Active extracts of black tea *(Camellia Sinensis)* induce apoptosis of PC-3 prostate cancer cells via mitochondrial dysfunction

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Abstract. Cancer of the prostate gland is the most common invasive malignancy and the second leading cause of cancer-related death in human males. Many studies have shown that black tea reduces the risk of several types of cancer. We studied the effects of active extracts of black tea and the black tea polyphenols theaflavins (TFs), on the cellular proliferation and mitochondria of the human prostate cancer cell line PC-3. Our studies revealed that Yinghong black tea extracts (YBT), Assam black tea extracts (ABT) and TFs inhibited cell proliferation in a dose-dependent manner. We also showed that TFs, YBT and ABT affected the morphology of PC-3 cells and induced apoptosis or even necrosis in PC-3 cells. In addition, it was observed that the samples significantly caused loss of the mitochondrial membrane potential, release of cytochrome *c* from the intermembrane space into the cytosol, decrease of the ATP content and activation of caspase-3 compared with the control. Taken together, these findings suggest that black tea could act as an effective anti-proliferative agent in PC-3 cells, and TFs, YBT and ABT induced apoptosis of PC-3 cells through mitochondrial dysfunction.

Introduction

Tea, one of the most widely consumed beverages in the world, has diverse pharmacological activities, including anti-hyperglycemia, antioxidative, anti-obesity and antitumor activities (1-4). Green tea, consumed at high levels in Asian countries, and black tea, consumed primarily in Western countries, are derived from leaves of *Camellia sinensis* (5).

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During the past decades, numerous *in vitro* and *in vivo* studies have showed the possible protective effects of tea and tea polyphenols on cancer and other diseases (6-8). Catechins are the most abundant polyphenol in green tea, whereas the typical pigments in black tea are formed from catechin oxidation during fermentation which includes theaflavins, thearubigins and theabrownins (9). Among them, the major theaflavins in black tea are theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B) and theaflavin-3,3'-digallate (TF3) (Fig. 1). Therefore, the monomeric polyphenol content of black tea is low. However, it is not diminished for black tea during partial polymerization or other alterations in the fermentation of tea leaves (10), as it was proved that TF3 showed higher antioxidative activity than EGCG (11).

Apoptosis can occur via the mitochondria. The mitochondria is essential for energy production, and is involved in reactive oxygen species (ROS) generation and induction of apoptosis (12). In most tissues, the mitochondria accounts for the generation of ~90% of the ATP needed by the cells (13). Mitochondrial dysfunction causes permeabilization of the outer mitochondrial membrane (14) and then leads to the release of the intermembrane space proteins such as cytochrome *c*, which ultimately triggers apoptotic cell death (15). Therefore, the mitochondrial permeability transition event could serve as an early indicator of the initiation of apoptosis. This mitochondrial permeability transition process results in the collapse of the electrochemical gradient across mitochondrial membrane and thus could be measured by noting the changes of the mitochondrial membrane potential (14).

Prostate cancer is one of the leading causes of human male deaths throughout the world (16). It is a group of cancerous cells (a malignant tumor) that grow mostly from the outer part of the prostate (17). In our previous study, we evaluated time- and dose-dependent cytotoxicity of EGCG and Zn^{2+} on PC-3 cells by the MTT assay (18). Whereas, in another study, we found that EGCG, Zn^{2+} and EGCG+Zn^{2+} induced apoptosis or even necrosis of PC-3 cells through the mitochondria-mediated pathway, and free Zn^{2+} enhanced the effects of EGCG on PC-3 cells due to its interactions with mitochondria (19). In the present study, we investigated effects of black tea extracts on the viability and morphology of PC-3 cells, the functions of mitochondria of PC-3 cells,

including mitochondrial membrane potential, cytochrome *c* release, ATP contents and caspase-3 activities.

Materials and methods

Materials. Yinghong black tea (YBT) was obtained from Tea Research Institute, Guangdong Academy of Agricultural Sciences in China. Assam black tea (ABT) was purchased from Premier's Tea Limited in India. Theaflavins (TFs, >50% purity) were obtained from Hangzhou Easily Biotechnology Co. Ltd (Hangzhou, China). HEPES and ATP were all purchased from Sigma (St. Louis, MO, USA). Mitochondrial membrane potential assay kit with JC-1 and caspase-3 activity assay kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). EdU solution was obtained from Guangzhou RiboBio Co. Ltd. (Guangzhou, China). The cell apoptosis Annexin V-FITC detection kit was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, China). Cytochrome *c* pAb was purchased from Bioworld Technology (Louis Park, MN, USA). Human prostate cancer cells (PC-3) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All other chemicals were extra-pure grade or analytical grade except those explained in the study.

Preparation of black tea extracts and cell culture. Black tea was minced and extracted three times by placing in the boiling distilled water for 10 min each time (tea/water, 1:5 w/v). These extracted solutions were combined and concentrated at 60°C with RE-2000 Rotary Evaporator (Yarong Biochemical Instrument, Shanghai, China). Finally, the solution was dried by lyophilization (Savant Novalyphe-NL500, USA) to obtain the aqueous extract.

The contents of catechin and theaflavin monomers were analyzed using a high performance liquid chromatography (HPLC) system (Shimadzu LC-2010, Kyoto, Japan). The TFs, YBT and ABT extracts were analyzed on a Diamonsil C18 column (4.6x250 mm, 5 μm particle size, Japan). The eluate was monitored at 280 nm. Mobile phase A and B were made of acetic acid/acetonitrile/water (A: 0.5:3:96.5 and B: 0.5:30:69.5, by volume). The flow rate was set at 1 ml/min and 10 μl sample was injected into the column. The elution was performed using a linear gradient from solvent A to solvent B in 45 min followed by an isocratic step of solvent B for 15 min. The column temperature was set to 28°C. Peaks were identified in comparison with the retention time of authentic standards.

