Effect of Green Tea Extract on Reactive Oxygen Species Produced by Neutrophils from Cancer Patients

KATSUHIKO SUZUKI[1,2,3], SATOSHI OHNO[2,4], YOKO SUZUKI[2,3], YUMIKO OHNO[2], RYUJI OKUYAMA[4], ATSUSHI ARUGA[5], MASAKAZU YAMAMOTO[4], KEN-O ISHIHARA[6], TSUTOMU NOZAKI[6], SHIGEKI MIURA[7], HIROSHI YOSHIOKA[7] and YUICHI MORI[7]

[1]*Faculty of Sport Sciences, Waseda University, Saitama, Japan;*
[2]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan;*
[3]*Cooperative Major in Advanced Health Science, Graduate School Collaborative Education Curriculum, Tokyo University of Agriculture and Technology/Waseda University, Tokyo, Japan;*
[4]*Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan;*
[5]*Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan;*
[6]*BHN Co., Ltd, Tokyo, Japan;*
[7]*Mebiol Inc., Kanagawa, Japan*

Abstract. *Background/Aim: Oxidative stress in cancer patients has been demonstrated to be partly mediated by neutrophils. Although it is reported that natural antioxidants, such as green tea extract, reduce oxidative stress, there is limited evidence of their effects in cancer patients. This study aimed to determine the effect of green tea extract on reactive oxygen species produced by neutrophils from cancer patients. Materials and Methods: Peripheral blood samples were obtained from eighteen patients with advanced cancer. Green tea extract was added to the blood samples with luminol on Mebiol gel, and luminol-dependent chemiluminescence was measured to monitor the production of reactive oxygen species from migrated neutrophils into the gel, at 37°C. Results: Luminol-dependent chemiluminescence was significantly down-regulated in the presence of green tea extract in a concentration-dependent manner. Conclusion: These results indicate the antioxidant effect of green tea extract on reactive oxygen species produced by neutrophils, which may be effective in reducing oxidative stress in cancer patients.*

Chronic inflammation is associated with cancer development and may advance disease progression and negatively affect prognosis in cancer patients (1, 2). Inflammatory cells infiltrate into the tumor microenvironment and play an important role in the neoplastic process, fostering proliferation, survival and migration (2). The main inflammatory cells which infiltrate tumor tissues are neutrophils and macrophages. Neutrophils are a primary source of production of reactive oxygen species (ROS), and cellular oxidative stress has long been associated with carcinogenesis. Furthermore, the role of chronic inflammation and oxidative stress in the onset of cancer cachexia has been reported (3, 4), and we have demonstrated that oxidative stress, as evaluated using ROS production by neutrophils, is greater in patients with advanced cancer (5).

Recent research has focused on the effectiveness of anti-inflammatory drugs for the treatment of cancer-related cachexia. Various anti-inflammatory drugs and strategies against cancer-related cachexia have been reported *in vitro* and *in vivo* (3, 4). Furthermore, clinical trials have been performed with the cyclooxygenase (COX)-2 inhibitor celecoxib, infliximab, various antioxidants, megestrolacetate and dexamethasone (6-10). Among these studies, anti-inflammatory treatment of cancer patients reduced inflammation and oxidative stress parameters, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP) and ROS, and were also demonstrated to be useful for improving both cachexia and the quality of life of cancer patients.

Green tea and its major constituent epigallocatechin-3-gallate (EGCG) have been extensively studied as potential therapeutic agents for a variety of diseases, including cancer. Numerous investigations have indicated that green tea and EGCG possess antioxidant, anti-inflammatory, anti-mutagenic

Correspondence to: Katsuhiko Suzuki, Faculty of Sport Sciences, Waseda University, 2-579-15 Mikajima, Tokorozawa, Saitama 359-1192, Japan. Tel/Fax: +81 429476898, e-mail: katsu.suzu@waseda.jp

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and anticarcinogenic properties (11, 12). Moreover, certain studies demonstrate that EGCG has a potent preventive effect against cancer-related cachexia. Wang *et al*. demonstrated that EGCG effectively attenuates skeletal muscle atrophy caused by cancer cachexia in mice (3). EGCG has been reported to inhibit inflammatory responses mediated by neutrophils (13), however, the effects of green tea or EGCG on neutrophil activation in cancer and the related cachexia have not been investigated, to date.

