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Effects of epi-gallocatechin gallate on PC-3 cell cytoplasmic membrane in the presence of Cu^{2+}

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Abstract

Catechins, which exist in green tea, are excellent antioxidants and metals enhance their pro-oxidative character. In this study, we investigated damage to the cell cytoplasmic membrane of the androgen-insensitive prostate cancer cell PC-3 from rearrangement of EGCG (epigallocatechin gallate), the main bioactive component of catechins. In a cell culture system containing Cu^{2+}, the generation of free radicals from EGCG rearrangement was measured. Electron microscopy, flow cytometry, gel electrophoresis, and ESR measurements showed that the cytoplasmic membrane of PC-3 cells was disrupted in this culture system and that the death of most cells followed within a few minutes. Hydroxyl radicals, detected in the ESR experiment using the spin trapping method, may cause damage to the cytoplasmic membrane. Analysis of F-12 medium after 6 h, by HPLC, showed small amounts of EGCG when PC-3 cells were absent, and no detectable levels when PC-3 cells were present. In addition to the concentration and order in which EGCG and Cu^{2+} are added to the culture medium, oxides, polymers, or some compounds integrated with the cytoplasmic membrane of the PC-3 cells themselves may have a key role in damage to the PC-3 cell cytoplasmic membrane.

Keywords: Epigallocatechin gallate; Cu^{2+}; Rearrangement; Cytoplasm membrane; Free radicals; Prostate cancer cells

1. Introduction

Tea is consumed worldwide and is second only to water in popularity as a beverage. Many health benefits have been ascribed to consumption of this beverage. These include anti-cancer, anti-oxidant, and bactericidal effects (Anna et al., 2003; Campanella, Bonanni, & Tomassetti, 2003; Joshua & Chung, 2003; Martin, Vlasta, & Andrej, 2003; Sami et al., 2003; Shengrong, Haining, & Chaofang, 2002). In recent years, epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent present in green tea (Kada, Kaneko, Matsuzaki, Matsuzaki, & Hara, 1985), has shown remarkable effects in inhibiting cancer cell growth in both cell culture systems and in tumor models in vivo (Nihal, Vaqar, Sanjay, Pingyan, & Hasan, 2002; Sosamma, Sanjay, Charles, David, & Hasan, 2001). It has been demonstrated that the viability of human prostate carcinoma cells and the growth of prostate tumor cells decrease with EGCG treatment (Sanjay, Tajamul, & Hasan, 2003; Shutsung, Umekita, Jingtao, Kokontis, & Hiipakka, 1995; Steven & Wang, 2002).

Trace elements have been implicated in the etiology of cancer. Copper ion is related to prostate cancer (Daniel, Gupta, Harbach, Guida, & Dou, 2004; Yilmaz et al., 2004). Hence determination of copper ion may be of value in the early diagnosis of prostate cancer. Nayak, Bhat, Upadhyay, and Udupa (2003) found that copper and ceruloplasmin levels were increased significantly in cancer patients compared to controls.

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EGCG is also excellent an antioxidant. Several epidemiological reports indicate that high consumption of green tea decreases the risk of many types of cancer (Arts, Jacobs, Gross, Harnack, & Folsom, 2002; Guni, Nasu, Yamamoto, & Nomura, 1988). Catechins could suppress the growth of certain cancer cells and induce their apoptosis (Uesato et al., 2001). It has been reported that catechins, in the presence of trace levels of elements (Desong, Shengrong, Xun, Yuyan, & Chongyang, 2004.) in particular Cu^{2+}, have pro-oxidative activity. This has been observed in the generation of free radicals, damage to DNA, and the peroxidation of lipids under certain conditions (Fumiko et al., 1997; Hisashi, Yasushi, Kieko, Takahide, & Fumiko, 2001; Yamanaka, Oda, & Nagao, 1997). Nobuo, Takahide, Akira, and Takashi (1999) reported the bactericidal effects of catechins in the presence of Cu^{2+} and cell surface-associated hydrogen peroxide of affected cells of *Escherichia coli* ATCC11775 in cell culture systems. Recycling redox reaction of Cu^{2+} and Cu^{+} occurred with damage to the cytoplasmic membrane and cell death. It is probable that the free radical products of these reactions cause damage of the cytoplasmic membrane. Catechin and Cu^{2+} interaction in a cell culture system and the effect of catechin on the growth of various cells in the presence of Cu^{2+} have not been explained. The interaction of copper ion, a necessary element for human health, with catechins found in green tea has been a concern for this reason. In this paper, we investigate the interaction of EGCG with Cu^{2+} in the PC-3 prostate cancer cell culture system, a common model used in studies on the disease process (Kaighn, Lechner, Narayan, & Jones, 1978).