PC-3 cells were cultured in F-12 medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. The cells were cultured in a 5% CO_2 humidified atmosphere at 37°C.

MTT and EdU assays. PC-3 cells were seeded on 96-well plates for 24 h and then incubated with 0.4, 0.8, 1.2, 1.6, 2.0 mg/ml TFs, YBT and ABT for 12, 24 and 36 h, respectively. Thereafter, 20 μl of 5 mg/ml MTT was added to each well from a stock solution and cultured for another 4 h. Finally, the reaction was stopped by addition of 150 μl of dimethyl sulfoxide (DMSO). The absorbance in each well was measured with the microplate reader (Thermal Labsystem, Helsinki, Finland) at 490 nm.
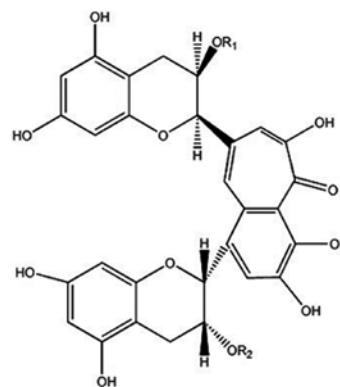

Figure 1. Chemical structures of theaflavins. TF1=Theaflavin R_1=R_2=H; TF2A=Theaflavin-3-gallate R_1=galloyl R_2=H; TF2B=Theaflavin-3'-gallate R_1=H R_2=galloyl; TF3=Theaflavin-3,3'-digallate R_1=R_2=galloyl.

The proliferation of PC-3 cells was estimated using the EdU incorporation assay. Briefly, the PC-3 cells ($1x10^5$) were exposed to 0.4 mg/ml YBT, ABT and TFs for 24 h, respectively. The cells were cultured in 24-well plates and exposed to 50 μmol/l EdU for 2 h at 37°C. The cells were then fixed in 4% formaldehyde for 30 min at room temperature and permeabilized in 0.5% TritonX-100 for 10 min. Cells were washed with PBS, and each well was incubated with 100 μl 1X Apollo® reaction cocktail for 30 min. DNA was then stained with 100 μl 1X Hoechst 33342 for 30 min and imaged under a fluorescent microscope.

Morphological changes of adhesive PC-3 cells. PC-3 cells were cultured in 6-wells plates (20x20 mm) and then exposed to 0.4 mg/ml YBT, ABT and TFs for 24 h. After exposure, the images of adhesive PC-3 cells were made through the inverted microscope at 10x10 magnifications (XDS-1B, Chongqing, China). The number of cells adhering on the plate and the changes of cell morphology indicated the cytotoxicity of YBT, ABT and TFs on PC-3 cells.

Apoptosis of PC-3 cells. The apoptosis of PC-3 cells was measured by flow cytometry. PC-3 cells were treated with 0.4 mg/ml YBT, ABT and TFs for 24 h, and then cell apoptosis was analyzed by Annexin V-FITC apoptosis detection kit following the manufacturer's instructions. Briefly, PC-3 cells were gently trypsinized, washed twice with PBS, suspended in binding buffer ($1x10^6$ cells/ml). Then, 0.5 ml suspension with 1.25 μl Annexin V-FITC and 10 μl PI was incubated at room temperature in the dark for 15 min, and analyzed immediately by flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA).

Isolation and purification of mitochondria. The lowest effective concentration for suppressing PC-3 cells measured by the MTT assay was 0.4 mg/ml in our previous study (unpublished data). Therefore, in the present study, YBT, ABT and TFs were prepared at equal concentrations (0.4 mg/ml) to investigate the bioactivity of black tea. The PC-3 cells were seeded on 50-cm² plates at a density of $6x10^5$ cells/ml and allowed to grow for 24 h. After treatment with 0.4 mg/ml YBT, ABT and TFs for 24 h, the cells were harvested by centrifugation at 1,500 rpm for 5 min at 4°C, and washed twice with cold PBS and

Table I. Catechin and theaflavin monomers contents of TFs, YBT and ABT as analyzed by HPLC (mg/g).

Sample	C	EC	GC	EGC	CG	ECG	GCG	EGCG	TF1	TF2A/TF2B	TF3
TFs	15.66±2.65[b]	32.01±9.20[b]	140.35±20.19[a]	30.23±7.26[a]	1.97±0.04[a]	22.33±2.03[b]	5.58±0.26[b]	9.68±0.07[b]	199.08±44.96[b]	118.89±39.54[c]	193.34±53.61[c]
YBT	5.46±0.32[a]	7.61±1.04[a]	151.29±23.78[a]	32.37±7.39[a]	2.30±0.06[a]	20.07±1.88[a]	2.74±0.04[a]	7.32±0.04[a]	4.31±0.13[a]	1.41±0.02[a]	3.23±0.02[a]
ABT	17.36±2.97[b]	43.15±12.31[b]	192.52±31.16[b]	88.46±13.11[b]	3.91±0.10[b]	22.64±2.15[a]	6.22±0.39[b]	19.92±0.15[c]	5.80±0.28[a]	6.40±0.17[b]	8.20±0.08[b]

The value of catechin and theaflavin monomer contents are the means ± SD (n=3). [a-c]Means followed by the same letter are not significantly different at $p<0.05$.

finally re-suspended in the cold PBS. These cells were lysed by Ultrasonic Cell Disruption System (JY92-II, Chongqing, China) and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was centrifuged at 9,000 rpm for 10 min to obtain mitochondrial pellets that were then washed twice with cold PBS. The final mitochondrial pellets were re-suspended in the test medium (220 mmol/l Mannitol, 70 mmol/l Sucrose, 5 mmol/l HEPES, pH 7.2) for studies. The final protein concentrations of the mitochondrial suspension were adjusted to 0.3 mg/ml.

