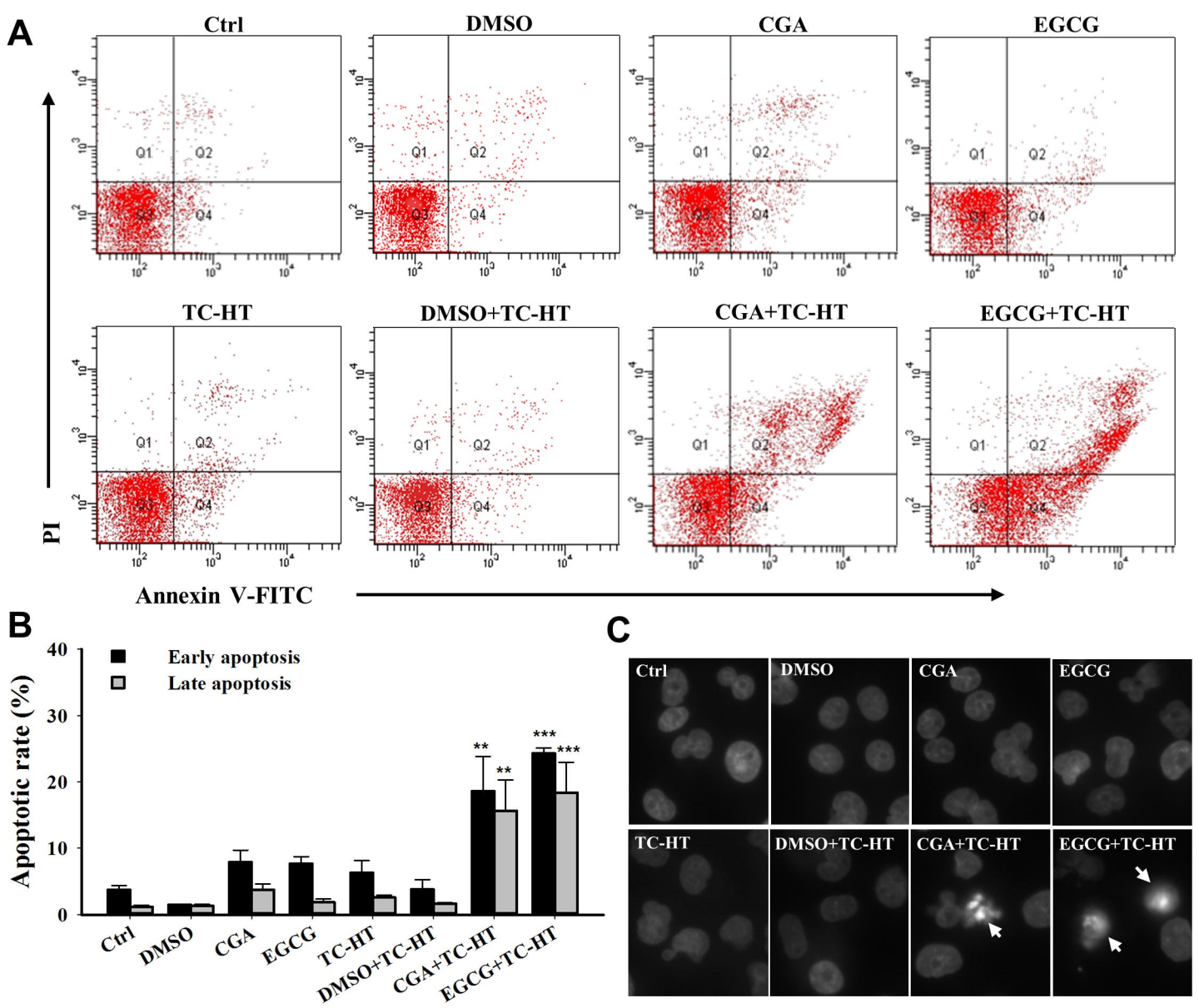
