

Research Article

The Peroxidation of Leukocytes Index Ratio Reveals the Prooxidant Effect of Green Tea Extract

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Despite tea increased plasma nonenzymatic antioxidant capacity, the European Food Safety Administration (EFSA) denied claims related to tea and its protection from oxidative damage. Furthermore, the Supplement Information Expert Committee (DSI EC) expressed some doubts on the safety of green tea extract (GTE). We performed a pilot study in order to evaluate the effect of a single dose of two capsules of a GTE supplement (200 mg × 2) on the peroxidation of leukocytes index ratio (PLIR) in relation to uric acid (UA) and ferric reducing antioxidant potential (FRAP), as well as the sample size to reach statistical significance. GTE induced a prooxidant effect on leukocytes, whereas FRAP did not change, in agreement with the EFSA and the DSI EC conclusions. Besides, our results confirm the primary role of UA in the antioxidant defences. The ratio based calculation of the PLIR reduced the sample size to reach statistical significance, compared to the resistance to an exogenous oxidative stress and to the functional capacity of oxidative burst. Therefore, PLIR could be a sensitive marker of redox status.

1. Introduction

The Supplement Information Expert Committee (DSI EC) indicated that consumption of green tea extract (GTE) could induce liver damage [1]. In fact, there are an increasing number of case reports of hepatotoxicity in humans associated with intake of green tea (GT) dietary supplements [2–7]. The types of preparation responsible for the adverse effects were hydroalcoholic extract and aqueous extract of GT, consumed as tea or in capsules [7]. However, there are also cases reporting hepatotoxicity after GT infusion [2–7]. In particular, a case has been reported with features mimicking autoimmune hepatitis, with abnormal liver histology and elevated levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyl-transferase and bilirubin, associated with hypergammaglobulinemia, and the transient presence of anti-smooth-muscle antibodies (ASMA) and anti-neutrophil cytoplasmic antibodies (ANCA) [8]. GT withdrawal resulted in a slow and continuous improvement with a complete resolution after 7 months [8].

Furthermore, the Food and Drug Administration (FDA) and the European Food Safety Administration (EFSA) have

denied the proposed health claims for GT and decreased risk of noncommunicable diseases [9]. In particular, despite GT increased plasma nonenzymatic antioxidant capacity (NEAC) [10], the EFSA denied claims related to tea and protection of DNA and lipids from oxidative damage [9].

GT contains several flavonoids with antioxidant properties, in particular the flavanol monomers known as catechins, where epigallocatechin-3-gallate (EGCG) is the most effective antioxidant compound [11]. However, tea catechins could have also prooxidant activity [11]. Besides, some of the protective effects of EGCG have been ascribed to its capability to reduce excessive uric acid (UA) level [12]. In particular, flavanols of *Camellia sinensis* modulate both xanthine oxidase and urate transport [13]. UA is the major plasma antioxidant and contributes to plasma nonenzymatic antioxidant capacity [10].

The peroxidation of leukocytes index ratio (PLIR) measures the resistance of leukocytes to exogenous oxidative stress and their functional capacity of oxidative burst upon activation [14].

Therefore, we performed a pilot study in order to evaluate the effect of a single dose of a GTE supplement on the PLIR,

in relation to plasma UA and ferric reducing antioxidant potential (FRAP) [15], as well as the sample size to reach statistical significance.

2. Material and Methods

2.1. Subjects and Treatment. Participants (6 men and 4 women, 19–35 years old) to the study, who volunteered in response to advertisements, were healthy, nonsmokers and were taking no supplements.

For two days prior to each feeding study the subjects followed a low antioxidant diet (washout) by avoiding all fresh fruit, vegetables, tea, coffee, cocoa, fruit juices, and wine.

On the day of the study, after an overnight fast, venous blood samples were collected (in EDTA-tubes) before (T0), 30 minutes (T0.5), and 3 hours (T3) after a single dose of two capsules of a GTE (200 mg × 2), commercially available in Italy (cod. 1820, REGISTRO INTEGRATORI https://www.salute.gov.it/imgs/C_17_pagineAree_3668_listaFile_itemName_1_file.pdf).

