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The Effects on Cell Growth of Tea Polyphenols Acting as a Strong Anti-peroxidatant and an Inhibitor of Apoptosis in Primary Cultured Rat Skin Cells

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Studies during the past few years have indicated an inhibitory effect of green tea or tea polyphenols on tumorigenesis in animal and even in human. The purpose of this study was to observe the possible effects of tea polyphenols on skin cell growth and on apoptosis in rat primary cultured keratinocytes and fibroblasts. The release of a cell plasma enzyme (LDH), lipid peroxidation products (MDA production), and GSH-Px (glutathione peroxidase) into the medium in cultured cells was determined after treatment with tea polyphenols in a primary culture of skin cells. The percentage of cells in each cell cycle phase and in apoptosis were assayed by flow cytometry (FCM). Tea polyphenols may have a beneficial effect on skin cells at concentrations from 0.05% to 0.1%, showing a dose-dependent decrease in LDH, MDA (malondialdehyde) production, and a significant dose-dependent increase in GSH-Px and cell number. These effects were more obvious after exposure for 24 h than after 12 h. The results indicate that tea polyphenols may stabilize and protect the cell membrane against the release of cell plasma enzyme LDH, and its anti-peroxidation effect is also important for cell growth. FCM analysis revealed that treatment with 0.01% to 0.1% tea polyphenols decreased the percentage of cells in the G_1/G_0(quiescent) phase from 81.32% to 74.38%, and increased the percentage of cells in S and G_2/M phase from 9.87% to 15.26%, and from 6.51% to 10.36%, respectively. Tea polyphenols also increased the value of *PI* (proliferation index) from 18.17 to 25.62. At the same time it decreased the percentage of apoptosis from 27.10% to 17.97%, which indicates that green tea stimulates cell growth and inhibits the occurrence of apoptosis. Our results indicate that tea polyphenols are effective anti-oxidants and also inhibit apoptosis, which may improve the proliferative capacity of primary skin cells *in vitro*.

INTRODUCTION

Since its discovery in China nearly 5000 years ago, tea has become the most popular beverage consumed by people worldwide (Alexis, Jones and Matthew, 1999). Most of the polyphenols present in tea are flavonols, commonly known as catechins. The major catechins in green tea are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) (Weisburger *et al.*, 1996; Wang *et al.*, 1992). Although these chemicals have different roles in the anti-tumor process (Okabe *et al.*, 1997), Suganuma *et al.* (1999) found that the whole tea polyphenols are a more effective mixture for cancer prevention. In recent years, many laboratory studies have shown that the administration of green tea, specifically the polyphenolic fraction isolated from green tea

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leaves, protects against several forms of cancer induction in a variety of animal tumor bioassay systems (Suganuma *et al.*, 1998; Weisburger *et al.*, 1996; Javed, Mehrotra and Shukla, 1998; Wang *et al.*, 1992). In addition, green tea has fond to scavenge peroxidation agents and inhibit carcinogenesis of tumor growth (Hu, Han and Chen, 1995; Javed, Mehrotra and Shukla, 1998).

Pretreatment of cells with green tea polyphenols before each carcinogen application resulted in significant protection against skin tumors in a dose-dependent manner, indicating that tea polyphenols, specifically epigallocatechin-3-gallate, could be useful against some forms of animal or human cancers induced by environmental agents *in vivo* or *in vitro* (Fox, 1997; Liang *et al.*, 1999; Hu, Han and Chen, 1995; Fujiki *et al.*, 1999). The mechanisms may be linked to the reduction of EGF receptor and PDGF receptor autophosphorylation, and to the increase in level of some sex hormones, which may have some effect on tumor inhibition (Liang *et al.*, 1999; Fujiki *et al.*, 1999). The tea polyphenols may also block growth factor from binding to their receptors, thus suppressing mitogenic signal transduction (Hu, Han and Chen, 1995; Fujiki *et al.*, 1999; Okabe *et al.*, 1997). These results are of great interests for scientists in medicine, especially for biologists and dermatologists (Alexis, Jones and Matthew, 1999). Although the inhibition of tumor in mice or human by tea polyphenols had been demonstrated directly or indirectly (Fujiki *et al.*, 1999; Suganuma *et al.*, 1998), the effects on normal cells, especially skin cells, needs further study (Fox, 1997). Many studies have shown that tea polyphenols can scavenge free radicals generated by the ROS and Fe(Ⅱ)-H_2O_2 systems *in vitro* without cells (Wiseman, Balentine, and Frei, 1997; Fang *et al.*, 1998) leading some cosmetics manufacturers to add tea polyphenols to cosmetics products. Our works centered on the effect on two types of normal skin cells, keratinocytes and fibroblasts, which are the main cells present in the skin epidermis and dermis, respectively.