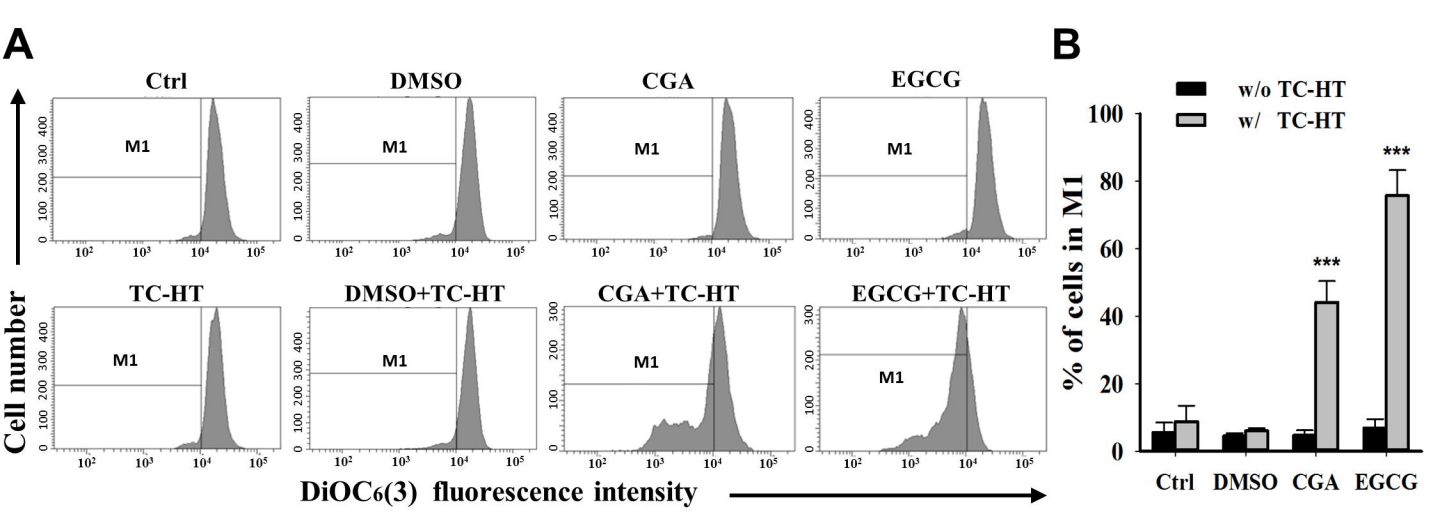
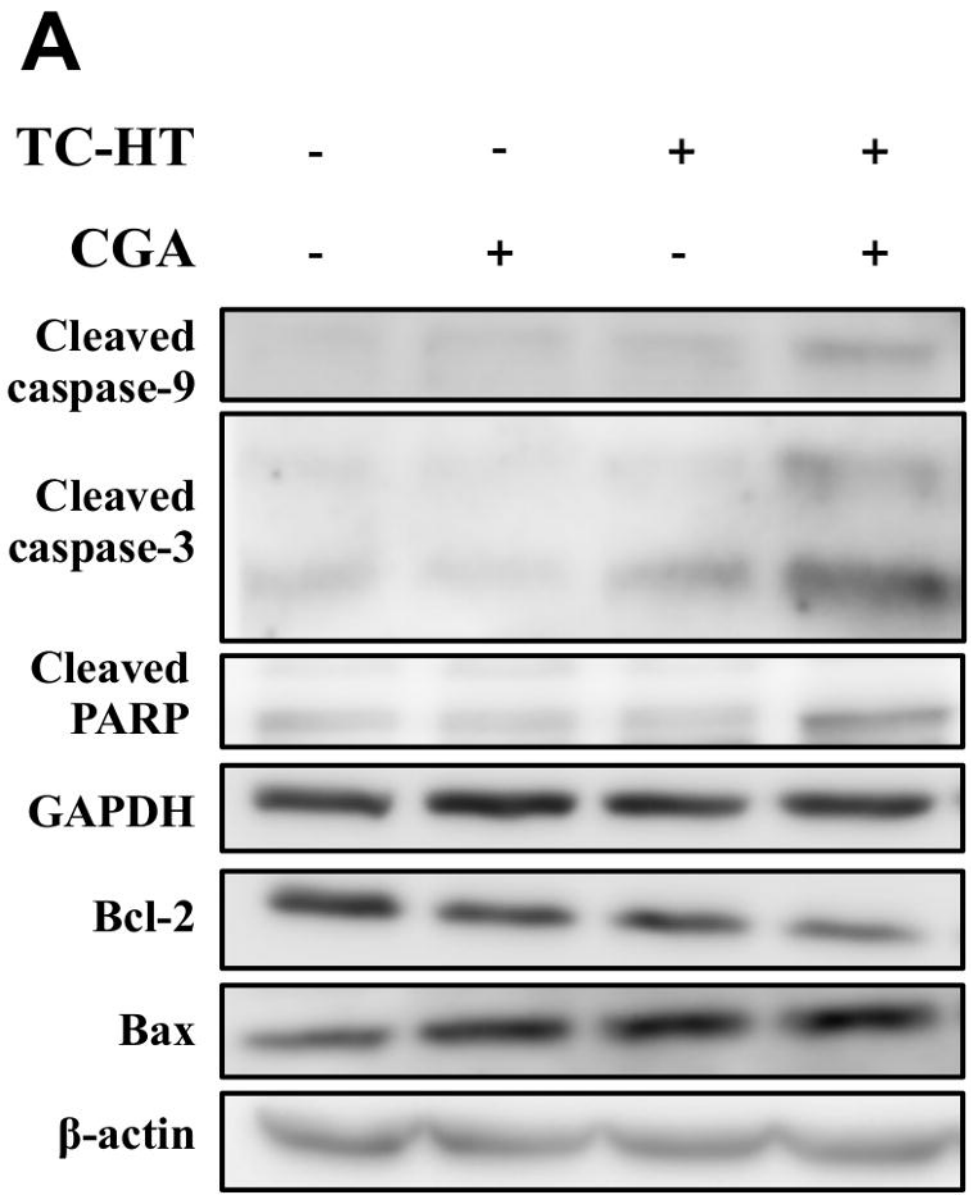