2.2. Plasma Uric Acid and TAC. The plasma was separated by centrifugation at 1300 ×g at 4°C for 15 min and stored at –80°C. Plasma levels of UA were quantified using colorimetric kits (Sentinel CH. SpA, Italy).

Plasma TAC was measured with the FRAP assay [15]. We calculated also the uric acid- independent FRAP (FRAP-UA) as previously described [16], applying the formula:

$$\text{FRAP-UA} = \text{FRAP } \mu\text{M} - 2 \text{ UA } \mu\text{M}. \quad (1)$$

2.3. PLIR Method. After red blood cells' lysis and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diazas-indacene-3-undecanoic acid (C11-BODIPY, Invitrogen, final concentration 1 μM) staining, leukocytes were treated as previously described [14, 17] with phorbol 12-myristate 13-acetate (PMA, Sigma, final concentration 1 μg/mL), 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH, Sigma, final concentration 10 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma, final concentration 10 μM), PMA 1 μg/mL + Trolox 10 μM, or AAPH 10 mM + Trolox 10 μM. After 30 min at 37°C cells were stored in ice, to stop reactions, and rapidly analyzed on an Accuri C6 BD cytometer.

Data acquired on the Accuri C6 was exported in FCS format and analyzed by FCS express software (De Novo Software) to calculate the ratio of oxidation of the probe C11-BODIPY (FL1/FL2).

PLIR was calculated as previously described [14, 17], applying the formula:

$$\text{PLIR} = \frac{(\text{ratio AAPH} \times \text{ratio PMA Trolox})}{(\text{ratio AAPH Trolox} \times \text{ratio PMA})}. \quad (2)$$

2.4. Statistics. Statistical analysis, carried out with Friedman RM ANOVA on Ranks, revealed a normal distribution for all markers (Normality Test Shapiro-Wilk and Equal variance test passed).

TABLE 1: Effect of GTE consumption on plasma antioxidant markers and PLIR.

	T0	T0.5	T3
UA μM	334.1 ± 7.4	324.9 ± 3.9	324.3 ± 3.7
FRAP μM	1061.5 ± 38.45	1061.48 ± 52.85	1083.33 ± 46.78
FRAP-UA μM	393.23 ± 41.63*	411.57 ± 53.38	434.59 ± 41.75*
PLIR L	2.09 ± 0.17	2.09 ± 0.17	2.37 ± 0.19
PLIR M	1.88 ± 0.13*	1.86 ± 0.12	2.19 ± 0.15*
PLIR G	1.80 ± 0.12*	1.90 ± 0.14	2.17 ± 0.15*

Plasma antioxidant markers in samples collected from 10 healthy subjects before (T0), 0.5 (T0.5), and 3 hours (T3) after the consumption of a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). UA: uric acid, FRAP: ferric reducing antioxidant potential, FRAP-UA: uric acid-independent FRAP, PLIR: peroxidation of leukocytes index ratio, L: lymphocytes, M: monocytes, and G: granulocytes. RM ANOVA, with time as within-subjects factor, followed by Student-Newman-Keuls post hoc analysis: T3 versus T0: * $p < 0.05$.

Therefore, statistical analysis was carried out with repeated measures analysis of variance (RM ANOVA), with time or treatment as within-subjects factors. Student-Newman-Keuls post hoc analysis (all pairwise multiple comparison procedure) was used to isolate differences between groups. Spearman correlation was used to evaluate relationships between variables. All statistical evaluations were performed using the SigmaStat and SigmaPlot software (Jandel Scientific, Inc.).

3. Results

3.1. Plasma Uric Acid and TAC. RM ANOVA, with time as within-subjects factor, followed by Student-Newman-Keuls post hoc analysis (all pairwise multiple comparison procedure), revealed that GTE consumption did not affect FRAP values, whereas 3 hours (T3) after treatment both a nonsignificant decrease in UA and a significant increase in FRAP-UA were found (Table 1).

