http://dx.doi.org/10.22537/jksct.14.2.78
pISSN: 1738-1320 / eISSN: 2508-6332

Journal of The Korean Society of Clinical Toxicology

원 저

글라이포세이트 노출로 인한 DNA손상에 대한 녹차의 예방적 효과

계명대학교 의과대학 동산의료원 응급의학교실[1], 계명대학교 의과대학 해부학교실[2]

박정민[1] · 최우익[1] · 진상찬[1] · 이재호[2] · 최인장[2]

In vitro Effects of Epigallocatechin Gallate on Sister Chromatid Exchange in the Lymphocytes Exposed to Glyphosate

Jung-Min Park, M.D.[1], Woo-Ik Choi, M.D.[1], Sang-Chan Jin, M.D.[1], Jae-Ho Lee, M.D.[2], In-Jang Choi, M.D.[2]

Department of Emergency Medicine, School of Medicine, Keimyung University, Dongsan Medical Center, Daegu[1], Department of Anatomy, College of Medicine, Keimyung University, Daegu[2], Korea

Purpose: Green tea is known as a potent anti-oxidant, anti-carcinogen, and genetic protector. Glyphosate (N-phosphonomethyl glycine) is a widely used non-selective herbicide that causes DNA damage. The present study was conducted to investigate the protective effects of green tea in human blood lymphocytes exposed to glyphosate using the Sister Chromatid Exchange (SCE) frequency method.

Methods: Peripheral blood was obtained from 10 volunteers and cultured through four different conditions. Four groups were divided into control, glyphosate only (300 ng/mL), glyphosate and low (20 μm) concentrations of epigallocatechin gallate (EGCG) and glyphosate and high (100 μm) concentrations of EGCG.

Results: The glyphosate exposed groups had a higher mean SCE frequency (10.33±2.50) than the control group (6.38±2.28, p<0.001). The low concentrations of EGCG groups had a lower mean SCE frequency (9.91±1.93) than the glyphosate-only group, although this difference was not significant (p=0.219). However, the high concentration group (9.49±1.85) had a significantly lower SCE frequency than the glyphosate-only group (p=0.001).

Conclusion: EGCG has a gene protective effect in human lymphocytes exposed to the genotoxicity of glyphosate in the case of high concentrations.

Key Words: EGCG, Glyphosate, Sister chromatid exchange

Introduction

Green tea (GT), one of the most widely consumed beverage in the world, has been consumed by Eastern Asian people as a medicinal beverage to promote health and stabilize body and soul. The commonly known effects of GT are anti-diabetic activity[1,2], the lowering of plasma cholesterol and triglyceride levels[3] and anti-oxidant activity[4-6]. The 4 major catechins in GT are epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epicatechin (EC) and epigallocatechin (EGC)[7]. In addition to commonly known effects of GT above, EGCG have anti-inflammatory and antiviral activities[8] and prevent cardiovascular diseases[9], neurological problems[10,11] and

책임저자: 진 상 찬
대구광역시 중구 달성로 56
계명대학교 동산의료원 응급의학과
Tel: 053) 250-7610, Fax: 053) 250-7028
E-mail: jchan98@hanmail.net

투고일: 2016년 8월 22일 1차 심사일: 2016년 9월 2일
게재 승인일: 2016년 9월 19일

cancer as a potent genetic protector[12,13].

Glyphosate, chemically N-(phosphonomethyl) glycine, is widely used non-selective herbicide for both agricultural and non-agricultural purpose. Since then, constantly, glyphosate intoxicated patients frequently visited emergency room and their severity was dependent on intake concentration. Large amount of ingestion may cause gastrointestinal tract injury, such as erosion or ulcer or haemorrhage, and severe systemic effects. Severe systemic symptoms may occur from cardiotoxicity, hepatotoxicity, renal toxicity, non-cardiogenic pulmonary edema, mental change, metabolic acidosis and even to cardiac arrest and death[14]. Small amount of oral intake may be asymtomatic or cause nausea, vomiting, and diarrhea, however, we cannot guarantee its safety. Therefore, many previous studies about the safety of glyphosate formulation have been performed. These studies on this herbicide suggested its minimal genotoxic activity[15,16] and a review on glyphosate also concluded that there is no strong evidence to pose a health risk to humans tissues[17]. However, latest studies showed a harmful effect of glyphosate variously as a potential endocrine disruptor and inducing reproductive disability on placental cells[18,19]. And, occupational exposure to glyphosate is a risk factor of cancers by comet assay or Sister Chromatid Exchange (SCE) test[20-22]. It is difficult to know the detail mechanisms of both 'genotoxic effect of glyphosate' and 'genetic protective effect of EGCG'. This study was done to clarify protective effect of EGCG in human blood lymphocyte exposed to genotoxicity of glyphosate by SCE frequency method.