Determination of mitochondrial membrane potential. PC-3 cells were cultured in 6-well plates (20x20 mm) and then exposed to 0.4 mg/ml YBT, ABT and TFs for 24 h. The mitochondrial membrane potential ($\Delta\Psi m$) was estimated using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodide (JC-1, Molecular Probes) (20).

Analysis of cytochrome c release. PC-3 cells were cultured and exposed to 0.4 mg/ml YBT, ABT and TFs for 24 h, then isolated mitochondria. Contents of the cytochrome *c* were measured by western blotting (21). The experiments were repeated at least three times.

Assay of cellular ATP contents. The 0.4 mg/ml YBT, ABT and TFs-treated PC-3 cells were washed twice with cold PBS and homogenized with 600 μl cold PBS and 80 μl 0.4 μmol/l perchloric acid by mechanical homogenization at the high speed for 10 min. These samples were adjusted to pH 6.0 with 0.2 μmol/l K_2CO_3 and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through the Millipore filter with 0.45-μm pore diameter and then injected into HPLC system (LC-2010A, Shimadzu). The samples were analyzed on a C_{18} column (200x4.6 mm i.d., particle size 5 μm), where the mobile phase (pH 6.0) was methanol/potassium dihydrogen phosphate/potassium hydrogen phosphate (5: 47.5: 47.5). The flow rate was 1.0 ml/min, the effluent was monitored at 280 nm, and the sample injection volume was 20 μl. All instruments and the columns were operated at room temperature (23-25°C). All the experiments were repeated at least three times.

Determination of caspase-3 activity. PC-3 cells were cultured and exposed to 0.4 mg/ml YBT, ABT and TFs for 12, 24 and 36 h. The cells were harvested and carried out according to caspase-3 activity assay kit instructions. The assay was based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after its cleavage by caspase-3 from the labeled substrate, Ac-DEVD-*p*NA. Briefly, cell lysates were prepared after their respective treatment. Assays were performed on 96-well plates by incubating 50 μl protein of cell lysate per sample in the 50 μl reaction buffer containing 5 μl caspase-3 substrate (Ac-DEVD-*p*NA). Lysates were incubated at 37°C for 4 h. Samples were measured with an ELISA reader (Thermal Labsystem) at an absorbance of 405 nm.

Statistical analysis. Results were expressed as the means ± SD of at least three independent replications of each experiment. Statistical significance was determined by pair t-test analysis using Origin 7.5 software for Windows.
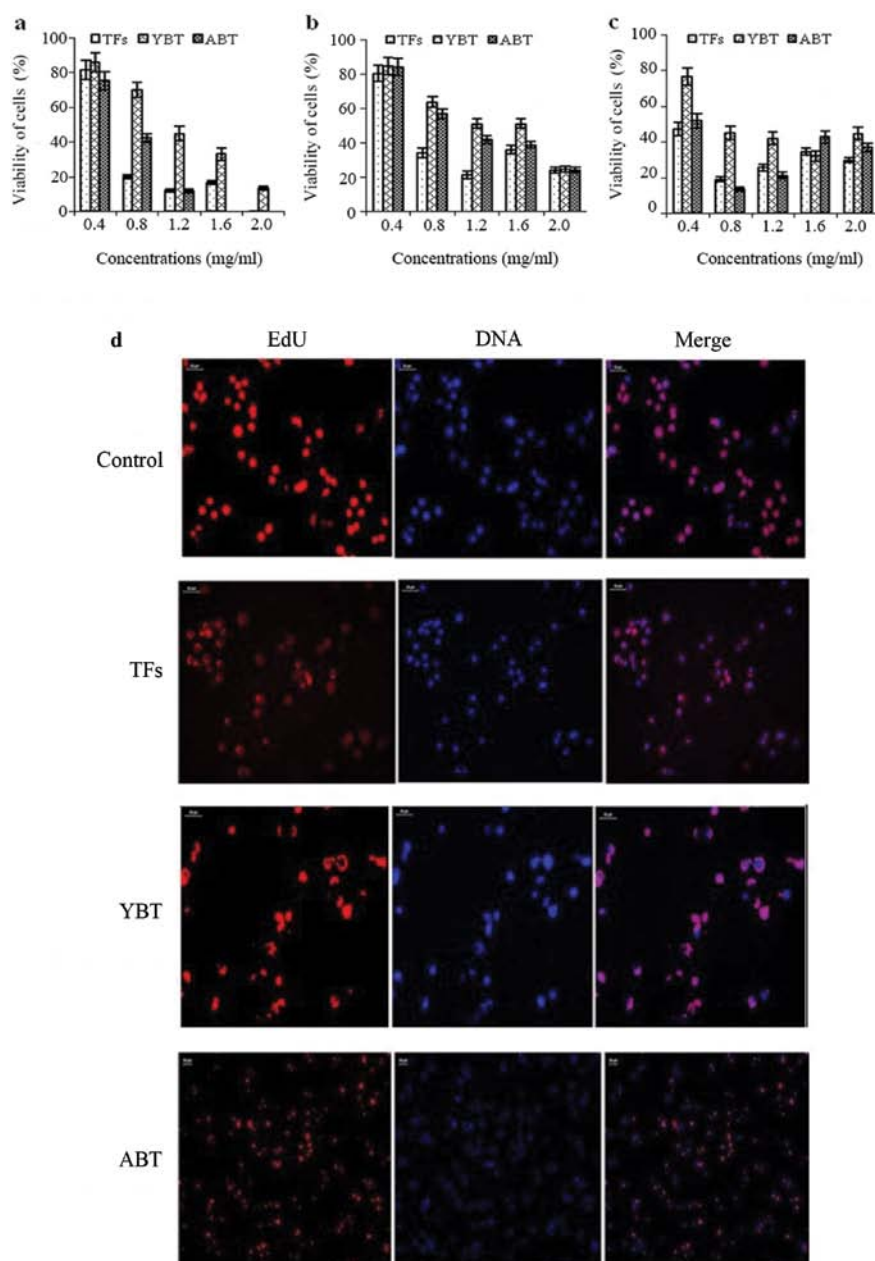
Figure 2. Effects of TFs, YBT and ABT on viability of PC-3 cells. The cells were exposed to TFs, YBT and ABT of different concentrations (0, 0.4, 0.8, 1.2, 1.6, 2.0 mg/ml) for (a) 12 h, (b) 24 h and (c) 36 h, respectively, and the cell viability was measured by the MTT assay as showed in Materials and methods. The values represent the percent of viable cells, where untreated cells were regarded as 100% viable. Data are means ± SD, n=7. (d) Proliferation was estimated using the EdU incorporation assay. Scale bars, 50 μm. The cells were exposed to TFs, YBT and ABT (0.4 mg/ml) for 24 h. The proliferation was significantly inhibited.