From the difference of means and the standard deviations (power 0.8; alpha 0.05) we calculated a sample size (to reach statistical significance) of 106 for UA.

3.2. PLIR Method. Treatment with GTE significantly increased PLIR of monocytes and granulocytes at T3, whereas a nonsignificant increase was observed for PLIR of lymphocytes (Table 1). We calculated a sample size (to reach statistical significance) of 80 for PLIR of lymphocytes.

Pearson Product Moment Correlation revealed an inverse correlation of UA with PLIR L (CC = –0.383, $p = 0.0368$), PLIR M (CC = –0.474, $p = 0.008$), and PLIR G (CC = –0.545, $p = 0.001$) and a direct correlation of FRAP-UA with PLIR L (CC = 0.451, $p = 0.012$), PLIR M (CC = 0.398, $p = 0.029$), and PLIR G (CC = 0.434, $p = 0.016$).

3.3. Ratio of Oxidation of the Probe C11-BODIPY. Typical overlay dot plots of the four treatments used for PLIR calculation and ratio of fluorescence (FL1/FL2) on single cells, before GTE consumption and 3 hours after, are presented in Figures 1 and 2, respectively.

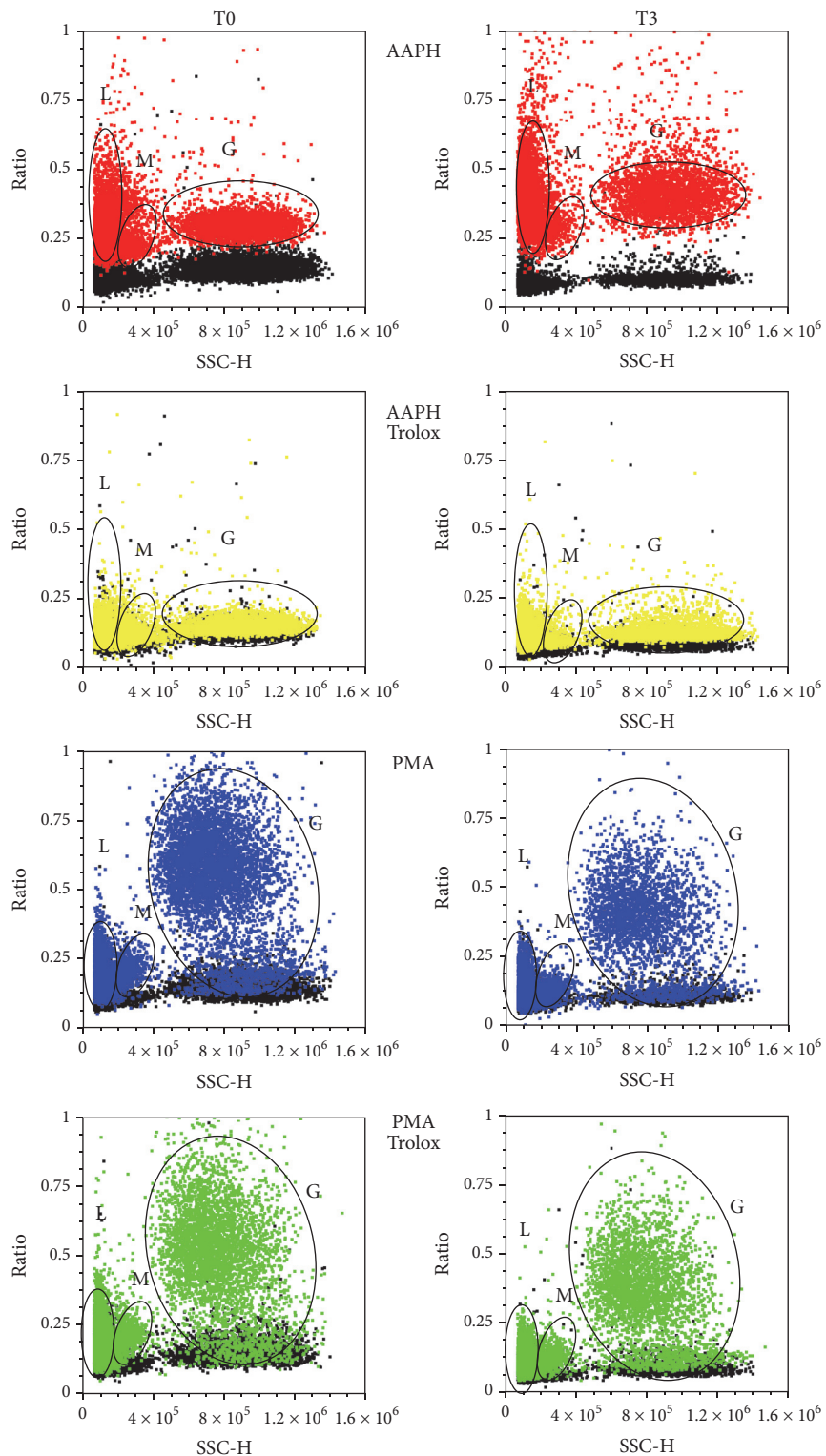