Although the effectiveness of green tea against cancer has been reported, there is no report of the effect of green tea with respect to neutrophil activation in patients with advanced cancer. To address this issue, we investigated the effect of green tea extract on blood neutrophils from cancer patients, by application of luminol-dependent chemiluminescence (LmCL), which largely detects myeloperoxidase (MPO)-dependent formation of highly toxic ROS such as hypochlorous acid (HOCl) (14).

Materials and Methods

Patients. The entry criteria were as follows: 16-79 years of age; the presence of inoperable, chemoresistant and radioresistant cancer; estimated survival of more than three months; performance status 0-3; no severe organ function impairment and the written informed consent of the patient. At least four weeks prior to sampling, the patients were free of antitumor treatments, such as surgery, chemotherapy and radiation. The protocol was approved by the Ethical Committee at Tokyo Women's Medical University (approval number: 1692) and Waseda University (approval number: 08, 589).

Green tea extract. The green tea-leaf was first extracted using hot water for filtration, the extract was then applied to a column (polyvinyl alcohol) and eluted using 80% ethanol. This fraction was concentrated in a rotary evaporator and the total green tea fraction (GREEN TEA PE, BHN Co., Tokyo, Japan) was formed using a spray drier. Forty milligrams of GREEN TEA PE were dissolved with 30 ml distilled water, and transferred to 100 ml volumetric flask. After pipetting 0.4 ml of the GREEN TEA PE solution to the flask, 2 ml of ferrous tartrate were added and mixed before adding 2 ml buffer solution of 1/15 M H_3PO_4 (pH 7.5). The solution was incubated for 5 min at room temperature and was further clarified by centrifugation at 3000 rpm for 10 min. The absorbance was determined at 540 nm using a standard solution (ethyl gallate) as a reference. The samples were analyzed by high performance liquid chromatography (HPLC) and the catechins and (–)-epigallocatechin-3-gallate (EGCG: Nacalai Tesque Co. Ltd. Kyoto, Japan) were quantified against (+)-catechin (Sigma-Aldrich Co. Ltd., Tokyo, Japan), (–)-epicatechin (EC: Sigma-Aldrich Co. Ltd., Tokyo, Japan), (–)-epicatechin gallate (ECG: Nagara Science Co. Ltd., Gifu, Japan), (–)-epigallocatechin (EGC: Nagara Science Co. Ltd.), and gallocatechin-3-gallate (GCG: Nagara Science Co. Ltd.), used as external standards. The HPLC column was a capcellpak C18, UG120 4.6×250 mm (Shiseido Co. Ltd., Tokyo, Japan), and the mobile phase (A=distilled water, B=*N-N* dimethylformamide: methanol: acetic acid, 40:2:1.5) was used in a gradient elution. Detection was set at 278 nm. It was confirmed that the polyphenol content was more than 98%, and catechin content was more than 80% (EGCG: more than 50%, EGC: 0.6%, Catechin: 0.3%, EC: 0.3%, GCG: 5%, ECG: 9%) in the GREEN TEA PE.

Dilution of green tea extract. In a clean-air laminar hood workbench, 40 mg of GREEN TEA PE (BHN Co., Ltd., Tokyo, Japan) were dissolved in 10 ml of Hanks' balanced salt solution (HBSS) with calcium chloride and magnesium chloride. The solution was then filtered with a membrane (Syringe filters 0.22 μm TPP; Millipore, MA, USA). The concentration of green tea extract was adjusted to 0 μg/ml (HBSS only), 10 μg/ml, 100 μg/ml, and 1000 μg/ml in HBSS for further use in experiments.

