Thermal cycling as a novel thermal therapy to synergistically enhance the anticancer effect of propolis on PANC-1 cells

WEI-TING CHEN[1,2], YI-KUN SUN[2], CHUEH-HSUAN LU[1,2] and CHIH-YU CHAO[1-3]

[1]Department of Physics, Laboratory for Medical Physics and Biomedical Engineering, National Taiwan University, Taipei 10617; [2]Biomedical and Molecular Imaging Center, National Taiwan University College of Medicine, Taipei 10051; [3]Institute of Applied Physics, National Taiwan University, Taipei 10617, Taiwan, R.O.C.

Abstract. Hyperthermia (HT) has shown potential in cancer therapy. In particular, it appears to sensitize cancer cells to chemotherapy. However, a major concern associated with HT is that the thermal dosage applied to the tumor cells may also harm the normal tissue cells. Besides, the drugs used in HT are conventional chemotherapy drugs, which may cause serious side effects. The present study demonstrated a novel methodology in HT therapy called thermal cycle (TC)-HT. With this strategy, a therapeutic window with a maximum synergistic effect was created by combining TC-HT with natural compounds, with minimal unwanted cell damage. The natural compound propolis was selected, and the synergistic anticancer effect of TC-HT and propolis was investigated in pancreatic cancer cells. The present results demonstrated for the first time that TC-HT could enhance the anticancer effect of propolis on PANC-1 cancer cells through the mitochondria-dependent apoptosis pathway and cell cycle arrest. Combined treatment greatly suppressed mitochondrial membrane potential, which is an important indicator of damaged and dysfunctional mitochondria. Furthermore, the cell cycle-regulating protein cell division cycle protein 2 was downregulated upon combined treatment, which prevented cellular progression into mitosis. The present study offers the first report, to the best of our knowledge, on the combination of TC-HT with a natural compound for pancreatic cancer treatment. It is anticipated that this methodology may be a starting point for more sophisticated cancer treatments and may thereby improve the quality of life of many patients with cancer.

Correspondence to: Dr Chih-Yu Chao, Department of Physics, Laboratory for Medical Physics and Biomedical Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan, R.O.C.
E-mail: cychao@phys.ntu.edu.tw

Introduction

Temperature serves an important role in regulating biological reactions. The human body has the remarkable ability to maintain its core temperature between 36.5 and 37.5°C. Occasionally, the body raises its temperature to support the immune system, making the environment less favorable for replicating viruses and bacteria. Scientists have been interested in the profound effects of heat on cells for a long time, and have utilized it in various types of therapies. The most popular, called hyperthermia (HT) therapy, is used in the treatment of cancer. Multiple attempts have been made to uncover the biological effects of HT on tumors in recent years (1-3). Although the treatment of cancer with HT has been explored in previous studies (4-6), research on the effect of HT alone as a cancer treatment is limited.

Previous results indicated that a direct cancer cell killing effect could occur when cells were heated to >42°C for ≥1 h (1). This made HT less feasible in clinical treatment, since damage to the central nervous system occurs within a few minutes of exposure to 42°C (7). Therefore, a second line of HT research, which focused on its use in combination with chemotherapy or radiotherapy, quickly emerged (8). Previous clinical trials or *in vitro* studies showed that HT improves the effect of anticancer drugs and radiation (9-11). For example, Schaaf *et al* (8) demonstrated that HT synergizes with cisplatin or doxorubicin by inhibiting poly(ADP)-ribose polymerase (PARP)1-dependent DNA replication arrest. Mild HT also improves drug delivery by breaking the stromal barrier in pancreatic cancer xenograft mouse models and sensitizes cancer cells to PARP1 inhibition (9,12). These studies suggested that HT could be an adjuvant method to cancer chemotherapy. However, little attention has been paid to discussing the optimal treatment temperature and time sequences that provide the maximum potentiating effects and the minimum unwanted cell damage. Mild HT could have limited potentiating effects, while too high temperatures may cause unwanted cell damage. In fact, previous *in vitro* studies revealed that HT is not tumor selective and could also damage normal tissue cells (7,13). Therefore, it is of utmost importance to select the correct temperature and duration so that the combination of HT and chemotherapeutic drugs can provide an optimal anticancer effect while minimizing the unwanted

cell damage caused by HT. Furthermore, the drugs used in HT combination therapies are conventional chemotherapy drugs, which may also cause serious side effects. There is currently an emerging area of research on cancer prevention and cure focused on natural compounds, particularly dietary products, due to their low toxicity and potent efficacy. The present study focused on the effects of propolis, which is a resinous substance produced by honeybees. It has historically been used to treat or alleviate several maladies in traditional medicine (14-16), and it has been the focus of numerous studies due to its anticancer, anti-inflammatory and antioxidant activities (17-19). Frión-Herrera *et al* (20) reported that Brazilian propolis induced apoptosis in human lung cancer A549 cells through the mitochondria-mediated pathway. Demir *et al* (21) also demonstrated the antiproliferative and proapoptotic activity of propolis on human lung cancer cells. Therefore, the objective of the present study was to investigate the synergistic anticancer effect of thermal cycle (TC)-HT and propolis.

The present study reports a refined method of cycling high and low temperatures to achieve a synergistic anticancer effect with natural compounds while minimizing the damage caused by HT. In this strategy, high temperatures markedly enhance the anticancer effect of the natural compounds while the cooling process prevents cell damage caused by an excessive thermal dosage. Various time-temperature combinations were examined to achieve the most marked synergistic cell-killing effect when combined with propolis. Notably, our TC-HT parameters alone did not damage the cells, which makes thermal therapy safer and more feasible.

In the present study, the results demonstrated for the first time that TC-HT has a synergistic cytotoxic effect with a natural compound, propolis, on the human pancreatic cancer cell line PANC-1. The results indicated that TC-HT augmented propolis-induced apoptotic cell-killing and cell inhibition through the mitochondria-dependent apoptosis pathway and G2/M phase arrest. The TC methodology was introduced as an efficient manner to avoid unwanted cell damage in HT therapy. These findings indicated that combining TC-HT with propolis is a promising thermal therapy strategy, which sheds light on novel anticancer treatments combining TC-HT with other natural compounds.

Materials and methods

Cell culture and treatment. PANC-1 and AsPC-1 pancreatic cancer cells, and the normal human embryonic skin cell line Detroit 551, were obtained from the Bioresource Collection and Research Center. Normal human pancreatic duct H6c7 cells were obtained from Kerafast, Inc. PANC-1, AsPC-1 and Detroit cells were maintained in DMEM (PANC-1), RPMI-1640 medium (AsPC-1) or EMEM (Detroit 551) (all from HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin. H6c7 cells were maintained in keratinocyte-serum free medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with human recombinant epidermal growth factor, bovine pituitary extract (Invitrogen; Thermo Fisher Scientific, Inc.), and 1% (v/v) penicillin and streptomycin. All cells were maintained in a humidified incubator with 5% CO_2 and 95% air at 37˚C.

Liquid bee propolis was purchased from Grandhealth™. The Thermal Cycler (model 2720) was purchased from Applied Biosystems; Thermo Fisher Scientific, Inc. Cells were plated in 24-well plates or 3-cm culture dishes 24 h before treatment with or without TC-HT and/or propolis. Propolis was added for 1 h before TC-HT treatment. In the present study, TC-HT was performed using a modified PCR system. The cells were heated to the desired high temperature followed by a cooling period, and this protocol was repeated for different numbers of cycles. The actual temperatures sensed by the cancer cells were measured by a needle thermocouple. During the TC-HT treatment (~30 min), the control and treated groups were in ambient conditions at room temperature (RT). Upon treatment, the cells were maintained in the cell culture incubator for an additional 72 h.

