Original Research

Polyphenols enhance total oxidant-scavenging capacities of human blood by binding to red blood cells

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Abstract

The present study offers a new look at the role of erythrocytes and of erythrocytes–polyphenol complexes as potent 'sinks' for reactive oxygen species. We hereby show that human erythrocytes have the capacity not only to carry oxygen, but also to bind avidly to their surfaces a large variety of polyphenol antioxidants, which endows upon such complexes enhanced total oxidant-scavenging capacities (TOSC). This was proven by using confocal microscopy, 2,2-diphenyl-1-picrylhydrazyl radical, Folin-Ciocalteu's reagent, cyclic voltammetry and chemiluminescence techniques. The results presented suggest that the true TOSC of blood is the sum of intracellular antioxidants of red blood cells and other blood cells (mainly due to catalase), the polyphenols bound to their surfaces and the antioxidant agents present in plasma. Since erythrocytes can avidly bind and rapidly remove circulating polyphenols, the rule of the thumb to quantify antioxidants in health and disease processes exclusively in plasma as customary in clinical settings, does not represent the true TOSC of whole blood. We also postulate that circulating erythrocytes and possibly also other blood cells might be constantly coated by polyphenols from supplemented nutrients, which act as antioxidant depots and can thus act as protectors against the harmful consequences of oxidative stress. Further studies are needed to determine the faith of polyphenols in the circulation and their sequestration in the spleen.

Keywords: erythrocytes, plasma, polyphenols, total oxidant-scavenging capacity, antioxidant supplementation

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Introduction

There is general consensus that oxidative stress plays a pivotal role in the pathophysiology of cardiovascular disorders, diabetes, atherosclerosis, neurodegenerative disorders, asthma, cataract, irritable bowel syndrome and in senescence.[1] Therefore, there is a clinical importance to evaluate the total oxidant-scavenging capacities (TOSC) of body fluids and cells in these disorders. If found significantly low, it might be considered to recommend antioxidant supplementation, which might protect against further cell and tissue damage.[2] Plant polyphenols with more than 8000 identified compounds represent one of the largest and most ubiquitous agents in the human diet. It has been reported that consumption of polyphenol-rich foods is associated with lower incidences of heart disease, ischemic stroke, cancer and other chronic manifestations.[3–5] An example for the beneficial nutritional protective effects against the onset of cardiovascular disorders is the Mediterranean diet and the famous paradigm of the 'French Paradox'.[6] However, the use of antioxidants to treat various clinical disorders may not be as simple as it originally sounded.[7] One reason is that even after hefty meals, rich in polyphenol antioxidants, their maximal plasma concentrations reach levels not greater than 1–7 μmol/L.[8] Also, chronic long-term consumption of flavonoid-rich foods, including the polyphenol quercetin, failed to accumulate significantly in plasma (1 μmol/L).[9] This low bioavailability is obviously not expected to be of any value to prevent oxidation of plasma lipids.[10] Therefore, it severely limits the capability of dietary polyphenols to act as significant antioxidants in plasma *in vivo*, especially when compared with the much higher levels of other antioxidants (e.g. vitamin C, uric acid and vitamin E) present in plasma at high steady-state concentrations (15–450 μmol/L).[11,12] The low levels of polyphenols in plasma might either be due to an extensive metabolism in the gastrointestinal (GI) tract or to strict regulatory mechanisms developed over evolution intended to prevent the toxic effects of flavonoids. This might also be due either to their

pro-oxidant properties, mitochondrial toxicity or to their interactions with drug-metabolizing enzymes.[13] Lotito and Frei[14,15] suggested that the increased plasma antioxidant capacity in humans, following consumption of polyphenol-rich foods (e.g. fructose in apples is a known stimulator of purine metabolism), did not result in an increase in plasma levels of polyphenols but was due to enhanced uric acid levels.

In a recent study,[16] it was shown that the overall antioxidant capacities of cells in culture could not be enhanced by supplementation with exogenous antioxidants pointing to a tight regulation analogous to that of acid/base balances.[17] Thus, it is not clear whether plant polyphenols might have any direct antioxidant effects in plasma, although they might be capable of exerting such effects locally within the oral cavity or in the GI tract.[18] It is therefore intriguing whether long-term supplementation with large amounts of polyphenol antioxidants might enhance TOSC of body fluids or of cells.[1] Moreover, it was also reported[19] that supplementation of large amounts of the dietary antioxidants vitamin E and β-carotene in humans failed to affect cardiovascular disorders and even significantly enhanced all-cause mortality.

To shed light on the possible beneficial role of antioxidant polyphenols as protectors against oxidative stress, we have recently proposed[20] that there might be a way to increase TOSC of microbiota and of mammalian cells due to their ability to bind avidly polyphenol antioxidants to their surfaces. Many polyphenols, present in colored vegetables and in fruit beverages, were found to form stable complexes with a variety of Gram-positive, Gram-negative, anaerobic microbial species from the oral cavity and of *Candida albicans*, rendering upon such complexes a marked enhanced TOSC.[20] Therefore, the possibility was explored that similarly to microorganisms, blood cells (mainly red blood cells [RBC]) in the circulation might also avidly bind polyphenols to their surfaces resulting in enhanced TOSC.

It is paradoxical that although both RBC and plasma possess strong antioxidant capacities, the quantification of TOSC in various clinical disorders is exclusively performed either in plasma or in serum, but surprisingly not in whole blood. Human erythrocytes possess antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase/reductase and NADH–metHgb reductase) and non-enzymatic antioxidant agents (lipophilic vitamin E, carotenoids, ubiquinon, melatonin, etc. and water-soluble vitamin C, glutathione, uric acid, ceruloplasmin, transferrin, haptoglobulin, etc.).[21] On the other hand, plasma is rich in albumin and in low molecular weight antioxidants (LMWA) glutathione, ascorbate, α-tocopherol, uric acid and bilirubin. Therefore, it stands to reason that the TOSC of whole blood is the sum of the intracellular antioxidants present in RBC and in other blood cells, the antioxidants bound to their surfaces[20] and those found in plasma. This probably allows blood cells and plasma antioxidants to act in concert to scavenge reactive oxygen species (ROS) in the circulation.