2. Materials and methods

2.1. Materials

A purified preparation of EGCG was obtained from Sigma (St. Louis, MO, USA). The human prostate cancer cell, PC-3, was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All other chemicals were extra-pure grade or analytical grade. ANNEXIN V kit was purchased from Caltag Laboratories (USA). The spin trap, 5,5-dimethyl-1-1-pyrroline *N*-oxide (DMPO) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

PC-3 cells were cultured in F-12 medium (GIBCO, Invitrogen Corporation) supplemented with 10% fetal bovine serum and 100 U/ml penicillin–streptomycin. Cells were maintained at 37 °C and 5% CO_2 in a humid environment (Shellab, USA).

2.3. Flow cytometry and cell damage studies

The cells were grown to a density of 2×10^5 in 50 cm^2 plates for 48 h, and were treated with different concentrations of EGCG and $CuSO_4$ for 24 h. The cells were washed with phosphate-buffered saline (PBS), pH 7.4. Cells were trypsinized and collected by centrifugation (300*g*, 10 min). Dead Cells were counted with ANNEXIN V kit and analyzed by flow cytometry (FACS sort, BD, USA).

2.4. Gel electrophoresis and DNA fragmentation studies

Cells (1×10^5) were seeded onto 25 cm^2 plates for 48 h and incubated with different concentrations of EGCG and $CuSO_4$ for 24 h. Following this treatment, the cells were washed twice with PBS, pH 7.4, and 1 ml trypsinase was added after 5 min; cells were collected by centrifugation (300*g*) at 4 °C for 5 min, and washed twice with PBS. Cells were incubated in 200 μl of cytolysis solution (0.1 M NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS) and proteinase (50 μg/ml) for 10 h at 50 °C. Five hundred micro liter of ethanol were added and the DNA was collected by centrifugation (12,000*g*) for 10 min at 4 °C. The DNA was washed twice with 75% ethanol. After ethanol was entirely volatilized, DNA was incubated with 30 μl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and 4 μl RNase A (dissolved with TE, 50 μg/ml) for 1 h at 37 °C. The sample (10 μl) was mixed with 2 μl of loading solution (Bromophenol Blue 0.25%, xylene cyanol FF 0.25%, sucrose 40%), The DNA samples were loaded onto a 1.2% (w/v) agarose gel (containing 0.5 μg/ml of ethidium bromide). The DNA fragments were separated by electrophoresis (Amersham Biosciences) at 5 V/cm for 2.5–3 h in TAE buffer (40 mM Tris–HCl, 20 mM acetic acid, 1 mM Na_2EDTA). The DNA was visualized using an imaging system (Tianneng Shanghai, China) and photographed using an Alphaimager 2000 digital camera.

2.5. Morphology of membranes of PC-3 cells

Cells were treated with different concentrations of EGCG and $CuSO_4$, and collected by centrifugation (300*g*) for 10 min. The samples were fixed overnight in 2.5% glutaraldehyde, buffered at pH 7.0 PBS and washed three times for 15 min. Cells were then post-fixed in 1% osmium tetroxide for 1 h, and washed three times with phosphate buffer (pH 7.0), again for 15 min each time. The samples were then dehydrated through a graded ethanol series (15 min in each of 50%, 70%, 80%, 90%, and 95%). This was followed by treatment with 100% ethanol and 100% acetone for 20 min each. The samples were infiltrated for 1 h in epon–ethanol (1:1, v/v), and infiltrated for 3 h in epon–ethanol (3:1, v/v). The samples were then infiltrated overnight in

epon, immersed in epon and left in a 70 °C oven overnight. The samples were cut with a Du Pont diamond knife and a Sorvall Porter-Blum MT2-B ultramicrotome. The samples were examined with an electron microscope (JEM-1200EX, JEOL, Japan).

