Differential Effects of Green Tea Polyphenol in the γ-irradiation Induced Human Leukemic and Lymphoblastic Cell Damage

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녹차 폴리페놀이 감마선조사에 의한 백혈병과 림프구모세포의 손상에 미치는 영향의 차이

정환정[1,2,3], 김은미[1,2,3], 민정준[4], 범희승[4], 김영호[5], 정영도[6], 김창근[1,2,3]

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Abstract

Purpose: The green tea polyphenol (GTPP) has been known to exert antioxidant activity as a radical scavenger as well as cancer preventive and cancer growth inhibition effect. The aim of this study was to identify whether GTPP not only potentiate the growth inhibition effect in γ-irradiated human cancer cell but also exert protection action for irradiated human normal cell. **Materials and Methods:** GTPP (80% catechin including >45% EGCG) added in the HL60, human leukemia, and NC37, human lymphoblast, before irradiation. After establishing the amount of GTPP and the dose of radiation, the cells were treated with the GTPP for 6 hours and irradiated with the determined doses. **Results:** Viability when 10 $\mu g/ml$ GTPP added before γ-irradiation with 1 Gy to NC37 cells was not different in comparison with control but it when was irradiated with 3 Gy significantly different (1 Gy;P=0.126, 3 Gy;P=0.010). 20 $\mu g/ml$ GTPP did not show significant difference in both NC37 cells irradiated with 1 Gy and 3 Gy (1 Gy;P=0.946, 3 Gy;P=0.096). Viabilities were significantly decreased with concentration of additional GTPP in HL60 with 1 or 3 Gy (1 Gy; 69.0±1.7% vs 42.4±1.3%, 3 Gy; 66.9±3.9% vs 44.2±1.6 %). **Conclusion:** In vitro study, we certified that when the cells were irradiated with dose below 3 Gy, GTPP provide not only anticancerous effect against cancer cells but also radioprotective effect in normal cells simultaneously. Theses results suggest the possibility that consumption of green tea could give the radioprotective effect and maximize the effect on internal radiation such as radioiodine therapy concomitantly.

Key words: Green tea polyphenol, Radioprotection, radiation, radical scavenger

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Introduction

The green tea is the most widely consumed beverage in the world, and the possible beneficial health effects have received a great deal of attention. Through epidemiological and animal studies, the cancer protective effect of green tea has been suggested in the organs involved cancers, such as the lung, skin, esophagus, liver and stomach, but this result is not conclusive. The green tea polyphenols (GTPP) have also been shown the inhibition of the proliferation of cultured mammalian cancer cells, including colon carcinoma, lung carcinoma, breast carcinoma, melanoma and leukemic cells.[1-3] In general, green tea is flavan-3-ols, commonly known as catechins, which include (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG), and caffeine (Fig. 1). Of these, the most active is (-)-epigallocatechin-3-gallate (EGCG), the major constituent in GTPP. It has been reported that constituents of green tea extract together have synergistic or additive effects on cancer-preventive activity.[4]

Radiotherapy that has used to the patients developed the cancer with external X-ray source or beta-emitting isotope such as I-131, cause their therapeutic effects by generating free radicals. But simultaneously radiotherapy results in damage of normal tissues in the radiation port or taken the isotope physiologically. For prevention of this complication, many workers have investigated the preventive materials from natural plants and artificial chemicals. The one of those results was reported that parenchymal damage in salivary glands induced by high-dose radioiodine therapy in patients with differentiated thyroid cancer could be reduced significantly by amifostine as a radical scavenger.[5]

The GTPP has antioxidant activity and so acts a radical scavenger. Steele et al. reported that most of the GTPP fractions used inhibited TPA-induced free radicals in human HL60 cells.[6] Parshad et al. reported the addition of GTPP, not EGCG, to the culture medium before X-irradiation (53 rads) significantly reduced chromtid break frequencies.[7] In