In the present communication, we argue that since erythrocytes can form stable complexes with polyphenols, they acquire the ability to act in concert with LMWA and with albumin to enhance TOSC. It is also postulated that human erythrocytes play a pivotal role in the distribution and bioavailability of circulating polyphenols and in protection against cell damage induced by ROS. This implies that the minute quantities of polyphenols detected in plasma do not reflect the true amounts of polyphenols which gain access to the circulation, since these agents might also be complexed with circulating erythrocytes, removed and therefore not accounted for if TOSC is tested exclusively in plasma. This clearly justifies that the quantification of the true total antioxidant capacities should be quantified not in plasma or serum, but in whole blood.

Material and methods

Biochemicals

Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St Louis, MO, USA). Resveratrol, tannic acid, gallic acid, caffeic acid, morin, rutin, catechin, epigallocatechin gallate, nordihydroguaiaretic acid, curcumin, hesperitin, quercetin and luminol were first dissolved in absolute ethanol at 100 mmol/L and then further diluted in normal saline. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH; Sigma-Aldrich) was dissolved in methanol. Human serum albumin (HSA), sodium selenite, $CoCl_2 \cdot 6H_2O$, morpholino syndonimine (SIN-1, a donor of nitric oxide, superoxide and peroxynitrite), glucose oxidase (GO, a generator of H_2O_2), *N*-ethylmaleimide (a glutathione peroxidase inhibitor), sodium azide (a catalase inhibitor) and superoxide dismutase (SOD) were all prepared in normal saline. Folin-Ciocalteu's phenol reagent, phosphate-buffered saline (PBS) (pH 7) 4% formaldehyde (for RBC fixation) and Dulbecco's PBS/glycerol 80% mixture with 2% DABCO (as mounting solution in confocal microscopy analysis) were also used. Red wine was obtained from a local market. Hank's balanced salt solution (HBSS) was obtained from Beit Haemek Industries, Israel.

Blood preparations

Freshly collected non-fasting human blood in EDTA was obtained by consent from 11 healthy volunteers. By using each sample as its own internal control, the authors were capable of reducing individuals' daily diet variabilities and increasing the significance of this model. To prepare complexes between whole blood and polyphenols, 100 μL samples of blood ($\sim 5 \times 10^8$ erythrocytes) were first incubated for 10 min at room temperature with various amounts of the polyphenols (100 μmol/L for single compounds and 10 μL for beverages) and then washed three times by centrifugation at 500 *g* for five minutes to remove unbound agents. The sedimented cells were re-suspended in 0.5 mL of HBSS and were analyzed for their antioxidant capacities by the various analytical methods described below. The same amounts of erythrocytes pellets were also lysed by exposing them to 0.5 mL of sterile distilled deionized water. Whole plasma was obtained following centrifugation of whole blood. A similar procedure was

carried out in experiments with washed erythrocytes. In this case, whole blood was first centrifuged at 500 *g* for five minutes; the pelleted cells were washed in saline to remove plasma, exposed to polyphenols, washed three times by centrifugation at 500 *g* for five minutes to remove unbound agents and were then re-suspended in HBSS and analyzed.

Confocal laser microscopy

A computerized confocal laser microscope (Zeiss Confocal Axiomate LSM410), using ×63 objective with double excitation at 410 and 543 nm, was used to visualize the autofluorescence of washed RBC exposed to polyphenols. The polyphenol-coated erythrocytes were fixed by 4% formaldehyde in buffered PBS (pH 7), placed on a microscope slide to which a mounting medium, consisting of PBS/glycerol 80% and 2% DABCO was added, sealed with micro-cover glasses and then scanned by the confocal microscope. The visual images (phase and differential interference contrast according to Nomarski) and the fluorescent slice scans were processed for overlap slice summation or three-dimensional presentation.

Folin-Ciocalteu's phenol reagent

The Folin-Ciocalteu's reagent assay was modified and used for the colorimetric measurements of phenolic and polyphenolic antioxidants absorbed upon erythrocytes in whole blood.[22] Briefly, to 600 μL normal saline, whole blood erythrocyte suspensions ($\sim 2 \times 10^8$ cells) and 100 μmol/L of polyphenols were incubated for five minutes. This was followed by washing the suspensions to remove unbound agents and by the addition of 50 μL of Folin reagent. Five minutes later, 150 μL of a saturated sodium carbonate solution (25% w/v) were added. After an additional five minutes at room temperature, the test tubes were centrifuged at 500 *g* for two minutes and the optical densities in the supernatant fluids were determined at 760 nm. The amounts of polyphenols bound to the washed RBC were expressed as gallic acid equivalents (GAE).

Luminol-dependent chemiluminescence assay

The quantification of the TOSC of whole blood, washed RBC and of complexes formed between RBC and polyphenols was performed with a chemiluminescence assay.[23,24] This assay is based on the ability of a large variety of antioxidant agents to quench, in a dose-dependent manner, the luminescence generated by a special 'cocktail'. It is comprised of a mixture of luminol (10 μmol/L), glucose oxidase (2.3 U/mL), sodium selenite (IV) (2 mmol/L) and $CoCl_2 \cdot 6H_2O$ (II) (10 μmol/L), which when added in this sequence to 950 μL HBSS, at room temperature, generates a constant flux of H_2O_2 and of cobalt-catalyzed hydroxyl radical ($OH^{\bullet}$).[25] The luminol-dependent chemiluminescence (LDCL) generated was measured by a Lumac 2500M Biocounter (Landgraaf, The Netherlands) and expressed as counts per minute (cpm). The TOSC of the various agents tested is expressed as their ability to quench the luminescence generated by the cocktail due probably to both their antioxidant properties and chelating activities for cobalt (II).

