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Mechanism of Free Zn(2+) Enhancing Inhibitory Effects of EGCG on the Growth of PC-3 Cells: Interactions with Mitochondria

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Abstract Green tea and its major constituent epigallocatechin gallate (EGCG) are known for their chemopreventive effects including those against prostate cancer, which could be mediated by metal ions. Zn^{2+} is an essential trace element that is required for human health and plays an important role in the normal function of the prostate gland. In the present study, the effect of EGCG on cell membrane and mitochondria of PC-3 (prostate carcinoma) cells in the presence and absence of Zn^{2+} was studied. These studies revealed that EGCG, Zn^{2+}, or EGCG + Zn^{2+} affected the morphology of PC-3 cells and induced apoptosis in PC-3 cells. It was observed that effects of treatment with EGCG, Zn^{2+}, or EGCG + Zn^{2+}on mitochondria showed EGCG + Zn^{2+} > Zn^{2+} > EGCG, including cytochrome C release from the intermembrane space into the cytosol, inhibited the synthesis of ATP, loss of mitochondrial membrane potential, and activation of caspase-9. However, the order of effect on depressing membrane fluidity of PC-3 cells was EGCG > EGCG + Zn^{2+} > Zn^{2+}. In summary, these findings suggest that EGCG, Zn^{2+}, and EGCG + Zn^{2+} induce necrosis or apoptosis of PC-3 cells through mitochondria-mediated apoptotic pathway and free Zn^{2+}-enhanced effects of EGCG on PC-3 cells due to its interactions with mitochondria.

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Introduction

Zinc ions (Zn^{2+}) play an important role in maintaining normal function of the prostate and development of prostate malignancy [1, 2]. Total Zn^{2+} levels in the prostate are ten times higher than in other soft tissues, and this ability of Zn^{2+} to accumulate in the prostate is lost during prostate carcinogenesis [3, 4]. During the development and progression of prostate malignancy, normal zinc-accumulating citrate-producing cells are transformed into citrate-oxidizing malignant cells that resulted from the loss of the ability of prostate tissue to accumulate zinc. During the malignant transformation process, the inhibitory effect of zinc on terminal oxidation (inhibitory effect of zinc on *m*-aconitase that permits citrate oxidation via a functional Krebs cycle) is depressed that allows malignant cells to become more energy efficient and simultaneously, the proapoptotic actions of zinc is eliminated thereby permitting proliferation of the malignant cells [1]. This is supported by the observation that zinc treatment restricted growth of prostate cancer cells via cell-cycle arrest and by inducing apoptosis and necrosis [5–7].

Epigallocatechin gallate (EGCG) is an important bioactive component of green tea. Some of the well-documented biological activities of EGCG include its antimutagenic, antibacterial, hypocholesterolemic, antioxidant, antitumor, and cancer preventive properties [8–10]. Recent studies showed that the bioactivity of EGCG against PC-3 cells was significantly affected by metal ions [11, 12]. Previously, we observed that the inhibitory effects of EGCG on the growth of PC-3 cells are mediated by Zn^{2+} [11, 13]. However, their mechanisms remain unknown. It is believed that cell membrane is the primary target of EGCG including the 67-kDa laminin receptor (67LR) and membrane androgen receptor [14, 15]. Some investigations have demonstrated that agents that act on laminin and androgen receptors could induce actin cytoskeleton polymerization, redistribution of microfilaments [16], and cause apoptosis [15].

Apoptosis may occur via mitochondrial pathway. Mitochondria are essential for energy production, involved in reactive oxygen species generation and induction of apoptosis. These three functions of mitochondria could account for its close association with tumorigenesis. In most tissues, mitochondria account for the generation of about 90% of the ATP needed by the cells [17]. Mitochondrial dysfunction causes permeabilization of the outer mitochondrial membrane to large molecules including ions that could lead to the initiation of apoptosis [18]. The permeabilization of outer mitochondrial membrane leads to the release of the intermembrane space proteins such as cytochrome C, which ultimately trigger apoptotic cell death [19]. Thus, detection of the mitochondrial permeability transition event could serve as an early indicator of the initiation of apoptosis. This mitochondrial permeability transition process that results in the collapse of the electrochemical gradient across mitochondrial membrane can be measured by noting the change in the mitochondrial membrane potential [18].