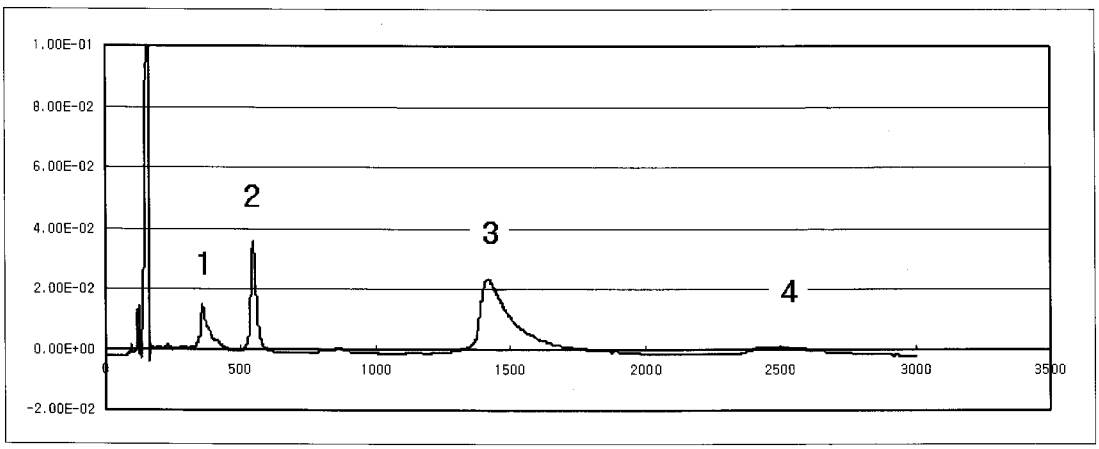

Fig. 1. The results of HPLC by catechin standard
Green tea extract (0.1g) was dissolved in 100 *ml* of distilled water. The HPLC system set at 280 nm and stationary phase was Luna C18 column. EGC (1), EC (2), EGCG (3), and ECG (4) were identified sequentially. The amount of EGC, EC, EGCG, and ECG were 94, 87, 456, and 164 mg per 1 g catechin repectively.

the present study we intended to examine the different effects of GTPP on normal and tumor cell damage induced by irradiation.

Materials and Methods

1. Green tea polyphenol (GTPP) preparations and analysis using HPLC

GTPP was generous gift from the Tea Research Lab. Pacific R&D Center in Korea. GTPP was composed of above 80% catechin including >45% EGCG in the 95~98% polyphenols and below 3% caffeine. The components were analyzed with high-performance liquid chromatography (HPLC). Green tea extract (0.1g) was dissolved in 100 *ml* of distilled water. The aqueous extract was filtered through 0.45 *㎛* membrane filter and subjected to HPLC analysis. The HPLC system (Hewlett-Packard series 1100, Palo Alto, CA, USA) was equipped with an ultraviolet-visible detector and set at 280 *㎚*. Samples (20 *㎕*) were injected into the column (Luna 5 *㎛* C18 column, 150×4.60 *㎜*) and eluted with 22% THF(Tetrahydrofuran) solution. The flow rate was 0.8 *ml*/min.

2. Cell culture

Using a 95% air/5% CO2 humidified incubator set at 37℃, HL60 (Human leukemia, acute promyelocyte, KCLB, Korea) and NC37 (Human lymphoblast, KCLB, Korea) were grown in RPMI 1640 culture media supplemented with 10% FBS, and 1% penicillin-streptomycin.

3. Experimental procedure

HL60 and NC37 were plated at 3×10^4 cells per well in the 96 well plate. The effect of GTPP on the cell growth was assessed with MTT assay kit (Boehringer Mannheim, Mannheim, Germany) which resulted with the optical density (OD) value in the 570 *㎚*.

To decide the adequate concentration of GTPP mediated growth inhibition of cancer cells (HL60), but not normal cells (NC37), GTPP dissolved in 50 *㎕* RPMI 1640 was employed for the treatment of cells. The cells were treated with the various concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 *㎍/ml* per 100 *㎕* GTPP into the 96 well plate, and MTT assays were done for assaying cell proliferation.

To determine the maximal dose of radiation not affecting the normal cell, cells were irradiated with 1, 3, 5, 7, and 9 Gy using Cs-137 irradiator (Gammacell 3000 Elan. MDS Nordion Inc., Kanata, Ontario, Canada).

After determining the concentration of GTPP and the dose of radiation, the cells were pretreated with the GTPP for 6 hours and irradiated. After 24 or 48 hours incubation, each treated group consisted of 5 wells in the 96 well plates and the same experiment was repeated twice. The controls, which were treated each concentrations of GTPP only and each dose of radiation only were plated in the same plates.