In this study, the amount of LDH released from cultured cells was determined to determine the appropriate tea concentration which may not damage cells, followed by measurement of peroxidation production (MDA) and anti-peroxidant (GSH-Px) levels to study the anti-peroxidation effects of tea in normal skin cells. The cell growth and cell cycles were also determined to show whether tea polyphenols can improve cell growth. The other purpose of this study was to observe the changes in apoptosis of keratinocytes after they were treated with tea polyphenols.

MATERIALS AND METHODS

Chemicals

Tea polyphenols (a gift from Prof. ZHANG Ying, Zhejiang University, consist of EGCG 45%, EGC 10%, ECG 13%, EC 5% and total catechins 75%), a mild brown powder, separated and purified by Dept. of Food Sci. & Tech., Zhejiang University, was disolved in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, N. Y., U.S.A.) and filtrated with 0.22 μm filter for sterilization.

K-SFM (Keratinocyte-SFM), the specific keratinocyte culture medium, was purchased from Gibco Co., N. Y., U.S.A. Trypsin was purchased from Difco, U.S.A. NBS (new borine serum) was purchased from Hyclone, Utah, U.S.A. Ficoll 400 was purchased from Phamacia,

Uppsala, Sweden. DTNB (5,5′-dithiobis-p-nitrobenzoic acid) was purchased from Fluka, N. Y., U.S.A. The other agents not mentioned were procured form Sino-ABC, Shanghai, China, and in AR grade of purity. All of the chemicals were prepared immediately before use.

Cell Culture

Dorsal skin was removed from 3-day-old Wistar rats and keratinocytes were isolated by the modified method of Jin (1986) and Yin (1999). The skin was sterilized with 75% ethanol, and then it was stretched and floated in PBS containing 0.15% trypsin at 4℃ for 12 h. The epidermis was separated from the dermis with forceps and floated in PBS and DMEM medium with 10% NBS to wash and neutralize the trypsin. The epidermis sheet was gently brushed with a camel-hair brush and shaken in DMEM containing 10% NBS. The keratinocytes were fractionated on a discontinuous gradient formed by centrifugation in 10% Ficoll 400 and DMEM density gradients. An aliquor of 2 ml of cells were added to each 35 mm Corning (N. Y., U.S.A.) plastic dish at a density of 5×10^5 cells per ml. Afterwards the cells were suspended in K-SFM, supplemented with 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were then cultured in a humidified incubator with 5% CO_2/air at 37℃. The first medium was changed 12 h after cell plating, and then the culture medium was changed every 2 days.

Fibroblast were obtained and treated the same as the keratinocytes, except that the dermis separated from the whole skin was digested for another 30 min with 0.25% trypsin. When the confluence of the cultured cells reached 80%, the tea polyphenols disolved in DMEM without NBS was added.

Lactate Dehydrogenase Release Assay

Solution of tea polyphenols at concentrations of 0.01%, 0.05%, 0.1% to 0.5% were added to cultured keratinocytes and fibroblasts, then 12 h and 24 h later the LDH level in the medium was assayed (Bonnekoh *et al.*, 1990). The Wahlefeld UV assay method with L-lactate and NAD was used (Wahlefeld, 1983). The amount of L-lactate oxidized at 37℃ per unit time, measured by the continuously increased absorptance at 339 nm due to the reduction of NAD, is a measure of the catalytic activity of LDH (Wahlefeld, 1983).

