1 Study on the effect of a triple cancer treatment of propolis,
2 thermal cycling-hyperthermia, and low-intensity ultrasound
3 on PANC-1 cells

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22 **Abstract**

23 To reduce side effects and enhance treatment efficacy, study on combination therapy for

24 pancreatic cancer, a deadly cancer, has gained much attraction in recent years. In this study,

25 we propose a novel triple treatment combining propolis and two physical stimuli—thermal

26 cycling-hyperthermia (TC-HT) and low-intensity ultrasound (US). The study found that, after

27 the triple treatment, the cell viability of a human cancer cell line PANC-1 decreased to a level

28 80% less than the control, without affecting the normal pancreatic cells. Another result was

29 excessive accumulation of reactive oxygen species (ROS) after the triple treatment, leading to

30 the amplification of apoptotic pathway through the MAPK family and mitochondrial

31 dysfunction, while activating autophagy simultaneously. This study, to the best of our

32 knowledge, is the first attempt to combine TC-HT, US, and a natural compound in cancer

33 treatment. The combination of TC-HT and US also promotes the anticancer effect of the

34 heat-sensitive chemotherapy drug cisplatin on PANC-1 cells. It is expected that optimized

35 parameters for different agents and different types of cancer will expand the methodology on

36 oncological therapy in a safe manner.

37

38 **Introduction**

39 Cancer is one of the most dreadful diseases and the second leading cause of death
40 around the world. Among all kinds of cancers, pancreatic cancer is the most threatening one,
41 due to its high death rate and low five-year survival rate [1]. Existing therapies for pancreatic
42 cancer, including surgery, radiation, and chemotherapy, all involve major risks, such as tumor
43 recurrence, refractory, and serious side effects [2], as a result of which development of new
44 therapies is of the utmost importance. A popular option is combination therapy, administering
45 two or more anticancer agents to attain a synergistic effect [3]. However, the interaction
46 between drugs may lead to unexpected competition and even harmful side effects [4, 5],
47 jeopardizing patients' health, let alone improving therapeutic efficacy.

48 An emerging option in combination therapy is physical stimulus, whose effects on
49 cellular physiology have been reported in several studies [6-8]. Our team has also looked into
50 the feasibility of combining drug therapy and physical stimuli, such as heat [9], electric field
51 [10], and magnetic field [11]. Ultrasound (US) is also a therapeutic tool with extensive
52 application, such as developing internal images [12, 13], transporting liposomes to increase
53 agent-delivery rate [14, 15], and ablating tumors from normal tissues [16, 17]. The previous
54 study also found US as a helpful method in inhibiting significantly the viability of PANC-1

55 cells [18]. However, US may also entail a number of risks, such as harm to normal tissues
 56 around tumors due to overheating caused by high-intensity US [19, 20]. In addition,
 57 liposomes may induce myocardial injury during transport by US [21, 22]. In view of this,
 58 integrating low-intensity US [23, 24] with a non-hazardous agent is important for expanding
 59 the use of US in therapy.

60 Accordingly, the study employed natural compounds from herbal medicines as
 61 anticancer agent. Among natural compounds with therapeutic potential, propolis has been
 62 found to be effective in inhibiting several cancer cell lines [25-27]. In our previous study,
 63 propolis was applied, along with thermal cycling-hyperthermia (TC-HT) as a physical
 64 stimulus [28], in cancer treatment, but the effect still lags behind the in vitro efficacy of
 65 chemotherapy drugs.

66 In this paper, the study introduced low-intensity US as a secondary helper, on top of
 67 TC-HT, in order to further augment the anticancer effect of propolis. The novel triple
 68 treatment turned out to inhibit the viability of PANC-1 cells significantly, approaching the in
 69 vitro efficacy of chemotherapy drugs, without damaging the normal human pancreatic duct
 70 cells and skin cells. The study found that the triple treatment manipulated the phosphorylation
 71 levels of MAPK family, thereby activating the intracellular apoptotic signalling. Besides, the

72 autophagy signalling was noticed being greatly activated by the triple treatment. Moreover, it
73 was found in the upstream that intracellular reactive oxygen species (ROS) also increased
74 greatly, thereby boosting the death rate of PANC-1 cells.

