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Effects of environmental factors on the pro-oxidant properties of epigallocatechin gallate

(エピガロカテキンガレートの酸化促進特性に対する環境要因の影響）

ABSTRACT

Despite being a crucial molecule that sustains life, oxygen is toxic and can induce harmful effects ranging from cell and DNA damages, to mutation and carcinogenesis. Anti-oxidants, compounds that are innately present in the body system or obtained in external sources, have played crucial roles in keeping the balance between the benefits and hazards of oxygen. However, when innate anti-oxidants in the system are unable to cope with the levels of oxidation, a state called ‘oxidative stress’ occurs. This state is defined as when the oxidation in the system exceeds the ameliorative properties of anti-oxidants. Therefore, there is a need to obtain anti-oxidants from external sources like food. Polyphenols, a large group of compounds found mainly in plants and plant-derived products, have gained the interest of both researchers and the general public due to its reported health benefits, with their anti-oxidant property being one of the most explored. Currently, there are thousands of polyphenols discovered and identified by scientists, one of them being epigallocatechin gallate (EGCG) which is the predominant form of polyphenol found in green tea. EGCG was reported to have several benefits to human health such as anti-cancer, anti-diabetic, lipid lowering capability, and anti-oxidant property. However, considerable amounts of studies have also shown that EGCG functions as a pro-oxidant, driving the need to investigate such situations that trigger this effect. Tea is one of the most consumed beverage in the world, second only to water. Taking this into consideration, there is a significant amount of the population that can be exposed to the effects of tea and EGCG. In addition, the varying reports on the health effects of EGCG drives the

need to further clarify the specifics of how EGCG behaves in the body system. This study aims to explore the pro-oxidant effects of EGCG through the involvement of environmental factors, mainly heat, toxicants, anti-oxidants, and cancer cells.

Chapter 1 is on the introduction of the topics and the objectives of the study. In this chapter, the harmful effects of oxygen was introduced, followed by concepts related to it such as oxidation, oxidative stress, and anti-oxidants. Then, the compound of interest EGCG was introduced, as well as its benefits on several animal and human cell experiments. The contradicting findings of EGCG's harmful effects was also stated. Given the abundance of established studies that show that EGCG has varying effects, the overall objectives of the study was stated.

Chapter 2 focuses on the interaction of EGCG with L-ascorbic acid (commonly referred to as vitamin C) under the presence of cadmium (Cd), a known environmental toxicant. Cd is a heavy metal with no known health benefits to man, and can also bring about several harmful health effects including neurological impairments. The sources of Cd are often anthropological, and is introduced into the food system mainly through the contamination of agricultural lands. Several studies have shown that Cd is one of the most abundant heavy metal pollutant found in plants. This brings the issue of Cd contamination of food as a concern for food safety and human health. Given that vitamin C and EGCG are two known anti-oxidants found in plant and plant-based food products, it was hypothesized that the combined effect of the two compounds can be effective against the toxic effects of Cd. Pre-treatment of EGCG and vitamin C followed by Cd treatment was done on PC12 cells, which aims to replicate a scenario wherein an organism has a polyphenol-rich diet followed by heavy metal exposure. The results found that EGCG, vitamin C, and its combination does not pose cytotoxicity on PC12 cells. However, when Cd is present, the behavior of the chemicals, especially EGCG, changed significantly. Vitamin C did show ameliorative properties against Cd but unexpectedly, EGCG showed the

contrary when it came to combination treatments with Cd; EGCG-Cd groups having low cell viability and high LDH assay trends. This behavior of EGCG can be explained by its chemical structure; the trihydroxy groups in EGCG are able to exhibit its anti-oxidant properties through electron delocalization which could quench free radicals or ROS. However, certain conditions would also render these trihydroxy groups as pro-oxidants. pH, temperature, concentration of EGCG, oxygen level, and presence of other species such as anti-oxidants and metal ions affect the stability of EGCG which further results to its auto-oxidation and production of reactive oxygen species (ROS). How Cd brings about the cytotoxicity of EGCG, as well as the possibility of involvement of EGCG-induced ROS, warrants further investigation.

Vitamin C is an important factor in various cellular processes and is also a well-known anti-oxidant. Oxidative stress assessment via measurement of GSH level showed vitamin C had a significantly higher GSH level than Cd-only group, which meant pre-treatment of vitamin C lessened the oxidation caused by Cd. Studies have shown that during oxidative stress, moderate levels of ROS in cells can induce autophagy. The higher viability and GSH levels of cells exposed to vitamin C, along with the upregulation of autophagic proteins p62 and pBeclin1, implies that the moderation of oxidative stress and induction of autophagy was able to protect the cells from Cd-induced death.