Differential pulse voltammetry assay

An electrochemical method was used to measure the reducing capacity of erythrocytes coated by various polyphenols. A BAS model CV-1B cyclic voltammeter (West Lafayette, IN, USA) was used.[26] Differential pulse voltammetry (DPV) tracing was recorded at a range of 0 to 1.0 V and a rate of 100 mV/s versus an Ag/AgCl reference electrode. The sensitivity of the cyclic voltammeter was set to $1 \times e^{-5}$ (A/V). A three-electrode system was used throughout the study. The working electrode was a glassy carbon disk (BAS MF-2012) of 3.2 mm diameter. A platinum wire served as the counter-auxiliary electrode. The working electrode was polished prior to each measurement with a polishing kit (BAS-PK-1). DPV tracings were analyzed to determine peak potential ($E_{1/2}$) and anodic current (I_a). This potential (x-axis) is typical for specific tissues or compounds, and represents the ability of the samples to donate electrons to the working electrode. The I_a (y-axis) correlates with the concentration of the reducing equivalents. By this method, the current is measured at different times of the duration and the effect of the charging current is minimized. Therefore, a higher sensitivity can be achieved compared with standard cyclic voltammetry measurements.[26]

DPPH assay

The antioxidative potencies of whole human blood, plasma, washed erythrocytes and of complexes formed between RBC and polyphenols were also tested by the modified spectrophotometric DPPH assay.[27] DPPH, a purple-colored stable free radical, is reduced to the yellow-colored diphenylpicryl hydrazine. We used this assay to quantify the TOSC of whole blood, erythrocytes and plasma (Figure 1b), and also of complexes of erythrocytes with various polyphenols agents at 100 μmol/L concentration (Figures 3c and 5c). For whole blood, plasma and erythrocytes measurements, 50 μL of saline were added either to increasing amounts (1–25 μL) of whole blood, plasma (diluted 1:1) or washed RBC (an equivalent amount of erythrocytes present in whole blood samples). This was followed by the addition of 50 μL of DPPH solution (from 1 mmol/L stock). Two minutes later, 800 μL of absolute methanol were added, the samples were vortexed, kept at room temperature for two minutes and then centrifuged at 1500 *g* for two minutes. The supernatant fluids' absorbance was measured at 518 nm in a Cecil 1011 spectrophotometer (Cecil, London, UK). For polyphenols–RBC complexes measurements, 2.5×10^8 erythrocytes (washed or from whole blood) were pretreated for five minutes with 100 μmol/L amounts of polyphenols in saline solution and washed several times with normal saline to remove unbound agents. The pelleted cells were re-suspended in 200 μL of HBSS. Fifty microliters of the cells (approx. 8×10^7 erythrocytes) were mixed with 50 μL of DPPH solution (from 1 mmol/L stock). Two minutes later, 800 μL of

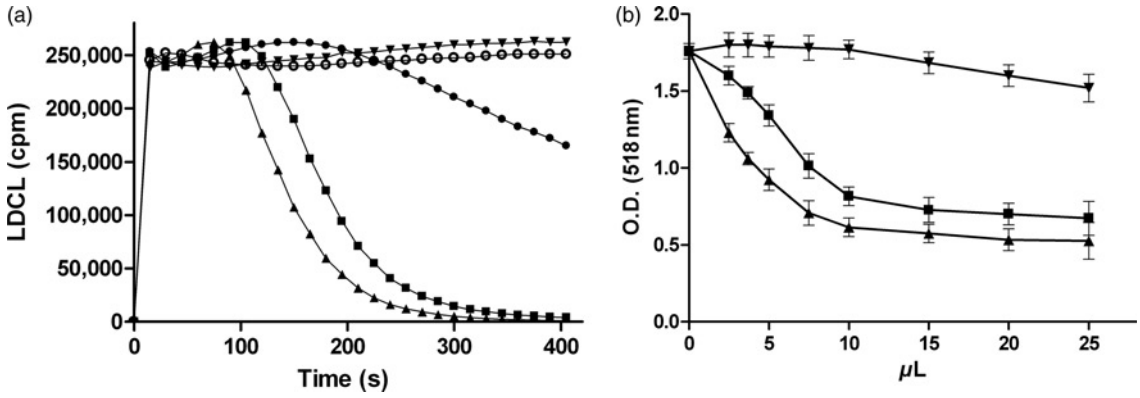

Figure 1 Whole blood TOSC quantification as measured by the luminescence and DPPH methods. LDCL generated by the GO cocktail (a) and DPPH assay patterns (b) under the effects of whole blood —▲— (5 μL, approx. 2.5×10^7 erythrocytes), plasma —▼— (2.5 μL), washed RBC —■— (~2.5×10^7 erythrocytes) and whole blood with sodium azide —●— (1 mmol/L). GO cocktail curve is marked as open circle —⊖—. Note the bell-shaped curve of luminescence (a) induced by whole blood and by washed RBC, the lack of inhibition of luminescence by plasma and the reversal of inhibition of luminescence by the catalase inhibitor sodium azide. In both assays, $n = 4$ (representative curves for LDCL assay). TOSC, total oxidant-scavenging capacities; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; LDCL, luminol-dependent chemiluminescence; GO, glucose oxidase; RBC, red blood cells

absolute methanol were added, the samples were vortexed, kept at room temperature for two minutes and then centrifuged at 1500 *g* for two minutes. The supernatant fluids' absorbance was measured at 518 nm in a Cecil 1011 spectrophotometer (Cecil), using a gallic acid calibration curve and calculated as GAE.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Results are presented as the mean ± standard deviation. Unpaired Student's *t*-test was performed as specified in the legends of figures (differences were considered significant when $P < 0.05$).