In our previous paper, we evaluated time- and dose-dependent cytotoxicity of EGCG and Zn^{2+} on PC-3 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In an extension of study, we chose the best exposure period (24 h) and optimal concentration (80 μmol/L) to investigate the effects of EGCG, free Zn^{2+}, and EGCG in the presence of Zn^{2+} on the morphology of PC-3 cells, membrane fluidity, membrane protein conformational changes, and the function of mitochondria of PC-3 cells, including mitochondrial membrane potential, ATP content, cytochrome C release, and casepase-9 activity and the results were reported here.

Materials and Methods

Materials

EGCG, 5- and 16-doxyl stearic acid, maleimide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ATP, cytochrome C, and EGCG were all purchased from Sigma (Saint Louis, MO, USA). Cell Apoptosis Rhodamine 123 Detection Kit and Caspase-9 Activity Assay Kit was purchased from Nanjing KeyGen Biotec. Co. Ltd. (Nanjing, China). Human prostate cancer cells (PC-3) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All other chemicals were extrapure grade or analytical grade.

Cell Culture

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

Morphology Detection of Adhesive PC-3 Cells

PC-3 cells were cultured in six-well plates (20×20 mm) and then exposed to 80 μmol/L EGCG, 80 μmol/L Zn^{2+}, and 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h. After exposure, the photographs of adhesive PC-3 cells were made through an inverted microscope at ×20 magnifications (XDS-1B, Chongqing, China). The number of cells adhering on plate and the changes of cell morphology indirectly indicated the cytotoxicity of EGCG and Zn^{2+} on PC-3 cells.

Detection of Apoptosis by Rhodamine 123

Rhodamine 123 is a fluorescent dye, which incorporated into mitochondria in a transmembrane potential-dependent manner. PC-3 cells were cultured in six-well plates (20×20 mm) and then exposed to 80 μmol/L EGCG, 80 μmol/L Zn^{2+}, and 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h. After exposure, the apoptosis of PC-3 cells was measured according to Cell Apoptosis Rhodamine 123 Detection Kit. Mitochondria of PC-3 cells were dyed by 50 μl Rhodamine 123 (5 μg/mL) for 10 min and washed twice with phosphate buffered saline (PBS). Then, images were obtained with a fluorescence microscopy (×20; excitation, 488 nm; emission, 516 nm; LEICA DMIRB and LEICA DFC300FX, Germany).

Analysis of Cell Membrane Fluidity by Electron Spin Resonance Labeling Using 5- and 16-SASL

Stearic acid spin labels (5- and 16-SASL) were used as a probe to quantify membrane fluidity of prostate cancer cells. After treatment, the suspension of PC-3 cells in PBS solution was labeled with 5- and 16-SASL at a final concentration of 100 μM incubated for 30 min at 37°C with gentle shaking to incorporate the spin label into the membranes. The labeled cells were washed three times with PBS to remove unbounded spin label and resuspended in PBS. Finally, 20 μL of suspension was transferred to a glass capillary for electron spin resonance (ESR) experiments. All ESR experiments were performed at

9.852 GHz using a Bruker ESR A300 spectrometer (Bruker, Germany) and operating at a center field strength of 3,509 with 60 G as scan range, a modulation amplitude of 4.0 G and 20 mW microwave power. Samples were analyzed three times and the typical examples are shown in the figures.

Isolation and Purification of Mitochondria

Esparza et al. [20] found that catechin forms 1:1 complex with either Zn^{2+} or Cu^{2+}. Therefore, in the present study, EGCG, Zn^{2+}, and EGCG + Zn^{2+} were prepared at equal molar concentrations (80 μmol/L) and used to investigate the bioactivity of EGCG. PC-3 cells were seeded on 50 cm^2 plates at a density of 6×10^5 cells/mL and allowed to grow for 24 h. After treatment with 80 μmol/L EGCG, 80 μmol/L Zn^{2+}, or 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h, the cells were harvested by centrifuging at 1,500 rpm for 5 min at 4°C and washing twice with cold PBS and finally resuspended in PBS. 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 thus obtained was centrifuged at 9,000 rpm for 10 min to obtain mitochondrial pellets that were washed twice with cold PBS. The final mitochondrial pellets were suspended in PBS for ESR assay or suspended in test medium (220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, pH 7.2) for other studies. The final protein concentrations of the mitochondrial suspension were all adjusted to 0.3 mg/mL.

Assay of Cellular ATP Contents

Eighty-micromole per liter EGCG-, 80-μmol/L Zn^{2+}-, or 80-μmol/L EGCG + 80 μmol/L Zn^{2+}-treated PC-3 cells were washed twice with cold PBS and homogenized with 600 μl PBS and 80 μl 0.4 μmol/L perchloric acid by mechanical homogenization at high speed during 10 min. The 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 a Millipore filter of 0.45 μm pore diameter and injected into HPLC system (LC-2010A, Shimadzu, Japan). The samples were analyzed on a C_{18} column (200×4.6 mm i.d., particle size 5 μm), wherein 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, and effluent was monitored at 280 nm. The sample injection volume was 20 μL. All instruments and the columns were operated in laboratory at room temperature (23–25°C). The experiment was repeated three times.

