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Dietary supplementation with green tea extract promotes enhanced human leukocyte activity

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Abstract

Background: Leukocytes play a vital role in the host defence and inflammatory systems, the latter being responsible for the pathogenesis of a wide spectrum of acute and chronic diseases. Green tea is a popular beverage, which is consumed worldwide and its active ingredients are epicatechin derivatives, which possess distinct anti-inflammatory properties. The purpose of this study was to investigate if a green tea extract could enhance leukocyte function in humans.

Methods: Volunteers were asked to take 300 mg of the green tea extract daily for 14 days and the capacity of circulating leukocytes to release both myeloperoxidase and lactoferrin was assessed. Whole blood from volunteers was stimulated with the bacterial peptide Formyl-Methionine-Leucine-Phenylalanine (fMet-Leu-Phe). Myeloperoxidase an enzyme that converts hydrogen peroxide to hypochlorous acid and is stored and secreted from the granules of neutrophils and monocytes and was measured as well as lactoferrin which is an iron-binding protein stored and secreted from the neutrophils. In conjunction the antioxidant capacity of the blood of the volunteers was also determined using a chemiluminescence method that measures the capacity of plasma to scavenge superoxide.

Results: After 14 days of treatment there was a significant increase in the release of myeloperoxidase and lactoferrin when whole blood was stimulated with fMet-Leu-Phe ($p < 0.05$), which activates a number of leukocytes including mature neutrophils and monocytes. This was mirrored by a significant increase in the total antioxidant status after 14 days of green tea ingestion ($p < 0.05$). After the "wash-out" period of 4 weeks, all parameters were consistent with those observed at the start of the trial (day 0). Treatment with the green tea extract also caused a slight but non-significant decrease in the number of circulating leukocytes, but the counts remained within published "normal" ranges for healthy human adults.

Conclusions: This study indicates that a green tea extract when taken as a dietary supplement for 14 days can increase the leukocyte activity and the total plasma antioxidant status and may have role to play in the prevention of inflammatory disease.

Keywords: green tea extract, lactoferrin, monocyte, myeloperoxidase, neutrophil

Introduction

In humans, both neutrophils and monocytes provide defensive mechanisms against bacterial infections. This defensive process requires a number of mechanisms which can be divided into oxygen dependent and independent pathways. The oxygen-dependent pathway is reliant upon both the activation of NADPH oxidase, an enzyme complex present on the plasma membrane that converts oxygen to superoxide, and also the release of myeloperoxidase from granules of the neutrophils or monocytes [1–4]. Myeloperoxidase is responsible for the conversion of hydrogen peroxide to hypochlorous acid, a very potent oxidant [5]. The oxygen independent pathways are dependent upon phagocytosis, and the generation of a low pH within the phagolysosome [6]. This is supported by granule movement, and secretion of their contents into the phagolysosome [7]. The killing of bacteria is aided by many mechanisms including the binding of iron by lactoferrin [8], which is present in the secondary granules of neutrophils and the direct action of lysozyme on the bacterial cell. These are only two examples of the mechanisms adopted by leukocytes, leading to bacterial killing.

Neutrophil priming by agents such as tumour necrosis factor (TNFα), GM-CSF (granulocyte/macrophage-colony stimulating factor) and lipopolysaccharide [9, 10] cause a dramatic increase in the response of these cells to an activating species, such as the bacterial peptide analogue Formyl-Methionine-Leucine-Phenylalanine (fMet-Leu-Phe). The principal consequence of priming is to potentiate superoxide anion generation, degranulation and the

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production of lipid mediators such as leukotriene B_4 upon stimulation of the neutrophil [11].

Green tea has a rich polyphenol content, of which there are three subclasses: the flavonoids, the flavones and the flavonols. The major flavonols or catechins are (-) –epigallocatechin-3-gallate, (-)-epicatechin, (-)-epicatechin gallate, and these constitute about a third of the dry weight of green tea. Quercitin, kaempferol, myrietin and apigenin are also present at lower concentrations [12]. Previous research indicated that isolated components such as epicatechin gallate and hydrophobic components of green tea tend to inhibit neutrophil activity [13–15]. However, previous work in this laboratory demonstrated that a green tea extract could prime isolated human neutrophils. This resulted in a twofold increase in the production of superoxide and a 5.5-fold increase in the release of myeloperoxidase, following stimulation with fMet-Leu-Phe [16].