FIGURE 1: Typical overlay dot plots of ratio (ratio of oxidation of the probe C11-BODIPY: FL1/FL2) versus side scatter (SSC): before (T0) and 3 hours (T3) in leukocytes collected after a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). L: lymphocytes, M: monocytes, and G: granulocytes. Unstimulated samples (black) and leukocytes treated with 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH, 10 mM, red), AAPH (10 mM) + 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 μM, yellow), phorbol 12-myristate 13-acetate (PMA, 1 μg/mL, blue), or PMA (1 μg/mL) + Trolox (green).

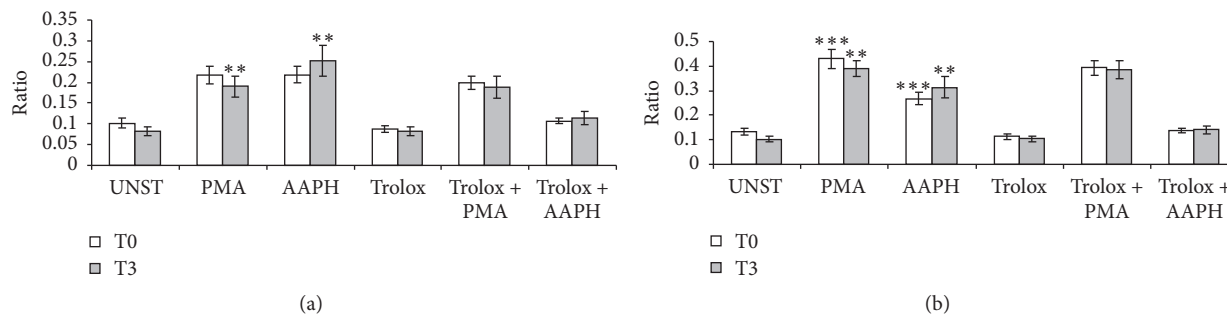


FIGURE 2: Ratio (ratio of oxidation of the probe C11-BODIPY: FL1/FL2) of monocytes (a) and granulocytes (b), in samples collected from 10 healthy subjects before (T0) and 3 hours (T3) after the consumption of a single dose of two capsules of a green tea extract (GTE) supplement (200 mg \times 2). Cells unstimulated (UNST) or treated with phorbol 12-myristate 13-acetate (PMA, 1 μ g/mL), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 10 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 μ M), Trolox + PMA, or Trolox + AAPH. Two-way (2W) RM ANOVA, with time and treatment as within-subjects factors, followed by Student-Newman-Keuls post hoc analysis: AAPH versus PMA within time: ** $p < 0.01$, *** $p < 0.001$.