Anti-peroxidation and Lipid Peroxidation Test

GSH-Px was assayed by DTNB spectrometry methods (Sugawara *et al.*, 1991). This method depends on the ability of GSH-Px 's to catalyze the oxidation of GSH, which reacts with DTNB (5,5′-dithiobis-p-nitrobenzoic acid). After the keratinocytes and fibroblasts reaching 70% confluence, the cells were treated with tea polyphenols. Twelve and 24 h later, 50 μl of supernatant medium was used in this assay. After 0.4 ml of heated (37℃) H_2O_2 and 1.67% perphosphate were added, the reaction products were centrifuged for 10 min at 3000 r/min. 2.0 ml of this supernatant was added to 2.5 ml of 0.32 mol/L Na_2HPO_4, and 0.5 ml DTNB. The results were determined spectrophotometric at wavelength in 422 nm, and calculated as U per ml.

The TBA spectrometry assay was used to determine MDA production. To 0.3 ml of cell

culture supernatant medium, 3.0 ml HCl, and 1 ml of 0.67% TBA were added individually, then heated for 30 min in boiling water. The reaction products were extracted in a mixture of methanol and n-butyl alcohol, and the supernatant was measured at 535 nm, with the result given in μmol per L (Smith *et al.*, 1982).

Cell Counting Assay

After the cultured cells were nearly confluent, the cells were treated with 0.25% trypsin and 0.05% EDTA, and put in an incubator until some cells detached from the growth surface. PBS was added to remove the enzyme and wash the cells, by shaking and pushing down all unattached cells from the dish wall. Cell counts were performed in a standard cell counting chamber (Wille *et al.*, 1984). The cell viability was assayed using Trypan Blue Exclusion test (Benson, Belton, and Scheve, 1986). All determinations were performed in triplicate.

Cell Cycle and Cell Apoptosis

About 2×10^6 cells were fixed with 75% ethanol for 30 min, then washed with 0.1% Triton 100 and centrifuged for 7 min. The precipitates was incubated in 2 ml of 0.1% RNase at 37℃ for 30 min, then 0.05% Propidium iodide (PI) was added. Excitation was provided by a laser tuned to 488 nm, and 630 nm filter was used to detect the red fluorescence emitted by PI in EPICS VI (U.S.A.). The DNA content was used to determine the cell cycle phase (G_1, G_2, S, M). The sub-peak of DNA content before the G_1 phase indicated that there was chromatin loss in the nucleus which could be defined as cell apoptosis, for DNA fragments can leak from the nucleus (Dean, and Jett, 1974; Arends, Morris and Wyllie, 1990; Monti *et al.*, 1992). The value of PI (proliferation index) was calculated according to the formula: $PI = [(S + G_2/M)/(G_0/G_1 + S + G_2/M)] \times 100\%$ (Yin *et al.*, 1999).

Statistical Analysis

The data were analyzed with ANOVA in SAS statistical package 6.12 (N. C., U.S.A.). To compare the differences among the groups, the Student-Newman-Keuls test was applied. The alpha level was set at 0.05, two tailed.

RESULTS

Effect on LDH Release Level

The release of LDH from the different cells after exposed to tea polyphenols at different time intervals are presented in Fig. 1 (a) and Fig. 1 (b). Statistical evaluation of this data demonstrated that a significant dose dependent decrease in the amount of LDH release could only be observed at concentrations between 0.01% to 0.1%. At the 0.1% concentration, the LDH level was similar to the VC 's (also at 0.1%), which was defined as the positive control group. In the group treated with 0.5% showed the LDH release increased abruptly, which indicates that

at this concentration there may be some adverse effects on cells. Therefore we determined that the test doses below 0.1% were the proper concentration for the following experiments. The statistical differences are indicated in Fig. 1 with asterisks. The results also showed that the LDH level was higher at 24 h after exposure than at 12 h after exposure.