A supplementation study was designed to assess if the immunomodulatory active components of the green tea extract could enhance the release of both myeloperoxidase and lactoferrin from human leukocytes following stimulation with fMet-Leu-Phe. There have also been several observations that isolated neutrophils have different properties to those found in whole blood. Some studies report a greater expression of adhesion receptors on the neutrophil plasma membrane upon isolation than in whole blood [17, 18]. Since the isolation of human neutrophils is associated with some priming, such a procedure may introduce complications when analysing the results. One solution to avoid this would be to simply stimulate whole blood with fMet-Leu-Phe and then quantitate the released myeloperoxidase and lactoferrin. This approach is certainly not specific for neutrophils but also for other leukocytes such as monocytes that express the FMLP receptor and release myeloperoxidase upon stimulation [19, 20], but lactoferrin is only present in exocrine fluids and neutrophils [21]. The stimulation of whole blood will be regarded as a measure of a total leukocyte response.

In this study, we report the results from a supplementation study that was designed to determine if the green tea extract could enhance human leukocyte function. This was assessed by stimulating whole blood with fMet-Leu-Phe and assessing the concentration of myeloperoxidase and lactoferrin released into the plasma. The results indicated that the green tea extract does potentiate leukocyte function without detrimental effects on the white cell count.

Materials and methods

The green tea extract, was prepared into pills, each containing 100 mg of the extract and was kindly donated by Quest Vitamins Ltd (Birmingham UK). This is a commercial product, and its release to the public is dependent upon stringent quality control and a manufacturer's guarantee that each pill contained 60 mg of total polyphenols.

Subjects

Healthy subjects who were not taking any medication for any known disease participated in the study, which had the approval of the Ethics Committee of Liverpool John Moores University. Informed consent was obtained from every volunteer. The cohort consisted of 10 males and 10 females between the ages of 20–55 (mean age: 35.2 ± 7.3). On day 0 of the trial, all the volunteers donated 10 mL of blood to establish baseline values for lactoferrin and myeloperoxidase release, white cell count and an indirect measurement of water soluble antioxidant capacity. All blood samples were obtained by venepuncture, and 9 parts of blood were added to 1 part of 3.8% (w/v) tri-sodium citrate. Granulocyte counts were performed using an improved Neubauer counting chamber. The subjects then took 3×100 mg green tea extract (Quest Vitamins Birmingham, UK) pills per day, this is the dose recommended by manufacturer. The subjects carried out their usual lifestyles, with no alterations in diet or alcohol intake. On day 14, another blood sample was donated by the volunteers. All subjects were then requested to discontinue taking the green tea extract for a further 4 weeks after which a final blood sample was obtained. These samples were analysed for lactoferrin, myeloperoxidase, white cell counts and total antioxidant status.

Whole blood was aliquoted into 2 mL minifuge tubes and phenylmethanesulphonyl fluoride was added (final concentration 1 mM), to inhibit any protease activity from plasma or secreted proteases. This was followed by the addition of fMet-Leu-Phe at a final concentration of 1 μM. The samples were incubated at 37°C for 10 min and were then centrifuged at 13,000 rpm for 5 s. The plasma layer was removed and stored at –80°C prior to analysis.

Enzyme immunoassay of myeloperoxidase and lactoferrin

Following neutrophil stimulation with fMet-Leu-Phe, released concentrations of myeloperoxidase and lactoferrin were determined using commercially available enzyme linked immunoassay kits (Calbiochem, UK).

The assays were carried out in accordance with the manufacturer's instructions. The participant plasma was thawed and 100 μL aliquot was placed in duplicate into a microtitre wells previously coated with anti-myeloperoxidase. The plate was covered and incubated for 2 h at 37°C. The plate was then washed extensively followed by the addition of 100 μL biotinylated goat anti-myeloperoxidase. The plate was again incubated for 1 h at 37°C and

following washing 100 µL of avidin-alkaline phosphatase was added. This was followed by a final incubation for 1 h at 37°C after which the substrate p-nitrophenylphosphate was added. The reaction was allowed to proceed for 25 min at 37°C and was stopped by the addition of a stop solution and the absorption at 405 nm was then determined. A standard curve was prepared using serial dilutions of a myeloperoxidase standard.