2.6. Measurement of EGCG by HPLC

Cells were seeded into 25 cm^2 plates at 1×10^5 for 48 h. Cells were incubated with different concentrations of EGCG and $CuSO_4$. The concentration of EGCG in F-12 medium was determined by HPLC analysis (LC-2010A, Shimadzu, Japan) at 6, 12 and 24 h. F-12 medium without cells was incubated with different concentrations of EGCG and $CuSO_4$ for 6, 12 and 24 h, and concentrations of EGCG were determined by HPLC analysis. The samples were analyzed on an ODS column. A gradient elution was carried out using the following solvent systems: mobile phase A, double-distilled water/acetonitrile (97:3, containing 0.5% acetic acid); mobile phase B, double-distilled water/acetonitrile (70:30, containing 0.5% acetic acid). The linear gradient elution system was: from 100% A to 100% B in 45 min, standing at 100% B at 10 min and returning to 100% A after 5 min. The flow was 1.0 ml/min, and effluent was monitored at 280 nm (0.001 sensitivity).

2.7. Analysis of free radicals by ESR method

The control F-12 medium was maintained at 37 °C and 5% CO_2 in a humid environment. DMPO and $CuSO_4$ were added first; ESR data were collected at 5, 15, 30, 60 min, and 24 h after mixing with EGCG. The final concentrations of DMPO, $CuSO_4$ and EGCG were: 200 mM, 100 μM and 200 μM, respectively. Then in reverse of the addition order, DMPO and EGCG were added to F-12 medium first, and data were collected at 5, 15, 30, 60 min and 24 h after mixing with $CuSO_4$. The final concentrations of DMPO, EGCG and $CuSO_4$ were: 200 mM, 200 and 100 μM, respectively. Conventional ESR spectra were obtained with a Varian E-109 X-band spectrometer at room temperature. ESR signals were recorded with 10 mW incident microwave power and 100 kHz field modulation of 1 G measurement. Each ESR spectrum was recorded 5 min after sample preparation.

3. Results and discussion

3.1. Configuration of PC-3 cells

Concentrations of EGCG and Cu^{2+} were chosen according to results of our earlier experiments (Haining, Junjie, & Shengrong, 2004) and fixed for all treated groups. Electron microscopy was employed to detect ultra-structure and the cytoplasmic membrane of PC-3 cells. In the control culture, electron microscopy revealed that the PC-3 cell has a clear cytoplasm, intact sub-cellular organelles, a nuclear membrane and normal nucleoli (Fig. 1A). Following treatment with 10 μM EGCG, the nuclear membrane and chromosome of PC-3 cells were intact, and the cytoplasm became slightly vacuolated; however, the cytoplasmic membrane was damaged (Figs. 1B and C). Heavy metals, including copper ion, were toxic to cells, as previously described (Bjorn, Anders, & Anders, 1997; Yasuo et al., 2002). We investigated the effects of Cu^{2+} on PC-3 cells. The cytoplasm of PC-3 cells incubated with 0.0064 μM Cu^{2+} and those treated with 10 μM EGCG were similar; however, the cytoplasmic membrane became smooth and intermittent, an indication of damage (Fig. 1D). When EGCG coexisted with Cu^{2+}, the cytoplasmic membrane was damaged. This was observed with all concentrations and orders of addition (Figs. 1E–H). In these instances, the cytoplasmic membrane was extensively vacuolated. The cytoplasmic membrane of PC-3 cells incubated with 320 μM Cu^{2+}, following pretreatment with 200 μM EGCG, was damaged to the extent that the sub-cellular organelles were difficult to detect (Fig. 1E). Conversely, analysis of DNA fragments of PC-3 cells treated with EGCG and Cu^{2+} by gel electrophoresis and the DNA fragmentation laddering effect showed no indication of apoptosis for any treatment (data not shown). Flow cytometry showed high mortality of PC-3 cells treated with Cu^{2+} and EGCG, but levels of apoptosis were low (Table 1, Fig. 2). It is well known that detecting of apoptosis with flow cytometry, by the ANNEXIN V kit, is dependent upon an intact cytoplasmic membrane and damage by EGCG and Cu^{2+} was considered. Results of electron microscopy were consistent with this.