Cell viability assay. Cells were seeded at a density of $2x10^4$ cells/well in 24-well plates. After treatment, cell viability was determined by MTT assay. In brief, the medium was replaced with MTT solution (0.5 mg/ml in DMEM) and incubated at 37˚C for 4 h. The supernatants were discarded, and dimethyl sulfoxide was added to dissolve the formazan crystals. The optical density in each well was then evaluated by the measurement of absorbance at 570 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Ltd.). The cell viability was calculated based on the intensity of the formazan, and was expressed as a percentage of the untreated controls, which were set at 100%.

Cellular growth assay. The long-term cell killing effect of the combination treatment was assessed by colony formation area. PANC-1 cells were seeded at a density of $2x10^4$ cells/dish in 3-cm culture dishes 24 h before being treated with or without TC-HT and/or 0.2% propolis. Upon treatment, the cells were continuously cultured for 10 days. Cells were stained with 0.5% crystal violet in methanol for 5 min at RT, washed with PBS, and images were captured. The colony area was measured using the 'ColonyArea' plugin in ImageJ software (version 1.49j; National Institutes of Health) (22). The sums of the pixel depth over the region of interest were calculated and are represented as arbitrary units. The actual units of colony formation area were cm^2.

Flow cytometric detection of apoptotic cells. Apoptotic cells were analyzed by flow cytometry with an Annexin V-FITC and propidium iodide (PI) double-staining kit (BD Biosciences). Cells used for flow cytometry were collected by trypsinization and resuspended in 100 μl 1X binding buffer containing Annexin V and PI. Cells were stained for 15 min at 25˚C in the dark before being analyzed by flow cytometry. All the cytometry data in the present study were acquired with a BD FACSVerse flow cytometer and analyzed using FlowJo software (version 10.0.7; Tree Star, Inc.).

Immunofluorescence microscopy. Cells were seeded on 20 mm coverslips in 6-well plates at a density of $2x10^4$ cells/well for 24 h and then treated with TC-HT and/or propolis. Next, cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) in PBS for 20 min at RT. Fixed cells were then permeabilized with 0.1% Triton X-100

in PBS for 20 min. Nonspecific protein binding was blocked with 2% BSA (BioShop Canada, Inc.) in PBS for 30 min at RT. The cells were then incubated with anti-β-tubulin (cat. no. ab21057; 1:1,500 dilution; Abcam) and anti-active caspase-3 (cat. no. 9661; 1:800 dilution; Cell Signaling Technology, Inc.) primary antibodies overnight at 4°C. After washing three times in PBS, the cells were incubated with Alexa Fluor 488-conjugated donkey anti-goat (cat. no. 705-545-003) and Alexa Fluor 647-conjugated donkey anti-rabbit (cat. no. 711-605-152) secondary antibodies (both 1:500 dilution; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 37°C in the dark. The coverslips were mounted to slides using mounting medium with DAPI (Abcam). The mounted samples were examined with an inverted laser scanning confocal microscope with a x20 objective (Zeiss LSM 880; Zeiss AG). Images of randomly selected areas were captured for each sample. The integrated fluorescence density was calculated using the ImageJ software. The sums of the pixel density over the region of interest were calculated and are represented as arbitrary units. The actual units of the integrated fluorescence density were candela.

Measurement of mitochondrial membrane potential (MMP). The loss of MMP was determined using the lipophilic cationic fluorescent dye 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$; Enzo Life Sciences, Inc.] (23). Depolarization of MMP results in the loss of $DiOC_6(3)$ from the mitochondria and a decrease in intracellular fluorescence. Cells were harvested and suspended at a density of $1x10^6$ cells/ml in dye working solution (1 μM dye in culture medium) in the dark. After 15 min of culture at 37°C, the supernatant was removed by centrifugation, and the cells were gently resuspended in pre-warmed (37°C) culture medium. Cells labeled with $DiOC_6(3)$ were detected by flow cytometer with the FL1 channel.

Cell cycle analysis. Upon treatment, the cells were collected by trypsinization and fixed in 70% ethanol overnight at 4°C. Prior to the analysis, the cells ($1x10^6$ cells/ml) were washed with cold PBS and treated with RNase A (0.1 mg/ml) for 20 min at 37°C. Subsequently, the cells were stained with PI (0.2 mg/ml) for 30 min at RT. The distribution of cell cycle stages was then determined by flow cytometry.

Western blot analysis. The protein expression levels of PANC-1 cells were investigated by western blot analysis. Cells were scraped off from culture dishes in RIPA lysis buffer (EMD Millipore). After centrifugation, the supernatants were collected and the protein concentrations were determined by Bradford protein assay (BioShop, Inc.). Equal amount of proteins (30 μg) were resolved on 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. For the detection of cytochrome *c* release, the cytosolic fractions were collected via the REAP method (24). Nonspecific antibody binding sites were blocked in 5% nonfat dry milk in TBS with Tween-20 (TBST; 20 mM Tris-base, pH 7.6; 0.15 M NaCl; and 0.1% Tween-20) for 1 h at RT. The blocked membranes were probed with anti-cell division cycle protein 2 (cdc2; cat. no. GTX108120; 1:1,000), anti-actin (cat. no. GTX109639; 1:10,000) (both from GeneTex, Inc.), anti-Bcl-2 (cat. no. 2872; 1:1,000), anti-Bax (cat. no. 2772; 1:1,000), anti-cytochrome *c*

(cat. no. 4272; 1:1,000) (all from Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. GTX100118; 1:10,000; GeneTex, Inc.) antibodies overnight at 4°C. The membranes were washed three times with TBST solution and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (cat. no. 111-035-003; 1:5,000; Jackson ImmunoResearch Laboratories, Inc.) in a blocking solution. Immunoreactivity was visualized with an enhanced chemiluminescence substrate (Advansta, Inc.) and detected with an imaging system (Amersham Imager 600; GE Healthcare Life Sciences). The images were analyzed with Image Lab software (version 6.0.1; Bio-Rad Laboratories, Inc.)

Statistical analysis. The results are expressed as the mean ± standard deviation, and each data point represents the average from three independent experiments. Analyses were performed using OriginPro 2015 software (OriginLab). Differences in statistical significance were determined by one-way analysis of variance followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

In vitro-applied TC. To apply a TC with a rapid temperature change, modified PCR equipment was used as the TC controller (Fig. 1B) in the following *in vitro* experiments. In this design, some protruding parts of the PCR machine and plastic well were cut off so that the bottom of the well could touch the heat sink. The schematic TC settings are shown in Fig. 1A, where the temperature was elevated to the desired HT temperature followed by a cooling period. The actual temperatures sensed by the cancer cells were measured with a needle thermocouple. Fig. 1C and D represent the tumor cell temperature by TC between 46°C and three different cooling temperatures and cycle numbers; the temperature was measured every 20 sec. As shown in Fig. 1C, the temperature in the tumor cells could be raised from 37 to 44°C within 5 min and returned to a relatively safe low temperature (~42°C) rapidly. The 46-37°C parameter setting was selected to mimic the passive cooling process in the human body. The actual cycling temperature of the cancer cells measured by the needle thermocouple was 44-42°C. In practice, the heating device can be switched off in the cooling process to achieve similar results. For other cycling parameters that require a higher thermal dissipation rate, active cooling devices could be used as pre-cooling, such as liquid cooling blankets containing circulating water with antifreeze (25).