A similar method was employed for lactoferrin determination, except that the Horseradish Peroxidase linked secondary antibody was used, and o-phenylenediamine was used as the substrate. The coloured product 2,3-diaminophenazine was monitored at 420 nm. The sensitivity of both assays is reported to be approximately 1 ng/mL.

Antioxidant status

Antioxidant status of the volunteers was also assessed using a Pholasin based kit (Abel-21, Knight Scientific, Plymouth, UK). If the volunteers had been taking the green tea supplements then there should be an increase in antioxidant status as the green tea extract is rich in polyphenols.

Superoxide was generated according to instructions supplied by the kit and chemiluminescence was generated when the radical was scavenged by pholasin. When plasma is used in conjunction with the assay water soluble antioxidants compete with pholasin to quench the light emitted. A reduced chemiluminescence is thus associated with an increased antioxidant status in the plasma. The manufacturer's instructions were followed, and chemiluminescence was monitored using a LKB Wallac 1251 Luminometer. The antioxidant capacity of the sample was expressed as % inhibition of peak light emission.

Statistics

Results from the green tea supplementation study were analysed using repeated measures ANOVA using the post hoc Bonferroni correction. A p-value less than 0.05 was considered statistically significant. All statistical tests were performed using SPSS v20.

Results

White cell count

The volunteers were healthy adults with no apparent inflammatory diseases and reported no adverse problems during the supplementation study. However, the green tea extract appeared to reduce the white cell count in some volunteers (Figure 1). Statistical treatment using a repeated measures ANOVA indicated there was no significant difference ($p > 0.05$) across the three time points and the white cell count remained within range of $4–11 \times 10^9$/L [22]. The mean neutrophil count at day 0 was $(6.73 \pm 1.14) \times 10^9$/L, at day 14 was $(6.1 \pm 1.55) \times 10^9$/L, after washout period was $(6.7 \pm 1.95) \times 10^9$/L.

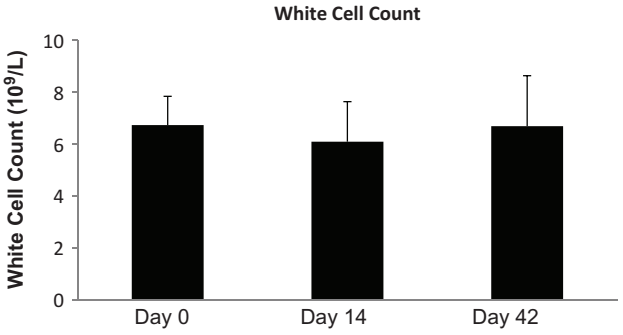

Figure 1: Human blood samples were taken at days 0, 14 and 42. These were diluted, the red cells were lysed and the leukocytes were stained and counted using an improved Neubauer counting chamber. Each count was performed three times and the average was taken. The results indicate no significant decrease in total white cell count at day 14 or 42 compared to the baseline.

Lactoferrin and myeloperoxidase

Both myeloperoxidase and lactoferrin were measured following stimulation of leukocytes in whole blood by the bacterial peptide analogue fMet-Leu-Phe. Green tea supplementation for 14 days significantly increased the release of both myeloperoxidase and lactoferrin when compared to day 0 (12.4 ± 2.3 vs. 15.2 ± 4.2 ng/10^7 white cells) and (16.3 ± 2.9 vs. 23.1 ± 4.9 ng/10^7 white cells) respectively ($p < 0.05$) (Figure 2A, B). After the washout period, the level of myeloperoxidase (12.3 ± 2.9 ng/10^7 white cells) and lactoferrin (17.6 ± 4.2 ng/10^7 white cells) release was comparable to that observed on day 0. On an individual basis the effect ranged from a modest increase to approximately double that of the initial response. However no increase in the release of either lactoferrin or myeloperoxidase was observed in 15% of the subjects.