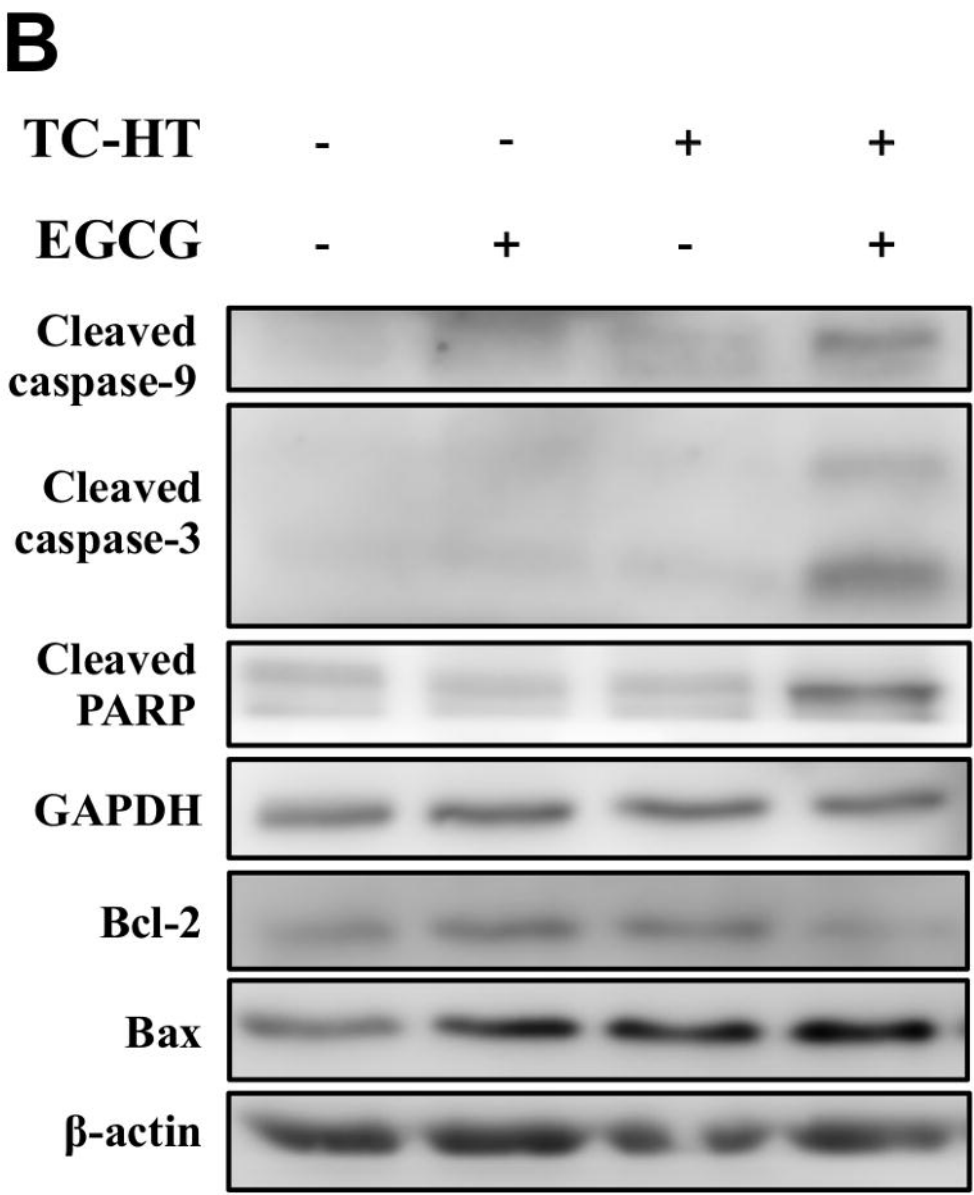