TC-HT enhances the anticancer effect of propolis via the apoptosis pathway in PANC-1 cells. To examine whether TC treatment could enhance the anticancer effect of honeybee propolis, PANC-1 cancer cells were treated with increasing doses of propolis with or without TC-HT treatment. In the TC-HT groups, 5 different TC parameters for 6 cycles were applied 1 h after propolis administration. The viability of the cancer cells was examined by MTT assay 72 h after treatment. As shown in Fig. 2A, the viability of PANC-1 pancreas cancer cells decreased in a dose-dependent manner, except at markedly low doses. As shown in Fig. 2B, treatment with TC-HT alone or low doses of propolis had little effect on cell
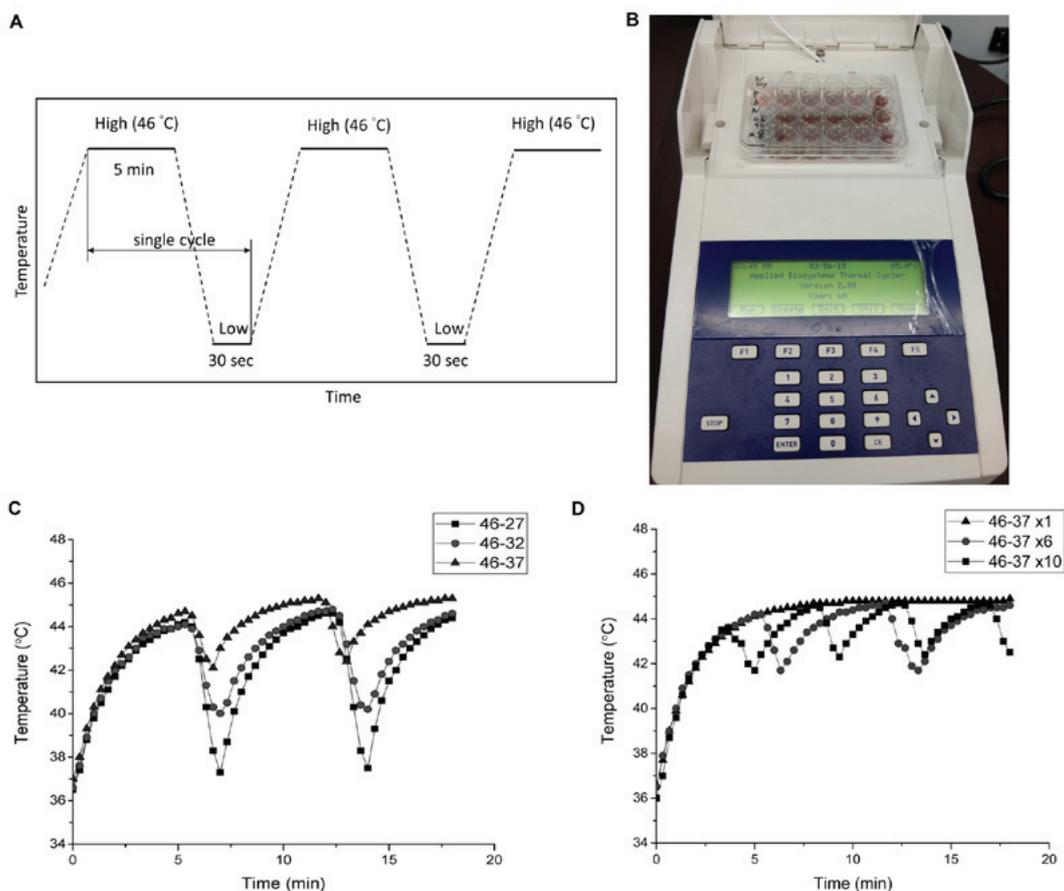
Figure 1. TC-HT with a modified PCR machine. (A) Schematic representation of the TC settings. (B) Image of the TC controller setup. (C) Cell temperature with different cooling temperatures (as monitored by a needle thermocouple located in the bottom of the well). (D) Cell temperature with different cycle numbers. TC-HT, thermal cycle-hyperthermia.
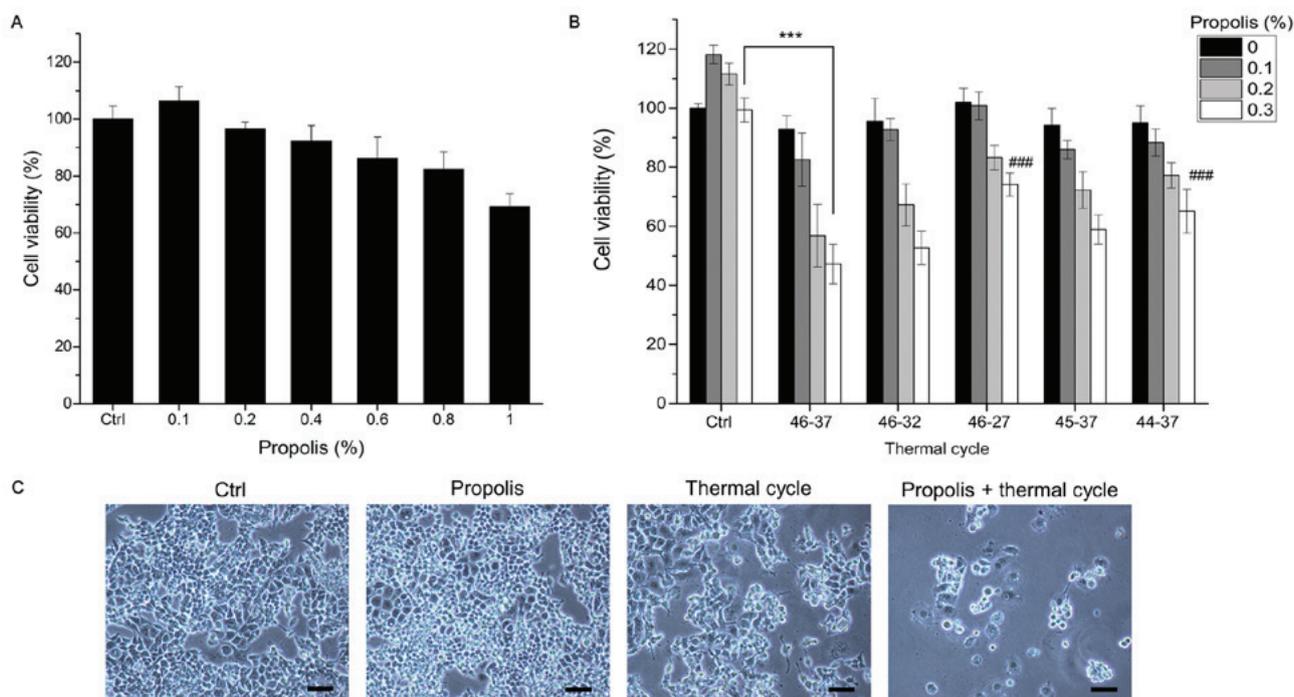
Figure 2. Viability and light microscopy images of PANC-1 cells. (A) Dose-response curve of PANC-1 cells treated with different concentrations of propolis for 72 h. (B) PANC-1 cells were treated with different TC-HT high temperatures and low temperatures with or without propolis for 72 h and then the residual cell viability was measured by MTT assay. (C) Representative bright field images of PANC-1 cells after treatment with TC-HT and 0.2% propolis for 72 h. Scale bar, 100 μm. Data represent the mean ± standard deviation (n=3). ***P<0.001; ###P<0.001 vs. cells treated with combined 46-37°C TC-HT and 0.3% propolis. TC-HT, thermal cycle-hyperthermia; Ctrl, control.
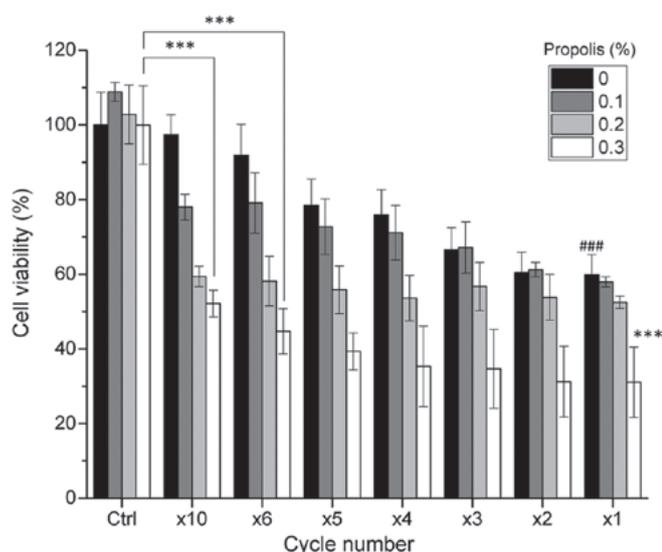
Figure 3. Effect of different TC cycle numbers on the inhibitory effect of TC-HT in PANC-1 cells when combined with propolis. The total thermal dosage was divided into different cycles. In the x1 cycle group, a high temperature of 46°C was sustained for 30 min continuously. Data represent the mean ± standard deviation (n=3). ***P<0.001; ###P<0.001 vs. control without treatment. TC-HT, thermal cycle-hyperthermia; Ctrl, control.