4. Statistical Analysis

Data are given as mean±SD. To evaluate statistical differences between each groups, one-way ANOVA test was used with SPSS version 10.0, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

1. HPLC Chromatographic Analysis of Green Tea Polyphenol (GTPP)

To determine the components of GTPP, HPLC analysis was done. As shown in Figure 1, a characteristic HPLC chromatogram of GTPP in which mainly composed of four components such as diffrent (-)-epicatechin (EC), (-)-epicatechin-3-gallate

Table 1. Dose-dependent Effects of GTPP and Gamma-irradiation on the Growth of NC37 and HL60 Cell Lines

		GTPP (n=4)				Gamma-Irradiation (n=4)			
		0	10	20	30	0	1	3	5
Viability	NC37	100±2.9	94.4±3.9	94.5±3.1	72.3±3.1*	100±2.9	96.1±4.1	89.2±2.8*	74.3±5.7*
	HL60	100±4.1	51.9±4.1†	32.8±4.0	25.5±1.9†	100±1.2	89.5±2.5	58.3±3.2†	43.5±2.6†

MTT assays were done after 48 hours incubation with indicated treatment. Each data represents mean±SD.
*$P < 0.05$, †$P < 0.0001$

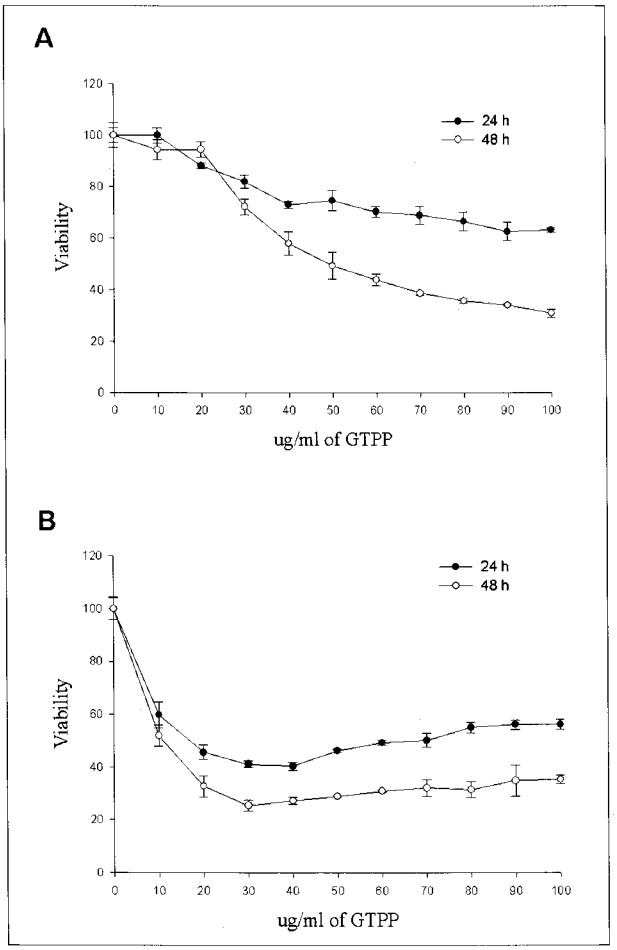

Fig. 2. Differential effect of GTPP on the growth of NC37 (A) and HL60 (B)
3×10^4 cells were plated in 96 wells, and incubated overnight. Cells were, then, treated with GTPP as indicated concentration for 24 or 48 hours and MTT assays were performed. The cytotoxicity was dose-dependent, and HL60 cells (B) were more sensitive to GTPP than NC37 cells (A).

(ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG), as known by commercial internal standard. When the catechin was included above 80% totally in the polyphenol, the percentage of compositions were as follows: EGCG (45.4%), ECG (16.4%), EGC (9.4%), and EC (8.7%).