Trolox inhibited the peroxidation of C11-BODIPY in leukocytes exposed to AAPH free radicals generating system, but not the PMA-induced oxidation in monocytes and granulocytes, both at T3 and at T0 (Figure 1).

Considering the major components of PLIR affected by treatment, compared to baseline, the AAPH-induced (exogenous) oxidation appeared greater, whereas the PMA-induced oxidative burst appeared lower (Figure 1).

Despite the differences of ratio PMA and ratio AAPH between times did not reach significance, the statistical significance between ratio AAPH and ratio PMA was different at baseline and at T3 on granulocytes and monocytes (Figure 2). We calculated a sample size (to reach statistical significance between times) of 17 and 33 for ratio AAPH and of 21 and 51 for ratio PMA, for granulocytes and monocytes, respectively.

Ratio PMA was not related to neither UA nor FRAP-UA, whereas ratio AAPH was inversely correlated with UA on all cells (L: CC = -0.477 , $p = 0.007$; M: CC = -0.514 , $p = 0.003$; G: CC = -0.511 , $p = 0.003$), but not with FRAP-UA.

4. Discussion

4.1. Effect of GTE on Plasma Antioxidants. Previous studies reported decreased, increased, or unchanged UA and NEAC levels after bolus consumption of EGCG or GTE [18–22]. EGCG increased in plasma from 30 min to 2.6 hours after GTE consumption, depending on the dose and on the formulation [21–25]. However, the FRAP value did not increase when free EGCG concentration was at its peak [21], probably due to the decrease in UA levels observed after GTE consumption [22].

In agreement with these results, in our study GTE consumption did not affect FRAP values whereas a nonsignificant decrease in UA and a significant increase in FRAP-UA were found 3 hours after treatment. The increase in FRAP-UA, probably due to the catechins, could counterbalance the reduction in FRAP induced by the UA decrease. However, the FRAP assay matches the antioxidant capacity to the reducing ability [15] and the reduced iron is critical in the onset of oxidative stress due to the Fenton reaction that generates the hydroxyl radical initiator of lipid peroxidation [26].

Therefore, an increase in the metal reducing power could be more likely detrimental than beneficial.

4.2. Effect of GTE on AAPH-Induced Lipoperoxidation. The increase in FRAP-UA was temporally associated with an increased oxidation of the fluorescent probe C11-BODIPY incorporated into the leukocytes. In this context, the prooxidant effects of tea catechins on cells are supported by the molecular mechanisms involved in their induction of antioxidant enzymes, through the antioxidant responsive elements (ARE) pathway [11, 27]. In particular, it has been suggested that some derivatives of catechins can oxidize highly reactive cysteine thiol groups of Kelch-like ECH-associated protein-1 (Keap1), resulting in disulfide bond formation and nuclear factor-erythroid 2-related factor 2 (Nrf2) release [27].

However, the ratio AAPH was inversely correlated with UA on all cells, but not with FRAP-UA. In agreement with our results the consumption for 112 days of a lutein (12 mg/d) plus GTE (200 mg/d) supplement did not reduce the oxidation of the C11-BODIPY incorporated into the lipid compartment of plasma [28].

4.3. Effect of GTE on PMA-Induced Oxidative Burst. We have found a nonsignificant decrease of PMA-induced lipoperoxidation after GTE consumption, contrarily with the increase of the oxidative burst of granulocyte, observed in cyclists when quercetin was administered with the tea flavanol epigallocatechin 3-gallate, by using dihydrorhodamine 123 (DHR123) as fluorescence probe [29]. However, the post-exercise-induced decrease in oxidative burst was unaffected after bolus consumption of the same supplement [29, 30], when hydroethidine was used as probe [30]. Therefore our results confirm that the plasma membrane C-11 BODIPY is a suitable probe in the evaluation of the effects on the oxidative burst of flavonoids, which increase DHR123 accumulation [31]. Though the effect of GTE consumption on oxidative burst requires more subjects to reach statistical significance, our results are in agreement with the reduction of the p22phox subunit of the NADPH oxidase observed in hemodialysis patients after 6 months of treatment with GT [32].