Analysis of Cytochrome C Release

Content of cytochrome C was measured at 520 nm in UV–vis spectroscopy (HP 8453, Hewlett-Packard, USA) in isolated mitochondria in a reaction medium containing 10 mg sodium dithionite and 0.5 mL of 0.3 mg/mL mitochondrial protein. Sample concentrations were determined based on a standard curve. Each of the experiments was replicated three times.

Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured by suspending 0.5 mL of 0.3 mg/mL mitochondrial protein in 1.5 mL Rhodamine 123 assays buffer and 10 μL Rhodamine 123

(1 mg/mL) and incubating the reaction mixture at 25°C for 20 min. Fluorescence intensity of the reaction medium was measured at the 488/525 nm wavelength pair by Cary Eclipse fluorescence spectrophotometer (VARIAN, USA). All the experiments were repeated at least three times.

Determination of Caspase-9 Activity

Caspase-9 activity was assayed using Caspase-9 Activity Assay Kit according to the manufacture's instructions (Keygen, Nanjing, China). After treatment, 5×10^6 cells were harvested by centrifuging at 1,500 rpm for 5 min at 4°C and washing twice with cold PBS, then resuspended in a cell lysis buffer. After incubation on ice for 60 min, the lysates were centrifuged for 20 min at 12,000 rpm, then the supernatants were collected and protein concentrations were determined. Cell lysates (100 μg) were mixed with reaction buffer containing the LEHD-pNA, then cells were incubated with caspase-9 substrate for 4 h at 37°C, and the absorbance was measured in the wells at 405 nm using an ELISA reader (Thermal Labsystem, Helsinki, Finland).

Statistical Analysis

Results were expressed as the mean ± standard deviation (SD) of at least three independent replications of each experiment. Statistical significance was determined by pair *t* test analysis using Origin7.5 software for windows.

Results

Morphological Changes of PC-3 Cells

Effects of EGCG, Zn^{2+}, and EGCG + Zn^{2+} on PC-3 cells were observed through an inverted microscope. The number of cells adhering on plate and the changes of cell morphology indirectly indicated the cytotoxicity of EGCG and Zn^{2+} on PC-3 cells. The number of PC-3 cells adhering on plate decreased after exposed to EGCG, Zn^{2+}, and EGCG + Zn^{2+} (Fig. 1), especially treatment of free Zn^{2+}. What is more, Zn^{2+} treatment made PC-3 cells become round globular cells and smaller than control cells.

Apoptotic and Necrotic Cells

Apoptotic PC-3 cells treated with EGCG, Zn^{2+}, and EGCG + Zn^{2+} were examined with fluorescence microscopy. According to the manufacture of the detection kit, after cells were stained by Rhodamine 123, normal cells exhibit faint green fluorescence, apoptotic cells exhibit strong green fluorescence, and necrotic cells exhibit negligible fluorescence (as shown in Fig. 2). From our results, 80 μmol/L Zn^{2+} and 80 μmol/L EGCG + 80 μmol/L Zn^{2+} could induce apoptosis of PC-3 cells and cause sufficient damage to the cells to result in necrosis.

Changes of Cell Membrane Fluidity

Typical ESR spectra obtained with 5- and 16-doxyl stearic acid in mitochondrial membrane of PC-3 cells at 25°C is shown in Fig. 3. The ESR spectrum of 5- and 16-doxyl stearic acid spin labels in mitochondrial membranes was further characterized by calculating the values of