75

76 **Materials and Methods**

77 **Cell culture and propolis treatment**

78 The human pancreatic cancer cell line PANC-1 and the normal human embryonic skin cell
79 line Detroit 551 were obtained from Bioresource Collection and Research Center (Hsinchu,
80 Taiwan). Normal human pancreatic duct H6c7 cells were purchased from Kerafast, Inc.
81 (Boston, MA, USA). PANC-1 and Detroit 551 cells were cultured respectively in DMEM and
82 EMEM (both from Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine
83 serum (FBS) (Hyclone) and 1% penicillin-streptomycin (Gibco Life Technologies, Grand
84 Island, NY, USA). H6c7 cells were maintained in keratinocyte-serum free medium
85 (Invitrogen, Life Technologies, Grand Island, NY, USA) supplemented with human
86 recombinant epidermal growth factor, bovine pituitary extract (Invitrogen), and 1%
87 penicillin-streptomycin (Gibco Life Technologies). All cells were maintained in a humidified

88 5% CO₂ incubator at 37 °C and subcultured by 0.05% trypsin–0.5 mM EDTA solution (Gibco
89 Life Technologies). Once the confluences reached suitable percentages, cells were plated in
90 96-well or 35-mm-diameter culture dishes (Thermo Fisher Scientific, Inc., Waltham, MA,
91 USA) for in vitro experiments after 24 h. Propolis was purchased from Grandhealth™ (Grand
92 Health Inc, Richmond, BC, Canada), and cisplatin was obtained from Sigma-Aldrich (St.
93 Louis, MO, USA). All agents were mixed with culture medium to the desired concentration
94 and were incubated with cells for 1h before treating physical stimuli.

95

96 **Ultrasound exposure**

97 The US exposure system consisted of a function generator (SG382; Stanford Research
98 Systems, Sunnyvale, CA, USA), a power amplifier (25a250a; amplifier research, Souderton,
99 PA, USA), and a planar transducer (A104S-RM; Olympus NDT Inc., Waltham, MA, USA).
100 Continuous pulses were produced using the function generator with the following parameters:
101 -10 dBm amplitude, 1 ms pulse period, and 0.5 ms pulse width. The cell culture plate or dish
102 was placed on the ceramic transducer (resonance frequency 2.25 MHz), which converted
103 electrical signals into acoustic power (**Fig 1A**). To avoid undesirable thermal effects induced

104 by US, the output power of the spatial average intensity of the US exposure was adjusted to
105 be 0.3 W/cm² according to the previous studies [29, 30].

106

107 **Fig 1. Experimental setups for the triple treatment.**

108 35-mm culture dishes were placed on (A) a ceramic transducer and (B) a modified PCR
109 machine for the exposures of US and TC-HT, respectively. (C) The schematic representation
110 of the TC-HT temperature settings. (D) Cell temperature detected by a needle thermocouple
111 when TC-HT was implemented. (E) Experimental schedule of the triple treatment with
112 different exposing order of US and TC-HT.

113

114 **Thermal cycling-hyperthermia (TC-HT) treatment**

115 A modified polymerase chain reaction (PCR) system was used to perform TC-HT (**Fig 1B**).
116 Thermal cycler (model 2720) was purchased from Applied Biosystems (Thermo Fisher
117 Scientific). The system was repeatedly brought to the desired high temperature state and
118 followed by a cooling stage to achieve a series of short period of heat exposure within the
119 desired time (**Fig 1C**). The experimental setup and administration of TC-HT (10-cycles) have
120 been previously described [28] with optimum results. The actual temperatures the cancer cells

121 sensed were measured by a needle thermocouple, ranging in 45~40.5 °C (Fig 1D). Ultrasound
122 exposure was applied either before or after the TC-HT treatment, for different combination
123 tests. During the TC-HT treatment (~ 45 min), the control and propolis-treated groups were
124 under room temperature (RT) without a 5% CO₂ environment. After the treatments, cells were
125 maintained in the cell culture incubator for the following experiments.