Results

Blood as a scavenger of ROS

Quantification of TOSC of whole blood by the LDCL and DPPH methods

Using the LDCL assay, Figure 1a shows that while the flux of luminescence induced by the GO cocktail is kept at a high steady level for more than six minutes, 5 μL of whole blood (approx. 2.5×10^7 erythrocytes) failed to affect luminescence for about two minutes. However, this was then followed by a sharp and steady decline in luminescence due to the consumption of the bulk of oxidants generated, yielding a typical bell-shaped curve of light emission. It was also found that light quenching by whole blood paralleled the consumption of the bulk of H_2O_2 generated by the GO cocktail as determined quantitatively by the Thurman assay[28] (not shown). Since no hemolysis and release of intracellular catalase from RBC occurred, the lag of about two minutes observed before luminescence started to decrease, was attributed to the time needed for the ROS generated by the cocktail to freely diffuse through the RBC membrane to later undergo decomposition by intracellular antioxidants. Figure 1a also shows that since light quenching by RBC was significantly prevented by sodium azide (a potent catalase inhibitor), but neither by *N*-ethylmaleimide (a glutathione peroxidase inhibitor) nor by SOD (not shown), it indicated that intracellular catalase was probably the main scavenger of ROS, generated by the cocktail. However, in contrast to the results obtained with the ROS-generating GO cocktail, whole blood totally failed to quench luminescence generated by the SIN-1 cocktail,[23,24] which mainly generates nitric oxide, superoxide and peroxynitrite (not shown). Using the DPPH assay, Figure 1b shows similar patterns of reducing properties for whole blood, plasma and erythrocytes compared with the patterns shown in Figure 1a. DPPH and LDCL results confirm that different blood ingredients contribute to the TOSC of whole blood.

Role of RBC lysates as scavengers of ROS

As indicated above, quenching of LDCL by whole blood was not accompanied by any visible hemolysis. However, it is expected that in various pathological conditions, hemolysis of RBC can occur, resulting in the release into the surrounding medium of the bulk of the intracellular antioxidants of RBC (mostly catalase). Contrary to intact RBC, hemolysates might rapidly scavenge ROS and thus change the TOSC of the cell surroundings. Figure 2 shows that while about two minutes were needed for 2.5 μL of whole blood (approx. 1.25×10^7 erythrocytes) to commence quenching of LDCL generated by the GO cocktail, hemolysates derived from RBC ($0.25–1.25 \times 10^7$ cells) induced a very significant immediate inhibition of luminescence, indicating that the intracellular antioxidants released were able to scavenge ROS rapidly. Similarly to the results with intact RBC (Figure 1a), neither *N*-ethylmaleimide nor SOD could prevent the luminescence decrease (not shown), again pointing to the major role played by catalase as the main scavenger of ROS. Thus, both intact RBC and RBC hemolysates probably act as protectors of other cells against oxidative stress.[29]

To test the nature of the agents in plasma responsible for acting in concert with RBC, plasma containing mainly the antioxidants albumin and LMWA was subjected to

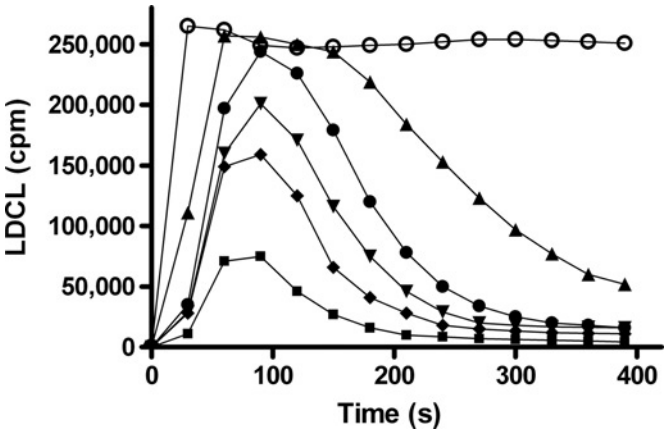

Figure 2 Role of RBC lysates as scavengers of reactive oxygen species. LDCL, generated by the GO cocktail, under the effects of whole blood —▲— (2.5 μL; approx. 1.25×10^7 erythrocytes) and by increasing concentrations of RBC lysates (1, —●—; 1.5, —▼—; 2, —◆—; and 2.5 μL, —■— lysates from whole blood; approx. $0.25–1.25 \times 10^7$ erythrocytes, respectively). GO cocktail curve is marked as open circle —○—. Note that compared with whole blood, a much stronger and faster inhibition of LDCL is induced by the lysates, presumably due to the rapid release of intracellular catalase. Representative experiment ($n = 4$). LDCL, luminol-dependent chemiluminescence; RBC, red blood cells; GO, glucose oxidase

an exhaustive dialysis against normal saline for 15 h at 4°C. It was found that removal by dialysis of LMWA did not diminish the activity of plasma, indicating that LMWA were not significantly involved in the quenching of luminescence (not shown). This assumption was also supported by experiments (not shown) showing that while a mixture of LMWA (ascorbate, uric acid, bilirubin, α-tocopherol, reduced glutathione), at the concentrations normally found in plasma, failed to quench luminescence, the antioxidant activity of dialyzed plasma remained unaltered, indicating the role of albumin as the major plasma antioxidant.

Polyphenols binding to erythrocytes

In previous communications,[23,24] it was shown that a large assortment of polyphenols and antioxidants in colored fruit beverages (red wind, cranberry, tea, coffee, pomegranate) were able to inhibit strongly LDCL generated by the GO cocktail and also to avidly and irreversibly bind to surfaces of microbial and yeast cells. This endowed upon such complexes enhanced TOSC, which could also be proven by using other analytical methods.[20] It was, therefore, of interest to establish whether RBC could also bind polyphenols to enhance their TOSC and whether RBC–polyphenol complexes might act in concert with plasma proteins to enhance TOSC and the scavenging of ROS.