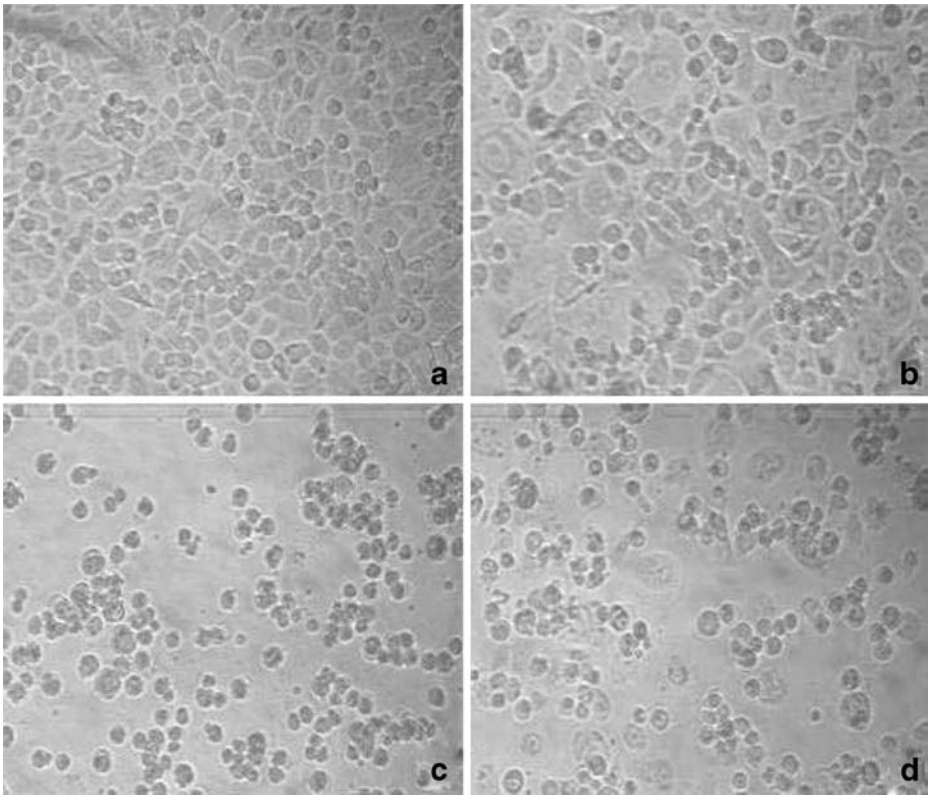

Fig. 1 Microscopic images of PC-3 cells treated with EGCG, Zn^{2+}, and EGCG+Zn^{2+}. PC-3 cells were exposed to **a** control, **b** 80 μmol/L EGCG, **c** 80 μmol/L Zn^{2+}, and **d** 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h

the order parameter *S* and effective rotation correlation time *t*. A decrease in membrane fluidity produces an increase in the value of *S* and *t* that can be detected as a decrease in spin label mobility, while a decrease of the value suggests that the spin label is in a more fluid environment. Results shown in Table 1 revealed that treatments with EGCG and EGCG in the presence of Zn^{2+} produced a decrease in mitochondrial membrane fluidity as evidenced by an increase in order parameter *S* and effective rotation correlation time *t*, while the effect of Zn^{2+} was not significant compared to control (EGCG > EGCG + Zn^{2+} > Zn^{2+}).

ATP Contents in PC-3 Cells

In aerobic organisms, the mitochondria are the main source of energy and site of the tricarboxylic acid cycle, a metabolic network involved in the generation of reducing factors that power the production of ATP [21]. In the present study, it was noted that EGCG, Zn^{2+}, or EGCG + Zn^{2+} inhibited ATP synthesis (Fig. 4b) in particular ATP content in PC-3 cells treated with Zn^{2+} was significantly different from the control. The ATP content in EGCG + Zn^{2+}-treated PC-3 cells was lower than those exposed to EGCG and higher than exposed to Zn^{2+}. The results showed that effect of EGCG in decreasing ATP content in PC-3 cells was enhanced in the presence of Zn^{2+}, while effect of Zn^{2+} was inhibited in the presence of EGCG. The results suggested that EGCG, Zn^{2+}, and EGCG + Zn^{2+} might induce the damages of mitochondria in PC-3 cells.