Combination treatments of vitamin C – EGCG – Cd had upregulated autophagy-linked proteins but low cell viability, indicating that induction of autophagy has ultimately led to cell death. In addition, the lower cell viability of this group as compared to vitamin C-Cd indicated that addition of EGCG had decreased the effectivity of vitamin C in lessening Cd damage. This antagonism of EGCG and vitamin C under the presence of Cd can be associated with EGCG's reactivity due to its chemical structure. Further studies need to be done to investigate the interaction of these anti-oxidants, as well as the progression of autophagy towards cell death. The findings from Chapter 2 show that the combination of two anti-oxidants does not

necessarily mean an enhanced effect against a toxicant, rather it can lead to an antagonistic effect. From such findings, the researcher decided to further explore the behavior of EGCG in order to fully understand its effect in the context of oxidation and cellular stress and toxicity.

Chapter 3 addresses previous findings that polyphenols like EGCG oxidizes under the presence of metal ions (such as Cd), which explained EGCG's toxicity when combined with Cd as seen in Chapter 2. As mentioned earlier, there are other factors which can induce the oxidation of EGCG, one of them being heat. Studies have shown that high temperature can induce oxidation of EGCG leading to its degradation and production of ROS. By inducing EGCG oxidation through heat, prior to Cd co-exposure, it was hypothesized that EGCG-Cd cytotoxicity would decrease since the metal-polyphenol combination treatment will not be able to generate enough ROS to induce oxidative stress. EGCG solutions were heated at 50 ºC up until 72 h. Upon heating of EGCG solutions, a color change was observed from colorless to a brown colored solution. This color change is known to be a good indicator of the occurrence of the polyphenol's oxidation. In order to limit sample size, unheated EGCG and 72 h-heated EGCG (the longest heating duration) was used for the experimentations involving Cd combination treatments. For Chapter 3, PC12 cell line was also used. After 48 h incubation of PC12 cells to EGCG and Cd, results have proven the contrary to the hypothesis. Cell viability of 72 h-heated EGCG with Cd was comparable to that of unheated EGCG with Cd. Membrane damage as seen from LDH activity of the two treatment groups was also found to be similar. ROS generation and the cells' anti-oxidant system gave different results on Cd with unheated EGCG or 72 h-heated EGCG. Wherein, 72 h-heated EGCG with Cd induced high glutathione reductase (GR) expression. The upregulation of GR in 72 h heated EGCG-Cd group may indicate that there is high oxidative stress that is triggering the enzyme to be more expressed and reduce more GSSG to glutathione. However, ROS generation in the cells treated with the Cd-combination did not show any significant difference.

Western blot was performed in order to determine the expression of relevant proteins such as 67-kDa laminin receptor, which has been found to be activated under the presence of EGCG. As expected, there was an increase in 67LR expression in EGCG-only treatment, with 72 h-heated EGCG having lower expression than unheated EGCG. On the other hand, despite having the highest expression of 67LR, unheated EGCG when combined with Cd had a low 67LR expression. In fact, all Cd treated groups had the same 67LR expression regardless if there is a presence or absence of EGCG. This indicates that the expression of 67LR is highly dependent on the native form of EGCG. Changes in EGCG structure due to heat, whether it led to EGCG degradation or dimer formation, did not upregulate the expression of 67LR. The lack of 67LR expression by EGCG under the presence of Cd may either indicate that there is an interaction between EGCG and Cd so that 67LR expression is not induced; or that 67LR expression requires that EGCG is in the native form and that there is an absence of other species. Downregulation of Akt, pAkt, mTOR, and NF**κ**B at Cd-treated groups indicate the inhibition of cell proliferation, as well as the survival mechanisms by the cell. ERK1/2, a pro-apoptotic protein, was found to be downregulated in all Cd-treated groups indicating that ERK1/2-led apoptosis did not occur in the cells.