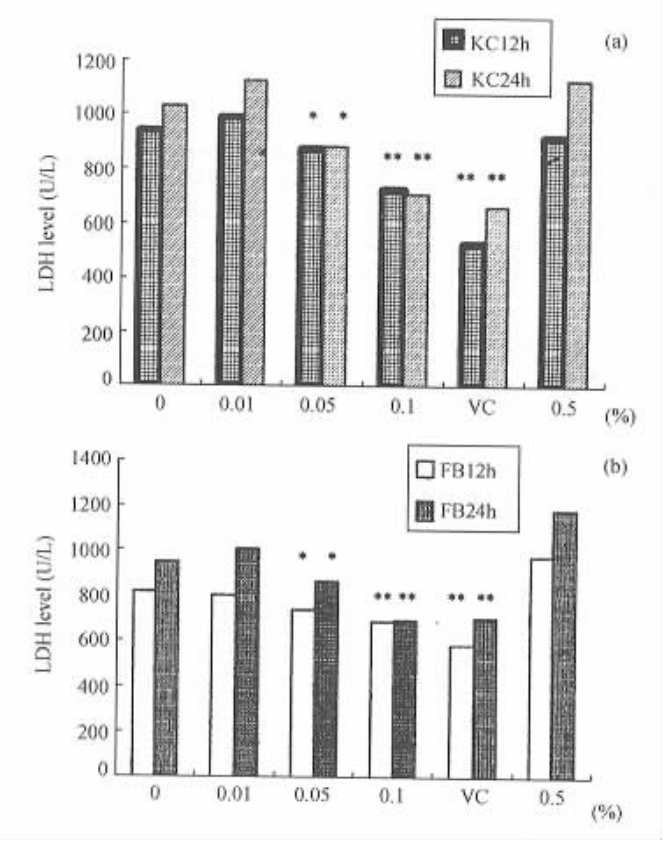

FIG. 1. The effects of tea polyphenols on LDH release in primary cultured rat skin cells. (a) The effect on the release of LDH from skin keratinocytes. At concentrations from 0.01% to 0.1%, there were dose-dependant decreases of LDH, the LDH level at 0.1% was as low as the positive group (VC at 0.1%). At a concentration of 0.5%, the release of LDH increased greatly. (b) Skin fibroblasts showed the same changes as those of keratinocytes. Significant difference in comparison to the control: * $P < 0.05$, ** $P < 0.01$ ($n = 6$).

Anti-peroxidation of Tea Polyphenols

The anti-peroxidation ability of the tea polyphenols are shown in Tables 1 and 2. The dose-dependent increase in GSH-Px release from keratinocytes from 28.98 U/ml to 35.91 U/ml, at tea polyphenols concentration from 0.005% to 0.1% after 24 h exposure. For fibroblasts the concentration of GSH-Px ranged from 15.325 U/ml to 33.994 U/ml. The changes at 24 h were higher than at 12 h (Table 1). MDA production decreased in a dose-dependant manner. In keratinocytes MDA production decreased from 12.96 to 7.22 μmol/L at 24 h; in fibroblasts from 12.76 to 6.38 μmol/L at 24 h. All the results of the 0.1% group were similar to those of the positive group (VC at 0.1%) (Table 2).

TABLE 1

Effects of Tea Polyphenols on Concentration of GSH-Px (U/ml) in Primary Cultured Skin Cells

Concentration (%)	Keratinocyte		Fibroblast	
	12 h	24 h	12 h	24 h
0	25.121 ± 1.871	29.860 ± 3.374	11.781 ± 2.915	12.540 ± 7.054
0.005	26.118 ± 3.790	28.988 ± 3.650	14.226 ± 5.163	15.325 ± 4.598
0.01	28.071 ± 4.138	29.572 ± 4.281	19.525 ± 4.234	21.943 ± 5.481
0.05	29.746 ± 3.630	29.370 ± 5.123	21.807 ± 4.625*	26.513 ± 6.031*
0.1	30.258 ± 5.274*	35.901 ± 5.088*	29.253 ± 6.038**	33.994 ± 5.452**
VC	24.547 ± 3.597	34.858 ± 2.548	30.274 ± 5.296	42.050 ± 7.962

Note. The VC (vitamin C) was set as positive control. Significant difference in comparison to the control: * $P < 0.05$; ** $P < 0.01$. The data are $\bar{x} \pm s$ ($n = 6$).