viability compared with control cells. However, when TC-HT treatment was combined with a low dosage of propolis, the viability of PANC-1 cells was significantly reduced. The most effective parameter was the 46-37°C cycling, showing a >50% enhancement in cell killing compared with the single treatment. Light microscopy images also revealed a marked inhibitory effect in cells subjected to the combination of TC-HT and 0.2% propolis after 72 h of treatment (Fig. 2C). To elucidate the effect of different TC parameters on cell viability, different high temperatures and low temperatures were used in the experiments and the results are shown in Fig. 2B. The results revealed that 46-37°C (which led to an actual cycling temperature in the cancer cells of 44-42°C, as measured by needle thermocouple) was the best temperature cycling parameter against PANC-1 cancer cells. For other cancer cell lines, one could modulate the temperature cycle parameters to achieve the desire therapeutic effect. The present study also compared the effect of different cycle numbers in combination with propolis on the viability of PANC-1 cells (Fig. 3), in which the total thermal dosage was divided into different cycles. For example, 6 cycles (x6) means that the high temperature of 46°C was sustained for 5 min (with the actual PANC-1 cell temperature being 44°C), followed by a cooling period, and this process was repeated six times. In the x1 cycle group (or the HT group), a high temperature of 46°C was sustained for 30 min uninterruptedly. The results revealed that the viability of PANC-1 cells was cycle-dependent and decreased as the cycle number decreased. Although HT (x1 cycle) in combination with propolis induced the maximum cell death, heat alone exerted marked cytotoxicity towards the cells. In the x10 and x6 groups, the cell viability was >90% when the cells were treated with TC-HT alone, while TC-HT in combination with propolis also caused a notable decrease in PANC-1 cell viability (Fig. 3). It was found that the x10 TC-HT combined with propolis caused a >45% decrease in PANC-1 cell viability.

Notably, the combination of x6 TC-HT and propolis further caused >50% inhibitory effect on PANC-1 cells. Therefore, the x6 cycle was selected for subsequent experiments. A dose of 0.2% propolis was selected for subsequent analyses because excessive cell loss would adversely affect the subsequent experiments. To compare the short-term (compared to 72 h) and long-term effects of the combination treatment, an MTT assay at 36 h and a cellular growth assay at 10 days after the combination treatment were performed. The results revealed that the combination treatment suppressed the growth of PANC-1 cancer cells (Fig. 4A), and apoptosis proceeded after 36 h (Fig. 4B). To further demonstrate the specificity of the combination treatment, another human pancreatic cancer cell line, namely AsPC-1, normal human embryonic skin Detroit 551 cells and normal human pancreatic duct H6c7 cells were selected for comparison with the pancreatic cancer cell line PANC-1. The results revealed that the combination treatment also had inhibitory effects on AsPC-1 cancer cells (Fig. 4C) but was unharmful to the normal human cell lines Detroit 551 and H6c7 (Fig. 4D). It is worth noting that the effect of the TC parameters used in the present study was less pronounced in AsPC-1 cells than in PANC-1 cells. In order to examine the apoptotic signaling, the Bax/Bcl-2 ratio was also analyzed in AsPC-1 and H6c7 pancreatic cells (Fig. 4E). Notably, the apoptotic effect on AsPC-1 was less pronounced than that on PANC-1 cells, which is in accordance with the MTT results. Therefore, it was hypothesized that TC parameters are tissue-specific and should be optimized in different cell types. On the contrary, for normal human pancreatic duct H6c7 cells, the results showed that the Bax/Bcl-2 ratio was only slightly increased in the combined treatment group.

TC-HT enhances propolis-induced apoptosis in PANC-1 cells. To confirm whether TC and propolis treatments decreased cell viability via the induction of apoptosis, PANC-1 cells were cultured with propolis with or without TC-HT treatment and then assessed by flow cytometry. Cells were stained with Annexin V-FITC and PI for detecting apoptotic cells. The cycling parameters were 46-37°C for 6 cycles (x6). To elucidate the influence of the duration of high temperature within each cycle on the synergistic anticancer effect, the high temperature duration was doubled to 10 min and the cycle number was halved to 3 cycles (x3), so that the total high temperature duration was the same. As shown in Fig. 5, propolis alone did not cause apoptosis, which is consistent with the results of the MTT assay. When propolis was combined with TC (x6 or x3), it resulted in >70% Annexin V-positive apoptotic cells compared with 5.7% in the control group (upper right and lower right quadrants). Notably, the x3 TC protocol resulted in 40.2% apoptotic cells, while the x6 protocol did not notably harm the cells.

