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Dietary supplementation with green tea extract promotes enhanced human leukocyte activity

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10 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

20 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 (TNF α), 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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1 production of lipid mediators such as leukotriene B₄ upon
stimulation of the neutrophil [11].

Q1 Green tea has a rich polyphenol content, of which
there are three subclasses: the flavonoids, the flavones
5 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. Quercetin, kaemp-
ferol, myricetin and apigenin are also present at lower
10 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
15 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 myeloperoxi-
dase, following stimulation with fMet-Leu-Phe [16].

A supplementation study was designed to assess if
20 the immunomodulatory active components of the green
tea extract could enhance the release of both myelopero-
xidase and lactoferrin from human leukocytes following
stimulation with fMet-Leu-Phe. There have also been sev-
eral observations that isolated neutrophils have different
25 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 neu-
trophils is associated with some priming, such a proce-
30 dure 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 quan-
titate the released myeloperoxidase and lactoferrin. This
approach is certainly not specific for neutrophils but also
35 for other leukocytes such as monocytes that express the
FMLP receptor and release myeloperoxidase upon stimu-
lation [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
40 response.

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

50

Materials and methods 1

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
5 the public is dependent upon stringent quality control and a man-
ufacturer's guarantee that each pill contained 60 mg of total poly-
phenols.

Subjects 10

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
15 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
20 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
25 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, myelopero-
oxidase, white cell counts and total antioxidant status.

Whole blood was aliquoted into 2 mL minifuge tubes and phenyl-
methanesulphonyl fluoride was added (final concentration 1 mM), to
30 inhibit any protease activity from plasma or secreted proteases. This
was followed by the addition of fMet-Leu-Phe at a final concentra-
tion 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. 35

Enzyme immunoassay of myeloperoxidase and lactoferrin 40

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

The assays were carried out in accordance with the manufac-
turer's instructions. The participant plasma was thawed and 100 μL
45 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 exten-
sively followed by the addition of 100 μL biotinylated goat anti-
myeloperoxidase. The plate was again incubated for 1 h at 37°C and

50

1 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.

5 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

15 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.

20 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

30 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

40 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 $4\text{--}11 \times 10^9/\text{L}$ [22]. The mean neutrophil count at day 0

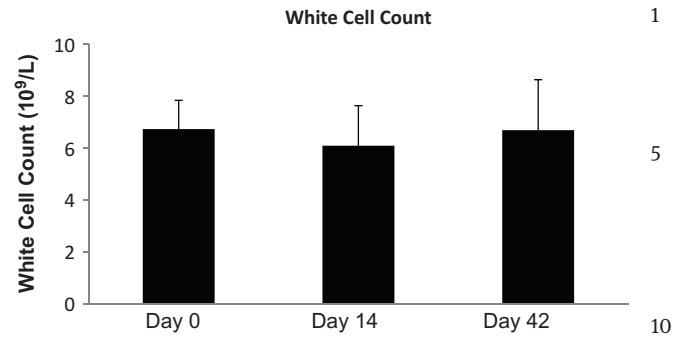


Figure 1: Human blood samples were taken at days 0, 14 and 42. These were diluted, the red cells were lysed and the leukocytes were stained and counted using an improved Neubauer counting chamber. Each count was performed three times and the average was taken. The results indicate no significant decrease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

was $(6.73 \pm 1.14) \times 10^9/\text{L}$, at day 14 was $(6.1 \pm 1.55) \times 10^9/\text{L}$, after washout period was $(6.7 \pm 1.95) \times 10^9/\text{L}$.