Synthesis of peptide-bound temperature-responsive polymer (G-TRP). Twenty-four grams of collagen peptide (SCP-5100; Nitta Gelatin Co., Osaka, Japan) were dissolved in 96 g of distilled water at 37˚C, followed by a reaction with 3.26 g of *N*-acryloylsuccinimide (Kokusan Kagaku, Tokyo, Japan) for 4 days, at 37˚C to obtain polymerizable collagen peptide. *N*-Isopropylacrylamide (108.5 g; Kojin, Tokyo, Japan) and *n*-butylmethacrylate (4.26 g; Wako Chemical, Osaka, Japan) were dissolved in 600 ml of ethanol and then 123 g of the above aqueous solution of polymerizable collagen peptide was added. Under a nitrogen atmosphere, 1 ml of *N, N, N', N'*-tetramethylethylenediamine (Wako Chemical) and 10 ml of 10 wt% ammonium persulfate (Wako Chemical) aqueous solution were added to the mixed solution, and then reacted for 5 h at 4˚C, maintaining the nitrogen atmosphere. After the reaction, 30 l of cold (4˚C) distilled water were added and the mixture was concentrated to 3 l using an ultrafiltration membrane (molecular weight cut-off of 100,000) at 4˚C. This dilution and concentration process was repeated five times in order to remove impurities and low molecular species. Lyophilization and sterilization of the final concentrated solution gave 105 g of peptide-bound temperature-responsive polymer (G-TRP).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel. Under a clean-air laminar hood workbench, 0.5 g of G-TRP and 0.5 g of TGP (Mebiol gel; Mebiol Inc, Kanagawa, Japan) were dissolved in 16.7 ml of HBSS, at 4˚C, overnight, yielding a viscous transparent S-TGP gel of uniform liquid without any bubbles for use in the experiments (15). Mebiol gel is a pure synthesized biocompatible co-polymer composed of thermoresponsive polymer blocks and hydrophilic polymer blocks. It is characterized by its temperature-dependent dynamic viscoelastic properties and is used as a biocompatible scaffold for three-dimensional cultures without any toxicity (15). S-TGP gel is a peptide-bound thermoreversible gel formed by mixing G-TRP with the Mebiol gel. It liquefies at low temperature, turns to gel immediately upon warming, and returns to a liquid state again when cooled.

LmCL assay. Peripheral blood samples were obtained from patients using 2 ml Na-heparin tubes (Venoject II, Terumo Co, Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. Then, 150 μl-luminol-blood samples were set on 50 μl S-TGP prepared in a tube at 37˚C, and chemiluminescence was promptly measured as 0 point using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Japan), then 50 μl of green tea extract solution were added. The samples were incubated at 37˚C, and the production of ROS from neutrophils was monitored by a luminometer in a kinetic mode

at 0.5, 1.0, 1.5, 2.0 and 2.5 h. Neutrophils migrate from the blood into the S-TGP gel in the tube at 37˚C, and LmCL can be detected through the transmissive gel, thereby there is no need to separate neutrophils from blood to determine ROS production, reducing any delay in sample processing that is associated with conventional methods (14). After LmCL was measured at 2.5 h, luminol-blood samples in the tubes were removed and the tubes with 50 μl S-TGP, in which neutrophils migrated, were washed three times with PBS warmed at 37˚C. Tubes with gel were then cooled on ice, and 50 μl reagent A and 50 μl reagent B (Chemometec A/S, Allerød, Denmark) were added and mixed well. This process effectively makes the cell membrane permeable to the DNA staining dye, and is effective in the dispersion of cell aggregates. The samples were aspirated into a NucleoCassett and the cell number was counted by the NucleoCounter (Chemometec A/S).

Statistical analysis. Differences between the control, medium (HBSS: 0 μg/ml) and green tea extract (10 μg/ml, 100 μg/ml, and 1000 μg/ml) were tested with the Friedman repeated measures analysis of variance on ranks and with post-hoc multiple pairwise comparison for Friedman. Calculations were performed using the statistical software package IBM SPSS Statistics ver. 19 (SPSS Japan Inc., Tokyo, Japan). Statistical significance was accepted at the 5% level.

Table I. *Patients' characteristics*.