4.4. Effect of GTE on PLIR. In a postprandial study [17], we observed that a functional food covered by dark chocolate and containing glucomannan, inulin, fructooligosaccharides, and *Bacillus coagulans* strain GanedenBC30 significantly improved postprandial metabolic stress (insulin, glucose, and triglycerides), reduced the postprandial increase of UA, and improved PLIR of lymphocytes, but not of monocytes and granulocytes. We suggested that, although PLIR is a functional index that is independent of baseline levels of oxidation, measuring the ratio between the resistance to exogenous and the resistance to endogenous ROS injury, this ratio calculation could mask the effect of foods that inhibit both the exogenous ROS injury and the oxidative burst [17].

On the contrary, in the present study, treatment with GTE significantly increased PLIR of monocytes and granulocytes at T3 after ingestion, whereas a nonsignificant increase was observed for PLIR of lymphocytes. An inverse correlation of UA with PLIR and a direct correlation of FRAP-UA with PLIR of all leukocytes were found. Therefore, though some of the protective effects of catechins have been ascribed to their capability to reduce excessive UA level [12], in our study the inverse correlation of PLIR with UA levels, in particular with the ratio AAPH component of PLIR, confirms that UA is a major circulating antioxidant as suggested by Fabbrini et al. [33]. Authors [33] reported that rasburicase treatment, in subjects who had high serum UA concentrations, caused a marked decrease in plasma FRAP and a significant increase in urinary isoprostanes/creatinine ratio and in skeletal muscle protein carbonylation.

On the other hand, though the effects of GTE consumption on oxidative burst and AAPH-induced lipoperoxidation require more subjects to reach statistical significance, the statistical significance between ratio AAPH and ratio PMA was different at baseline and at T3 on granulocytes and monocytes. Therefore, the ratio based calculation of the PLIR is able to appreciate differences also with a low number of subjects in monocytes and granulocytes.

5. Conclusion

In conclusion our study suggests that PLIR, in particular PLIR of granulocytes where the differences between ratio AAPH and ratio PMA are more evident, reveals the prooxidant effect of GTE. The direct correlation of FRAP-UA with PLIR suggests that the iron reducing power of GTE could be more likely detrimental than beneficial. This result is in agreement with the FDA, the EFSA, and the DSI EC conclusions [1, 9]. Furthermore, the inverse correlation of UA with PLIR confirms the primary role of UA in the antioxidant defences. Therefore, PLIR could be a sensitive marker of redox status.

Abbreviations

AAPH:	2,2'-Azobis(2-methylpropionamidine) dihydrochloride
C11-BODIPY:	4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid

DHR123:	Dihydrorhodamine 123
DSI EC:	Supplement Information Expert Committee
EFSA:	European Food Safety Administration
FDA:	Food and Drug Administration
FRAP:	Ferric reducing antioxidant potential
GT:	Green tea
GTE:	Green tea extract
NEAC:	Nonenzymatic antioxidant capacity
PLIR:	Peroxidation of leukocytes index ratio
PMA:	Phorbol 12-myristate 13-acetate
Trolox:	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UA:	Uric acid.

Ethical Approval

Approval for the study was obtained from the Ethics Committee for Human Non-Clinical Research of the Department of Physiology and Pharmacology "V. Erspamer," "Sapienza," University of Rome, and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000.

Consent

Written informed consent was obtained from all the participants in accordance with the Italian law (law number 196/2003, Ministry of Health Circular Letter GU number 76/2008).

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Ilaria Peluso designed the research, analyzed the data, and drafted the paper. Hussein Manafikhi, Anna Raguzzini, and Raffaella Reggi performed the analyses. Yaroslava Longhitano and Christian Zanza performed the blood sampling. Maura Palmery critically reviewed the paper and supervised the whole project.

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