Polyphenols binding to erythrocytes in whole blood

Using the luminescence technique, Figure 3a shows that incubation of whole blood with eight antioxidant agents followed by removal of unbound materials by washing resulted in a decrease in luminescence. It indicated that RBC in whole blood could bind polyphenols and that gallic acid, curcumin and resveratrol were the most potent polyphenols attached. In addition, using the Folin-Ciocalteu's and

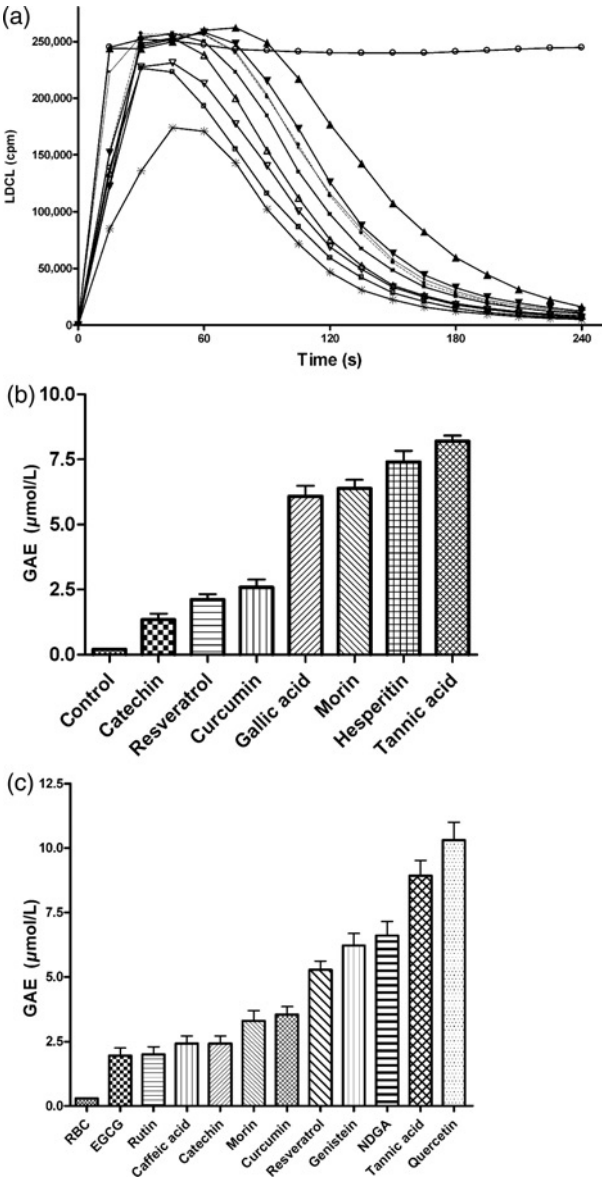

Figure 3 TOSC analysis of polyphenols binding to erythrocytes in whole blood using LDCL (a), Folin-Ciocalteu's phenol reagent (b) and DPPH (c) methods. Luminescence, generated by the GO cocktail (—○—), under the effects of whole blood (—▲—), treated by the polyphenols morin (—▼—), catechin, red wine, hesperitin, tannic acid, resveratrol, curcumin and gallic acid (—✳—), respectively (a) and GAEs of polyphenols bound to RBC, as determined by the Folin-Ciocalteu's phenol reagent (b) and DPPH (c) methods. Fifty microliters of whole blood (2.5×10^8 erythrocytes) were pretreated for five minutes with 100 μmol/L amounts of polyphenols. The reaction mixtures were washed several times with normal saline to remove unbound agents and the pelleted cells were re-suspended in 200 μL of HBSS. Fifty microliters of the cells (approx. 8×10^7 erythrocytes) were used for LDCL analysis. For Folin assay approx. 8×10^7 erythrocytes were mixed with 50 μL of Folin reagent, followed two minutes later by the addition of 150 μL of saturated sodium carbonate solution. For DPPH assay, 50 μL washed erythrocytes (originally from whole blood–polyphenol mixtures) were used as described in Materials and methods. Note that all polyphenols tested demonstrated a higher TOSC of the erythrocyte–polyphenol complexes compared with uncoated RBC ($n = 4$, $P \leq 0.05$). Representative experiment ($n = 4$) for luminescence assay. However, different polyphenols presented different TOSC values, suggesting different analysis mechanisms and various affinities of polyphenols to erythrocytes membranes. TOSC, total oxidant-scavenging capacities; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; LDCL, luminol-dependent chemiluminescence; GO, glucose oxidase; RBC, red blood cells; GAE, gallic acid equivalents

DPPH assays, Figures 3b and c show the intensity of binding of the various agents to the erythrocytes in whole blood, expressed as GAEs. These findings indicate that polyphenols are able to bind avidly to human erythrocytes and also to platelets and lymphocytes in whole blood (not shown, to be published) to enhance cells' TOSC.

Polyphenols binding to washed erythrocytes

To demonstrate the capacity of polyphenols to bind to washed erythrocytes surfaces, we employed a fluorescence confocal microscopy technique. Figure 4 shows that all four autofluorescing polyphenols: morin, resveratrol, curcumin and tannic acid were bound to the RBC.

The binding of polyphenols to washed erythrocytes was also confirmed by additional methods. Figure 5a shows the luminescence patterns of complexes formed among RBC and eight different polyphenols agents. The most potent quenchers of luminescence in a descending order were wine/tannic acid, curcumin, resveratrol and morin. The binding of the same polyphenols to erythrocytes in whole blood was not as strong as that shown for washed RBC due probably to their binding to plasma proteins, indicating the partial binding of polyphenols to human plasma constituents. Figure 5b shows the dose-dependent binding (100–500 μmol/L) of the polyphenol resveratrol to RBC, as measured by the luminescence assay and Figure 5c shows the binding of 11 polyphenols to RBC by the DPPH method (expressed as GAE).

Binding of polyphenols to the washed erythrocytes was also confirmed using the DPV method (see Materials and methods). Figure 6 shows four voltammograms of washed erythrocytes following incubation with the polyphenols morin, resveratrol, curcumin and tannic acid. When the polyphenol–erythrocyte complexes were analyzed, all four samples showed the typical anodic wave for each polyphenol (0.45 V for curcumin, 0.42/0.65 V for resveratrol, 0.19 V for morin and 0.15/0.27 V for tannic acid), indicating that the polyphenols remained attached to the erythrocyte surfaces to enhance their reducing capacities.

Discussion

The present communication offers a new look at the role of erythrocytes as oxidant 'sinks' capable of acting in concert with albumin, LMWA and with antioxidant polyphenols to scavenge ROS. It suggests therefore that the TOSC of whole blood should be expressed as the sum of the antioxidants' capacities in plasma and in erythrocytes. It is hypothesized that erythrocytes and possibly other blood cells in the circulation might always be coated, in a cumulative manner, by polyphenols, which gain access to the circulation from nutrients. This enhances the TOSC of whole blood, enabling it to deal more efficiently with oxidative stress processes.