No.	Age (years)	Gender	Diagnosis	PS
1	55	F	Colorectal cancer	2
2	45	F	Pancreatic cancer	2
3	65	M	Pancreatic cancer	1
4	61	F	Colorectal cancer	2
5	63	F	Colorectal cancer	2
6	61	F	Lung cancer	1
7	71	M	Lung cancer	2
8	54	F	Duodenal papilla cancer	0
9	52	F	Ovarian cancer	0
10	35	M	Pancreatic cancer	0
11	47	M	Gastric cancer	3
12	53	M	Colorectal cancer	2
13	74	M	Colorectal cancer	2
14	63	F	Pancreatic cancer	3
15	46	M	Lung cancer	0
16	76	M	Gastric cancer	2
17	50	F	Ovarian cancer	0
18	62	F	Unknown	0

PS: Performance status, M: male, F: female.

Results

Patients' characteristics. Eighteen patients were enrolled in this study. The mean age of the 18 enrolled patients was 57.4 years (range 35-76 years). Gender, diagnosis and the Eastern Cooperative Oncology Group performance status of patients are shown in Table I.

The LmCL values at each point. The LmCL values measured at 0.5, 1.0, 1.5, 2.0 and 2.5 h increased up to 1.0 h and plateaued thereafter (Figure 1 A-E). The LmCL value of samples was markedly ($p<0.001$) reduced by adding the green tea extract, in a concentration-dependent manner at each time point. At all time points, the LmCL values of the samples with green tea extract at 100 μg/ml and 1000 μg/ml were significantly lower than those of samples without green tea extract or those with HBSS (medium) (Figure 1 A-E). At 1.5 h, the LmCL values of the samples with green tea extract at a concentration of 10 μg/ml were significantly lower than samples without it ($p=0.044$) (Figure 1C).

Peak values in LmCL. The peak LmCL value of samples was markedly reduced by adding the green tea extract, in a concentration-dependent manner ($p<0.001$). The peak values of the samples with green tea extract (at 10 μg/ml, 100 μg/ml, and 1000 μg/ml) were significantly lower than those of the samples without it (Figure 1 F). The peak values of the samples with green tea extract at 100 μg/ml and 1000 μg/ml were significantly lower than those of samples with HBSS only.

Sum of values in LmCL. The total LmCL was determined from the sum of LmCL values at 0.5-1.5 h and at 0.5-2.5 h. Total LmCL at 1.5 h and 2.5 h was significantly reduced by adding the green tea extract, in a concentration-dependent manner ($p<0.001$). Total LmCL at 1.5 h and 2.5 h, of the samples with green tea extract (at 10 μg/ml, 100 μg/ml, and 1000 μg/ml), was significantly lower than those without it (Figure 1 G and H). Total LmCL at 1.5 h and 2.5 h was significantly lower with green tea extract at 100 μg/ml and 1000 μg/ml when compared to those with HBSS only.

Number of migratory neutrophils. The number of migrated cells was not significantly influenced by adding the green tea extract per se (Figure 1I). When adjusted by migrated cell count, the LmCL values per cell basis were still inhibited by the green tea extract (at 100 μg/ml and 1000 μg/ml) (Figure 1 J).

Discussion

This study demonstrated that the green tea extract significantly reduced markers of ROS production from neutrophils of cancer patients in a concentration-dependent manner. Neutrophil activation may be affected by green tea extract based on the antioxidative or anti-inflammatory actions of the green tea extract. Numerous studies have reported anti-inflammatory effects of EGCG, and several studies demonstrated an anti-inflammatory effect of EGCG on cancer (11, 12). Our findings that green tea extract reduced the neutrophil production of ROS are consistent with