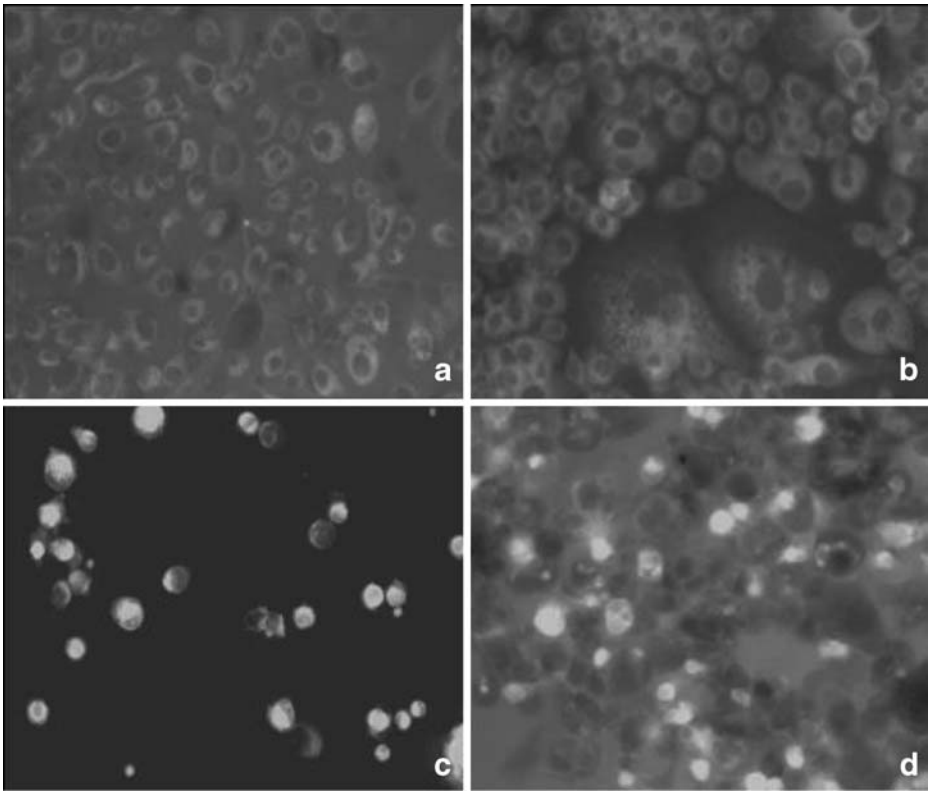

Fig. 2 Images of apoptotic cells stained with Rhodamine 123. PC-3 cells were exposed to **a** control, **b** 80 μmol/L EGCG, **c** 80 μmol/L Zn^{2+}, and **d** 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h, treated with Rhodamine 123 and observed with a fluorescence microscopy

Concentration of Mitochondrial Cytochrome C

Cytochrome C from the intermembrane space into the cytosol was investigated. Results given in Fig. 5 showed that concentrations of cytochrome C in mitochondria of PC-3 cells treated with EGCG, Zn^{2+}, or EGCG + Zn^{2+} were all significantly decreased, which suggested that cytochrome C was released from mitochondria into cytosol.

Loss of Mitochondrial Membrane Potential

EGCG, Zn^{2+}, or EGCG + Zn^{2+} caused a significant loss of mitochondrial membrane potential (EGCG+ Zn^{2+} > Zn^{2+} > EGCG), as detected by measuring the fluorescent intensity of the reaction medium (see Fig. 6).

Effects of Caspase-9 Activity

Caspases comprise a class of cysteine protease, many members of which are involved in apoptosis. Based on their order of activation, caspases are classified into two families: the initiator caspases and the effector caspases [22]. Caspase-9 belongs to the initiator caspase family. Once activated, caspase-9 specifically cleaves and activates an effector caspase

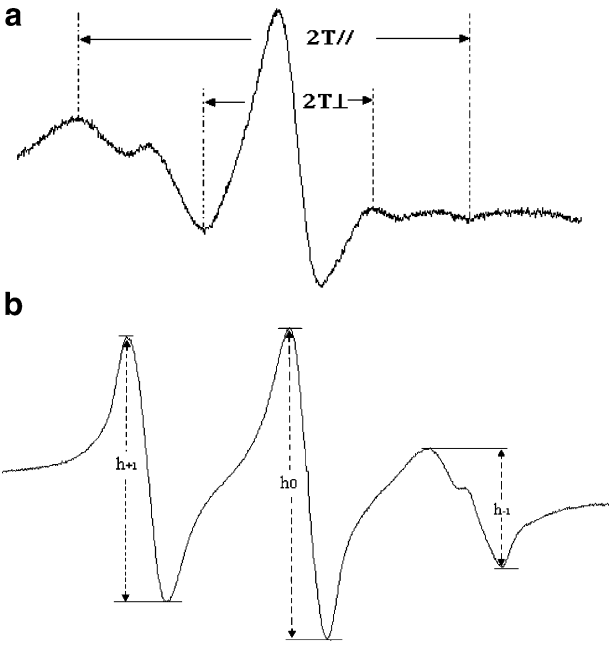

Fig. 3 Typical ESR spectra of 5-doxyl stearic acid (**a**) and 16-doxyl stearic acid (**b**) in membrane of PC-3 cells at 25°C. The spectra were recorded at 9.852 GHz at 25°C; microwave power, 20 mW; modulation amplitude, 4.0 G; center field strength, 3,509 G; scan range, 60 G

zymogen, which degrades a large number of cellular proteins that ultimately kill a cell. As shown in the Fig. 7, treatment with EGCG, Zn^{2+}, and EGCG + Zn^{2+} significantly increased the activity of caspase-9 in PC-3 cells, and the effects of treatments were EGCG+Zn^{2+} > Zn^{2+}>EGCG. The results indicated that Zn^{2+} had more potent effects than EGCG on stimulation of caspase-9.