The results presented address an important dilemma of whether or not consumption of antioxidants, especially polyphenols, has any significant value to counteract oxidative stresses. Antioxidant consumption has been critically evaluated and debated as a potential strategy to ameliorate various clinical disorders[30] and the phenomenon of RBC–polyphenol complexes formed *in vivo* might be a clue to their protective effects. This new approach takes into account that while RBC are rich in the intracellular antioxidants catalase, glutathione peroxidase and superoxide dismutase, which are considered as 'sinks' for toxic oxygen species,[1] plasma possesses the antioxidants albumin, uric acid, ascorbate, α-tocopherol, reduced glutathione, bilirubin and micromolar amounts of polyphenols. Therefore, the rule of the thumb to conveniently and exclusively use plasma or serum to quantify TOSC in various clinical disorders should be reconsidered since the antioxidant properties of RBC, other blood cells and also polyphenols bound to their surfaces are not accounted for. This implies therefore that cross-talk between antioxidants associated with blood cells and those antioxidant agents found in plasma represent the true TOSC of whole blood. The applicability of total antioxidant capacity data obtained from plasma measurements to human health issues was recently discussed.[31] It was suggested that plasma levels of urate, ascorbate and also α-tocopherol might represent better expression of TOSC, but the possible role of RBC and other blood cells in the antioxidant balance was not considered.

To further clarify the role of whole blood, RBC and complexes formed among RBC and polyphenols as scavengers of ROS, we have employed several analytical methods.

Measured by the luminescence assay, Figure 1a shows that the oxidant-scavenging capacity of whole blood is due to the combined effect of erythrocytes and plasma.

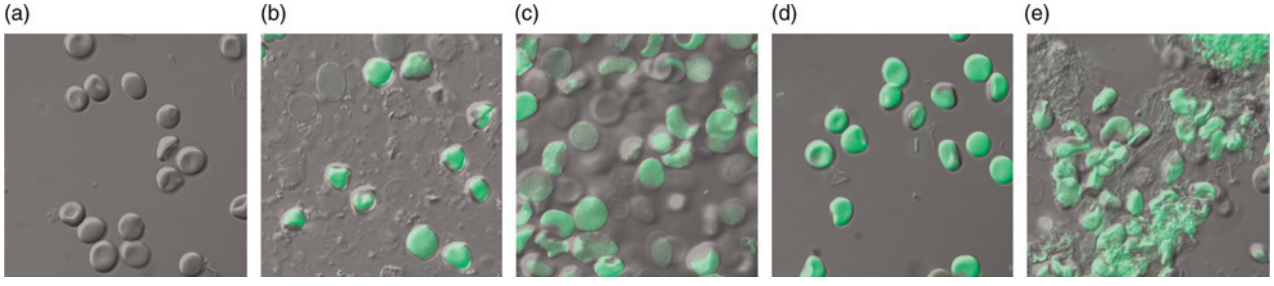

Figure 4 Confocal laser microscopy images of red blood cells, precoated by 100 μmol/L of four polyphenols, and then washed to remove unbound agents. The pictures presented represent untreated erythrocytes (a) and erythrocytes coated with morin (b), resveratrol (c), curcumin (d) and tannic acid (e) (for details see Materials and methods section). Note that polyphenol autoflorescence is shown in all four specimens, confirming the ability of polyphenols to bind to erythrocytes