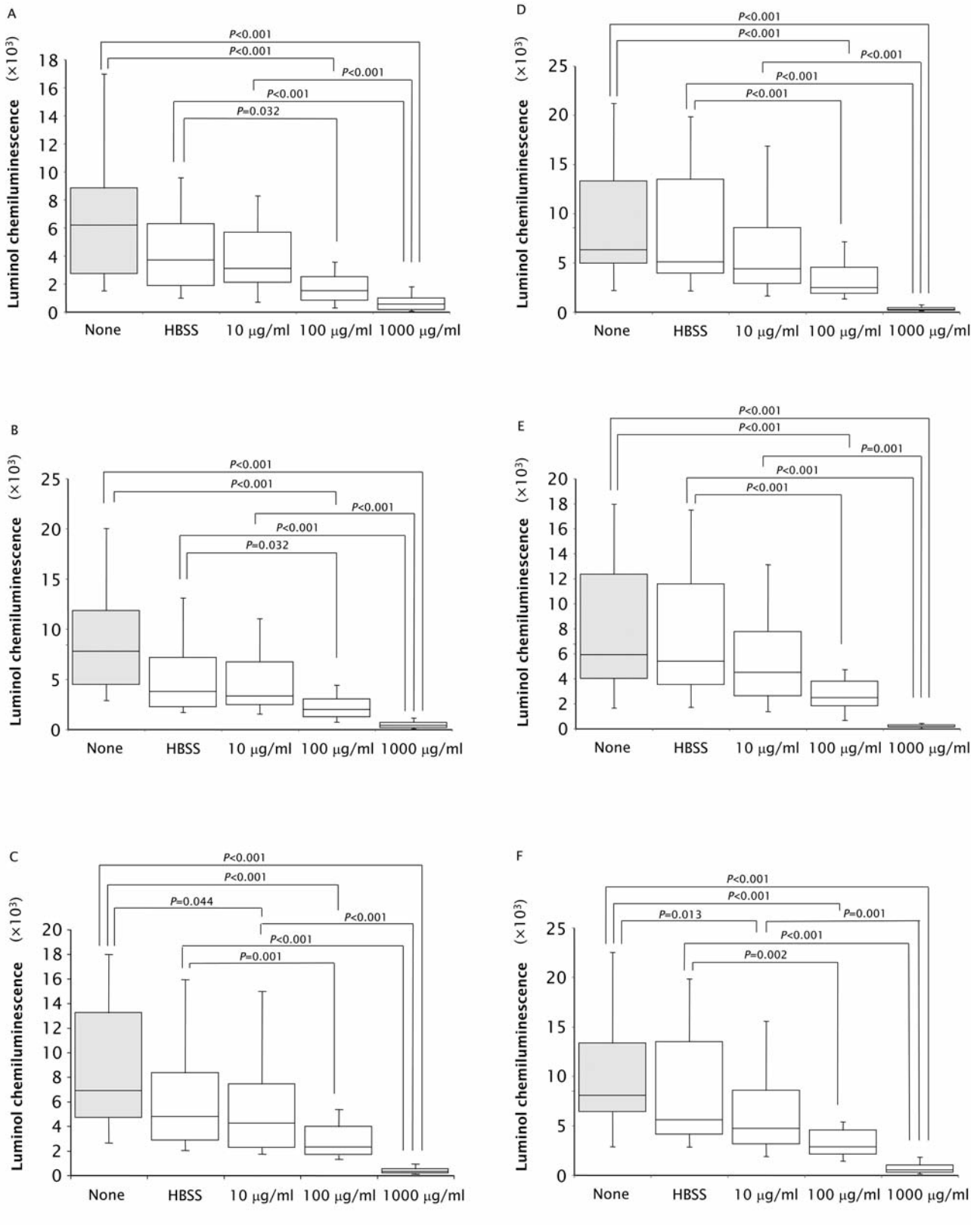

Figure 1. *continued*

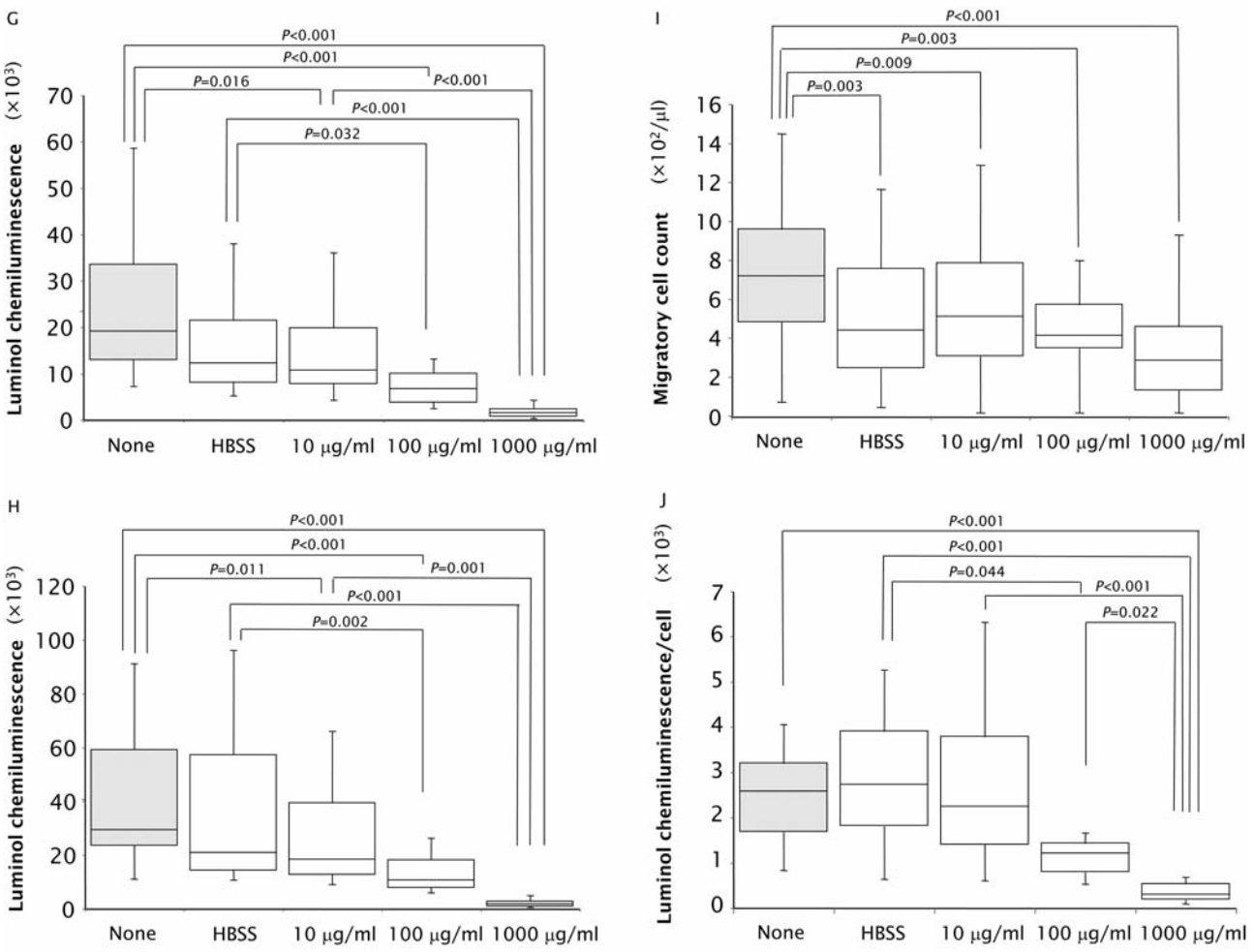

Figure 1. *Box and whisker plots demonstrating neutrophil luminol-dependent chemiluminescence (LmCL) (A-H, J) and cell counts (I), according to the concentrations of green tea extract: LmCL at 0.5 h (A), 1.0 h (B), 1.5 h (C), 2.0 h (D), 2.5 h (E) after addition of green tea extract; peak value during measurement (F), sum of value at 1.5 h (G) and 2.5 h (H); count of cells migrating into gel at 2.5 h (I), and sum of value per a cell at 2.5 h (J). Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles. HBSS: Hanks' balanced salt solution.*

previous research, supporting an antioxidant and anti-inflammatory effects of EGCG.