Results

Catechin and theaflavin monomers contents. Catechin and theaflavin monomer contents of TFs, YBT and ABT extracts were analyzed by HPLC. As shown in Table I, four kinds of theaflavin monomer contents of TFs were high in comparison with YBT and ABT, respectively. The active ingredients in black tea are theaflavins, thearubigins and theabrownins. The activity of TF3 theaflavin monomer is even more than that of EGCG. TF1, TF2 and TF3 content in three samples is in order of the TFs > ABT > YBT. The theaflavin monomer TF3 content was 193.34, 8.20, 3.23 mg/g in TFs, ABT and YBT, respectively.

Cell viability of PC-3 cells. The PC-3 cells were exposed to 0.4-2.0 mg/ml YBT, ABT and TFs for 12, 24 and 36 h, respectively. The MTT assay demonstrated that all the treatment caused suppression of the growth of PC-3 cells. As shown in Fig. 2, after the treatment, the cell viability of PC-3 cells decreased (untreated cells = 100%) in a dose-dependent manner and resulted in a maximum loss when the concentration is 2.0 mg/ml. At 12, 24 or 36 h, the decrease of the cell viability of PC-3 cells exposed to TFs was more significant than the other two black tea extracts. In addition, the decrease of the cell viability exposed to ABT was more significant than that exposed to YBT. These results suggested that TFs and black tea extracts could significantly inhibit the growth of PC-3 cells.
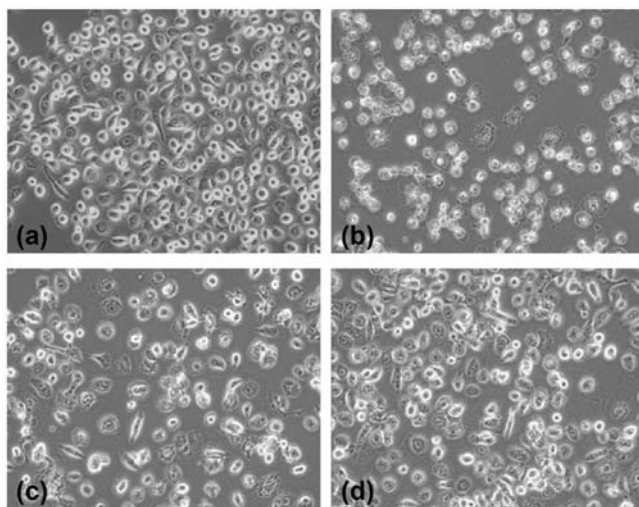
Figure 3. Microscopic images of PC-3 cells treated with TFs, YBT or ABT. PC-3 cells were exposed to (a) control, (b) 0.4 mg/ml TFs, (c) 0.4 mg/ml YBT, and (d) 0.4 mg/ml ABT for 24 h.

| Gated	UL	UR	LL	LR	UR+LR
Control	0.10%	1.54%	96.73%	1.63%	3.17%
TFs	0.66%	56.56%	13.00%	29.78%	86.34%
YBT	0.61%	2.77%	93.54%	3.08%	5.85%
ABT	0.47%	2.12%	93.97%	3.44%	5.56%

Figure 4. Apoptosis. PC-3 cells were exposed to (a) control, (b) 0.4 mg/ml TFs, (c) 0.4 mg/ml YBT, and (d) 0.4 mg/ml ABT for 24 h, and analyzed by flow cytometry. TFs, YBT or ABT treatment induced PC-3 cell apoptosis when compared with that of control group.

Proliferation of PC-3 cells. The proliferation of PC-3 cells was estimated using the EdU incorporation assay. The PC-3 cells ($1x10^5$) were exposed to 0.4 mg/ml YBT, ABT and TFs for 24 h. EdU incorporation assay showed that YBT, ABT and TFs treatment significantly inhibited PC-3 cell proliferation when compared to that of the control group.