TABLE 2

Effects of Tea Polyphenols on Concentration of MDA (μmol/L) in Primary Cultured Skin Cells

Concentration (%)	Keratinocyte		Fibroblast	
	12 h	24 h	12 h	24 h
0	12.12 ± 1.87	13.18 ± 2.30	11.78 ± 2.15	12.09 ± 1.41
0.005	13.11 ± 1.79	12.96 ± 1.92	12.26 ± 1.63	12.76 ± 1.54
0.01	10.71 ± 2.18	9.72 ± 2.76*	10.25 ± 2.34	10.10 ± 1.27*
0.05	9.76 ± 2.63*	8.12 ± 1.26*	9.87 ± 1.65*	8.76 ± 2.50*
0.1	9.58 ± 1.74*	7.22 ± 2.16**	9.03 ± 2.08*	6.38 ± 1.28**
VC	8.25 ± 2.06	5.66 ± 1.87	7.82 ± 1.76	5.09 ± 2.19

Note. The VC was set as positive control. Significant difference in comparison to the control: * $P < 0.05$; ** $P < 0.01$. The data are $\bar{x} \pm s$ ($n = 6$).

Influence on Cell Cycle and Cell Apoptosis

Table 3 shows the effect of tea polyphenols on cell growth as determined by cell counting (cytometry) assay. The effect on fibroblasts which increases from 1.26 to 7.03%, was stronger than on keratinocytes, which increased from 1.24 to 5.25%. The results of the cell cycle and apoptosis studies were shown in Table 5. At concentrations from 0.01% to 0.1%, the the number of cells in the S and G_2/M phases increased. The PI (proliferation index) increased from 18.17 to 25.62, and the cell detachment, and indication of apoptosis, decreased from 27.10 to 17.97% (Table 4). Tables 3 and 4 showed that tea polyphenols may stimulate to cell growth.

TABLE 3

Percentage of Live Cells and Cell Growth in Primary Cultured Rat Skin Cells Treated by Tea Polyphenols

Cell Number		Control	0.005%	0.01%	0.05%	0.1%
Live cell (%)	Keratinocytes	91.25	91.31	91.91	93.03	93.82
	Fibroblasts	93.18	93.24	94.15	95.28	95.47
Cell growth (%)	Keratinocytes	–	1.24 ± 0.75	1.32 ± 0.84	2.33 ± 1.25	5.25 ± 2.36
	Fibroblasts	–	1.26 ± 0.62	1.41 ± 0.82	4.88 ± 1.26	7.03 ± 1.75

Note. The live cell was calculated as percentage of viable cells by Trypan Blue Exclusion. Cell growth was calculated by the increase percent of cells *vs*. control group (0 dose group). The data are $\bar{x} \pm s$ ($n = 6$).

TABLE 4

Effects of Tea Polyphenols on Cell Cycle and Apoptosis of Primary Cultured Keratinocytes

Concentration (%)	$G_0 - G_1$ (%)	$G_2 - M$ (%)	S (%)	*PI*	Apoptosis (%)
0	81.81	6.67	11.53	18.20	19.98
0.01	81.32	6.51	9.87	18.17	27.10
0.05	81.76	8.37	10.17	18.24	20.45
0.1	74.38	10.36	15.26	25.62	17.97
VC	80.39	13.61	8.25	21.86	23.90

Note. The VC (vitamin C) was set as positive control. Proliferatinve index (*PI*) was calculated by $PI = [(S + G_2/M)/(G_0/G_1 + S + G_2/M)] \times 100\%$.