2. Differential effects of GTPP on the growth of NC37 and HL60 (Fig. 2)

We decided the incubation time as 48 hours after adding the GTPP into the each wells in NC37 and HL60 cell lines. The differential cytotoxicity of GTPP between normal and tumor cell were examined

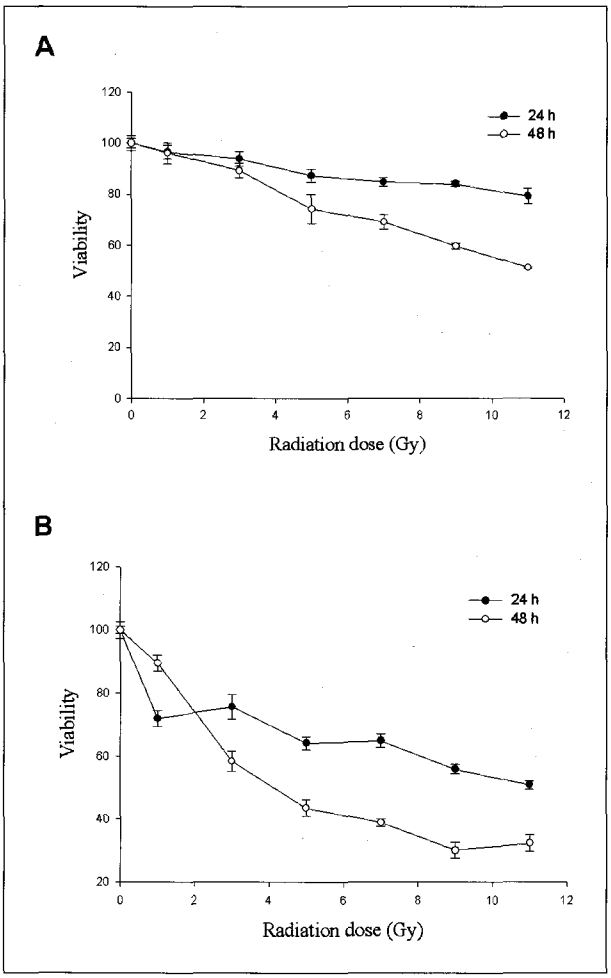

Fig. 3. The effects of γ-irradiation on the growth of NC37 (A) and HL60 (B) cell lines.
3×10^4 cells were plated in 96 wells, and incubated overnight. Cells were irradiated with each radiation dose, and then incubated for 24 or 48 hours and MTT assays were performed. The higher doses than 1 Gy were irradiated to normal cell, the more normal cells were non-viable (A). HL60 cell lines showed less viable above 1 Gy with compared to control (B).

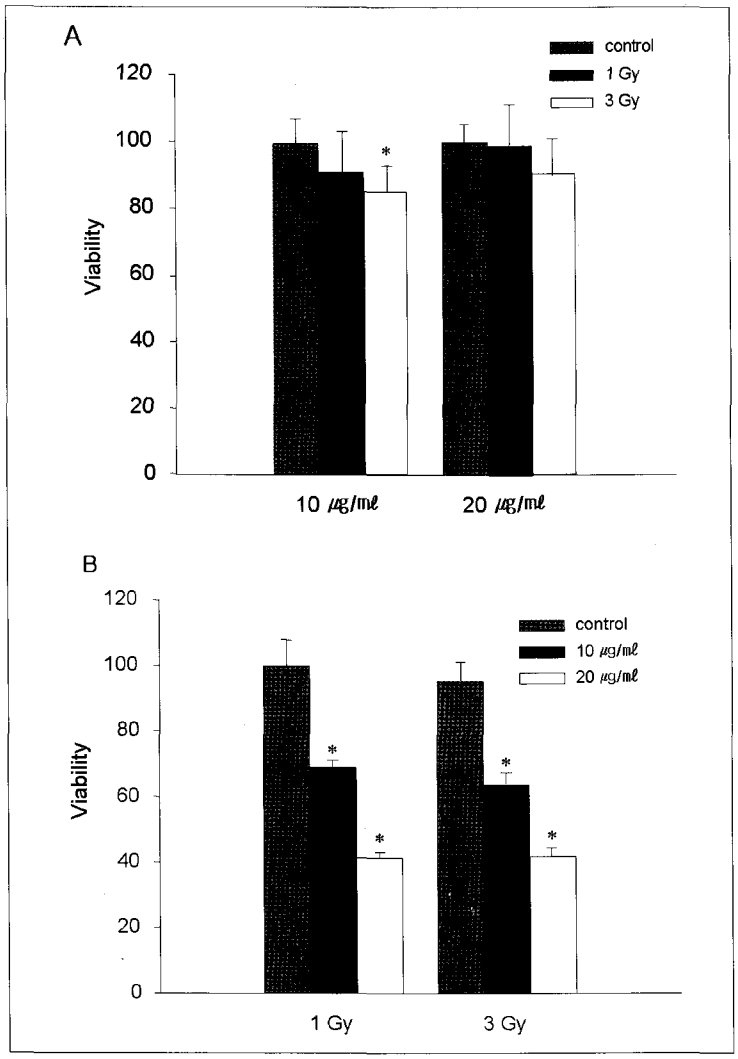

Fig. 4. Differential effects of additional GTPP before γ-irradiation on the radioprotection in the NC37 (A) and growth inhibition in the HL60 (B) cell lines simultaneously