126

127 **MTT assay**

128 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was
129 dissolved in distilled water to prepare a 5 mg/ml stock solution. The treated PANC-1 cells
130 were incubated for 4 h at 37 °C with a final MTT concentration 0.5 mg/ml in DMEM culture
131 medium to assess the cell viabilities. The formazan crystals were dissolved by equal volume
132 of the solubilizing buffer of 10% sodium dodecyl sulfate (SDS) (Bioshop Canada Inc.,
133 Burlington, ON, Canada) solution in 0.01 N hydrochloric acid (HCl) (Echo Chemical Co. Ltd.,
134 Taiwan) at 37 °C overnight. The absorbance of each well was detected by Multiskan GO
135 microplate Spectrophotometer (Thermo Fisher Scientific), and the quantity of formazan was
136 determined by the absorbance at 570 nm, with a background subtraction at 690 nm. The cell
137 viabilities were expressed in percentage and the untreated control was set at 100%.

138

139 **Treatment with ROS scavenger**

140 PANC-1 cells were seeded into 96-well or 35-mm-diameter culture dishes overnight. For

141 ROS inhibition analysis, cells were pretreated with 5 mM N-acetyl-cysteine (NAC)

142 (Sigma-Aldrich) in culture medium for 1 h and subsequently treated with propolis and/or

143 physical stimuli.

144

145 **Apoptotic analysis by flow cytometry**

146 PANC-1 cells were collected 24 h after treatments and then rinsed twice with ice-cold

147 phosphate buffered saline (PBS) (Hyclone). The apoptotic rates were analyzed by the

148 Annexin V-FITC and propidium iodide (PI) double detection kit (BD Biosciences, San Jose,

149 CA, USA), and the rinsed cells were resuspended in binding buffer containing Annexin

150 V-FITC and PI and then incubated at RT for 15 min in the dark. Apoptotic signals were

151 detected by FACSCanto II system (BD Biosciences).

152

153 **ROS and mitochondrial membrane potential (MMP) analyses by flow cytometry**

154 ROS was detected using the fluorescent dye dihydroethidium (DHE) (Sigma-Aldrich), and
155 the loss of MMP was determined using the lipophilic cationic fluorescent dye
156 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)) (Enzo Life Sciences, Inc., Plymouth Meeting,
157 PA, USA). PANC-1 cells were harvested 24 h after treatments and rinsed with PBS before
158 staining. Rinsed cells were resuspended and then incubated with 5 μM DHE or 20 nM
159 DiOC₆(3) in PBS at 37 °C for 30 min in the dark. The fluorescence signals were measured by
160 FACSCanto II system (BD Biosciences) with the PE channel (for DHE staining) or FL1
161 channel (for DiOC₆(3) staining).

162

163 **Western blot analysis**

164 Protein expression levels of PANC-1 cells were quantified by western blot analysis. Cells
165 were rinsed with PBS and then lysed in the lysis buffer (50 mM Tris-HCL, pH 7.4, 0.15 M
166 NaCl, 0.25% deoxycholic acid, 1% NP-40, 1% Triton X-100, 0.1 % SDS, 1 mM EDTA)
167 (Millipore, Billerica, MA, USA), supplemented with active protease (Millipore) and
168 phosphatase inhibitor cocktail (Cell signaling Technology, Danvers, MA, USA). After
169 centrifugation, the supernatants were collected and the protein concentrations were quantified
170 by Bradford protein assay (Bioshop, Inc.). Equal amount of proteins (20 μg) were resolved by

171 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto
172 polyvinylidene fluoride (PVDF) membranes (Millipore). 5% skim milk powder or 5% bovine
173 serum albumin in TBST (20 mM Tris-base, pH 7.6, 0.15 M NaCl, 0.1% Tween 20) was used
174 to block nonspecific antibody binding sites for 1 h at RT. Afterwards, the blocked membranes
175 were probed with specific primary antibodies against p-ERK, p-JNK, PARP (Cell signaling),
176 LC3 and GAPDH (Gentex, Irvine, CA, USA) at 4 °C overnight. The membranes were rinsed
177 with TBST buffer three times and then incubated with horseradish peroxidase-conjugated
178 goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove,
179 PA, USA) in a blocking solution at RT for 1 h. Immunoreactivity signal was amplified by an
180 enhanced chemiluminescence (ECL) substrate (Advansta, San Jose, CA, USA) and detected
181 by an imaging system Amersham Imager 600 (GE Healthcare Life Sciences). GAPDH was
182 used as the loading control to normalize the relative folds of targeting proteins.