Morphological changes of PC-3 cells. Effects of TFs, YBT and ABT on PC-3 cells were observed through the inverted microscope. The number of PC-3 cells adhering on plate decreased after exposed to 0.4 mg/ml TFs, YBT and ABT (Fig. 3) respectively, and the number of PC-3 cells adhering on the plates decreased according to the sequence of the control > YBT > ABT > TFs. Moreover, these three treatment made PC-3 cells become round, globular cells and larger than control cells.

Apoptotic and necrotic cells. Apoptosis of the PC-3 cells treated with TFs, YBT or ABT, respectively, was examined using flow cytometry. As shown in Fig. 4, 0.4 mg/ml TFs, 0.4 mg/ml YBT and 0.4 mg/ml ABT induced apoptosis of PC-3 cells, respectively, when compared with that of the control group.

Loss of mitochondrial membrane potential. JC-1 is a fluorescent compound (excitation maximum, 490 nm) that exists as a monomer at low concentrations. At higher concentrations, JC-1 forms aggregates. Fluorescence of the monomer is green (emission, 527 nm), whereas that of the J aggregate is red (emission, 590 nm). Mitochondria with intact membrane potential (>100 mV) concentrate JC-1 into aggregates that fluoresce red, whereas de-energized mitochondria cannot concentrate JC-1 and fluoresce green. JC-1 staining is widely used to measure mitochondrial membrane potential (ΔΨm). The change from red florescence to green represents the decrease of mitochondrial membrane potential. TFs, YBT and ABT caused a significant loss of mitochondrial membrane potential, as shown in Fig. 5.
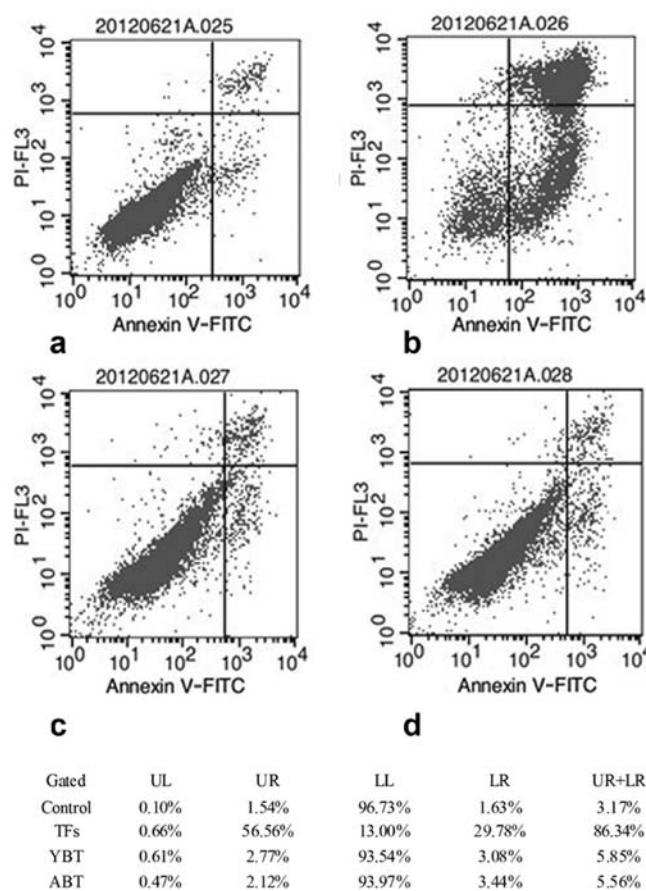
Concentration of mitochondrial cytochrome c. The cytochrome *c* from the intermembrane space into the cytosol was investigated. Western blot results (Fig. 6) showed that concentrations of the cytochrome *c* in mitochondria of PC-3 cells treated with TFs, YBT or ABT were all significantly decreased (TFs > YBT > ABT), which suggested that the cytochrome *c* was released from mitochondria into the cell cytosol.

ATP contents in PC-3 cells. In aerobic organisms, the mitochondria is the main source of energy and site of the tricarboxylic acid (TCA) cycle, a metabolic network is involved in the generation of reducing factors that power the production of ATP (22). In the present study, it was noted that TFs, YBT and ABT inhibited ATP synthesis (Fig. 7), and in particular ATP contents of PC-3 cells treated with TFs was significantly different from the control. The ATP contents in ABT-treated PC-3 cells were lower than that exposed to YBT and higher than that exposed to TFs. These results suggested that TFs, YBT and ABT could induce damage of mitochondria in PC-3 cells.