Lactoferrin and myeloperoxidase

25 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

45 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)

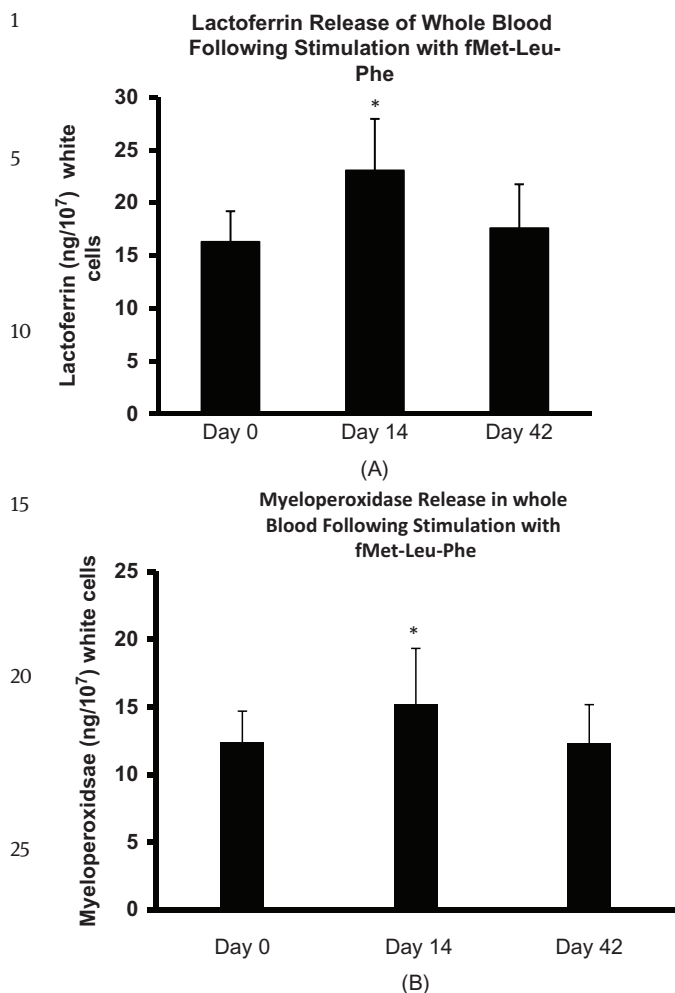


Figure 2: Neutrophils in human whole blood were stimulated by the addition of fMet-Leu-Phe. Plasma was obtained, and the release of (A) lactoferrin and (B) myeloperoxidase was assessed by immunoassay. The results indicate baseline measurements at day 0, significant increases at day 14 ($p < 0.05$). Following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

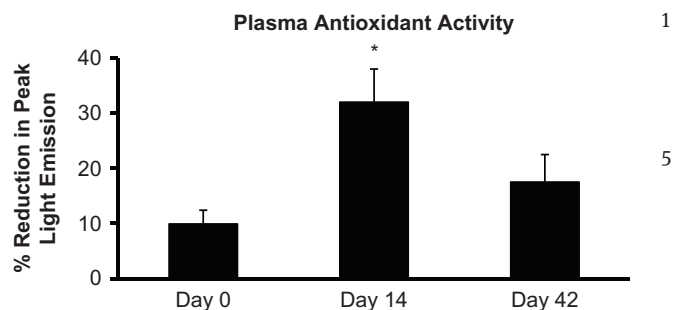


Figure 3: The total antioxidant activity of human plasma was determined by a chemiluminescence assay.

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

1 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
5 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
10 of myeloperoxidase in the presence of higher concentra-
tions 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 stud-
ies examined the antioxidative effects of the green tea
15 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
20 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 her-
bal mixtures may act at different points in the same
25 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 $6.8 \times 10^9/l$ to
30 $4.8 \times 10^9/l$. 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
35 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
40 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
45 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
50 diseases such as periodontitis or rheumatoid arthritis

diseases which involve leukocyte infiltration and activa- 1
tion [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, 5
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 TNF α
and IL-8 but not GM-CSF in the inflamed joint [36].

This small study suggests that green tea extract 10
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 proper-
ties of the green tea extract were approximately 50% of 15
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 compo-
nents of the green tea extract and mechanisms that are
responsible for the priming of mature human leukocytes. 20

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