TC-HT enhances propolis-induced apoptosis. To further examine the mechanism of TC and propolis-induced cell death, the present study investigated the expression of cleaved caspase-3, Bax and Bcl-2 in PANC-1 cells by confocal microscopy and western blotting. Since caspase-3 serves as a convergent downstream of apoptotic events in cells, it is a useful indicator in apoptosis assays. In the present study, PANC-1 cells were stained with anti-cleaved caspase-3 antibody to identify
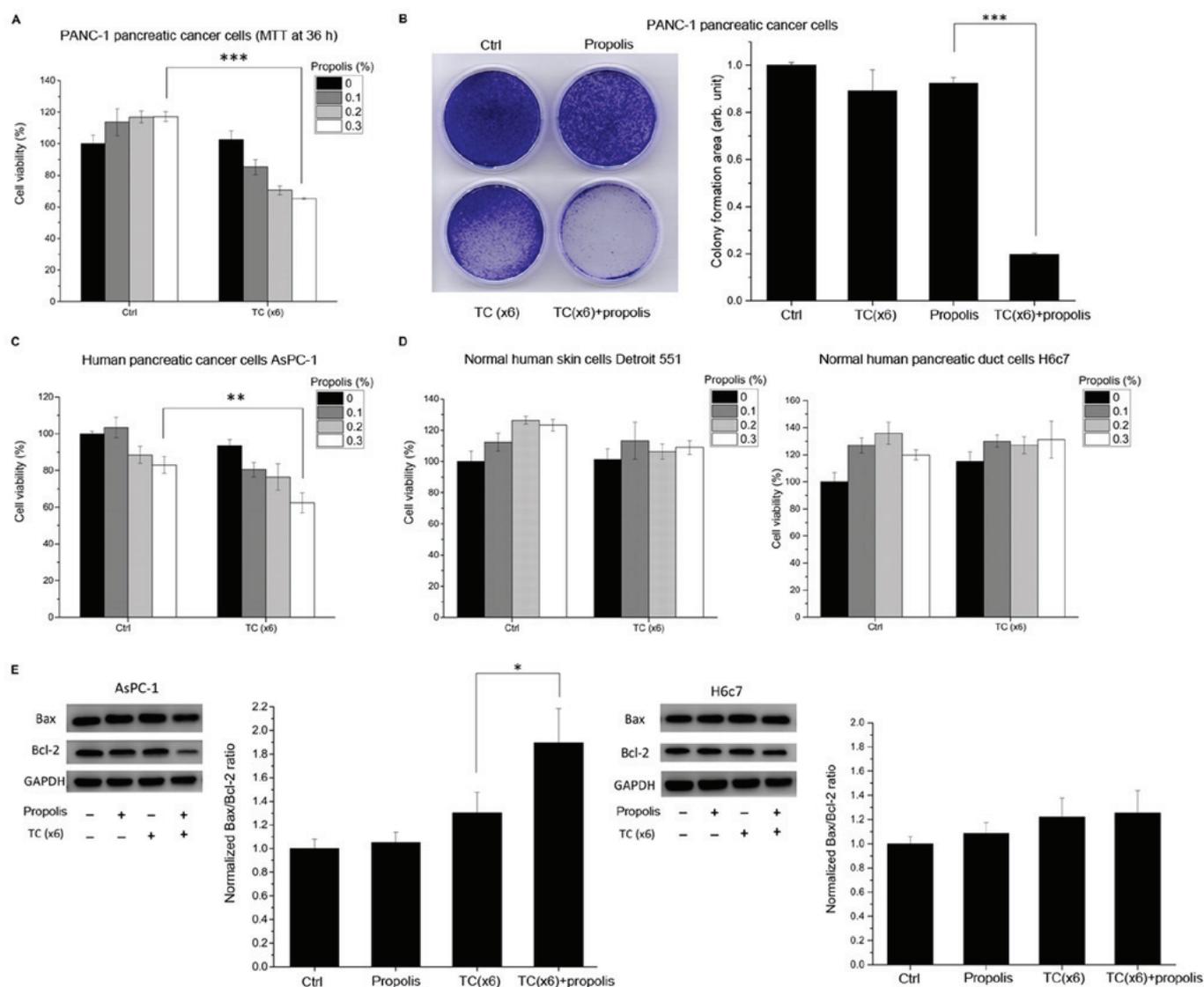
Figure 4. Viability assay and western blot analysis of cancer and normal human cells. (A) MTT assay for PANC-1 cells 36 h after the treatment. (B) Cellular growth assay for PANC-1 cells 10 days after treatment with propolis and/or TC-HT. (C) MTT assay for AsPC-1 cancer cells. (D) MTT assay for normal human skin cells Detroit 551 and normal human pancreatic duct cells H6c7 following treatment with propolis and/or TC-HT. (E) Western blot analysis of Bax and Bcl-2 protein expression in AsPC-1 and H6c7 cells. Data represent the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001. TC-HT, thermal cycle-hyperthermia; Ctrl, control.

the apoptotic cells, and the cell shape was outlined with a β-tubulin marker, while the nuclei of the cells were visualized with DAPI. As seen in Fig. 6A and B, the combined treatment of x6 TC-HT and 0.2% propolis resulted in a marked increase in cleaved caspase-3 immunostaining. By contrast, there was only a negligible increase in the cleaved caspase-3 signal with TC-HT or propolis treatment alone. The Bcl-2 family is an important regulator of the mitochondria-dependent apoptosis pathway. The Bax/Bcl-2 ratio can be used to assess the upregulation of the apoptotic signaling pathway. The results of the western blotting (Fig. 6C) indicated that the Bax/Bcl-2 ratio was significantly increased in the combined treatment group, suggesting that TC-HT and propolis could trigger the mitochondria-dependent apoptosis pathway.

Effects of TC-HT and propolis on MMP in PANC-1 cells. During apoptosis, the MMP decreases, causing the release of cytochrome *c* and other apoptotic factors (26).

To address the possibility that the synergistic anticancer effect of TC-HT in combination with propolis could be associated with the mitochondria-dependent apoptosis pathway, the MMP was assessed by flow cytometry and the cytochrome *c* release was detected by western blotting. Cells were pre-treated with propolis and/or TC-HT and then collected for further analyses. The MMP was detected using a mitochondria-specific probe, namely $DiOC_6(3)$. As shown in Fig. 7A and B, treatment with x6 TC-HT or 0.2% propolis alone did not affect the MMP. However, the combined treatment of TC-HT and 0.2% propolis markedly increased the number of cells exhibiting a loss in MMP, as indicated by a lower $DiOC_6(3)$ intensity. The collapse of the MMP leads to the opening of the mitochondrial permeability transition pores, and the subsequent release of cytochrome *c* in the cytosol. The results of the western blotting showed that the cytosolic cytochrome *c* level was significantly increased in the combined treatment group (Fig. 7C).
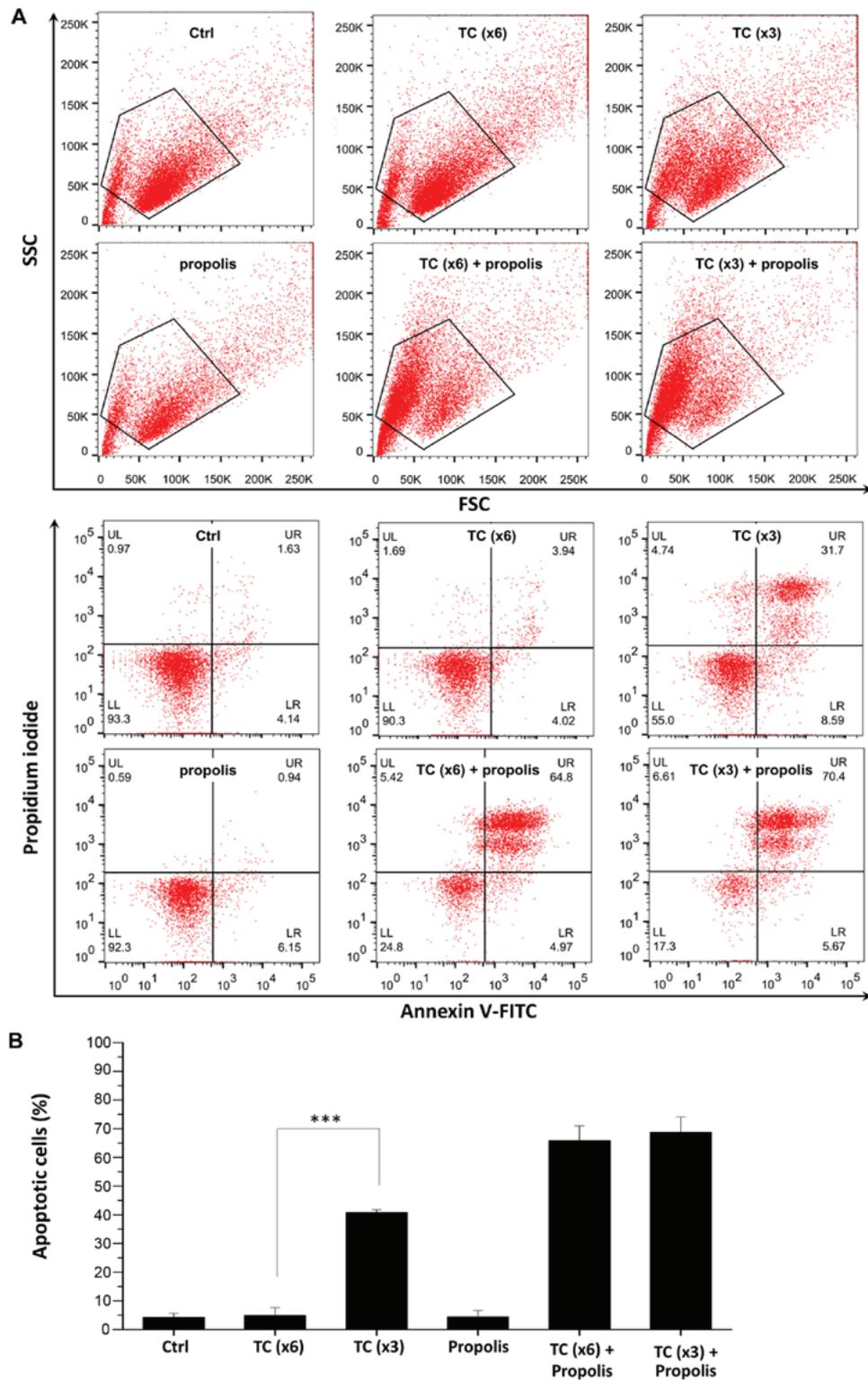
Figure 5. *In vitro* assessment of apoptosis via Annexin V-FITC/PI double staining. (A) PANC-1 cells were treated with 0.2% propolis and x3 or x6 TC-HT alone or in combination for 72 h, and then assessed by flow cytometric analysis of Annexin V-FITC and PI staining. Cells were gated by FSC/SSC plot to exclude cell debris. (B) Quantification of apoptotic cells. The cells in the LR and UR quadrants indicate Annexin V-positive apoptotic cells. Data represent the mean ± standard deviation (n=3). ***P<0.001. PI, propidium iodide; TC-HT, thermal cycle-hyperthermia; Ctrl, control; SSC, side scatter; FSC, forward scatter; LR, lower right; UR, upper right; LL, lower left; UL, upper left.