Methods

1. Preparation of the *in vitro* experiments

Four milliliters of Peripheral blood of 10 healthy volunteers aged from 21 to 26 years was collected because aging can affects SCE frequency. To control the factors that can change SCE frequency, regular drug users, smokers and alcoholics were excluded. In addition anybody who had cancer, chronic infection, history of chemotherapy, history of radiotherapy or radiation exposure history was also excluded. The regional institutional review board (IRB) approved the research proposal, and informed consent was obtained from all the individuals involved in the study.

Roundup UltraMax® (Monsanto, Roseville, CA, USA) was used as representative product of glyphosate herbicide. It contains 570 gram of active ingredient glyphosate in 1 liter and 2% ammonium sulphate as a surfactant. And EGCG made by Sigma (Saint Louis, MO, USA) was used.

All of blood samples were experimented together through four groups that divided by concentrations of glyphosate and EGCG. Group 1 is control group that contains no glyphosate and no EGCG. Group 2, 3 and 4 are experimental groups. Group 2 contains only 300 ng/mL of glyphosate and no EGCG and Group 3 contains 300 ng/mL of glyphosate and 20 μM of EGCG and Group 4 contains 300 ng/mL of glyphosate and 100 μM of EGCG.

2. Sister chromatid exchange (SCE) assay

Each sample of blood (1.0 ml) was mixed with 9 ml of culture medium that consists of RPMI-1640 (Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, Uxbridge, UK): 0.1 mL (1 g/mL) of Phytohemagglutinin (PHA, Gibco, Uxbridge, UK) was supplemented as a mitogen and then this was incubated at 37°C for 72 hours. At 24 hours of culture, 0.1 ml (1 g/ml) of 5-bromo-2-deoxyuridine (BrdU) was added each culture. Different concentrations of glyphosate and EGCG according to groups were added after 48 hours of incubation. At 70 hours of incubation, 0.1 ml (10 μg/mL) of colcemid (Gibco, Uxbridge, UK) was added to arrest mitosis at metaphase. All the chromosome preparations were stained using the BrdU-Hoechst-Giemsa technique. The SCE of the lymphocytes was microscopically examined and counted using the Cytovision Computer-Assisted Karyotyping System (Applied Imaging, Santa Clara, CA, USA). For each group in one subject, 20 of well-spread chromosome pairs in second division metaphase were included in results

by the same person. The results were used to determine the mean number of SCEs (SCEs/cell).

3. Statistical analysis

In this experiment, the statistical comparisons of the mean number of SCEs from each group were performed using the one-way ANOVA method. The TUKEY post-testing was utilized for multiple comparisons. All the statistical analyses were performed using SPSS software (version 18.0). A p value <0.05 was considered significant.

Results

The frequency of SCE was examined in the 10 healthy-male volunteers. The mean and standard deviation (SD) of each group were calculated and summarized in Table 1. Exposure group to glyphosate (Groups 2, 3 and 4) has a higher mean SCE frequency than Group 1, significantly. Compared to mean SCE frequency of Group 1 (6.38±2.28), Group 2 had extremely higher SCE (10.33±2.50, $p<0.001$). And Group 3 revealed decrease in mean SCE frequency (9.91±1.93) compared with that in group 2, however, this difference have no statistical value (p=0.219). The mean SCE frequency was lower in Group 4 (9.49±1.85) than group 2 with statistical significance (p=0.001). However, there was no significant difference between Group 3 and 4 (p=0.191), indicating no dose-dependent effect of EGCG in genotoxicity of glyphosate.

Average SCE frequency of each group in individual subject of this study was presented in Table 2. In all subjects, glyphosate increased SCE frequency significantly compared to control, respectively. EGCG treatment (Groups 3 and 4) reduced SCE frequency in the lymphocyte exposed to glyphosate, however statistical value was shown in only one subject (No. 5). Moreover, EGCG increased SCE frequency in two subjects (No. 7 and 8) though they did not have significance.

Discussion

The aim of this study was to know protective effect of EGCG in human blood lymphocyte exposed to genotoxicity of glyphosate by SCE method. GT is mainly comprised of EGCG, ECG, EGC and EC, therefore, their anti-genotoxic effects against glyphosate were analyzed in 3 subjects preliminarily. As a result, EGCG showed a lowest SCE frequency in the cytotoxicity by glyphosate compared to ECG, EGC and EC, though it did not have statistical significance. Of the catechins, polyphenolic components of GT, EGCG is the major constituent and also most active component with the highest antioxidant properties[23]. Therefore, our further main experiment was