DISCUSSION

The aim of our study was to investigate the effect of tea polyphenols on cell growth of keratinocytes and fibroblasts and to determine if apoptosis is changed in this process. Apoptosis, which is also known as programmed cell death, is a key event influencing the net rate of population expansion of cells, embryonic development and differentiation, and enables the qualitative and quantitative maintenance of cells in normal tissues (Tamada *et al.*, 1994; Medema, 1999). It is now established that a specialized form of apoptosis is part of the normal program of keratinocyte differentiation in the epidermis (Maruoka *et al.*, 1997; Arends, Morris and Wyllie, 1990; Sermadiras *et al.*, 1997). This program includes common biochemical events such as induction of the cross-linking enzyme transglutaminase, chromatin condensation and DNA fragmentation, in addition to regulation by factors such as calcium, retinoic acid, glucocorticoids and transforming growth factor (TGF)-β (Sermadiras *et al.*, 1997; Wyllie, 1980; Heenen *et al.*, 1998). Furthermore, apoptosis is also involved in abnormal and damaged cell death in various diseases and tissue injuries such as cell growth following ultraviolet (UV) irradiation and during aging (Aragane *et al.*, 1998; Monti *et al.*, 1992; Wrone-Smith *et al.*, 1997).

The LDH release test is a simple and sensitive indicator of chemical-induced membrane injury in cultured keratinocytes (Bonnekoh *et al.*, 1990). The aim of this study is to see if tea polyphenols can provide some benefits to skin cells. Therefore, it was necessary to first determine the safe concentration for cells. Our study found that LDH is a good indicator for this use, which was also demonstrated by Yin (1999).

Continuous exposure to endogenous and exogenous damaging agents plays a role in the aging process, for aging is probably the results of genetic and environmental factors (Monti *et al.*, 1992). Indeed, cells have to cope with the damage provoked by heat, radiation, glucose, and oxygen free radicals. Oxygen radicals are normally produced in the course of several biochemical reactions during cellular metabolism (Hebert *et al.*, 1998; Monti *et al.*, 1992). Some authors argue that antioxidant defense mechanisms, either enzyematic or nonenzymatic, must be considered all together because they are involved in complex redox reactions. Indeed, free radicals have many targets, including plasma membranes, organelles, proteins, and nucleic acids (Peterhans, 1997). As a consequence, a variety of defense mechanisms other than the release of antioxidants are triggered by oxidative stress (Monti *et al.*, 1992).

Green tea polyphenols are potent antioxidants. They have both anti-carcinogenic, anti-histaminic, anti-microbial and anti-inflammatory effects (Alexis, Jones and Matthew, 1999). The anti-inflammatory mechanism of tea polyphenols is mediated, at least in part, through down-regulation of TNF alpha gene expression by blocking NF-κB activation. It has been suggested that tea polyphenols may offer an effective therapy for a variety of inflammatory processes (Suganuma *et al.*, 1999; Yang *et al.*, 1998). Our findings demonstrate that green tea decrease lipid peroxidation (MDA) accompanied by a marked increase in the activities of the enzyme GSH-Px. These two changes demonstrate that tea polyphenols have an anti-peroxidation function in skin keratinocytes and fibroblasts, because of its ability to scavenge free radicals in vitro, as suggested by Fang (1998).

Flow cytometry DNA histogram analysis showed that there was no pronounced alteration in the cell cycle after exposure to low levels of tea polyphenols. With increasing concentration, the transportation of keratinocytes from quiescent G_0 and G_1 phase to DNA synthesis phase (S phase) and mitosis (M phase) indicates stimulation of cell growth, and inhibition of cell apoptosis to some extent. This may be related to the anti-peroxidation effect in that the tea was shown to decrease MDA production and increase GSH-Px levels. Its ability to inhibit apoptosis in normal skin cells results in a decrease cell in death and cell loss, thereby prolonging cell life to some extent. It should to be mentioned that tea polyphenols have been shown to inhibit cell growth and induce apoptosis in human tumor cell lines (Yang *et al.*, 1998). Their mechanisms may be different, for in normal cells it mainly works as an anti-peroxidant, but it may also block signal transduction, mediated by Fas/Fas ligands in tumor cell lines (Liang *et al.*, 1999; Fujiki *et al.*, 1999; Leverkus, Yaar and Gilchrest, 1997; Matsue *et al.*, 1995).

In conclusion, the present study indicates that tea polyphenols demonstrated a dose-dependent response for stabilizing cell membrane and enhancing the anti-peroxidation ability in cultured skin cells. In addition, these compounds may stimulate skin cell growth and inhibit cell apoptosis. Therefore, tea polyphenols are beneficial to skin cells, thus these may be possibilities for some prospective uses in cosmetics and other skin products.

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