(A) When the 20 $\mu g/ml$ GTPP was added, both the viability of irradiated NC37 cell with 1 Gy and 3 Gy were not significant difference (1 Gy;P=0.946, 3 Gy;P=0.096). The viability in which NC37 was added 20 $\mu g/ml$ before 3 Gy was same as in which 10 $\mu g/ml$ before 1 Gy (90.9±12.2 % vs 90.8±11.0 %). (B) Each concentration of GTPP was added before same dose of γ-irradiation in the HL60 cells to evaluate growth inhibitory capacity of GTPP against cancer cell. In the both cells irradiated with 1 or 3 Gy, viabilities were significantly decreased with concentrations of additional GTPP. All data were means±SD

* represent statistical significance compared to control (P<0.05).

by determined cell viability. The GTPP had the cytotoxicity in the normal and cancer cell lines at the different concentration. The cytotoxic activity was dose-dependent. As shown in Table 1 and Figure 2, the concentrations of GTPP were 10 and 20 $\mu g/ml$ inhibit cell growth of HL60, but did not normal NC37 cells.

4. The effects of γ -irradiation on the growth of NC37 and HL60 (Fig. 3)

To determine the sensitivity to radiation-induced normal and tumor cell death, the radiation dose, which was less effective to kill the NC37 in comparison with control, was 1 Gy. In the HL60 cell lines, all doses included 1 Gy were significantly effective on growth inhibition to control (Table 1, right column). We applied radiation doses such as 0, 1, and 3 Gy to the NC37 and HL60 for evaluation of the effects of GTPP on irradiated normal and tumor cells.

5. Differential effects of GTPP on γ -irradiation induced cell damage in normal and tumor cells (Fig. 4)

We investigated the effect of additional GTPP before and after γ-irradiation. After γ-irradiation adding the GTPP did not show the significant difference in comparison with group without adding the GTPP (data not shown).

When the 10 $\mu g/ml$ GTPP was added before γ -irradiation to NC37 cells, radiation with 1 Gy did not result in difference in comparison with control but 3 Gy resulted in significant difference (1 Gy;P=0.126, 3 Gy;P=0.010). But when the 20 $\mu g/ml$ GTPP was added, both 1 Gy and 3 Gy were not significant difference compared to the control (1 Gy;P=0.946, 3 Gy;P=0.096). When 20 $\mu g/ml$ GTPP before 3 Gy was added in the NC37 cells, the viability was same as that which 10 $\mu g/ml$ before 1 Gy (90.9±12.2 % vs 90.8±11.0 %) (Fig. 4A).

We performed that each concentration of GTPP was added before same dose of γ-irradiation in the HL60 cells to evaluate growth inhibitory capacity of GTPP against cancer cell. In HL60 cells irradiated with 1 and 3 Gy, viabilities were significantly decreased with concentration of additional GTPP (1 Gy;69.0±1.7% vs 42.4±1.3%, 3 Gy;66.9±3.9% vs 44.2±1.6 %) (Fig. 4B).

Discussion

The present study represents that the addition of GTPP significantly not only inhibit the growth of leukemic cells but also protect the radiation induced cell damage in human lymphoblasts. In the presence of 20 $\mu g/ml$ GTPP, the most strong effects of growth inhibition in the leukemic cells and radioprotection without inhibitory effect in the normal lymphoblasts appear concomitantly.