Effects of caspase-3 activities. Caspase-3 has been shown to play a pivotal role in the execution phase of apoptosis induced by diverse stimuli. In this study, we analyzed its activation in PC-3 cells treated by TFs, YBT or ABT for 12, 24 and 36 h. As shown in Fig. 8, treatment with TFs, YBT and ABT signifi-
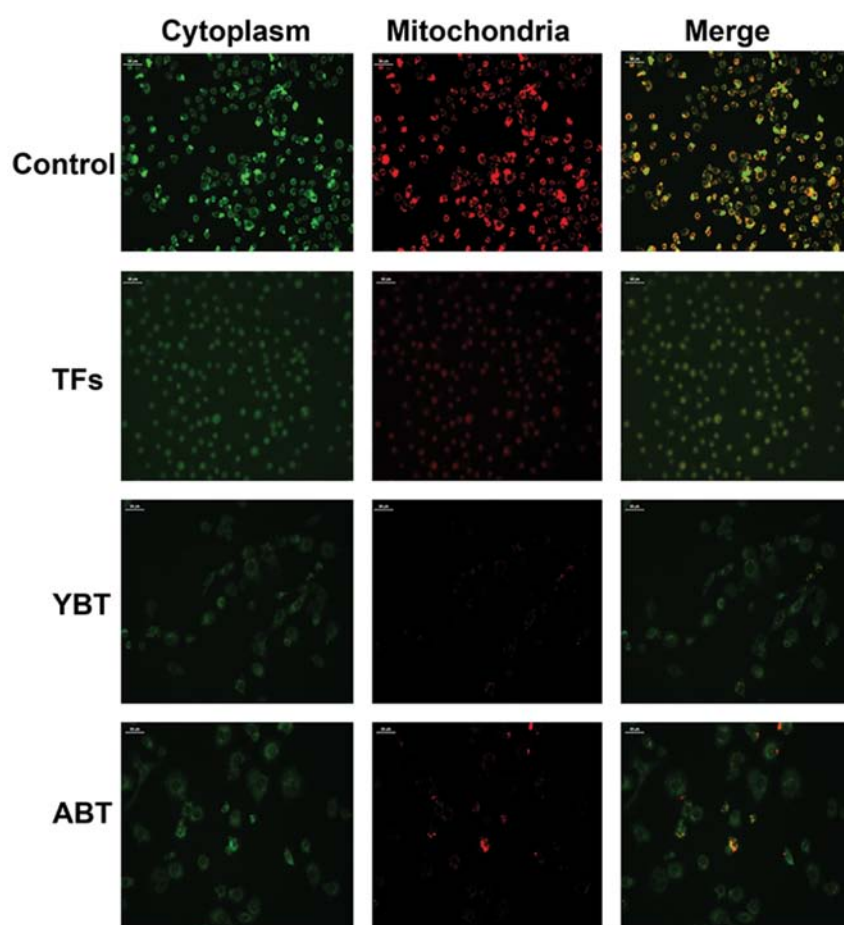
Figure 5. Effects of TFs, YBT and ABT on the mitochondrial membrane potential. PC-3 cells were treated with 0.4 mg/ml TFs, 0.4 mg/ml YBT and 0.4 mg/ml ABT for 24 h. The mitochondrial membrane potential was monitored using JC-1 staining. TFs, YBT or ABT treatment decreased mitochondrial membrane potential.
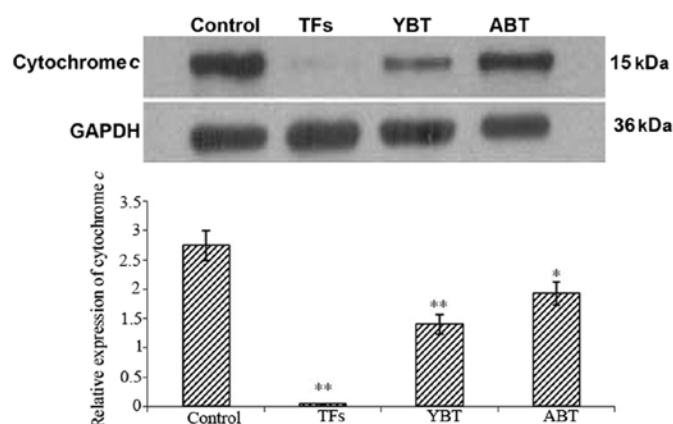

Figure 6. Concentration of cytochrome *c* in mitochondria of PC-3 cells. PC-3 cells were treated with 0.4 mg/ml TFs, 0.4 mg/ml YBT and 0.4 mg/ml ABT for 24 h and then the mitochondria was isolated. Concentration of cytochrome *c* in PC-3 cells was measured by western blotting. Data are means ± SD, n=3 (*p<0.05; **p<0.01).

cantly increased activities of caspase-3 in a time-dependent manner, and the effects of treatment were TFs > ABT > YBT. These results indicated that treatment with 0.4 mg/ml TFs, YBT or ABT stimulated the caspase-3 activities.

Discussion

Prostate cancer is one of the leading causes of human male death throughout the world. As estimated, more than 200,000 new cases of prostate cancer are detected in the United States in 2005, and ~30,000 men die each year from this disease (23). Prostate cancer is the most common type of cancer except skin cancer, diagnosed in American men (24). However, prostate disease is difficult to cure. The prostate gland has a nonporous capillary bed. In order to traverse the capillary endothelium, an antibiotic must pass through the endothelial cell membranes rather than the pores which are found in capillary beds elsewhere in the body. To reach the prostatic secretions, the drug must traverse the additional lipid barrier of the prostatic epithelium. However, drugs which are poorly lipid-soluble, such as penicillins, cephalosporins and aminoglycosides, traverse the capillary wall very slowly (25).