Total antioxidant status

A separate measure to monitor the effectiveness of the green tea supplement was to measure an increase in water-soluble antioxidant status. This was reflected in the results on day 14 when there was a significant increase in total antioxidant status ($p < 0.05$) (Figure 3)

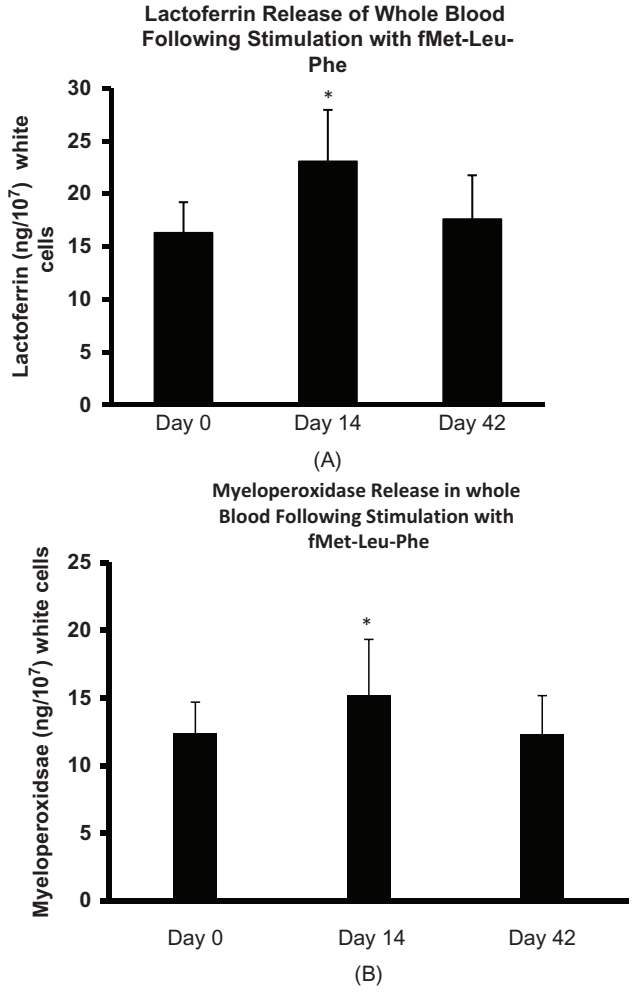

Figure 2: Neutrophils in human whole blood were stimulated by the addition of fMet-Leu-Phe.
Plasma was obtained, and the release of (A) lactoferrin and (B) myeloperoxidase was assessed by immunoassay. The results indicate baseline measurements at day 0, significant increases at day 14 ($p < 0.05$). Following a washout period, the levels of lactoferrin and myeloperoxidase release were comparable to those observed at baseline.

compared to that at day 0 (Day 0, mean 9.9 ± 2.5 vs. Day 14 mean $32 \pm 6\%$ reduction in light emission). However, three subjects had no increase in either myeloperoxidase, lactoferrin release or any increase in antioxidant status. This implies that these subjects were either not taking the supplements in the agreed manner or did not respond to the green tea extract. Interestingly, after the "wash-out" period (day 42), the total antioxidant status was consistent with that at the start of the supplementation study (Day 42 mean $17.5 \pm 5\%$ reduction in light emission). This would indicate the increase in total antioxidant status was largely due to the ingestion of the green tea extract.

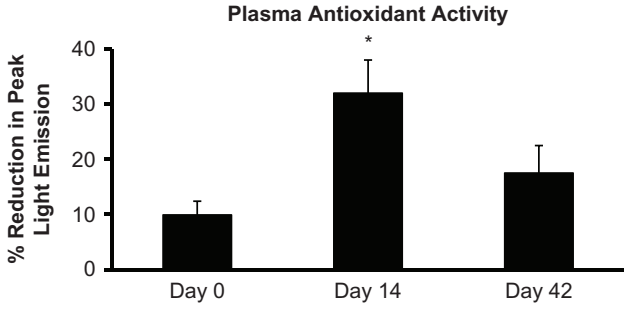

Figure 3: The total antioxidant activity of human plasma was determined by a chemiluminescence assay.
The plasma competes for chemically generated radicals, a reduction in chemiluminescence being regarded as having distinct antioxidant properties. The results indicate baseline measurements at day 0, significant increases in antioxidant activity at day 14 ($p < 0.05$). Following a washout period, the release was similar to baseline measurements.