Finally, pro-apoptotic proteins Bax, caspase 3, and caspase inhibitor XIAP were checked for their expressions. The Bax protein regulates the release of cytochrome c in the mitochondria which activates the caspase cascade pathway, which caspase 3 is part of. The Bax expression in Cd-treated cells were upregulated with the highest at 72 h-heated EGCG with Cd. However, caspase 3 expression was downregulated. This suggests that the apoptotic cell pathway of induced by Cd (and EGCG-Cd) is one that is Bax-dependent but caspase-independent,

It was hypothesized that inducing the oxidation of EGCG prior to Cd co-exposure can lead to a lesser degree of cytotoxicity compared to unheated EGCG-Cd. However, toxicity is

retained and EGCG-Cd groups even had a lower viability than Cd-only group. Comparing unheated and 72 h-heated EGCG, both exerted similar cytotoxic effects but activate different mechanisms as seen in the regulation of protein expressions. The findings of this Chapter 3 indicate that the effects of EGCG is not straightforward and can vary depending on whether EGCG is heated or unheated. Wherein, chemistry plays a crucial role towards induction of protein expression, and generation of ROS under the presence of Cd. The study is the first reported case of how polyphenol oxidation through heat can affect the cell death pathway of cells exposed to toxicants.

According to the global data of the World Health Organization from 2020, breast cancer has been found to be the world's most prevalent cancer. With over millions of women diagnosed and hundreds of thousands leading to deaths, breast cancer is a primary subject of many research in order to find the most effective therapy in curing the disease. Polyphenols have been a part of the research concerning breast cancer, however, mechanisms have mainly focused on cellular pathways rather than the involvement of polyphenol chemistry. Therefore, Chapter 4 focused on exploring the anti-cancer potential of EGCG on MCF-7 breast cancer cells. As it was proven by several journals that heat can degrade polyphenols, the anti-cancer activity of EGCG on MCF-7 cells was explored through treatment of cells by heated or unheated EGCG. If heat degradation can alter polyphenol structure, then it is hypothesized that the cellular effect of EGCG may also be altered. Following the methods of Chapter 3, EGCG solutions were also heated at 50 ºC up to 72 h. Compared to the previous chapters, however, no other chemicals apart from EGCG was used to treat the cells.

LC/MS analysis of EGCG solutions showed a decrease in EGCG peak intensity, showing that heating did cause changes in the polyphenol. In addition, several peaks that correspond to dimers were also observed, supporting previous studies that indicated that oxidation of EGCG leads to dimer formation. The peaks were found to have calculated masses

that correspond to literature values of two reported EGCG dimers, mainly theasinensin and P2. Theasinensin dimer had more pronounced peaks, which may imply that this dimer is the more preferred structure when it comes to heat-induced oxidation of EGCG. The role of heat on the production and predominance of dimers in EGCG solutions warrant further investigation.

Concerning the cellular aspect of Chapter 4, MCF-7 cells exhibited a decrease in viability, cell number, and membrane integrity after being treated with EGCG; with heated EGCG solutions having more potency to kill cells. Assessment of the morphology of the cells showed that EGCG-treated MCF-7 cells had a more shrunken appearance as compared to control. ROS generation of EGCG was confirmed through quantification of ROS, measurement of anti-oxidants, and assessment of lipid peroxidation products. After the measurement of ROS through DHE staining, it was confirmed that ROS was indeed generated by EGCG in MCF-7 cells, with all treatment groups having significantly higher values than control. However, after 48 h heating of EGCG, ROS generation significantly decreased. As for catalase and GSH, it was found that 48 h and 72 h-heated EGCG had lesser abilities to decrease the level of these anti-oxidants. For catalase activity, the obtained values had small differences with the control group, which can indicate that catalase activity is not greatly affected by the presence of EGCG. This may mean that the toxicity of EGCG on cancer cells is not just through the generation of hydrogen peroxide. Finally, for lipid peroxidation product malondialdehyde, there was no significant difference between the EGCG treated groups, indicating that unheated and heated EGCG had similar effects in inducing lipid peroxidation.

To summarize the results of Chapter 4, it was observed that heating of EGCG induced dimerization. In addition, EGCG was proven to be cytotoxic to MCF-7 cells through the exertion of ROS. However, heating of EGCG alters this cytotoxicity; wherein a longer heating time was found to generate lesser ROS and has decreased ability to deplete cellular anti-oxidants. From the current results, the cytotoxicity of EGCG can be ranked as follows:

unheated EGCG ≥ 24 h-heated > 48 h-heated > 72 h-heated. This trend can possibly be explained by the dimerization process of EGCG. As EGCG is heated, it is oxidized to produce dimers and ROS. As heating time progresses, more and more EGCG is dimerized until there is no longer any free EGCG to form dimers and produce ROS. Therefore, longer heating periods of EGCG have high amounts of dimers but low amount of ROS. Hence, EGCG may lose its cytotoxicity to cancer cells as it gets oxidized. The findings of this chapter is the first reported case of how heat oxidation can affect a compound's cytotoxicity to cancer cells.