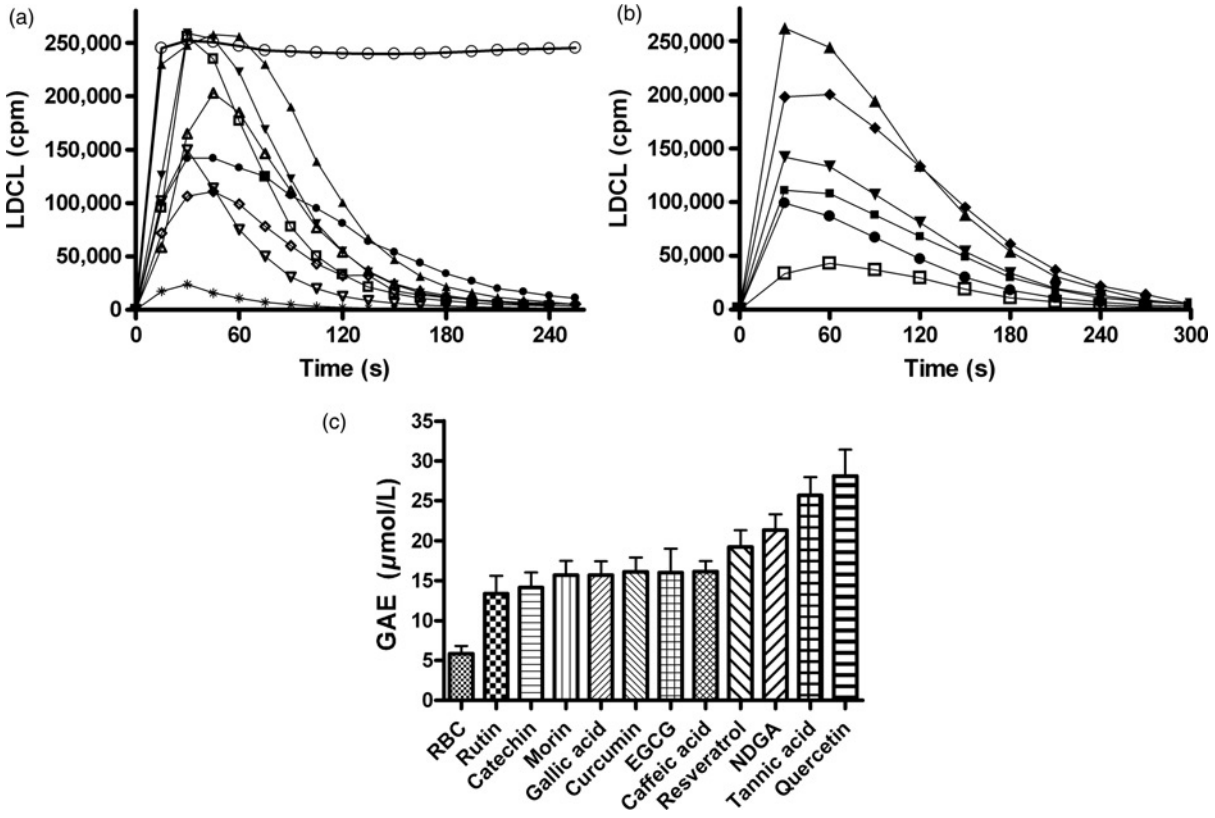

Figure 5 Effect of polyphenols on washed erythrocyte TOSC as measured by the luminescence and DPPH methods. Luminal-dependent chemiluminescence GO cocktail was used for assay of TOSC in various polyphenols agents (a) and several concentrations of the polyphenol resveratrol (b). DPPH assay (c) was also used for various polyphenols agents. Under the effects of washed erythrocytes, cells were pretreated by the different polyphenols at 100 μmol/L (a) (GO cocktail, —○—; plain RBC, —▲—; catechin/gallic acid, —▼—; hesperitin, —□—; morin, —△—; resveratrol, —●—; curcumin, —▽—; and by tannic acid/red wine (10 μL), —✳—) or by increasing concentrations of the polyphenol resveratrol (plain RBC, —▲—; 50 μmol/L, —◆—; 100 μmol/L, —▼—; 200 μmol/L, —■—; 300 μmol/L, —●—; 500 μmol/L, —⊟—) (b). (a and b) Decrease in luminescence, indicating polyphenols binding followed by enhanced antioxidant properties of the erythrocytes. Cells were washed extensively before testing. Binding of 11 polyphenols at 100 μmol/L to washed erythrocytes was also measured by the DPPH assay (c). Results for this assay are presented as GAE (μmol/L). Note that all polyphenols tested significantly increased TOSC of erythrocytes, but different polyphenols exerted different inhibitory effects when precoated with erythrocytes (a and c) ($n = 4$, $P \leq 0.05$). In addition, erythrocytes, precoated with resveratrol, showed a dose-dependent pattern using the LDCL method (b). TOSC, total oxidant-scavenging capacities; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; GO, glucose oxidase; LDCL, luminol-dependent chemiluminescence; RBC, red blood cells; GAE, gallic acid equivalents

It also suggests that intracellular catalase (inhibitable by azide) is probably the main intracellular antioxidant in RBC. The lag period (2–3 min) in the decline of luminescence (Figure 1a) can be explained on the grounds that both peroxide and $OH^{\bullet}$, generated by the cocktail, have first to traverse the erythrocyte membrane only to later undergo consumption by the intracellular antioxidants, mainly catalase. Thus, RBC antioxidants might function not only to decompose the accidental generation of peroxides species when hemoglobin interacts with oxygen[1] and as carriers of oxygen and removers of CO_2, but also as protectors of other cell types.[29] In addition, when combined with washed erythrocytes, albumin could totally replace plasma antioxidant properties (not shown). Albumin represents the major and predominant antioxidant in plasma[32] and a large proportion of total serum antioxidant properties can be attributed to this protein. The pivotal role of albumin as the major plasma antioxidant was also confirmed by exhaustively dialyzing fresh plasma to remove LMWA, which did not result in a decrease in the antioxidant activity of the dialyzed plasma (not shown). In addition, previous works have shown that more than 70% of the free radical-trapping activity of serum was due to HSA.[33] Therefore, it is also suggested that antioxidant status of whole blood also depends on the levels of the hematocrit, which might vary in different clinical disorders. The role of intracellular antioxidants as scavengers of ROS by whole erythrocytes is also demonstrated in lysed erythrocytes (Figure 2). It shows a much higher luminescence quenching, due to the release of the intracellular antioxidants of RBC (mainly catalase). This effect could also be reversed by blocking catalase action by azide. The data shown in Figures 1a, b and 2 also suggest that quenching of ROS by erythrocytes might have a dual function, as intact cells (a slow scavenging of ROS) or as an immediate catalase releaser from lysed cells, which are capable of protecting cells and body fluids against ROS. However, a publication by Wu *et al.*[34] has warned that the microenvironment can shift erythrocytes from a friendly to a harmful behavior. The release of iron-hemoglobin following RBC hemolysis can cause excess accumulation of free iron to catalyze the generation of the