Combination of TC-HT and propolis causes PANC-1 cell arrest at the G2/M phase. Cell cycle arrest is an important target in cancer therapy, since it is critical in the growth and development of tumors. In order to determine whether the cause of cell growth inhibition observed in the viability assay

was associated with cell cycle arrest, the DNA content of the cells was analyzed by PI staining followed by flow cytometry. After 72 h of treatment with TC and/or propolis, the cell cycle phase was determined by flow cytometry. As shown in Fig. 8A, the combination of TC-HT and propolis induced
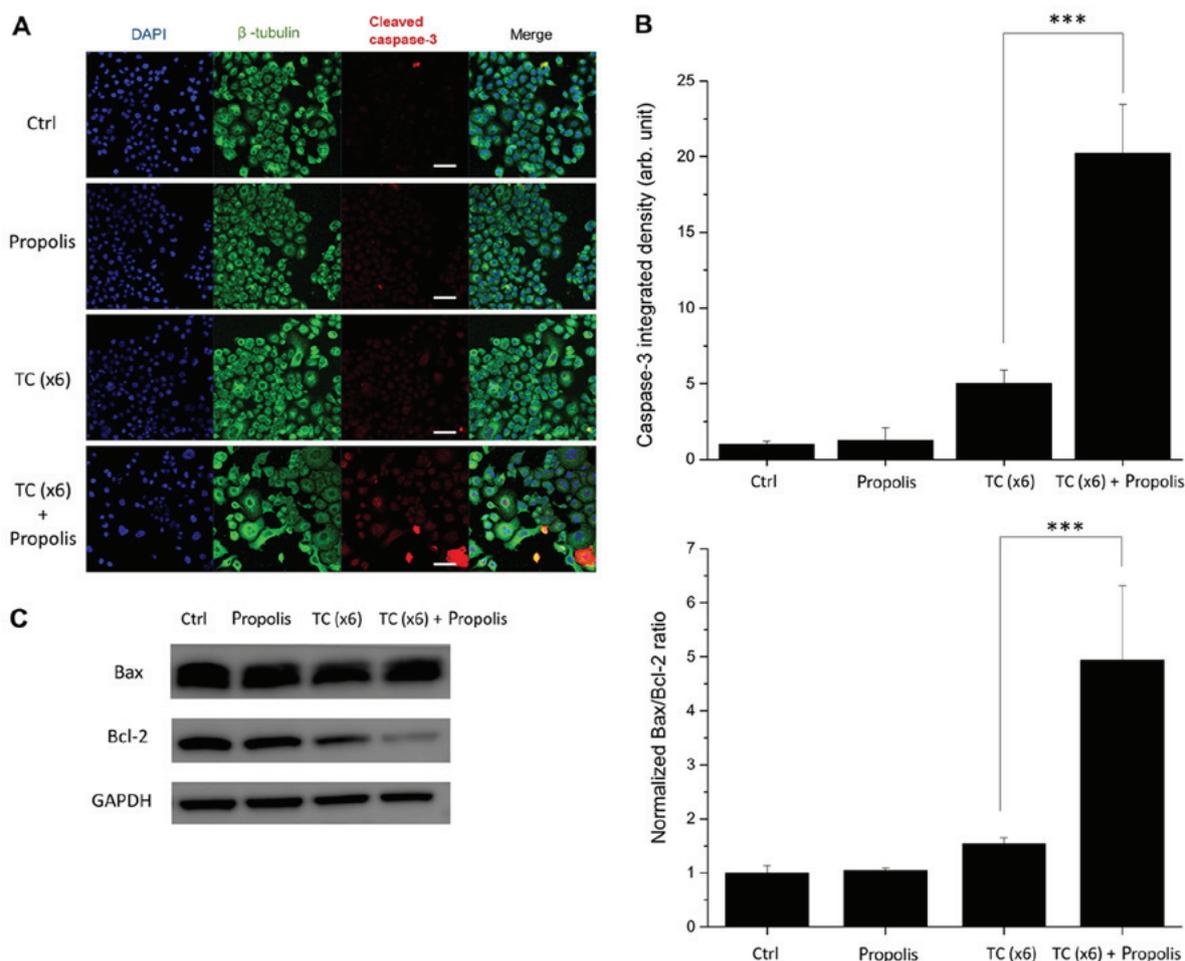
Figure 6. Effect of TC and propolis on active caspase-3 and Bax/Bcl-2 expression. (A) Confocal images of PANC-1 cells treated with 0.2% propolis and x6 TC-HT alone or in combination for 72 h. Red fluorescence represents active caspase-3 staining; cell shape was marked with a β-tubulin marker exhibiting green fluorescence, while the nuclei were labeled with DAPI. Scale bar, 50 μm. (B) Quantification of caspase-3 expression. (C) Western blot analysis of Bax and Bcl-2 protein expression. Data represent the mean ± standard deviation (n=3). ***P<0.001. TC-HT, thermal cycle-hyperthermia; Ctrl, control.

an accumulation of cells in the G2/M phase, changing from 20.1% in the control group to 33.2% in the group subjected to the x6 protocol, with a concurrent decline in the number of cells at the G0/G1 phase from 51.1 to 27.9%. In addition, an increase in the fraction of the sub-G1 population, which is an indication of dying cells, was observed in the TC and propolis co-treated group. To further examine the proteins that regulate cell cycle progression, the effects of TC and propolis on cyclin-dependent kinase 1, also known as cdc2, were subsequently investigated. It is known that the primary participant in the G2/M phase transition is cdc2 protein (27). In the present study (Fig. 8B), cdc2 protein expression was markedly reduced in the combined treatment with x6 TC-HT and 0.2% propolis group, while either treatment alone did not significantly affect cdc2 expression.

Discussion

The focus of the present study was to investigate the synergistic anticancer effect of TC-HT and propolis on PANC-1 cells. The anticancer effects of propolis were strongly potentiated by TC administration. This novel TC method was able to enhance the cytotoxicity of propolis by >10-fold, while being unharmful to normal cells and efficient as a combination therapy. In fact,

in the present results, >50% of cancer cells were inhibited or killed by <1 h of combination treatment, while the TC alone hardly harmed the cells.

HT is a promising strategy in combination with conventional therapies to halt tumor growth. It has been proposed that HT could enhance the sensitivity of cancer cells to drug treatment, thereby exhibiting a synergistic anticancer effect (28). The advantage of such combination treatments is the possibility of using minimal doses of chemotherapy and radiation, leading to a maximum curative effect with less unwanted cell damage. Numerous studies have demonstrated the beneficial effect of HT in chemotherapy (8,11,29). However, the unwanted damage caused by HT cannot be effectively controlled and avoided. One major concern is that the thermal dosage applied to tumor cells may also harm normal tissue cells. Previous studies revealed that a sustained temperature >42°C will cause necrosis of living cells (30,31). Besides, heat tolerance will be dissimilar in different tissues (32). Therefore, it is important to select the optimal thermal dosage, as different temperatures and durations may be necessary to achieve the desired outcomes.