The production of ROS, not only of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also of MPO-dependent HOCl production of neutrophils, can be measured by LmCL (14, 21). In the present study, green tea extract suppressed the neutrophil LmCL response, suggesting that ROS production was reduced. The major polyphenolic compound of green tea, EGCG, has demonstrated antioxidant potential, especially in detoxifying HOCl (16). The antioxidant effect of EGCG is well known and various *in vitro* and *in vivo* studies suggest that EGCG is associated with decreased risk and/or slower cancer progression (12, 19, 20). It is plausible that the beneficial effects of EGCG in cancer may be related partly to a reduction of neutrophil production of ROS.

Green tea is manufactured by drying fresh tea leaves. It contains characteristic polyphenolic compounds such as EGCG, EGC, ECG and EC. Catechin, gallocatechin, epigallocatechin digallates, epicatechin digallate, 3-O-methyl EC, catechin gallate, and gallocatechin gallate are present in smaller quantities (12). However, Kürbitz *et al*. reported that ECG and catechin gallate are superior to EGCG in anti-inflammatory activity in pancreatic tumor cells, and can have an equivalent action (17). Although the differential activity of these compounds is a matter of discussion, we did not fractionate the green tea extract into each component when assaying the effects on neutrophil functions. Thus, the results of our study might support that routine intake of green tea as a dietary supplement and drink is beneficial for the oxidative property of neutrophils in cancer patients.

In combination with antioxidant properties, extracts of green tea have anti-inflammatory effects and this may have contributed to the suppression of ROS. EGCG suppresses melanoma growth by inhibiting inflammasome and IL-1β secretion (11), and Gutierrez-Orozco *et al*. reported that green tea inhibited cytokine-induced IL-8 production and secretion in gastric cancer cells *via* inhibition of NF-κB activity (18). Although unrelated to cancer, certain reports have demonstrated that EGCG suppressed neutrophil activation. Zhu *et al*. demonstrated that EGCG inhibits leukocyte activation by bacterial formyl peptide through the formyl peptide receptor (13). The combined antioxidant and anti-inflammatory properties of green tea may have contributed to suppression of ROS production by neutrophils in our study.

Migration of neutrophils to the tissue microenvironment is a first step to evoking local inflammation. Green tea extract did not inhibit neutrophil migration, but ROS production from migrated cells was significantly suppressed. Various neutrophil functions, such as ROS production and/or chemotaxis, could work as a dual-edged sword on both sides of host defense and tissue injury (21). Thus, the balance between beneficial and harmful effects of neutrophil functions should be properly modulated. Considering that chronic oxidative stress occurs in cancer patients, suppression of ROS production from migrated neutrophils is advantageous. In addition, the ability of migration was not affected by the green tea extract, suggesting that the normal host defense response is retained in the presence of green tea extract. Although LmCL reflects oxidative stress based on the balance of production of oxidants and their removal by antioxidants, it can be used as a screening method for effective antioxidants for patients, in order to control oxidative stress *in vitro*, as well as for clinical monitoring of interventions *ex vivo*. The use of Mebiol gel, made it possible to mimic the *in vivo* microenvironment of neutrophil infiltration into tissues, and this approach may be used for the assessment of the antioxidant and anti-inflammatory actions of bioactive substances, such as green tea extract, using a small amount of blood, as demonstrated in the present study.

Cachexia in patients with terminal cancer is associated with pain, muscle atrophy, fatigue, anorexia, nausea, anemia, and immunodepression, resulting in a marked decrease in the patients' quality of life. Drugs with antioxidant and anti-inflammatory actions have been used in clinical trials. Mantovani *et al*. demonstrated their effectiveness for patients with cancer cachexia, by randomized phase III clinical trial of five different arms of treatment, which included polyphenols plus antioxidant agents (6). Our results in the present study might support the usefulness of green tea for patients with cancer cachexia in terms of increased protection against oxidative stress by reducing ROS production from neutrophils. Further studies are needed to investigate which constituents and regimens are more effective to reduce oxidative stress and risks of cancer development and related symptoms, by taking advantage of appropriate clinical monitoring systems.

Conclusion

This is the first investigation to support the antioxidant potential of green tea in whole blood from cancer patients. Green tea extract reduces neutrophil-derived oxidative stress and these findings provide further support for exploring the efficacy of green tea in clinical trials for cancer patients.

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