183

184 **Statistical analysis**

185 Experiments were repeated three times for validation, and statistical analyses were performed
186 using one-way analysis of variance (ANOVA) by OriginPro 2015 software (OriginLab).

187 Results were expressed as the mean $\pm$ standard deviation, and were considered to be
188 statistically significant when *p*-values were less than 0.05.

189

190 **Results**

191 **Triple treatment greatly inhibits the viability of PANC-1 cells**

192 The cell viability of PANC-1 cells versus the propolis concentration was performed in a
193 gradient manner. When the propolis was less than 0.5%, as shown in **Fig 2A**, there was no
194 notable inhibition effect on MTT results. However, when it exceeded 0.5%, the cell viability
195 dropped significantly. Therefore, a moderate propolis concentration 0.3% was chosen for the
196 following experiments. Next, the physical stimuli of TC-HT and low-intensity US were
197 introduced to affect the viability of PANC-1 cells. In our study, 10-cycles TC-HT and 2.25
198 MHz US with intensity 0.3W/m^2 and duration 30 minutes were chosen to avoid the
199 thermotoxicity on PANC-1 cells. As shown in **Fig 2B**, we found that the combination of
200 TC-HT and US was also innocent to PANC-1 cells, but when 0.3% propolis was involved in
201 the triple treatment, the viability of PANC-1 cells was greatly inhibited. It was also noted that
202 the implementation order of TC-HT and US in triple treatment was influential. When TC-HT

203 was performed prior to US (TC-HT + US) in the presence of 0.3% propolis, the cell viability

204 of PANC-1 cells was significantly suppressed down to 17.1%, cutting more than 80% of the

205 viability of the untreated control and thus approaching the in vitro efficacy of chemotherapy

206 drugs. In comparison, the treatment that US was performed prior to TC-HT (US + TC-HT)

207 showed a less inhibition effect (43.1% viability), and hence we adopted the implementation

208 order TC-HT + US as the protocol of the triple treatment in the subsequent experiments.

209 Furthermore, in all double treatments, only 0.3% propolis + TC-HT showed notable inhibition

210 effect (48.9% viability) on PANC-1 cells, which was consistent with our previous results [28].

211 However, 0.3% propolis + US performed a relatively poor inhibition effect (65.4% viability)

212 on PANC-1 cells, and as a result it was also not included in the following experiments. **Fig**

213 **2C** showed the light microscope images of PANC-1 cells 24 h after each treatment, and the

214 cell morphologies demonstrated an evident inhibition effect on PANC-1 cells after the triple

215 treatment. Moreover, normal cells such as the human skin cells Detroit 551 (**Fig 2D**) and

216 human pancreatic duct cells H6c7 (**Fig 2E**) were not significantly affected by the triple

217 treatment as well as all the other treatments. The result indicates that the triple treatment

218 could have a good selective effect on carcinoma cells and normal cells, which makes it safer

219 and more feasible in anticancer treatment.

220

221 **Fig 2. Viability inhibition effects of propolis on PANC-1, Detroit 551, and H6c7 cells.**

222 MTT assay was conducted to determine the viabilities of PANC-1 cells after the treatment of

223 (A) different propolis concentrations and (B) different combinations of physical stimulations.

224 (C) Representative light microscope images of PANC-1 cells after each treatment, scale bar =

225 100 μ m. The viabilities of (D) normal human skin cells Detroit 551 and (E) normal human

226 pancreatic duct cells H6c7 were measured 24 h after each treatment. Data were presented as

227 the mean $\pm$ standard deviation in triplicate. (**P<0.001).