The present study provides an efficient way of controlling the applied thermal dosage to cells. The heat-and-cool cycling used in the present study has advantages when combined
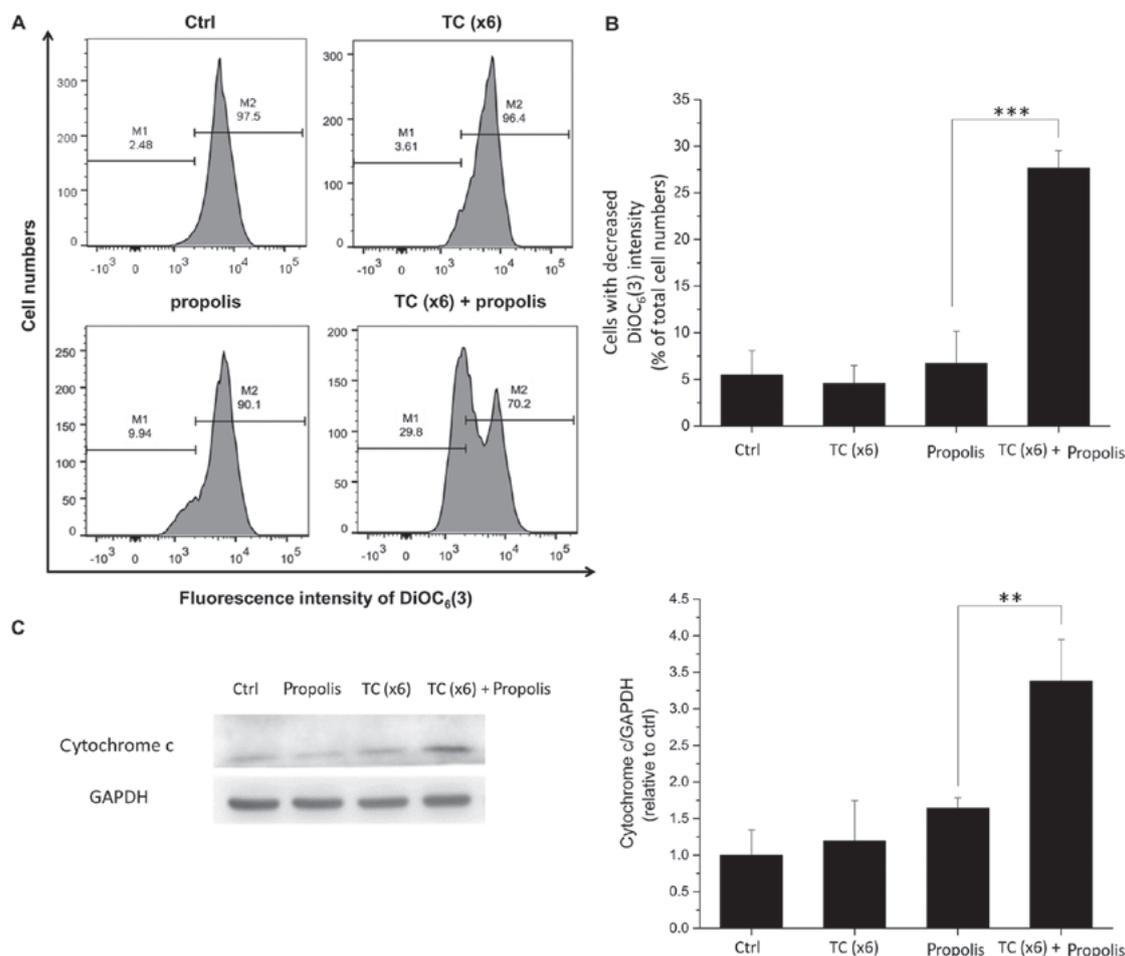
Figure 7. Flow cytometric analysis of MMP in PANC-1 cells treated with TC and propolis. PANC-1 cells were treated with x6 TC-HT and 0.2% propolis alone or in combination for 72 h. (A) After treatment, the MMP was studied with the use of $DiOC_6(3)$. (B) Quantification of the cells with lower $DiOC_6(3)$ intensity. (C) Western blot analysis of cytosolic cytochrome c. Data represent the mean ± standard deviation (n=3). $^{**}P<0.01$ and $^{***}P<0.001$. MMP, mitochondrial membrane potential; TC-HT, thermal cycle-hyperthermia; $DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine iodide; Ctrl, control.

with anticancer compounds or chemotherapy drugs. During the heating process, the temperature was elevated to a certain threshold and maintained for a specific period, which can synergize with anticancer drugs. In the cooling process, the tissue cell temperature was lowered to prevent excessive thermal dosage accumulation and subsequent cytotoxic cell damage. The heat-and-cool process can be repeated numerous times to achieve the desired anticancer effect. As shown in Fig. 2B, three different maximum thermal cycling temperatures for 6 cycles were applied upon administration of propolis. The most effective parameter setting was 46-37˚C cycling (notably, the actual cycling temperature of the cancer cells measured with the needle thermocouple was 44-42˚C), which mimics the passive cooling process in the human body. The results indicated that there was a specific threshold temperature for maximizing the cytotoxic anticancer effect of propolis. Furthermore, as shown in Fig. 2B, an excessively low temperature in the cooling process will diminish the synergistic effect, since the total thermal dosage is insufficient to sensitize PANC-1 cells in the cycling procedure. Therefore, it is important to determine the appropriate cycling parameters when combining TC-HT with different anticancer drugs. The results of the present study showed the advantages of TC in preventing cytotoxic

damage when the total thermal dosage was divided into different cycles. In the x1 cycle group, a high temperature of 46˚C was sustained for 30 min continuously. Although HT acts synergistically with propolis in killing cancer cells, heat alone also causes severe cytotoxicity. The aim of combination therapy is to avoid the latter. An earlier study analyzed the time-dependent modifications of cancer cells during exposure to HT, and revealed that the survival rate decreased with increasing exposure time (33). Namely, the short exposure of cancer cells to HT may induce cellular stress without affecting cell viability, while prolonged exposure may lead to cell death. The present study demonstrated that the viability of PANC-1 cells was >90% in the x6 group when treated with TC-HT alone. Notably, the combination of x6 TC-HT and propolis caused a >50% inhibitory effect on PANC-1 cell viability. Moreover, the same combination treatment exerted low cytotoxicity on the normal human cell lines Detroit 551 and H6c7. These data indicated that TC-HT and propolis are promising candidates for anti-pancreatic cancer treatment, with low toxicity towards normal cells.