Our previous studies indicated that EGCG could satisfactorily exhibit complex chemistry with metal ions, and this chelation between EGCG and metal ions changed their bioactivities and metabolisms, which suggested that EGCG could prevent or cure prostate cancer, and Zn^{2+} and Cd^{2+} could enhance the inhibitory effect of EGCG against PC-3 cells (26,27). In another study, we demonstrated that Zn^{2+} enhanced the action of EGCG on PC-3 cells, zinc-EGCG
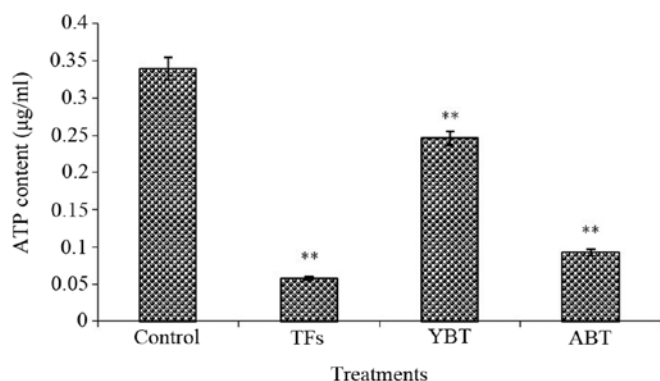
Figure 7. ATP contents of PC-3 cells. PC-3 cells were treated with 0.4 mg/ml TFs, 0.4 mg/ml YBT and 0.4 mg/ml ABT for 24 h, and then the mitochondria was isolated. ATP contents of PC-3 cells were measured by HPLC. Data are means ± SD, n=3 (**p<0.01).
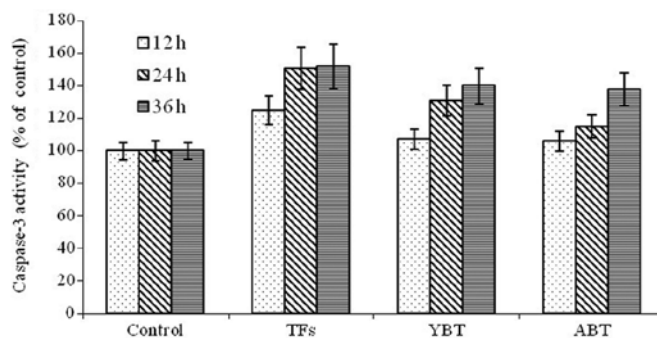

Figure 8. Caspase-3 activities of PC-3 cells treated with TFs, YBT or ABT for 12, 24 and 36 h. The cells were harvested and carried out according to caspase-3 activity assay kit instructions. The absorbance was measured in each well at 405 nm using the ELISA reader as showed in Materials and methods. Data are means ± SD, n=3.

complex was highly unlikely to be formed in the presence of Zn^{2+} and EGCG and our results also showed the potentiating action of Zn^{2+} on the growth inhibitory property of EGCG in PC-3 cells (18). In addition, we showed that EGCG induced apoptosis or even necrosis of PC-3 cells through the mitochondria-mediated pathway, and free Zn^{2+} enhanced effects of EGCG on PC-3 cells due to its interaction with mitochondria (19). However, studies elucidating the mechanisms involved in prostate cancer prevention by black tea are scarce in comparison with green tea. Therefore, in the present study, we report mechanism-based efficacy studies on black tea extracts in PC-3 cells.

Polyphenols present in food have been demonstrated to decrease various types of experimental carcinogenesis (28,29). In recent years, identification of effective chemopreventive polyphenols in diets or dietary supplements for human use is of great interest. Treatment with such polyphenols can result in cell cycle arrest (30), and therefore reduce the growth and proliferation through apoptosis or even necrosis, but only in cancerous, not in normal cells (31,32). In addition, tea polyphenols exert their potent anticancer activity and appear to be the ideal agents for chemoprevention. For the manufacture of black tea, the fermentation process causes green tea polyphenols to oxidise and form oligomeric flavanols, including theaflavins, thearubigin and other oligomers. Theaflavins, a mixture of TF1, TF2A, TF2B and TF3, are the most abundant phenolic fraction of black tea and their structures are not well characterized. Black tea is assumed to be less beneficial compared with green tea. However, studies have demonstrated that black tea could be as effective as green tea in cancer chemoprevention (10,33,34).

Among black tea components, theaflavins are generally considered to be the more effective components for the inhibition of carcinogenesis and account for 3-6% of dry weight of black tea (35). Black tea has also shown potent inhibition in animal systems, including the lung (36), colon (37) and skin (38). Other studies also provide evidence that black tea can significantly inhibit proliferation and enhance apoptosis in the established mouse skin tumor (39). Black tea polyphenols, especially TFs exert cancer chemopreventive activities by inducing apoptotic signals (39,40). In addition, lots of studies

have showed that the black tea and its polyphenol theaflavin could induce apoptosis in tumor cells, and such effect was not evident in normal healthy cells (41). This was possibly due to the difference in genetic programming in normal and tumor cells (42).

In the present study, we found that TFs, YBT and ABT all significantly suppressed the viability of PC-3 cells, made PC-3 cells become round, globular cells and bigger than control cells, induced apoptosis of PC-3 cells and even caused necrosis of PC-3 cells. One of the possibilities for why low cell viability at 2 mg/ml for 12 h was seen is that at the concentration of 2 mg/ml, the extracts cannot 100% inhibit cell proliferation and growth, the surviving cell proliferation might contribute to the higher cell viability at 24-36 h. The tea polyphenols or theaflavins *in vitro* have shown some activities against prostate cancer (18,19,43), and the polyphenol or theaflavin concentrations in our body is much lower than the micromole level (44,45). Therefore, drinking tea can probably prevent the occurrence of prostate cancer, but has almost no effect on curing the patient of prostate cancer.

Similar to green tea polyphenols, black tea and its major polyphenol has antioxidant (5,46) and pro-oxidant dual activities (47). TFs, the characteristic compositions in black tea, are formed via the co-oxidation of pairs of epimerized catechins, one with a vic-trihydroxyphenyl moiety, and the other with an ortho-dihydroxyphenyl structure. Different from epimerized catechins, four main TF derivatives reserve two A-rings, two C-rings from their precursors, and possess a characteristic element of the fused seven-member benzotropolone ring (Fig. 1). It has been suggested that the existence of resonance formed in the benzotropolone moiety might be responsible for electron donation (46). Jhoo *et al* suggested that the benzotropolone moiety of TFs might play an important role in affording antioxidant protection for the preferred oxidation site in the oxidant models of 2,2-diphenyl-1-picrylhydrazyl and H_2O_2 (48). However, theaflavins also exert pro-oxidant behavior (49). Sakagami *et al* suggested that the antioxidant activity of tea polyphenolics is the driving force to inhibit carcinogenesis, while the pro-oxidant activity accounts for their selective cytotoxicity, growth inhibitory and apoptosis-inducing activity towards tumor cells (50). In addition, Weisburg *et al* has shown that green tea catechins

generate ROS in the cell culture medium and increase the intracellular production of ROS (51), which may induce loss of the mitochondrial membrane potential leading to release of apoptotic factors (52,53).