228

229 **Triple treatment increases intracellular ROS levels synergistically**

230 Intracellular ROS is an important regulator of cell death. It has been reported that heat and

231 low-intensity US could elevate the intracellular ROS level [31, 32]. We further investigated

232 whether ROS was increased in response to the triple treatment, so the DHE was used in this

233 experiment to determine the level of superoxide radical anion ($O_2^{\cdot -}$) in PANC-1 cells after

234 each treatment. As shown in **Fig 3A** and **3B**, it was found that propolis hardly changed the

235 fluorescence signals. Although propolis + TC-HT significantly deformed the fluorescence

236 intensity distribution in an enhanced manner (1.6-fold to control), it did not significantly

237 differ from the enhancement induced by TC-HT alone. In addition, US elevated ROS levels
238 as well, though not as many as TC-HT. Noticeably, the triple treatment showed a significant
239 accumulation of the intracellular ROS (up to 2.1-fold of the control group), which was also
240 significantly higher than the TC-HT + 0.3% propolis treatment. The result suggested that the
241 triple treatment boosted up the generation of ROS in PANC-1 cells, and could result in
242 enhanced cell death rate after the treatment.

243

244 **Fig 3. The triple treatment raised the generation of intracellular ROS.**

245 (A) Intracellular superoxide radical anion ($O_2^{\cdot -}$) levels of PANC-1 cells were determined by
246 flow cytometry with the fluorescent dye DHE. (B) Quantification of the mean DHE
247 fluorescence levels after each treatment. Data were presented as the mean $\pm$ standard
248 deviation in triplicate. (**P<0.001).

249

250 **Triple treatment increases mitochondrial apoptosis and autophagy in PANC-1** 251 **cells**

252 It has been known that the enhanced intracellular ROS levels were positively correlated to
253 mitochondrial apoptosis [33]. In our work, the apoptotic rates of PANC-1 cells after various

254 treatments were analyzed by the flow cytometry with the fluorescence dye Annexin V and PI
255 (**Fig 4A & 4B**). The triple treatment caused 55.3% apoptotic rate, which was significantly
256 higher than the 23.5% of propolis + TC-HT. The cell apoptosis results observed here were
257 highly consistent with the results of the accumulated ROS levels in PANC-1 cells after the
258 same treatment, as described in **Fig 3**. Furthermore, the mitochondrial membrane potential
259 (MMP) was assessed using DiOC₆(3) fluorescence staining by flow cytometric analysis. As
260 shown in **Fig 4C** and **4D**, the ratio of the cells exhibiting MMP loss was significantly
261 promoted to 23.3% after the double treatment of propolis + TC-HT, and it was further
262 elevated significantly to 34.7% by adopting the triple treatment. These results showed that the
263 triple treatment could decrease MMP level, and hence caused mitochondrial dysfunction. The
264 decreased MMP level was an indicator of mitochondrial apoptosis, and since the results of
265 apoptosis assay (**Fig 4A & 4B**) and MMP assay (**Fig 4C & 4D**) were quite similar, we
266 believe that the mitochondrial dysfunction was implicated in the apoptosis of PANC-1 cells
267 via the triple treatment.

268

269 **Fig 4. Triple treatment increased apoptosis on PANC-1 cells via mitochondrial**
270 **dysfunction.**

271 (A) Apoptosis after treatment was analyzed via flow cytometry with Annexin V-FITC/PI
272 double staining, and (B) the apoptotic percentage (Q2+Q4) were calculated. (C) MMP level
273 after treatment was analyzed via flow cytometry with DiOC₆(3) staining, and (D) the
274 percentage of cells with the loss of MMP (M1) was calculated. Data were presented as the
275 mean $\pm$ standard deviation in triplicate. (***P<0.001, **P<0.01).

276
277 In addition to apoptosis of PANC-1 cells, our previous study has also shown that US alone or
278 accompanied with other treatments could promote autophagy in PANC-1 cells [18]. In this
279 experiment, autophagy was also examined after each treatment. The conversion of precursor
280 LC3-I to the product LC3-II (ratio of LC3-II/LC3-I) quantified the autophagy activity, and the
281 western blot result showed that both US and TC-HT could slightly elevate the autophagy
282 activity in PANC-1 cells (**Fig 5**). Moreover, propolis + TC-HT treatment further enhanced
283 this activity, but it was significantly less than the autophagy activity produced by the triple
284 treatment, as shown in **Fig 5**. Hence, the triple treatment in this study was also an effective
285 promoter of autophagy in PANC-1 cells.