Discussion

Mitochondria play an important role in cellular energy metabolism, free radical generation, and apoptosis [23]. Several mDNA-encoded proapoptotic proteins including cytochrome C, apoptosis inducing factor (AIF), endonuclease G, and smac/DIABLO normally reside in the mitochondria where they perform their physiological functions. Release of these factors

Table 1 The Values of Order Parameter (*s*) and Correlation Rotation Time (*t*) in PC-3 Cells Labeled with 5- and 16-SASL

		Control	EGCG	Zn^{2+}	EGCG + Zn^{2+}
5-SASL	*s*	0.478±0.0019	0.496±0.0011*	0.486±0.0027	0.494±0.0014*
6-SASL	*s*	0.193±0.0017	0.200±0.0008	0.189±0.0014	0.193±0.0027
	T	9.87±0.09	11.05±0.21*	9.41±0.17	10.45±0.12*

The order parameters were calculated from the ESR spectra by using the equation $S=0.5407\ (T_{//}-T_{\perp})/a_0$, where $a_0=(T_{//}+2T_{\perp})/3$. The rotation correlation time was calculated from the ESR spectra by using the equation $t=6.51\times10^{-10}\ \Delta H_0\ [(h_0/h_{-1})-(h_0/h_{+1})]$ *s*. ΔH_0 was the center line width, h_0, h_{-1}, and h_{+1} were measured from the ESR spectra as indicated in the figure

*$p<0.05$ (significant difference from control)

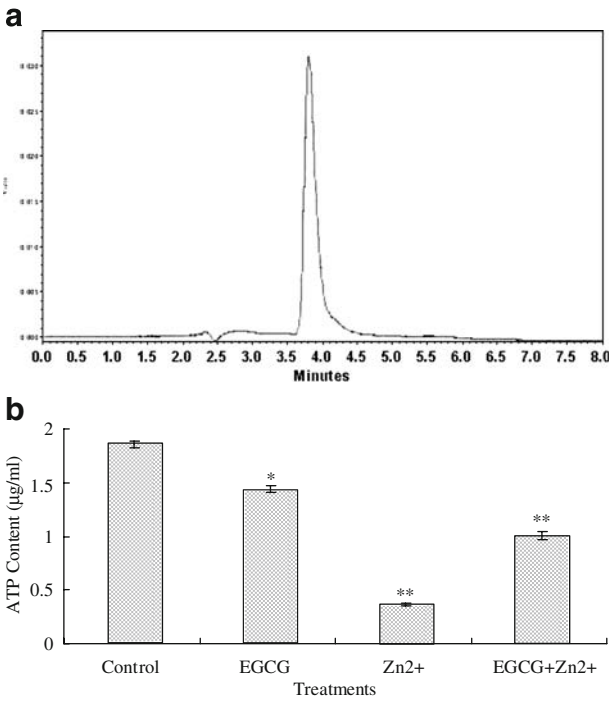

Fig. 4 ATP content in PC-3 cells. **a** HPLC chromatography of ATP standard. **b** PC-3 cells were treated with 80 μmol/L EGCG, 80 μmol/L Zn^{2+}, and 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h and the mitochondria were isolated. ATP content in PC-3 cells was detected by HPLC. Data are mean ± SD, n= 3. Significant difference from control *p<0.05; **p<0.01

from mitochondria triggers a series of biochemical events leading to activation of apoptotic signaling cascades [24].

It is evident from the results of the present study that treatments with EGCG, Zn^{2+}, and EGCG + Zn^{2+} could induce apoptosis of PC-3 cells through a mitochondria-dependent mechanism. This is supported by the observations that exposure of PC-3 cells to EGCG, Zn^{2+}, or EGCG + Zn^{2+} induced (a) release of cytochrome C from mitochondria and loss of mitochondrial membrane potential that play a critical role in mitochondria-dependent apoptosis pathway and (b) a decrease in ATP content and activation of caspase-9, an initiator caspase of apoptosis. These results thus suggest that mitochondrial dysfunction by EGCG, Zn^{2+}, or EGCG + Zn^{2+} could be responsible for induction of apoptosis of PC-3 cells, and the effects is EGCG + Zn^{2+} > Zn^{2+} > EGCG. In mitochondria, permeability transition pore (PTP) opening causes mitochondrial swelling and loss of the mitochondrial