Mitochondria plays an important role in cellular energy metabolism, free radical generation and apoptosis (54). Several mDNA-encoded pro-apoptotic proteins including cytochrome *c*, apoptosis inducing factor (AIF), endonuclease G, and smac/DIABLO normally reside in the mitochondria. Release of these factors from mitochondria triggers a series of biochemical events leading to activation of apoptotic signaling cascades (55).

It is evident from the results of the present study that treatment with TFs, YBT and ABT could induce apoptosis of PC-3 cells through mitochondrial dysfunctioning. This is supported by the observations that exposure of PC-3 cells to TFs, YBT or ABT induced: i) release of cytochrome *c* from mitochondria and loss of mitochondrial membrane potential that plays a critical role in mitochondria-dependent apoptosis pathway; and ii) a decrease in ATP contents and activation of caspase-3 that plays a pivotal role in the execution phase of apoptosis induced by diverse stimuli. These results suggest that mitochondrial dysfunction by TFs, YBT and ABT could be responsible for apoptosis of PC-3 cells, and the effects were TFs > extracts of black tea.

In mitochondria, permeability transition pore (PTP) opening causes mitochondrial swelling and loss of the mitochondrial membrane potential. As the matrix expands, the inner membrane keeps the membrane intact, but the outer membrane becomes ruptured. This leads to release of apoptotic factors such as cytochrome *c*, from the intermembrane space into the cytosol. The released cytochrome *c* complexes with Apaf-1 in the presence of dATP and ultimately cleave procaspase-3 to form active caspase-3. Activation of caspase-3 is an important step in the execution phase of apoptosis and its inhibition blocks cell apoptosis (56,57).

In addition, due to PTP opening, the inner membrane becomes permeable to protons which can lead to uncoupling oxidative phosphorylation. The F1F0-ATPase reverses and hydrolyzes ATP to maintain the mitochondrial membrane potential resulting in a decrease of intracellular ATP concentration. This can lead to the dysfunction of ATP-dependent processes such as disruption of ionic homeostasis, and activation of calcium-dependent degradative enzymes such as phospholipase, nucleases and proteases (58-60). It has been shown that a decrease in intracellular ATP level imposed apoptosis. Along with other evidence in our study, we hypothesized that the decrease of ATP after treatment might contribute to apoptosis (61). In TFs group, changes of ATP contents are proportional to the release of cytochrome *c* or activity of caspase-3. In the YBT and ABT treatment groups, although it seems that the ATP contents are not proportional to the release of cytochrome *c* or activity of caspase-3, there was no statistical difference.

The human prostate cancer cell lines include androgen-dependent LNCaP and androgen-independent PC-3 and DU145 cells. Kalra *et al* (62) reported that black tea polyphenol, theaflavin-induced apoptosis in human prostate carcinoma, LNCaP cells was mediated via modulation of two related pathways: upregulation of p53 and downregulation of NF-κB

activity, causing a change in the ratio of pro- and anti-apoptotic proteins leading to apoptosis. The altered expression of Bcl-2 family member proteins triggered the release of cytochrome *c* and activation of initiator capsase-9 followed by activation of effector caspase-3. Furthermore, TFs also affected the protein expression of mitogen activated protein kinases (MAPK) pathways. Their results demonstrated that TF treatment resulted in downregulation of phospho-extracellular signal-regulated protein kinase (Erk1/2) and phospho-p38 MAPK expressions. Therefore, they concluded that TFs induce apoptosis in LNCaP cells through shifting the balance between pro- and anti-apoptotic proteins and downregulation of cell survival pathways leading to apoptosis. They also showed that TFs induced apoptosis in the androgen-independent prostate cancer cell line PC-3 through caspases and MAPK-mediated pathways. Thus, effect of TFs on prostate cancer cell lines seems to be irrespective of their androgen status (62). Lee *et al* found that TF3 significantly reduced androgen-responsive LNCaP prostate cancer cell growth, suppressed expression of the androgen receptors and lowered androgen-induced prostate-specific antigen secretion and fatty acid synthase protein level (63). In this study, our findings suggest that black tea and TFs could act as an effective anti-proliferative agent in androgen-independent PC-3 cells.

Recently, Tachibana *et al* have reported that EGCG inhibited cell growth by inducing the myosin II regulatory light chain (MRLC) phosphorylation (64). It is likely that EGCG could disrupt the contractile ring by decreasing the MRLC phosphorylation resulting in the inhibition of cell proliferation (65,66). In addition, EGCG decreased MRLC phosphorylation by binding to 67LR (a cell surface receptor) (64). Therefore, further studies are needed to identify whether the inhibitory effects of black tea extracts, especially TFs are exerted by their binding to 67LR.

In conclusion, our results suggest that TFs, YBT and ABT could inhibit the growth and change the morphology of prostate cancer cells. Additionally, TFs, YBT and ABT induced apoptosis or even necrosis of PC-3 cells through mitochondrial dysfunctioning.

Acknowledgements

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