286

287 **Fig 5. Autophagy activation after the triple treatment in PANC-1 cells.**

288 (A) Representative western blots of autophagy related proteins LC3-I and LC3-II and (B) the
289 quantification of LC3-II/LC3-I ratio. GAPDH was used as loading control. Data were
290 presented as the mean $\pm$ standard deviation in triplicate. (**P<0.01, ***P<0.001).

291

292 **The apoptosis induced by triple treatment is regulated through MAPK pathway**

293 The activation of apoptotic signalling was examined by western blot analysis. As shown in
294 **Fig 6A**, we found that the PARP cleavage was significantly increased (2.9-fold of control)
295 after propolis + TC-HT treatment on PANC-1 cells. Noticeably, the PARP cleavage was
296 further promoted significantly to 6.2-fold of control by US in the triple treatment (**Fig 6A**).
297 Together with the previous flow cytometry results of apoptosis and MMP, it was pointed out
298 that propolis + TC-HT could activate the mitochondrial apoptosis signalling in PANC-1 cells,
299 and US in the triple treatment could further help this cascade to realize a near-chemotherapy
300 level treatment in vitro.

301 Moreover, it was known that the PARP cleavage could be modulated by MMP level,
302 and mitochondrial dysfunction could also be regulated by the excessive intracellular ROS via
303 MAPK family [34]. In MAPK family, the phosphorylated ERK (p-ERK) level represented the
304 activation of cell survival [35], while the phosphorylated JNK (p-JNK) and p38 (p-p38) levels

305 were the indicator of cell death [36, 37]. In this study, it was found that the p-ERK level was
 306 suppressed by propolis + TC-HT treatment (0.30-fold), and was further down-regulated when
 307 US was introduced in the triple treatment (0.15-fold) (**Fig 6B**). In addition, the p-JNK and
 308 p-p38 levels both exhibited a reverse performance, which were promoted the most in the
 309 triple treatment (8.7-fold & 9.2-fold, respectively) (**Fig 6C & 6D**). These results were
 310 consistent with the results of ROS and MMP assessments by flow cytometry. Therefore, we
 311 speculated that the excess intracellular ROS induced by the triple treatment regulated the
 312 activation of the MAPK family and thus caused mitochondrial dysfunction and the cascade of
 313 apoptosis.

314

315 **Fig 6. Triple treatment modulated apoptosis via regulating the MAPK family.**

316 Representative western blots of the apoptosis-related proteins and the quantification of (A)
 317 the PARP cleavage (ratio of cleaved PARP/full length PARP), the phosphorylation level of
 318 (B) ERK, (C) JNK, and (D) p38. GAPDH was used as loading control. Data were presented
 319 as the mean $\pm$ standard deviation in triplicate. (**P<0.01, *P<0.05).

320

321 **ROS scavenger attenuates the apoptosis induced by triple treatment**

322 To further confirm that the cell death after the triple treatment was regulated by the
323 generation of intracellular ROS, the ROS scavenger NAC was applied in the experiment [38].
324 5 mM NAC was incubated with PANC-1 cells 1 h prior to the triple treatment. As shown in
325 **Fig 7A**, the inhibitory effect of the triple treatment was restored by NAC, and NAC itself did
326 not affect the viability of PANC-1 cells. Similar results were also observed in the activation
327 of apoptotic pathway, as shown in **Fig 7B**. NAC alone did not affect PARP cleavage, but it
328 significantly down-regulated the triple treatment-promoted PARP cleavage (**Fig 7B**).
329 Therefore, the results supported our speculation that the triple treatment could induce
330 mitochondrial apoptosis of PANC-1 cells via the excessive increment of intracellular ROS.
331

332 **Fig 7. ROS inhibition blocked the cell death and the activation of apoptosis pathways.**
333 (A) Cell viabilities of PANC-1 cells and (B) the quantification of PARP cleavage (ratio of
334 cleaved PARP/full length PARP) after the triple treatment with or without 1h NAC
335 pretreatment. GAPDH was used as loading control. Data were presented as the mean $\pm$
336 standard deviation in triplicate. (**P<0.001, *P<0.01).
337

338 **Triple treatment can be applied with chemotherapy drug as a novel anticancer** 339 **treatment**