A
CGA
Cell viability (%)
0
DMSO
100 μM
200 μM
300 μM
Ctrl
10-cycles
6-cycles
3-cycles
1-cycle
B
EGCG
0
10 μM
20 μM
30 μM
C
Ctrl
DMSO
CGA
EGCG
TC-HT (-)
TC-HT (+)
D
None
TC-HT
Colony formation (%)

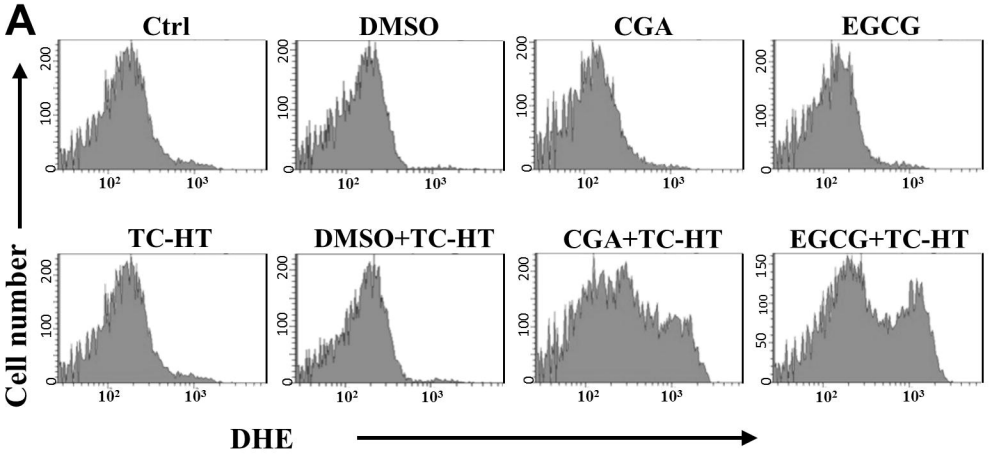

A
Ctrl
G1 59.67%
S 24.86%
G2/M 15.47%
DMSO
G1 56.06%
S 25.52%
G2/M 18.41%
CGA
G1 53.02%
S 27.09%
G2/M 19.89%
EGCG
G1 50.24%
S 26.64%
G2/M 23.12%
TC-HT
G1 57.21%
S 24.77%
G2/M 18.02%
DMSO+TC-HT
G1 53.99%
S 23.17%
G2/M 22.84%
CGA+TC-HT
G1 44.35%
S 21.60%
G2/M 34.05%
EGCG+TC-HT
G1 37.21%
S 20.87%
G2/M 41.92%
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