Irradiation causes a high degree of in vivo hydroxy radical generation by homolytic cleavage of body water or from the endogenous hydrogen peroxide formed by reduction of the superoxide anion. The hydroxyl radical is the major cytotoxic radical than others. Its high reactivity leads to an immediate reaction at the place where it is generated. The interaction of these radicals with phospholipid structures induces the peroxidation processes that increase hydroxyl radical activity in the DNA oxidative damage. Under these oxidative stress conditions, although radiation therapy is needed to patient with cancers, exogenous agents with a radical scavenging capacity need to be used to minimize the normal cell damage.[8]

Radiotherapy using radionuclide such as I-131, gamma and beta emitter, has the limitation that exposed dose to blood should not be in excess of 2 Gy to minimize complication like leukopenia or thrommbocytopenia.[9] This dose is so called as maximal permissible dose. In the case of patients

with differentiated thyroid cancer, recent effort reported high-dose I-131 therapy using maximal permissible dose shows significantly higher therapeutic effects as compared to low-dose therapy.[10-12] Although maximal permissible dose for internal radiation take the patients, radiation hazards by the dose should be interested.

Uchida et al. reported that EGCG as green tea component was an orally active radioprotector of very low toxicity and they had shown the potency of green tea against radiation hazard.[13] After then Steele et al reported that green tea fractions used inhibited TPA-induced free radicals in human HL60 cells and EGCG was ineffective, possibly because of inadequate uptake and/or retention in the cells.[6] Kim et al reported that the received 0.6 % (weight/volume) GTPP solution as sole source of drinking fluid for 8 days was distributed in the rodent tissue of heart, lung, kidney, liver, spleen, gastrointestinal tract, bladder, prostate gland, and thyroid gland.[14] Based on these reports, authors used the GTPP, not EGCG, to evaluate the radioprotective effect about lymphoblasts and to evaluate the growth inhibitory effect about leukemic cells composing blood components. Our results of study showed that 20 *μg/ml* GTPP had the powerful inhibition of growth in the tumor cells and radioprotective effect in the normal cells irradiated below the dose of 3 Gy.

Yang et al reported that GTPP displayed cell growth inhibition, showing IC50 values of about ~ 20 *μg/ml* in most cancer cell lines used in their study.[2] In our study we noticed that IC50 value of HL60 was also about 10~20 *μg/ml* and fortunately in this range GTPP about the normal blood component cells exerted radioprotective effect. Additionally the ratios of each composition of GTPP were nearly same between two studies.

With these results, further work should be done to determine whether the effective concentration shown in this study can effectively protect the radiation induced damage in animal or human studies and to know the exact cause of radioprotection in normal cell line and growth inhibition in tumor cell line at the same time.

In conclusion, when the cancerous and normal cells were irradiated in vitro, generally additional GTPP before irradiation had two advantages of follows: 1) a radioprotective effect on normal cells but not on cancerous cells 2) a pronounced growth inhibitory effect on cancerous cells but not on normal cells.

국문 초록

목적: 녹차 추출물(GTPP)은 암 예방과 암세포 성장 억제 효과 외에 항산화제의 효능이 있는 것으로 알려져 있다. 이번 연구에서는 암세포에 감마 방사선을 이용하여 치료하는 경우 GTPP를 첨가함으로써 암세포 억제 증폭 효과와 정상세포에서의 방사선방호 효과가 함께 나타나는지 여부를 확인하고자 하였다. **대상 및 방법**: GTPP (EGCG > 45%, catechin 80% 포함)를 사람 백혈병 세포주인 HL60과 사람 림프구 모세포인 NC37에 방사선을 쪼이기 전에 미리 첨가한 후 실험을 하였다. 두 세포주에서 각각의 GTPP 농도와 방사선양에 따라서 생존능을 평가하여 GTPP 농도와 방사능 양을 결정하였으며, 이를 이용하여 GTPP농도에 따른 NC37에서 방사선방호 효과와 HL60에서의 암세포 억제 효과에 대한 실험을 시행하였다. **결과**: NC37과 HL60 세포주에서 암세포 억제효과를 보이면서 정상세포에 큰 영향을 미치지 않는 방사선 조사량은 1 Gy와 3 Gy정도이고, GTPP의 농도는 10 *μg/ml*와 20 *μg/ml*였다. NC37 세포주에서 GTPP를 농도별로 첨가하고 1 Gy와 3 Gy의 방사선을 각각 조사하였을 때 10 *μg/ml*의 경우에는 3 Gy를 조사한 경우에만 대조군에 비해 유의한 차이를 보였으며(1 Gy;*P*=0.126, 3 Gy;*P*=0.010), 20 *μg/ml*를 첨가한 경우는 1 Gy와 3 Gy를 조사한 군 모두 대조군과 비교하여 유의한 차이를 보이지 않았다(1 Gy;*P*=0.946, 3 Gy;*P*=0.096). HL60 세포주에서는 방사선 조사량에 큰 상관없이 GTPP의 농도의