To summarize the findings from the three chapters of this study, it was found that EGCG alone posed no toxicity towards PC12 cells up to 100 μM. Due to the issues of EGCG's low bioavailability, it is best to explore its activities and cellular effects at the physiological concentration of ~1 μM. When this low concentration of EGCG is present with Cd, there is an increase in Cd cytotoxicity and even an antagonistic effects on anti-oxidants like vitamin C. This effect of EGCG is only observed under the presence of Cd, and EGCG does not antagonize vitamin C when Cd is absent (Chapter 2). The behavior of EGCG was found to be due to its oxidation under the presence of a metal ion. In addition, this sensitivity to degradation and oxidation of EGCG (and polyphenols in general) was also observed in factors like high temperature. Therefore, oxidation of EGCG was induced by heating prior to Cd co-exposure to check if this oxidation affects the cytotoxicity exerted by EGCG-Cd combination. Changes in EGCG brought about by heat was confirmed through spectrophotometric methods and cellular response of the EGCG protein receptor 67LR. However, in terms of cytotoxicity, heating of EGCG prior to Cd co-exposure did not have any effect and cytotoxicity was still retained. Therefore, despite heat change, EGCG still retained its cytotoxic ability under the presence of Cd (Chapter 3). Cancer cell lines, like MCF-7, was found to be more sensitive towards EGCG cytotoxicity. In fact, the viability of MCF-7 cells decreased at 50 μM as compared to the control-level viability of PC12 cells up until 100 μM. This cancer cytotoxicity

of EGCG was attributed to its ROS production that effectively inhibited cancer cell proliferation, induced membrane damage, and led to cell death. Following the hypothesis that oxidation of EGCG prior to treatment would cause changes in its cytotoxicity, MCF-7 cells were also exposed to heated EGCG solutions. Cell death and damage, as well as ROS generation was still observed, indicating the retention of EGCG cytotoxicity despite heating.. Comparing the findings from the three chapters with established studies, it was found that EGCG activity relies on its concentration, accompanying chemical, cell line, and condition (e.g., heat degradation).

Cell death induced by toxicants like metals and other environmental pollutants have always been the interest of many studies. Wherein, the end goal is to lessen the toxicity by applying compounds that can help increase the survival of cells damaged by these toxicants. However, it has been proven by a vast majority of studies that toxicants exert their harmful effects through ROS, which can damage DNA and lead to mutations and cancer. If not repaired, these DNA-damaged cells may actually be more harmful, than beneficial, if they survive and replicate. Therefore, elimination of these toxicant-damaged cells is a better option than letting them survive. The effect of EGCG when combined with Cd as found in chapter 2 and 3 of this study, as well as EGCG with other metals found in several established papers may actually support the earlier premise. One of the harmful effects of Cd is through DNA damage, serving as a factor toward carcinogenesis. Treating EGCG with Cd leads to further cell decline, and it can be that EGCG functions as stimulator of apoptosis (programmed cell death), in order for these damaged cells to no longer survive and replicate.

EGCG has been regarded as a potential anti-cancer treatment, both as a preventive and a therapeutic measure, the former being more popular even for those who are unfamiliar of the science behind it. The ‘anti-oxidant’ property of green tea has been lauded to prevent ageing, lipid disfunction, and cancer. However, it should also be emphasized that the anti-cancer effect

of EGCG also lies on its pro-oxidant effect. Chapter 3 of this study shows how EGCG induced oxidative stress by increasing ROS levels in MCF-7 cells, leading to the decrease of proliferation and viability. Therefore, the pro-oxidant capability of EGCG to be anti-cancer works both as a prevention – through its ability to prevent DNA-damaged cells to be potential factors for carcinogenesis by effectively inducing their deaths by induction of oxidative stress; and through therapeutics – through induction of oxidative stress leading to death of cancer cells.

Finding the points when EGCG becomes a pro-oxidant and an anti-oxidant is still debatable. What can be said from the findings of the study is that EGCG becomes a pro-oxidant in the presence of cancer cells; and also when the oxidative stress induced by toxicants is already high, that cell death would be the preferred outcome as a preventive measure against damaged cell replication. The findings of this study was able to contribute towards the growing studies exploring the benefits of pro-oxidation, a term that has always been avoided and tagged as harmful; but can actually be as beneficial and protective as anti-oxidation.