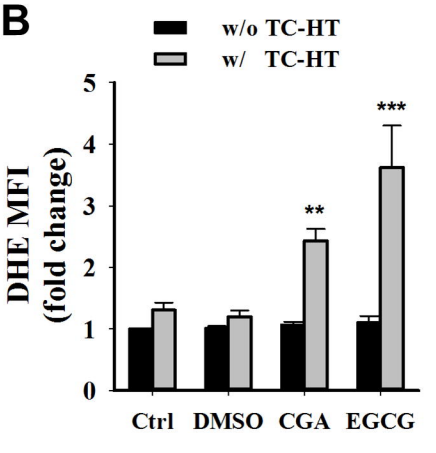

A
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
Q1
Q2
Q3
Q4
PI
Annexin V-FITC
B
Early apoptosis
Late apoptosis
Apoptotic rate (%)
40
30
20
10
0
**
**
***
***
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
C
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT

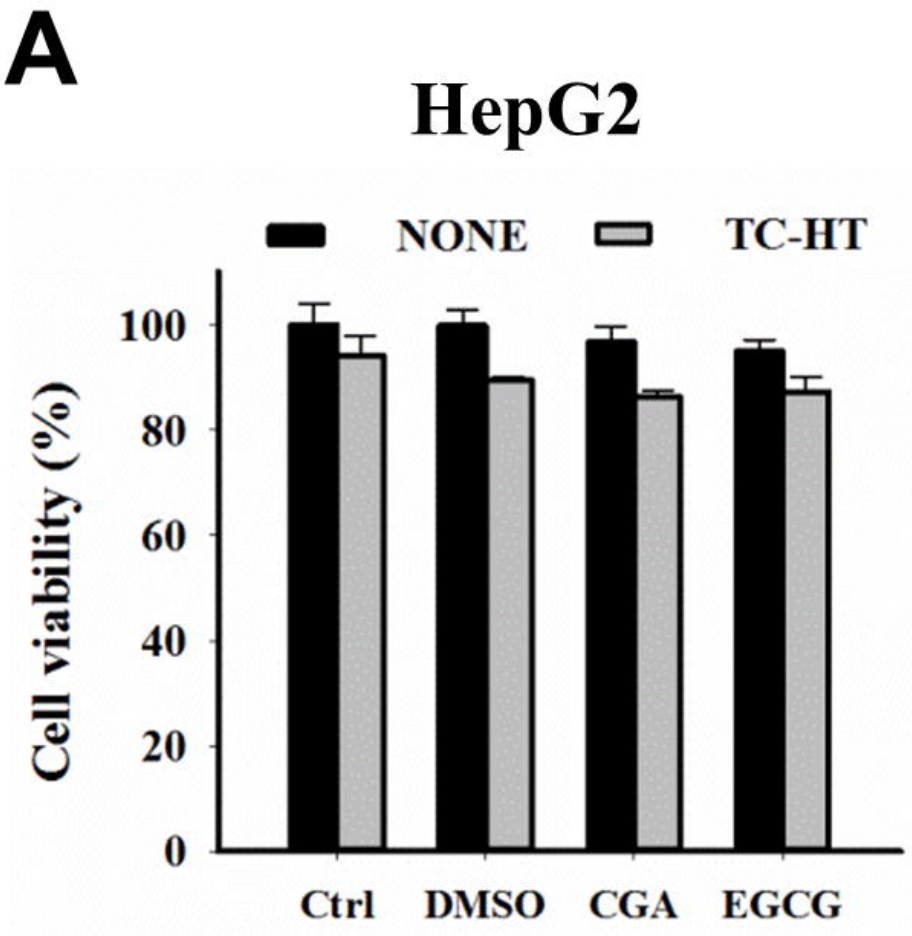

A
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
M1
Cell number
DiOC6(3) fluorescence intensity
B
w/o TC-HT
w/ TC-HT
% of cells in M1
***
Ctrl
DMSO
CGA
EGCG