증가에 따라 암세포 성장이 크게 억제됨을 알 수 있었다(1 Gy: 10 μg/ml; 69.0±1.7% vs 20 μg/ml; 42.4 ±1.3%, 3 Gy: 10 μg/ml; 66.9±3.9% vs 20 μg/ml; 44.2 ±1.6%). **결론**: 시험관 내 실험을 통하여 내부 방사선 치료를 시행하는 경우 GTPP를 첨가함으로써 정상세포에서 방사선방호 효과와 암세포에서 성장 억제 효과를 동시에 나타낼 수 있음을 확인할 수 있었다. 이러한 결과를 바탕으로 추후 생체 내 실험을 통한 녹차 추출물의 정상 세포에 대한 방사선방호 작용을 확인할 필요가 있을 것으로 사료된다.

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References

1) Otsuka T, Ogo T, Eto T, Asano Y, Suganuma M, Niho Y. Growth inhibition of leukemic cells by (-)-epigallocatechin gallate, the main constituent of green tea. *Life Sci* 1998;63:1397-403.
2) Yang GY, Liao J, Kim K, Yurkow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* 1998;19:611-6.
3) Chen ZP, Schell JB, Ho CT, Chen KY. Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett* 1998;129:173-9.
4) Suganuma M, Okabe S, Kai Y, Sueoka N, Sueoka E, Fujiki H. Synergistic effects of (-)-epigallocatechin gallate with (-)-epicatechin, sulindac, or tamoxifen on cancer-preventive activity in the human lung cancer cell line PC-9. *Cancer Res* 1999;59:44-7.
5) Bohuslavizki KH, Brenner W, Klutmann S, Hubner RH, Lassmann S, Feyerabend B, et al. Radioprotection of salivary glands by amifostine in high-dose radioiodine therapy. J Nucl Med 1998;39:1237-42.
6) Steele VE, Kelloff GJ, Balentine D, Boone CW, Mehta R, Bagheri D, et al. Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by in vitro bioassays. *Carcinogenesis* 2000 ;21:63-7.
7) Parshad R, Sanford KK, Price FM, Steele VE, Tarone RE, Kelloff GJ, et al. Protective action of plant polyphenols on radiation-induced chromatid breaks in cultured human cells. *Anticancer Res* 1998;18:3263-6.
8) Castillo J, Benavente-Garcia O, Lorente J, Alcaraz M, Redondo A, Ortuno A, et al. Antioxidant activity and radioprotective effects against chromosomal damage induced in vivo by X-rays of flavan-3-ols (Procyanidins) from grape seeds (Vitis vinifera): comparative study versus other phenolic and organic compounds. *J Agric Food Chem* 2000;48:1738-45.
9) Benua RS, Cicale NR, Sonenberg M, Rawson RW. The relation of radioiodine dosimetry to results and complications in the treatment of metastatic thyroid cancer. *AJR.* 1962;87:171-82.
10) Kim JC, Yoon JH, Bom HS, Jaegal YJ, Song HC, Min JJ, et al. Development and assessment individual maximum permissible dose method of I-131 therapy in high risk patients with differentiated papillary thyroid cancer. Kor J Nucl Med 2003;37:110-9.
11) Maxon HR, Thomas SR, Hertzberg VS, Kereiakes JG, Chen IW, Sperling MI, et al. Relation between effective radiation dose and outcome of radioiodine therapy for thyroid cancer. N Engl J Med 1983;309:937-41.
12) Karam M, Gianoukakis A, Feustel PJ, Cheema A, Postal ES, Cooper JA. Influence of diagnostic and therapeutic doses on thyroid remnant ablation rates. Nucl Med Commun 2003;24:489-95.
13) Uchida S, Ozaki M, Suzuki K, Shikita M. Radioprotective effects of (-)-epigallocatechin 3-O-gallate (green-tea tannin) in mice. *Life Sci* 1992;50:147-52.
14) Kim S, Lee MJ, Hong J, Li C, Smith TJ, Yang GY, et al. Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. *Nutr Cancer* 2000;37:41-8.