Discussion

Previous research established that chemical constituents in green tea extract can prime human neutrophils that were isolated from whole blood [16] and this study indicates that a similar effect exists *in vivo*. The twenty subjects, largely responded to the treatment, and yielded an increased release of both myeloperoxidase and lactoferrin, when whole blood was stimulated by the addition of FMet-Leu-Phe. The lactoferrin release data was more convincing than the myeloperoxidase. In the previous report [16], we used an assay that recorded myeloperoxidase activity, but in this study we used an immunological assay, and this may have contributed to this lack of discrimination with the myeloperoxidase data. This may be due to reactive oxygen species such as hypochlorous acid destroying antigenic sites on the enzyme. However the paired t-test data indicates that there was a significant difference between day 0 and day 14. This would suggest that if these volunteers encountered a bacterial infection whilst taking the green tea extract supplements, they may be able to mount an improved immunological response. The neutrophils in the supplemented volunteers would respond more effectively to bacterial peptides, have enhanced NADPH oxidase activity along with the enhanced release of myeloperoxidase inducing the formation of hypochlorous acid which can kill Gram-positive bacteria and an increased release of lactoferrin which chelates iron so helping to prevent the growth of bacterial species [23].

Several studies [10, 12, 14, 15] indicate that (-) epigallocatechin-3-gallate inhibit neutrophil function. The main

argument for this is that epicatechins inhibit neutrophil chemotaxis both *in vitro* and *in vivo* situations. This has been reported as a direct effect on neutrophils and an indirect effect on the inhibition of synthesis of chemokines by fibroblasts. Many of these studies were performed using rat blood [24, 25] or cell culture [26, 27] with a relatively short contact time of the epicatechin or green tea extract. In contrast to these studies it was recently reported that (-)-epicatechin may enhance the efficiency of myeloperoxidase in the presence of higher concentrations of hydrogen peroxide which would enhance the immune response to bacterial infections [28]. With regard to action of the NADPH oxidase the majority of the studies examined the antioxidative effects of the green tea extract rather than its priming properties [29, 30]. The present study employed a green tea extract over a 14 day period a rather short-term experiment, the *in vivo* observations could be explained by either a direct or an indirect action of the extract. The green tea extract used in this study is a complex mixture and it is possible that a synergistic action of some of the ingested components resulted in the observed priming effect. Efferth [31] reports that different components found in complex herbal mixtures may act at different points in the same signalling pathway (multi-target effects) to enhance observed effects.

There was a minor decrease in the total white cell count, at day 14 in some individuals (Figure 1). The most extreme case was reduction from 6.8×10^9/l to 4.8×10^9/l. This represented a 30% decrease, but the count was still within the normal range [22]. *Ex vivo* and *in vitro* studies indicated that green tea extract had very distinct leukocyte priming properties [12, 16]. In this case the green tea extract was delivered as a pill, with each containing 60 mg of polyphenols. The bioavailability of green tea extract in this form was previously investigated [32], the study investigated uptake and plasma antioxidant status following one dose of green tea as a beverage or as a gelatin capsule. It was found that the absorption of polyphenols from the green tea supplement in a gelatin capsule was delayed but was higher than that of green tea or black tea beverages. The supplement also increased the total plasma antioxidant status 2–3% greater than the beverages over an eight hour period. This study indicates that that green tea extract in a pill form is an efficient way of increasing polyphenols in the plasma.

The priming effect of the green tea extract may thus benefit the very young and the elderly against bacterial infections. However it could be argued that ingestion of the green tea extract may potentiate any inflammatory diseases such as periodontitis or rheumatoid arthritis diseases which involve leukocyte infiltration and activation [33, 34]. It has been suggested that immunoglobulins and other cytokines may prime these leukocytes, at sites of inflammation [35]. Green tea extract would not make any significant contribution to the inflammatory process, as priming of mature neutrophils in synovial fluid is a very efficient process. This could be the result of the presence of high levels of priming agents such as TNFα and IL-8 but not GM-CSF in the inflamed joint [36].

This small study suggests that green tea extract can potentiate neutrophil function through priming. Stimulation of primed neutrophils by bacteria would enhance degranulation and superoxide production. Previous work suggests that the *in vitro* priming properties of the green tea extract were approximately 50% of GM-CSF. Unlike GM-CSF the mechanism of green tea priming did not involve tyrosine kinase activation [16]. However, further work is needed to elucidate the components of the green tea extract and mechanisms that are responsible for the priming of mature human leukocytes.

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