340 In this study, we have shown that the method TC-HT followed by mild US exposure could
341 further amplify the anticancer effect of propolis. But, the question is whether the TC-HT +
342 US method can be expanded to the existing chemotherapy drugs. Cisplatin, for instance, was
343 a commonly used clinical chemotherapy drug for several kinds of cancers such as lung,
344 ovarian, breast, and brain cancer [39]. Besides, it has also been reported that cisplatin was
345 sensitive to heat [40]. However, the conventional therapeutic dosage of cisplatin could cause
346 severe side effects to the patients [41, 42], and therefore it was important to develop a new
347 method to reduce the effective dose of cisplatin. As a result, we applied the method of TC-HT
348 + US with cisplatin on the PANC-1 cells to investigate the potential of this triple treatment
349 method. A relatively low dose of 1 μ M cisplatin and short incubation time 24 h was chosen
350 [43, 44] for the MTT assay independently or in combination with the physical stimulations.
351 After the treatment, the viability of PANC-1 cells was just slightly inhibited by 7.5% by
352 individual cisplatin, and TC-HT + cisplatin treatment also did not alter the viability of
353 PANC-1 cells. The triple treatment, however, promoted the inhibitory effect significantly up
354 to 48.2% (**Fig 8**). Compared to the conventional results of cisplatin, our method could not

only reduce the effective dose but also boost up the anticancer effect of cisplatin. Therefore, the method of physical stimuli in the study should hold great potential to apply onto other heat sensitive chemotherapy drugs or anticancer agents to achieve a better anticancer effect.

Fig 8. The method of triple treatment promoted the inhibitory effect of the heat sensitive chemotherapy drug cisplatin.

Cell viabilities of PANC-1 cells treated with cisplatin, TC-HT + cisplatin, and the triple treatment of cisplatin. Cisplatin concentrations in all treatments were 1 μ M. Data were presented as the mean $\pm$ standard deviation in triplicate. (**P<0.001).

Discussion

Combination treatment augments the anticancer effect of individual agents by activating multiple pathways, thereby lowering the necessary dosage of the agents to a level harmless to normal cells and human health. However, research on combination anticancer agents is costly and time-consuming [45]. Moreover, unpredictable molecular interactions may be detrimental to patients' health [5]. Therefore, the application of physical stimuli has been considered as a potential candidate for combinative anticancer treatments in combating cancer. Following

372 several combination treatments of physical stimuli and herbal compounds proposed by our
373 team previously [46], the study put forth the combination treatment of propolis, TC-HT, and
374 low-intensity US, proving that it can inhibit pancreatic cancer cell line PANC-1 at a level
375 close to the in vitro efficacy of chemotherapy drugs.

376 In cancer cells, intracellular ROS levels have been known to be the main source of the
377 oxidative stress [47], a key factor for cell viability [48]. Elevated ROS level has been shown
378 to be able to activate some signalling pathways associated with cell proliferation, apoptosis,
379 autophagy, and cell cycle progression [49]. Heat treatment and low-intensity US both can
380 increase the intracellular ROS levels [31, 32]. In this work, the study shows that TC-HT
381 significantly augments the intracellular ROS levels of PANC-1 cells (**Fig 3A** and **Fig 3B**), at
382 an extent higher than the individual effects of propolis and low-intensity US. It was found that
383 the combination of propolis and TC-HT did not further elevate the levels in PANC-1 cells.
384 Nevertheless, the triple treatment showed a great improvement effect, doubling the
385 intracellular ROS levels of PANC-1 cells. It has been known that excessive intracellular ROS
386 level could activate the apoptotic pathway cascade, increasing the apoptosis in the carcinoma
387 cells [33]. Our study demonstrated that the apoptotic rate of PANC-1 cells was elevated along
388 with the increase of the intracellular ROS levels. The result showed that the triple treatment

389 induced the highest apoptotic rate, compared with other approaches, suggesting its ability to
 390 regulate the death of PANC-1 cells via excessive intracellular ROS accumulation. To
 391 demonstrate the crucial role of the intracellular ROS levels, NAC was employed in the
 392 following experiments on the triple treatment. While independent NAC pretreatment did not
 393 affect the viabilities of PANC-1 cells (**Fig 7A**), it protected the cells from cytotoxicity in the
 394 triple treatment. Hence, ROS elevation played a key role in the anticancer effect in the triple
 395 treatment.