The molecular mechanisms responsible for this potentiation were investigated by determining the MMP and cell cycle progression. The expression of downstream proteins of the apoptotic pathway and cell cycle regulatory proteins was also
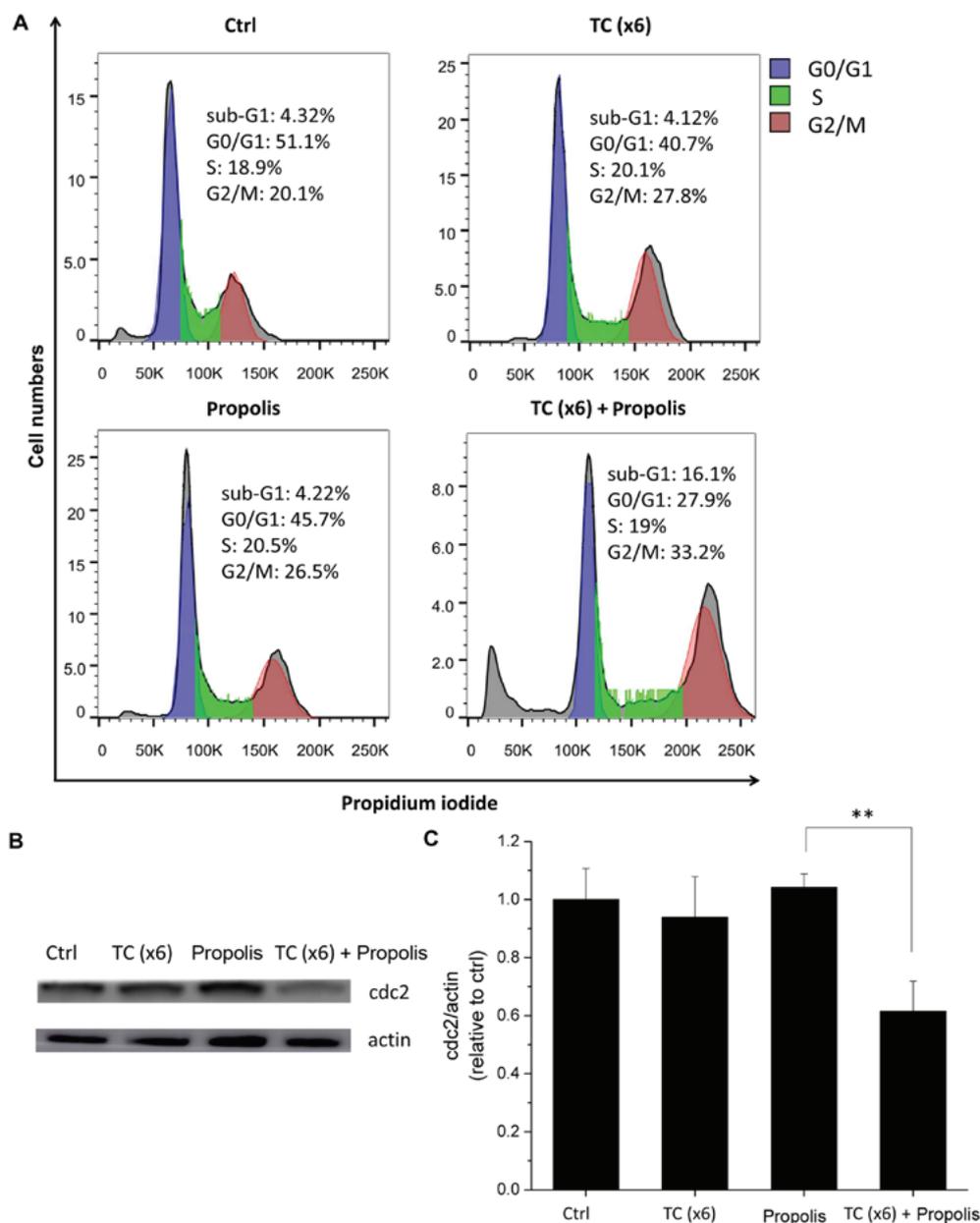
Figure 8. Effect of TC and propolis on PANC-1 cell cycle distribution. PANC-1 cells were treated with 0.2% propolis and x6 TC-HT alone or in combination for 72 h. (A) Cell cycle analysis by PI flow cytometry. (B) Western blot analysis of the G2/M phase-regulating protein cdc2. Actin was used to confirm equal protein loading. (C) Quantification of cdc2 expression. Data represent the mean ± standard deviation (n=3). **P<0.01. TC-HT, thermal cycle-hyperthermia; PI, propidium iodide; cdc2, cell division cycle protein 2; Ctrl, control.

evaluated. Previous *in vitro* and *in vivo* studies demonstrated that propolis exerts cytotoxic properties against cancer cells through the mitochondria-mediated apoptosis pathway and cell growth arrest (21). Mitochondria are associated with cell stress responses, including oxidative stress and cell death. During apoptosis, the MMP decreases, causing the release of cytochrome *c* into the cytosol and the activation of the subsequent caspase cascades (26). In the present study, the combined treatment of TC-HT and propolis greatly suppressed MMP, which is an important index for damage and dysfunction of the mitochondria. In addition, the western blotting results showed that the expression level of cytosolic cytochrome *c* was significantly higher in the combined treatment group. Moreover, it was found that caspase-3 expression was promoted by the combined treatment. These data indicated

that the enhancement of apoptosis mediated by the combined treatment was induced by a mitochondria-dependent apoptosis pathway.

Cancer is characterized by uncontrolled cell division, which is linked to the aberrant activity of various cell cycle regulators. Therefore, cell cycle regulatory proteins are considered attractive targets in cancer therapy. In this study, it was found that the combined treatment of TC-HT and propolis resulted in G2/M phase arrest. Cell cycle analysis by flow cytometry also demonstrated an increase in the fraction of dying cells, as indicated by the sub-G1 population in the combined treatment group. The main participant in the G2-M transition is CDK1, also known as cdc2. According to the western blotting results, cdc2 expression was downregulated in the combined treatment with propolis and TC group, while neither TC or propolis

alone interfered with cdc2 expression. These results indicated that the thermal enhancement of propolis cytotoxicity was mediated in part by inhibition of the kinase activity of cdc2, which prevents cell progression into mitosis. Although there is evidence that TC-HT and propolis result in G2/M phase arrest, it is not possible exclude the possibility that G0/G1-phase cells are more susceptible to treatment. Further studies are required to elucidate the detailed mechanism underlying the increased G2/M population.

Pancreatic cancer has the highest mortality rate of all cancer types, due in part to the lack of diagnostic tools for early detection. Treatment options are limited and mostly rely on chemotherapy or radiation. In pancreatic cancer, the effects of chemotherapy combined with HT using several heating methods and technologies have been investigated in clinical settings, such as whole body HT and HT intraperitoneal chemotherapy (34-37). Previous studies have demonstrated the benefits of HT, which can enhance the cytotoxicity of chemotherapy drugs towards pancreatic cancer (38-40). The present study provides a novel methodology in HT therapy, namely TC-HT therapy, which is safer and has more feasible administration when combined with anticancer compounds. The present results directly confirmed that, by thermally cycling the pancreatic cancer cells and administering propolis, significant cytotoxic and inhibitory effects were observed. The present study used PCR equipment to demonstrate TC-HT *in vitro*. For *in vivo* or clinical experiments, other heating strategies must be used and tested, such as high-intensity focused ultrasound (HIFU) (41). HIFU has been widely used as a hyperthermal technique. It can be used for thermal ablation as well as for producing mild HT in cancer (42). The thermal parameters may be finely tuned by modulating the heating power and the size of the heated volume to meet the specific requirement for the application of mild HT therapy *in vivo* (43). It was hypothesized that TC-HT will be a promising strategy that could be applied to pancreatic cancer, and thereby may be able to improve the quality of life of the majority of patients with pancreatic cancer.

In summary, the present study demonstrated a novel methodology in HT therapy, namely the TC-HT technique. The heat-and-cool cycling can create a therapeutic window with a maximum synergistic effect when combined with natural compounds, with minimal unwanted cell damage. This would allow for repeated and long-term treatments without the limitations associated with the accumulation of toxic cell damage. The present results confirmed that TC and propolis could synergistically inhibit PANC-1 cancer cell growth through the mitochondria-dependent apoptosis pathway and cell cycle arrest. It is thought that this strategy could be extended to other HT therapies in the fight against cancer. Further studies are required to examine the association between specific TC parameters and different anticancer drugs to optimize the curative effects.

Acknowledgements

Funding

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CYC initiated the study, conceived the experiments and managed the project. WTC and CYC wrote the manuscript. WTC, YKS, CHL and CYC performed experiments and analyzed the data. 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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