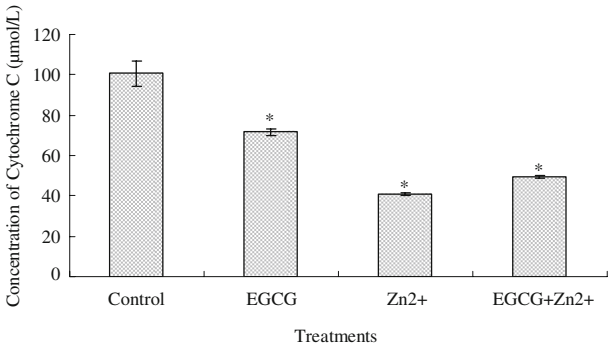

Fig. 5 Concentration of cytochrome C in mitochondria of PC-3 cells. PC-3 cells were treated with 80 μmol/L EGCG, 80 μmol/L Zn^{2+}, and 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h and the mitochondria were isolated. Concentration of cytochrome C in PC-3 cells was measured by UV–vis spectroscopy. Data are mean ± SD, n=3. Significant difference from control *p<0.05; **p<0.01

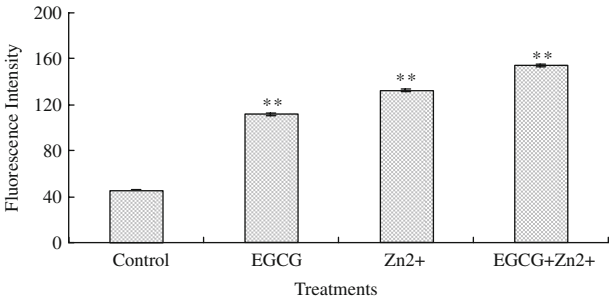

Fig. 6 Comparisons of effects of EGCG, Zn^{2+}, and EGCG + Zn^{2+} on mitochondrial membrane potential. PC-3 cells were treated with 80 μmol/L EGCG, 80 μmol/L Zn^{2+}, and 80 μmol/L EGCG + 80 μmol/L Zn^{2+} for 24 h and the mitochondria were isolated. Mitochondrial membrane potential was monitored using Rhodamine 123. Fluorescence intensity of the reaction medium was measured at the 488/525 nm wavelength pair. Data are mean ± SD, n=3. Significant difference from minimum concentration *p<0.05; **p<0.01

membrane potential. As the matrix expands, the inner membrane cristae unfold keeping the membrane intact, but the outer membrane becomes ruptured. That 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 recruit and activate caspase-9. Activated caspase-9 cleaves and activates caspase-3 and caspase-7. Known for their rapid catalytic turnover, caspase-3 and caspase-7 degrade a large number of cellular proteins that ultimately kill a cell [25]. In addition, due to PTP opening, the inner membrane becomes permeable to protons leading to uncoupling oxidative phosphorylation. The F_1F_0-ATPase reverses and hydrolyzes ATP to maintain the mitochondrial membrane potential resulting in a decrease in intracellular ATP concentration. This leads to the dysfunction of ATP-dependent processes such as disruption of ionic homeostasis, activation of calcium-dependent degradative enzymes such as phospholipase, nucleases, and proteases [26–28].

Normal human prostate accumulates zinc. Zinc content is increased in benign prostate hyperplasia but decreased in prostate cancer in comparison with normal tissue. A growing body of evidence suggested that the loss of this unique capacity to retain high levels of zinc is an important factor in the development and progression of malignant prostate cells [29]. Some reports suggested that zinc-induced apoptosis in PC-3 cells occurs due to the activation of caspase-9 and caspase-3 induced by poly (ADP)-ribose polymerase and/or a

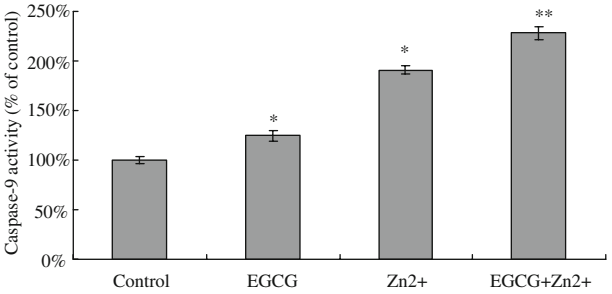

Fig. 7 Caspase-9 activity of PC-3 cells treated with EGCG, Zn^{2+}, and EGCG+ Zn^{2+}. Cells were harvested and carried out according to caspase-9 activity assay kit's instructions. The absorbance was measured in the wells at 405 nm using an ELISA reader (Thermal Labsystem, Finland). Data are mean ± SD, n=3. Significant difference from minimum concentration *p<0.05; **p<0.01