3.2. HPLC analysis and concentrations of EGCG studies

EGCG concentration in F-12 medium, with or without PC-3 cells, was determined by HPLC. In the absence of PC-3 cells, EGCG in F-12 medium declined quickly at 37 °C. There was no EGCG in F-12 medium at 6, 12 and 24 h when the initial concentration of EGCG was 10 μM. When F-12 medium was treated with 200 μM EGCG, concentration decreased to 5.98 μM after 6 h. It is interesting to note that EGCG was absent at 6 h with PC-3 cells, regardless of initial concentration. EGCG concentration in F-12 medium with PC-3 cells was significantly lower ($p < 0.01$) than without PC-3 cells. This indicates that change of EGCG concentration in F-12 medium depends upon the absence or presence of PC-3 cells. Effects of PC-3 cells on the transformation of EGCG were observed in the coexistive system of EGCG and Cu^{2+}. Similarly, we did not detect EGCG after 6 h when Cu^{2+} and PC-3 cells were present. In

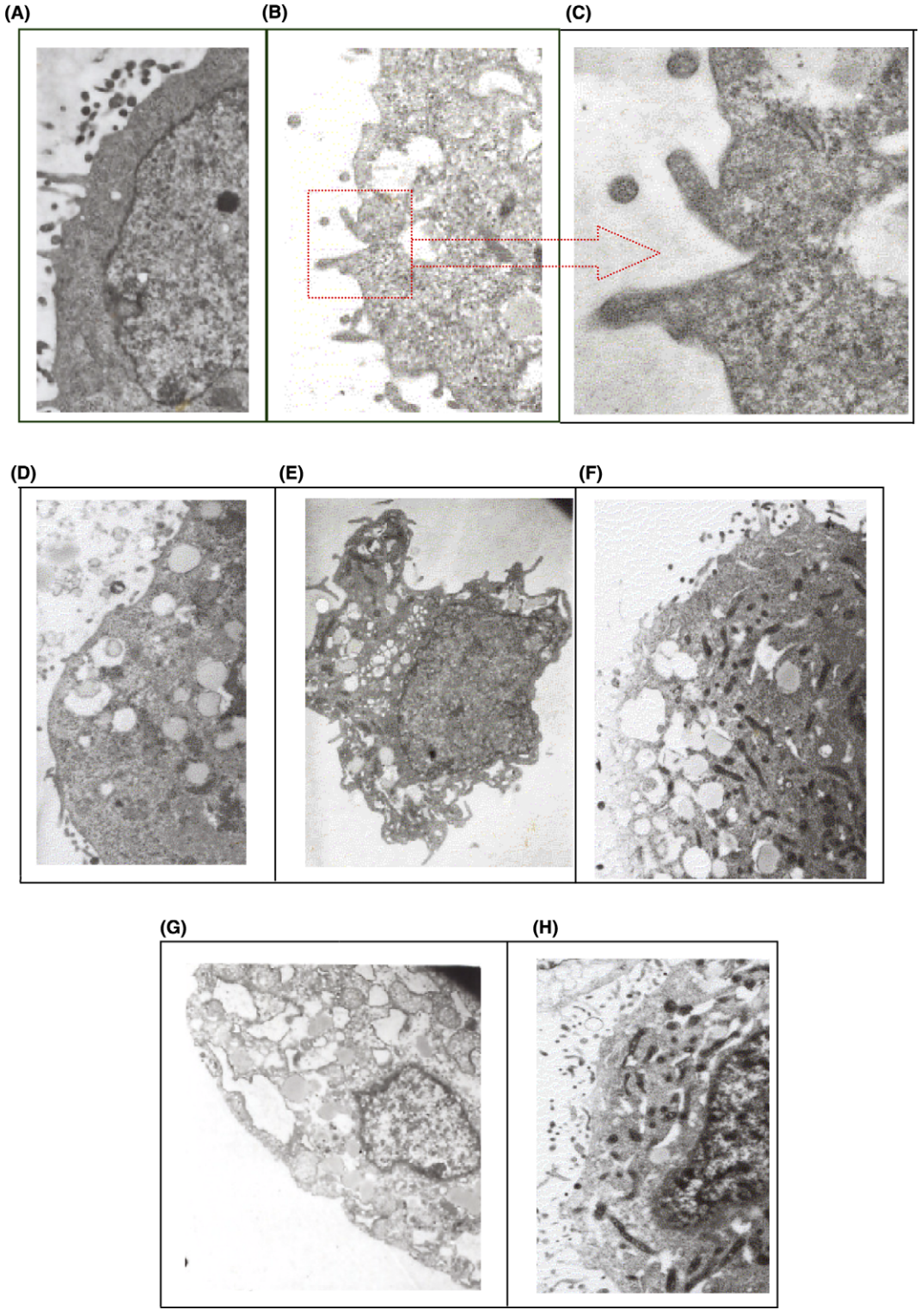

Fig. 1. Configuration of PC-3 cell treated with different concentrations of EGCG and $CuSO_4$. (A): Normal PC-3 cell; (B) and (C): treated with 10 μM EGCG; (D): treated with 0.0064 μM $CuSO_4$; (E): treated with 320 μM $CuSO_4$ after added 200 μM EGCG;)(F): put into 150 μM EGCG, then adding 32 μM $CuSO_4$; (G): before adding 200 μM EGCG, put into 320 μM $CuSO_4$; (H): treated with 150 μM EGCG in the presence of 32 μM $CuSO_4$.