396 The initiation of apoptosis, a common cell death mechanism, is closely related to the
 397 function of mitochondria, which is the chemical-energy source of cells and critical for the
 398 viability of cells [50]. Its dysfunction could induce a series of biochemical cascade of
 399 apoptosis, thereby blocking cell proliferation [51]. Meanwhile, the activated members of the
 400 MAPK family, such as ERK, JNK, and p38 were deemed to be capable of regulating the
 401 dysfunction of mitochondria, and the elevated ROS level was shown to be conducive to the
 402 activation of p38 and JNK but down-regulate the activation of ERK [52]. The results suggest
 403 that excessive ROS could cause the mitochondrial apoptotic rate via activating the MAPK
 404 family members. In this work, our study showed that the triple treatment raised significantly
 405 the apoptotic rate of PANC-1 cells (**Fig 4A and 4B**), while decreasing the MMP level, which

406 led to the dysfunction of mitochondria (**Fig 5A** and **5B**). Furthermore, the triple treatment
407 increased the phosphorylated levels of p38 and JNK significantly, while inhibiting the
408 phosphorylation of ERK (**Fig 6**), underscoring its ability to manipulate the function of
409 mitochondria via the ROS-activated MAPK family proteins.

410 The injured mitochondria would release cytochrome-c into the cytoplasm [53], cleaving
411 caspase 9 and thus activating caspase 3 in the downstream [54], which entered further the
412 nucleus and cleaved PARP. Then PARP would lose its enzyme activity, initiating apoptosis
413 irreversibly [55]. The study demonstrated that the triple treatment could induce mitochondrial
414 dysfunction via regulating the phosphorylation level of the members of MAPK family. In
415 addition, the triple treatment also increased PARP cleavage significantly, compared with the
416 combination treatment of propolis and TC-HT (**Fig 6A**). Furthermore, as shown in **Fig 7B**, it
417 was found that the triple treatment-promoted PARP cleavage was significantly suppressed by
418 NAC, which indicates that increased ROS level was the key regulator for the apoptotic effect
419 of the triple treatment.

420 The triple treatment in the study included two physical stimuli, TC-HT and US, which
421 could be integrated for simultaneous implementation via the high-intensity focused ultrasound
422 (HIFU). Being able to raise temperature in the exposed region for energy transfer [19, 20],

423 HIFU has been applied for tumor ablation for over a decade [16, 56]. The temperature
424 increase could be electrically controlled and directed to the targeted region [57]. Therefore,
425 with the help of HIFU, the triple treatment could be applied clinically. To augment the effect,
426 cisplatin, a common thermal sensitive chemotherapy drug, was incorporated as a substitute
427 for propolis into the triple treatment. It was shown that the inhibited viability of PANC-1 cells
428 by cisplatin was further suppressed with a large extent when both of TC-HT and US were
429 introduced into the treatment (**Fig 8**). The dosage of cisplatin can be reduced to a lower
430 concentration, without compromising the anticancer effect. As a result, the triple treatment
431 has the potential supplementing the administration of drugs, not only augmenting the effect
432 but also reducing the dosage of the latter.

433 In summary, this study proposed for the first time an effective triple cancer treatment
434 combining propolis, TC-HT, and low-intensity US, which could significantly suppress the
435 growth of PANC-1 cells via an ROS-modulated mitochondrial apoptosis, with a performance
436 comparable to chemotherapy. The study also showed that the triple treatment could induce
437 mitochondrial dysfunction via the regulation of MAPK family, resulting in apoptosis via the
438 up-regulated PARP cleavage. It also demonstrated that the ROS level plays a key role in the
439 performance of the triple treatment. In addition, chemotherapy drugs, such as cisplatin, can be

440 incorporated into the treatment as substitute for propolis. The triple treatment incorporating
441 cisplatin also exhibited a much higher effect in inhibiting cancer cell growth than the cisplatin
442 alone, promising to increase the performance and safety of the existing cancer therapy.
443 Overall, the study proposed employment of physical stimuli, as a promising option in cancer
444 therapy.

445

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454 **Competing Interests**

455 The authors have declared that no competing interests exist.

456

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Figure 1

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