decrease in mitochondrial membrane potential and Bcl-2 protein levels [30, 31]. Jiang et al. [32] showed in neuronal cells that, similar to Ca^{2+}, Zn^{2+} mediated its effects on mitochondria by opening the mitochondrial PTP and consequently releasing apoptotic mediators such as cytochrome C and AIF. These observations are in support of the results of the present study. Furthermore, Figs. 1 and 2 showed that Zn^{2+} induced morphological changes of PC-3 cells and apoptosis in PC-3 cells. These results contrast with previous findings that zinc inhibited apoptosis in normal mammalian cells. This suggests that metal ions can regulate the 67LR from surface of cancer cells to induce apoptosis in cancer cells [33]. Matheson et al. [34] reported no effect of Zn treatment on ATP level in PC-3 cells. The most likely reason for this is that the concentration of Zn was lower and exposure time of cells was shorter than those of the present work.

EGCG is the major and most effective anticarcinogenic constituent found in green tea [8, 9]. Many studies showed that EGCG inhibited the survival rate of malignant cells and induced apoptosis of malignant cells via the mitochondrial signal transduction pathway [35–38]. Roy et al. [39] reported that the increased ratio of Bax/Bcl-2 proteins after EGCG treatment might result in increased release of cytochrome C from mitochondria into cytosol, increase the expression of Apaf-1, and activate caspase-3 and poly (ADP-ribose) polymerase, which could lead to apoptosis in MDA-MB-468 cells. This is in agreement with the results of the present study, which revealed that effect of EGCG on mitochondrial signal transduction pathway could play a significant role in its anticancer action. Several other studies have identified specific targets modulated by EGCG, such as 67LR and membrane androgen receptor [14, 15], that indicate the interactions of EGCG with cell membrane. In the present work, ESR study results support the premise that EGCG could interact with cell membrane of PC-3 cells that is decrease membrane fluidity. Since fluid properties of biological membranes are essential for numerous cell functions including cell growth, solute transport, signal transduction, and membrane-associated enzymatic activities [40, 41], it is possible that even mild alterations in membrane fluidity could cause aberrant function and set in motion pathological processes [42]. These observations demonstrate that EGCG may have affected growth of PC-3 cells through interactions with cell membrane elicited by EGCG.

It is known that the biological behavior of EGCG can be influenced by metal ions, especially transition metal ions such as iron, zinc, and copper [43–45]. EGCG molecules have two rings, B- and D-ring, which have local structures that could form complexes with metals. It appear likely that the D-ring OH groups could occupy the first coordination sphere around metal ion, whereas the B-ring OH group may have a secondary effect on such a complex formation [46]. Thermogravimetric and atomic absorption spectrophotometric studies indicated that the 3-OH-4-oxo and 3′-OH-4′-OH moieties are the most likely binding sites of flavonoids for metals. Esparza et al. [20] reported that catechin could form 1:1 complexes with either Zn^{2+} or Cu^{2+} that could be regulated through the linearization algorithms as competitive with preconcentration of the metal ions on the mercury electrode. We observed that Zn^{2+} enhanced effects of EGCG on mitochondria of PC-3 cells (EGCG + Zn^{2+} > Zn^{2+} > EGCG) and treatments with EGCG + Zn^{2+} decreased the effects of EGCG (EGCG > EGCG + Zn^{2+} > Zn^{2+}), while effects of Zn^{2+} on cell membranes of PC-3 cells were not significant. Hence, we suggest that Zn^{2+}-enhanced effects of EGCG on the suppression of growth in PC-3 cells are due to strong interactions of Zn^{2+} with mitochondria.

In conclusion, the results of the present studies indicate that (a) EGCG may inhibit the growth of PC-3 cells through decreasing membrane fluidity and mitochondrial dysfunction, (b) free Zn^{2+} induces apoptosis of PC-3 cells through mitochondrial pathways involving

release of cytochrome C and activation of caspase-9, and (c) free Zn^{2+} increased effects of EGCG due to interactions of Zn^{2+} with mitochondria. Since Zn^{2+} enhanced the effect of EGCG on mitochondria of PC-3 cells, it is possible that this interaction could form the basis of development of EGCG as a novel and pharmacologically safe chemopreventive agent for prostate cancer.

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