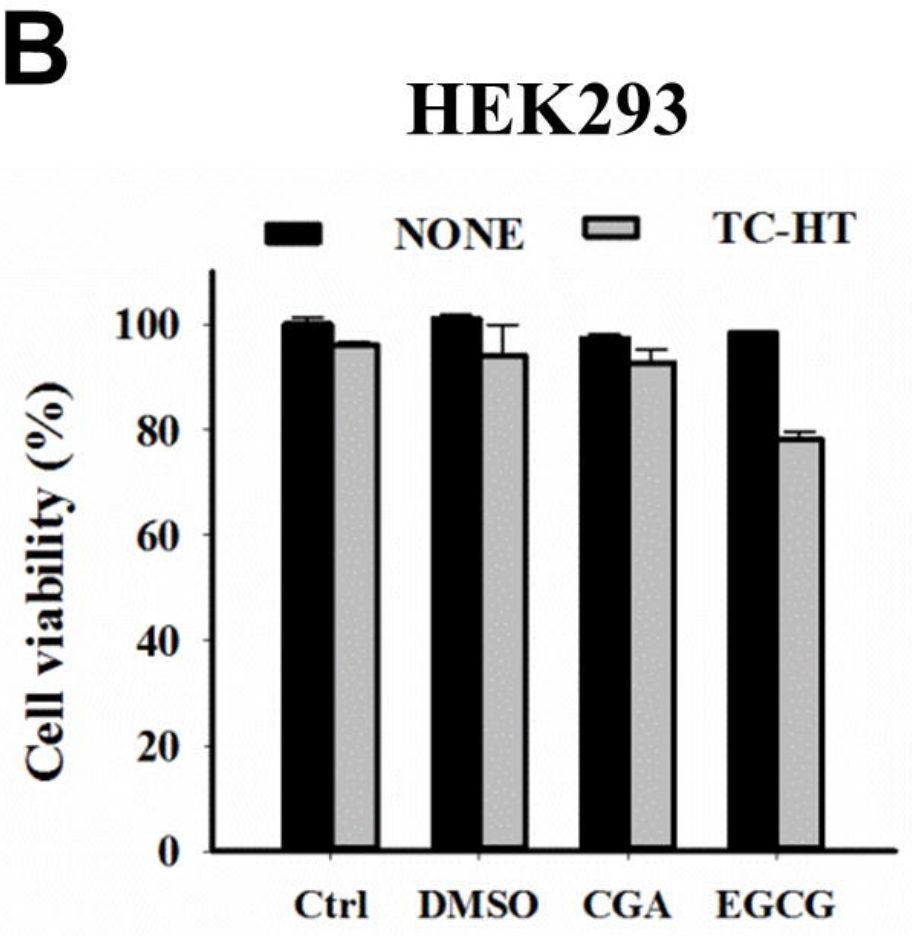

A
TC-HT - - + +
CGA - + - +
Cleaved caspase-9
Cleaved caspase-3
Cleaved PARP
GAPDH
Bcl-2
Bax
β-actin