the groups without PC-3 cells, EGCG was present in F-12 medium in small amounts after 6 h under all conditions, except in the presence of 32 μM Cu^{2+}. Here, concentration declined from 150 to 0 μM. EGCG concentration of F-12 medium declined from 200 to 6.25 μM following treatment with 320 μM Cu^{2+}, and

Table 1
Mortality of PCA cells treated with EGCG and Cu^{2+}

Groups	Mortality of PCA cells (%)
	PC-3
0.0064 μM $CuSO_4$	31.77 ± 1.3
64 μM $CuSO_4$	53.13 ± 7.0
320 μM $CuSO_4$	77.30 ± 5.9
50 μM EGCG	23.86 ± 1.2
150 μM EGCG	51.39 ± 3.0
200 μM EGCG	80.56 ± 6.1
320 μM $CuSO_4$ + 200 μM EGCG	94.32 ± 6.7
200 μM EGCG + 320 μM $CuSO_4$	55.23 ± 3.4
150 μM EGCG + 32 μM $CuSO_4$	20.06 ± 2.1
32 μM $CuSO_4$ + 150 μM EGCG	36.2 ± 0.3

LNCaP cells and PC-3 cells were treated with different concentrations of EGCG and $CuSO_4$.
At 24 h, mortality of PCA cells was detected by flow cytometry.
Data are means ± SD, $n = 5$.

was slightly higher than that without Cu^{2+}. However, in the presence of 32 μM Cu^{2+}, EGCG concentration of F-12 medium declined from 150 to 0 μM after 6 h. It was shown that changes in EGCG concentration, in coexistive systems with Cu^{2+}, depended on the concentration ratio of EGCG and Cu^{2+}. When the added order was reversed, the concentration of EGCG at 6 h differed for all concentration ratios evaluated. In F-12 medium treated with 200 μM EGCG and 320 μM Cu^{2+}, the concentration of EGCG decreased to 0.99 μM after 6 h, a value substantially below 6.25 and 5.98 μM. Higher concentrations were also detected when 150 μM EGCG was added first and not last. At 12 and 24 h, EGCG was not found in F-12 medium under any conditions evaluated in this study. This may be explained by the components of F-12 media, which include proteins and amino acids. These may react with EGCG in oxidation, chelation, or polymerization to rearrange EGCG, an important event in the process of forming a complex with Cu^{2+}.

3.3. Free radical analysis by ESR spectra

Employing DMPO as trapping agents, we evaluated the generation of free radicals in F-12 medium treated with Cu^{2+} and EGCG in the absence of PC-3 cells. Figs. 3A and B show the typical 1:2:2:1 4 line ESR signal (with hyperfine splitting parameter $a^N = a^H = 1.5$) of DMPO–ON· adducts. When 100 μM Cu^{2+} was added, we found a weak signal of DMPO–OH· in F-12 medium (Fig. 3A). The intensity of the signal of DMPO–OH· increased strongly when 200 μM EGCG was added, indicating that the generation of hydroxyl radicals might result from the interaction of Cu^{2+} with EGCG. In the data recorded at 15, 30, 60 min and 24 h after the final mixing with EGCG, the intensity of the signal of DMPO–OH· declined with time. At 24 h, the DMPO–OH· signal was absent in F-12 medium. When Cu^{2+} was added last, variations in intensity of the DMPO–OH· signal in ESR spectra were similar, i.e., the intensity decreased gradually with time. After DMPO and 200 μM EGCG were mixed with F-12 medium, no free radical signal was detected (Fig. 3B). The DMPO–OH· signal appeared in ESR spectra at 5 min following the addition of 100 μM Cu^{2+}. At 15 min, intensity began to decrease, and the DMPO–OH· signal was undetected at 24 h. Hydroxyl radical was the only free radical detected in our system and its intensity was different when the order of Cu^{2+} and EGCG addition was altered.