Table 1. Mean SCE frequency of individual subject

Subject	Groups			
	Group 1 Control	Group 2 GLY (+)	Group 3 GLY (+) & EGCG (+)	Group 4 GLY (+) & EGCG (++)
1	6.60	10.20*	9.95	9.40
2	6.00	10.50*	9.95	9.65
3	6.30	10.35*	10.10	9.70
4	5.80	10.35*	10.05	9.50
5	6.75	10.70*	9.60	8.75†
6	6.55	10.50*	10.10	9.50
7	6.30	9.95*	10.05	9.55
8	6.95	9.35*	9.50	9.15
9	6.85	10.70*	9.65	9.75
10	5.65	10.70*	10.20	9.90

GLY: glyphosate, SCE: sister chromatid exchange, EGCG: epigallocatechin gallate

* $p<0.001$ compared to Group 1

† $p<0.05$ compared to Group 2

Table 2. Effect of EGCG on genotoxic lymphocyte by glyphosate

Groups	Mean SCE frequency (Mean±SD)
Group 1 Control	6.38±2.28
Group 2 GLY (+)	10.33±2.50
Group 3 GLY (+) & EGCG (+)	9.91±1.93
Group 4 GLY (+) & EGCG (++)	9.49±1.85

GLY: glyphosate, SCE: sister chromatid exchange, EGCG: epigallocatechin gallate
p<0.001 between Group 1 and Group 2
p=0.219 between Group 2 and Group 3
p=0.001 between Group 2 and Group 4
p=0.191 between Group 3 and Group 4

performed by EGCG and its anti-genotoxic effect against glyphosate was evaluated.

SCE is the exchange of genetic material between two identical sister chromatids. SCE method in the lymphocyte is well-known experiment to examine genotoxicity of agent or environment[19]. After chromosomal double-strand breaks (DSBs), inter-strand cross-linking damage, and collapsed replication forks by DNA damage is occurred, the important pathway of genomic repair should be followed, named homologous recombination (HR). When demand of HR increased, available sequence from the sister chromatid is used and dysregulation of HR may occur. Therefore, SCE is originated from the result of above mechanism, so SCE frequency may correlated with the degree of DNA damage[20,21]. Until now, it is widely accepted that SCE is closely related to genotoxicity.

Our result showed that glyphosate causes DNA damage and genotoxicity that related to mutagenicity and carcinogenicity, as previously described[18-22]. Also, this study showed EGCG has a protective effect against genotoxicity of glyphosate in case of only high concentration (100 μM) not low concentration (20 μM). Interestingly, when calculating and analyzing this effect individually, EGCG decreased SCE frequency in only one subject exposed to glyphosate, moreover, EGCG treatment increased SCE frequency in two subjects. This data indicated that EGCG seems to be effective to genotoxic status at a different rate according to the bioavailability of each individual.

Previous studies demonstrated that GT is generally free of side effects. However, large amounts of green tea consumption may lead to insomnia, anxiety, and other symptoms caused by the caffeine content[24,25]. EGCG suppresses hepatic gluconeogenesis and insulin action[26-28]. Clinical studies in humans are lacking due to evident ethical considerations, some studies showed that EGCG may be hepatotoxic and nephrotoxic[29,30].

Considering general concentration of GT by oral intake, the effective concentration (100 μM) in present study was relatively high. It is not clear whether the result of this study can be applied to glyphosate acute exposure patient. Accordingly, the experiments in the lymphocyte of its acute exposure patient and in vivo studies might figure out its cytotoxicity. Therefore, further study with clinically available dose should be performed. And the effect of other catechins in GT also should be studied in various subject based on various kinds of personal consequences and side effects of EGCG.

Conclusion

It was suggested that EGCG may be a potential supplement for the genotoxicity of glyphosate. The numerous health benefits of EGCG as a prophylactic, but also as a therapeutic, agent acting through different pathways are well identified though there were conflicting results about its effect. Therefore, EGCG is still most attractive naturally available products. For its safe and effective use for anti-genotoxicity, concerning personal bioavailability and potential side effect of EGCG remain to be addressed.

REFERENCES

1. Iso H, Date C, Wakai K, Fukui M, Tamakoshi A, Group JS. The relationship between green tea and total caffeine intake and risk for self-reported type 2 diabetes among Japanese adults. Ann Intern Med 2006;144:554-62.
2. Wu LY, Juan CC, Ho LT, Hsu YP, Hwang LS. Effect of green tea supplementation on insulin sensitivity in Sprague-Dawley rats. J Agric Food Chem 2004;52:643-8.
3. Raederstorff DG, Schlachter MF, Elste V, Weber P. Effect of EGCG on lipid absorption and plasma lipid levels in rats. J Nutr Biochem 2003;14:326-32.
4. Frei B, Higdon JV. Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. J Nutr 2003;133:

3275-84.
5. Katiyar SK, Afaq F, Perez A, Mukhtar H. Green tea polyphenol (-)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. Carcinogenesis 2001;22:287-94.
6. Babu PV, Sabitha KE, Shyamaladevi CS. Therapeutic effect of green tea extract on oxidative stress in aorta and heart of streptozotocin diabetic rats. Chem Biol Interact 2006;162:114-20.
7. Sano M, Tabata M, Suzuki M, Degawa M, Miyase T, Maeda-Yamamoto M. Simultaneous determination of twelve tea catechins by high-performance liquid chromatography with electrochemical detection. Analyst 2001;126:816-20.
8. Cabrera C, Artacho R, Gimenez R. Beneficial effects of green tea--a review. J Am Coll Nutr 2006;25:79-99.
9. Jochmann N, Baumann G, Stangl V. Green tea and cardiovascular disease: from molecular targets towards human health. Curr Opin Clin Nutr Metab Care 2008;11:758-65.
10. Unno K, Takabayashi F, Yoshida H, Choba D, Fukutomi R, Kikunaga N, et al. Daily consumption of green tea catechin delays memory regression in aged mice. Biogerontology 2007;8:89-95.
11. Weinreb O, Mandel S, Amit T, Youdim MB. Neurological mechanisms of green tea polyphenols in Alzheimer's and Parkinson's diseases. J Nutr Biochem 2004;15:506-16.
12. Bushman JL. Green tea and cancer in humans: a review of the literature. Nutr Cancer 1998;31:151-9.
13. Cooper R, Morre DJ, Morre DM. Medicinal benefits of green tea: part II. review of anticancer properties. J Altern Complement Med 2005;11:639-52.
14. Talbot AR, Shiaw MH, Huang JS, Yang SF, Goo TS, Wang SH, et al. Acute poisoning with a glyphosate-surfactant herbicide ('Roundup'): a review of 93 cases. Hum Exp Toxicol 1991;10:1-8.
15. Vigfusson NV, Vyse ER. The effect of the pesticides, Dexon, Captan and Roundup, on sister-chromatid exchanges in human lymphocytes in vitro. Mutat Res 1980;79:53-7.
16. Li AP, Long TJ. An evaluation of the genotoxic potential of glyphosate. Fundam Appl Toxicol 1988;10:537-46.
17. Williams GM, Kroes R, Munro IC. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul Toxicol Pharmacol 2000;31:117-65.
18. Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Seralini GE. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 2009;262:184-91.
19. Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect 2005;113:716-20.
20. De Roos AJ, Blair A, Rusiecki JA, Hoppin JA, Svec M, Dosemeci M, et al. Cancer incidence among glyphosate-exposed pesticide applicators in the Agricultural Health Study. Environ Health Perspect 2005;113:49-54.
21. Paz-y-Miño C, Sánchez ME, Arévalo M, Muñoz MJ, Witte T, De-la-Carrera GO, et al. Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. Genetics and Molecular Biology 2007;30:456-60.
22. Lee SH, Kim SJ, Choi WI, Jin SC, Choi IJ, Lee JH. Genotoxicity of low-dose Glyphosate by Sister Chromatid Exchange. Journal of the Korean Society of Clinical Toxicology 2014;12:8-13.
23. Guo Q, Zhao B, Li M, Shen S, Xin W. Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. Biochim Biophys Acta 1996;1304:210-22.
24. Adachi N, Tomonaga S, Tachibana T, Denbow DM, Furuse M. (-)-Epigallocatechin gallate attenuates acute stress responses through GABAergic system in the brain. European journal of pharmacology 2006;531:171-5.
25. Vignes M, Maurice T, Lante F, Nedjar M, Thethi K, Guiramand J, et al. Anxiolytic properties of green tea polyphenol (-)-epigallocatechin gallate (EGCG). Brain Res 2006;1110:102-15.
26. Collins QF, Liu H-Y, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5′-AMP-activated protein kinase. Journal of Biological Chemistry 2007;282:30143-9.
27. Anton S, Melville L, Rena G. Epigallocatechin gallate (EGCG) mimics insulin action on the transcription factor FOXO1a and elicits cellular responses in the presence and absence of insulin. Cellular signalling 2007;19:378-83.
28. Li C, Allen A, Kwagh J, Doliba NM, Qin W, Najafi H, et al. Green tea polyphenols modulate insulin secretion by inhibiting glutamate dehydrogenase. Journal of biological chemistry 2006;281:10214-21.
29. Galati G, Lin A, Sultan AM, O'Brien PJ. Cellular and in vivo hepatotoxicity caused by green tea phenolic acids and catechins. Free Radical Biology and Medicine 2006; 40:570-80.
30. Lambert JD, Kennett MJ, Sang S, Reuhl KR, Ju J, Yang CS. Hepatotoxicity of high oral dose (-)-epigallocatechin-3-gallate in mice. Food and chemical toxicology 2010;48:409-16.