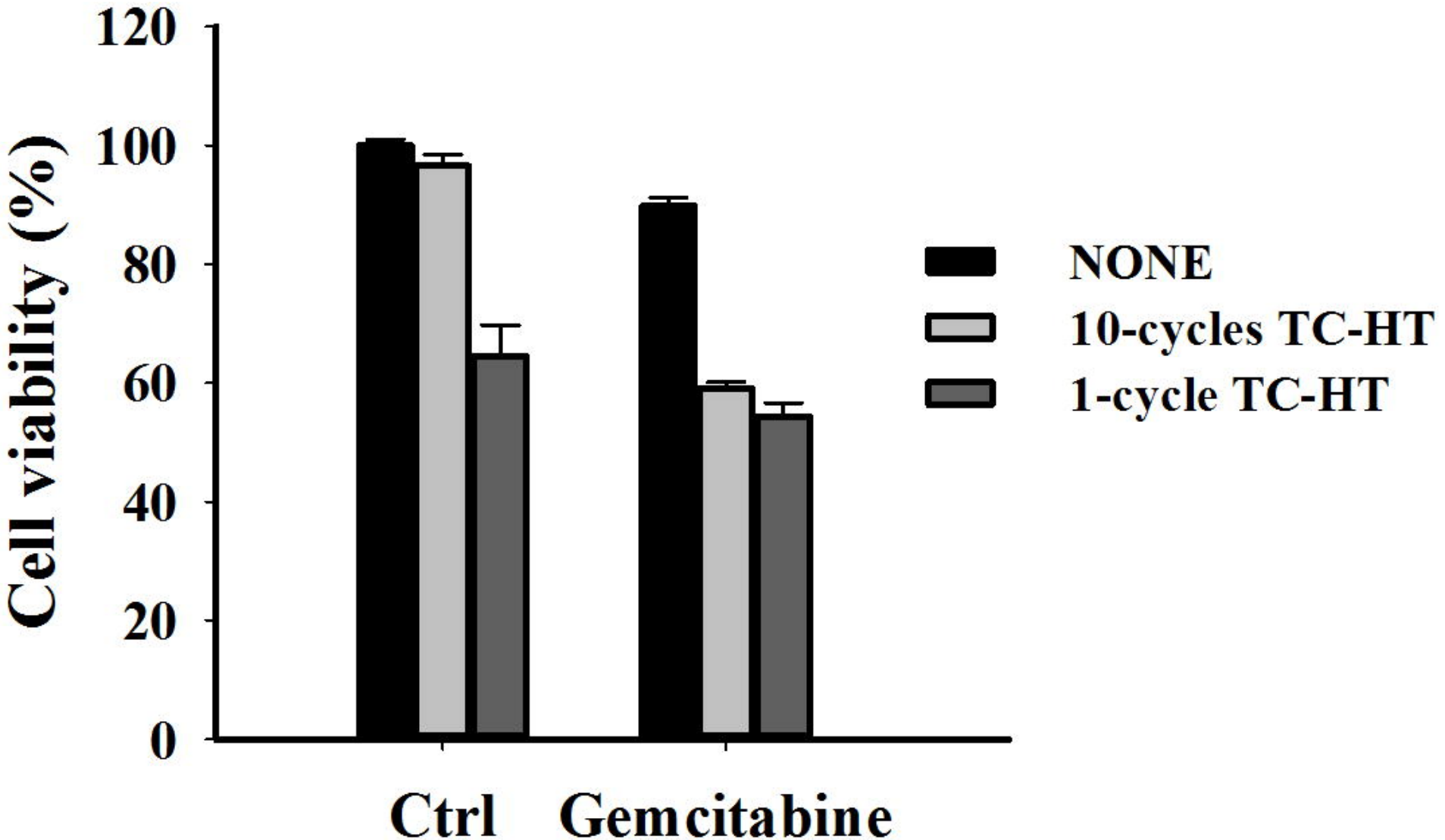

B
TC-HT - - + +
EGCG - + - +
Cleaved caspase-9
Cleaved caspase-3
Cleaved PARP
GAPDH
Bcl-2
Bax
β-actin


A
Ctrl
DMSO
CGA
EGCG
TC-HT
DMSO+TC-HT
CGA+TC-HT
EGCG+TC-HT
Cell number
DHE
0
100
200
50
150
10²
10³


B
w/o TC-HT
w/ TC-HT
DHE MFI
(fold change)
0
1
2
3
4
5
**
***
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 (%)
NONE
10-cycles TC-HT
1-cycle TC-HT
Ctrl
Gemcitabine