There are two possible mechanisms of EGCG with Cu^{2+} interaction. These are chelation and oxidation. In a recent report (Manabu, Shin-ichi, Kenji, & Tokuji, 2002), Cu^{2+} accelerated the autoxidation of catechins as a catalyst in pH 9 buffers, changing Cu^{2+} to Cu^{+}. The latter is responsible for the generation of hydroxyl radicals. The stability of EGCG was different when pH was changed. It is postulated that autoxidation of EGCG did not occur because EGCG was stable at pH 7.2–7.4 in our system. Hisashi et al. (2001) applied a DMPO-trapping method to examine the formation of free radicals in the EGCG–Cu^{2+} system (pH 9), and found signals of DMPO–OH· and DMPO–O_2^-. Their results showed that EGCG formed a stable complex with Cu^{2+} and generated the hydroxyl radical. This might be similar to the interaction of EGCG and Cu^{2+} in our system.

Some researchers found that DNA and the cytoplasmic membrane of *E. coli* were damaged by free radicals in the coexistive system of catechin and Cu^{2+} (Fumiko

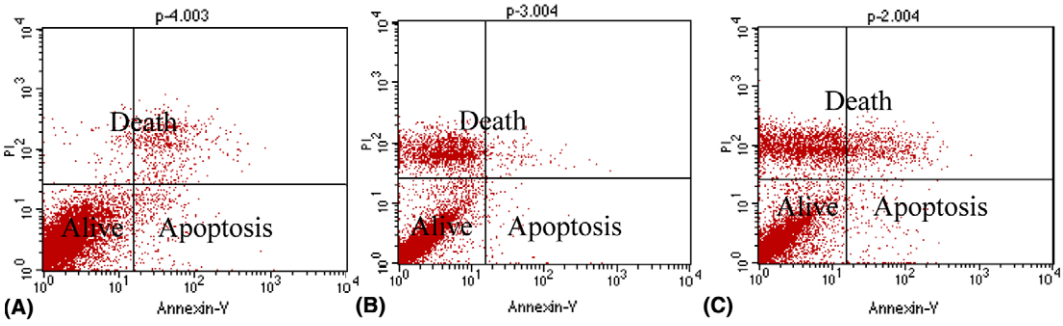

Fig. 2. Detection of apoptosis of PC-3 cells by flow cytometry. (A) Normal PC-3 cell; (B) PC-3 cells treated with 50 μM EGCG; (C) PC-3 cell incubated with 32 μM $CuSO_4$ after adding 150 μM EGCG.

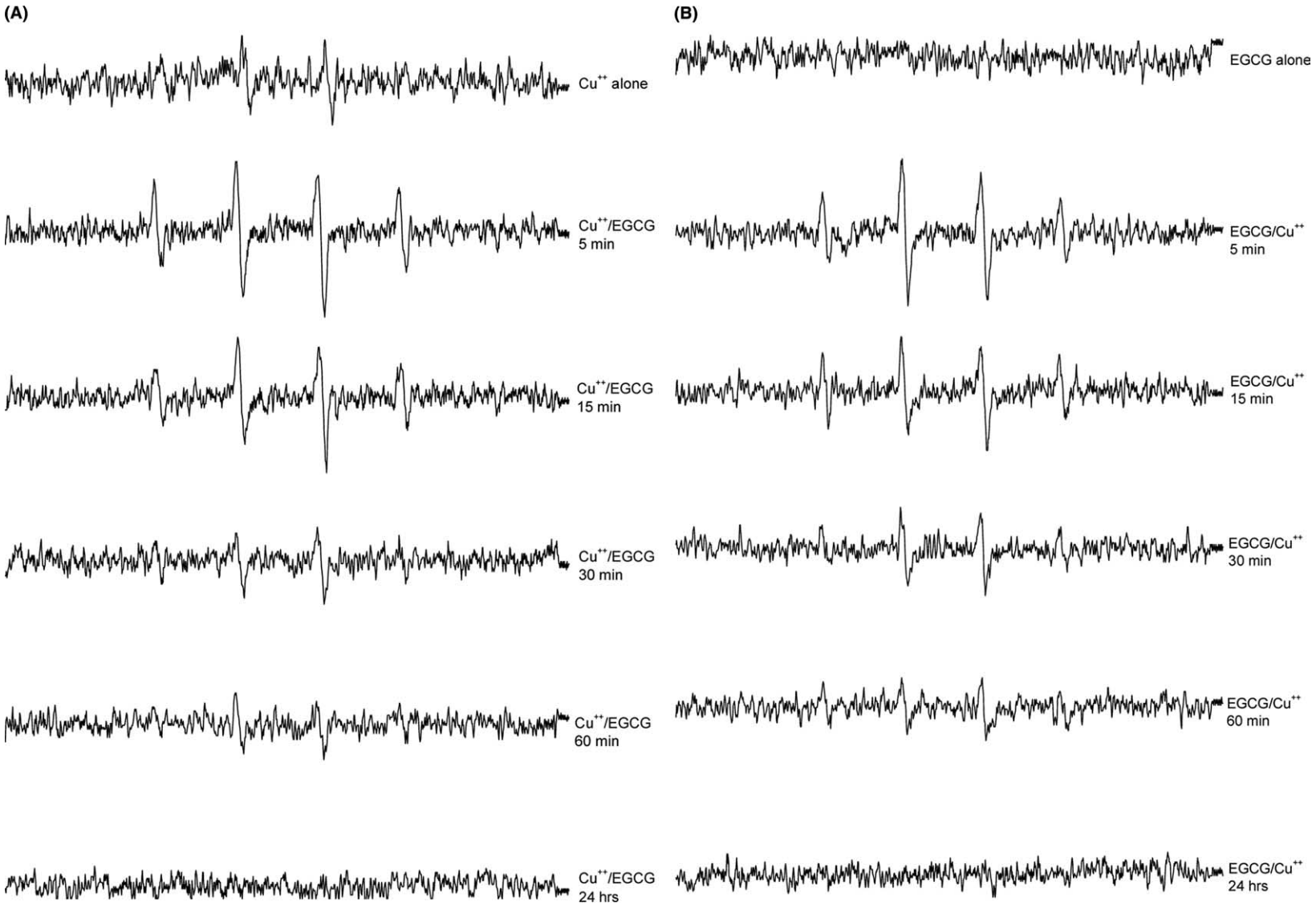

Fig. 3. ESR spectra of free radicals produced in the EGCG–Cu^{2+} system. (A) DMPO and Cu^{2+} were added to F-12 medium first; data were collected at 5, 15, 30, 60 min and 24 h after the final mixing with EGCG. The final concentrations for DMPO, Cu^{2+} and EGCG were 200 mM, 100 and 200 μM, respectively. (B) DMPO and EGCG were added to F12-medium first; data were collected at 5, 15, 30, 60 min and 24 h after the final mixing with Cu^{2+}. The final concentrations for DMPO, Cu^{2+} and EGCG were 200 mM, 100 μM and 200 μM, respectively.

et al., 1997; Nobuo et al., 1999). Results are similar with electron microscopy, which shows that the cytoplasmic membrane of PC-3 cells is damaged in our system. It is interesting that we found damaged cytoplasmic membrane without detecting free radicals in F-12 medium when mixed with EGCG. This observation is based on HPLC data only. Here, EGCG disappeared more quickly with PC-3 cells than without PC-3 cells. EGCG might contain at least one effective group, which interacts or absorbs on the cytoplasmic membrane of PC-3 cells. So, EGCG and some EGCG–Cu^{2+} complex, or other rearrangement of EGCG with an effective group, could damage the cytoplasmic membrane of PC-3 cell directly and independently of oxidative damage by free radicals. EGCG was absent in F-12 medium after 6 h. It is possible that rearrangement involving oxidation, polymerization or the formation of complex with Cu^{2+}, or other components of F-12 medium, in turn, react to damage the cytoplasmic membrane of PC-3 cells.

In vivo, effects of EGCG on prostate cancer were complex. On the one hand, EGCG could affect the growth of prostate cancer cells by modulating absorption of copper ion; on the other hand, EGCG could kill prostate cancer cells by producing free radicals when EGCG met with copper ion on the inside or outside of prostate cancer cells in vivo.

We conclude that derivatives of EGCG are important functional components, which suppress the growth of PC-3 cells, and that the cytoplasmic membrane is the site at which damage to the cell occurs in the PC-3 cell culture system used. The hydroxyl radical might be pivotal to damaging PC-3 cells in the coexisting system of EGCG and Cu^{2+}.

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