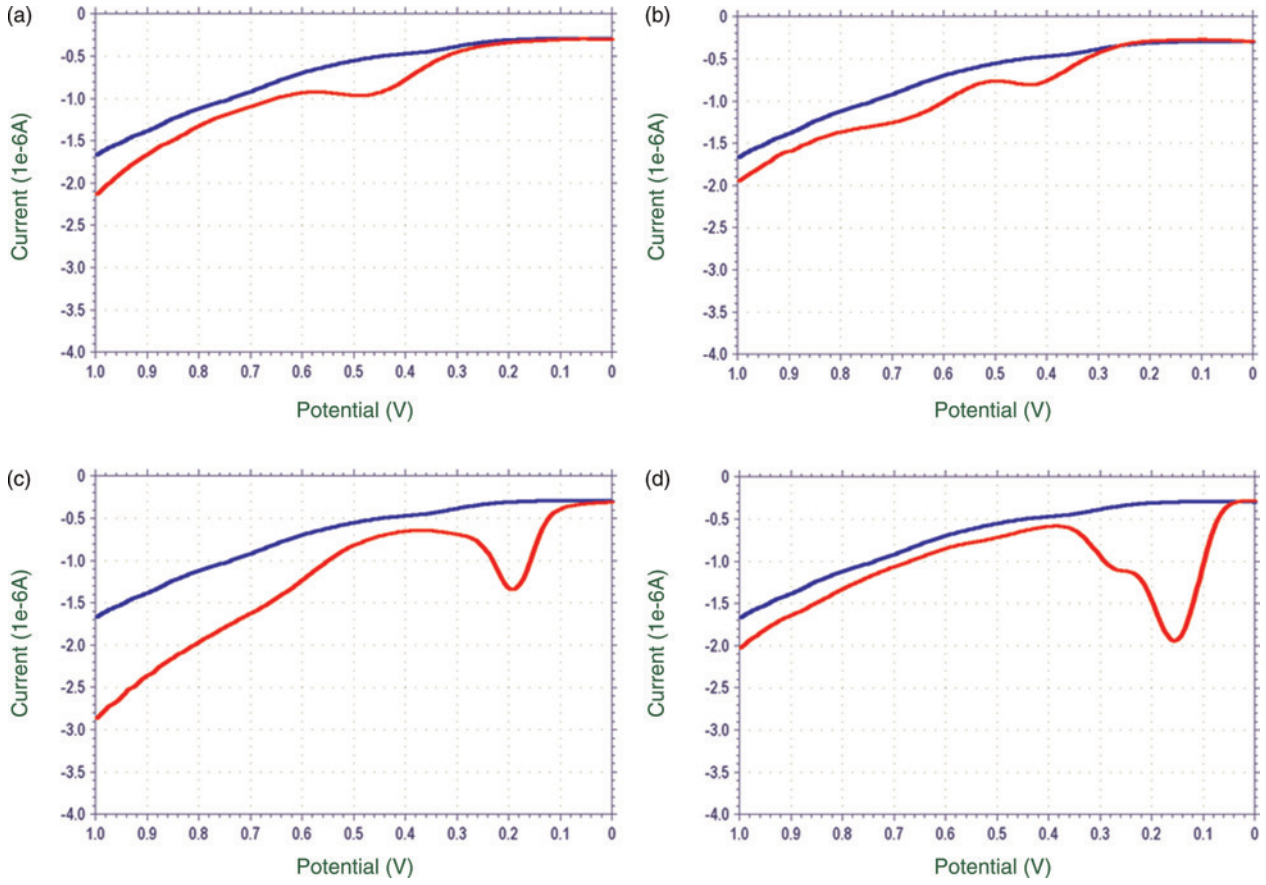

Figure 6 Four voltammograms of human erythrocytes complexed with curcumin (a), resveratrol (b), morin (c) and tannic acid (d) (100 μmol/L). Samples were analyzed using the differential pulse voltammetry analyzing technique (DPV) (see Materials and methods). Note that while uncoated erythrocytes did not show any anodic waves (upper curves), erythrocytes complexed with all four agents showed distinct and wide anodic waves (lower curves), indicating the firm bond between the polyphenols and red blood cells (RBC). These anodic waves appeared exactly at the same electrochemical potential displayed by the free polyphenols (not shown) (A color version of this figure is available in the online journal)

highly toxic OH$^{\bullet}$ or peroxyl/alkoxyl radicals. Therefore, by virtue of their antioxidant and chelating properties for divalent metals, both free and polyphenols bound to RBC (see below) might also act to neutralize the toxic effects of ROS.

In order to establish whether polyphenols and erythrocytes can form complexes, resulting in enhanced TOSC, we used several analytical methods. Since polyphenols possess autofluorescence properties, we used confocal fluorescence microscopy to prove the binding of polyphenols to RBC. Figure 4 shows the fluorescence patterns of the complexes formed between washed RBC and the polyphenols morin, resveratrol, curcumin and tannic acid. In addition, Figures 3a and c shows the degree of binding of various polyphenols agents to RBC in whole blood using luminescence and DPPH techniques, and Figure 3b shows the binding of seven polyphenols to RBC in whole blood by the Folin-Ciocalteu's method. Also, when tested with washed erythrocytes, Figure 5a shows the luminescence patterns of RBC and of RBC coated by seven polyphenols and by one sample of red wine. Using the luminescence assay, the dose-dependent binding of the polyphenol resveratrol to RBC is shown in Figure 5b. In addition, Figure 5c shows the formation of various polyphenol–RBC complexes as measured by the DPPH assay. Using the electrochemical method DPV, Figure 6 shows the binding of curcumin, resveratrol, morin and tannic acid to washed RBC. It clearly indicates that erythrocyte–polyphenol complexes possess a typical anodic peak confirming the ability of RBC to bind these specific polyphenols. Taken together, all the methods employed have shown that RBC coated by polyphenols acquired significantly higher antioxidant properties. It was also found that micromolar amounts of polyphenols could be detected by the various techniques (not shown). However, since not all the agents tested possessed significant effects in lower concentration, we only presented results with representative polyphenol concentrations tested at 100 μmol/L.

Although the nature of the binding of polyphenols to erythrocyte surfaces is still not fully clear, it is highly suggestive that they do so via hydroxyl groups, hydrophobic interactions, hydrogen bonding, their polymerization degree[35] or also by the presence of a methoxy group in the flavonoid C-ring.[36] Both in biological and in model membranes, the interaction between flavonoids and bilayers results either in their binding at the lipid–water interface or their distribution in the hydrophobic core of the membrane.[37] However, there might be major differences in the

binding mechanisms and affinities of different polyphenols to different types of cells as related also to their membrane structures, where erythrocytes membrane polysaccharides might act as binding sites for polyphenols.[38] As shown by Kure *et al.*,[39] the polyphenol kaempferol entered erythrocyte membranes immediately after incubation, resulting also in a decrease in membrane fluidity, which affected membrane proteins. This was followed by access of kaempferol from the membrane into the erythrocyte cytoplasm. In addition, the polyphenol quercetin was rapidly and avidly taken up by RBC via a passive diffusion mechanism.[40] Polyphenols were also shown to form complexes with proteins and polysaccharides,[41] and it is also probable that the highly polymerized polyphenol, tannic acid, binds to cell surface components rather than to intracellular targets.[42] Polyphenol's capacity to modify membrane-depending processes (e.g. free radical-induced membrane lipoperoxidation) and their ability to interact and enter the lipid bilayers were also found to be correlated.[43] It was also shown that quercetin and naringenin exhibited a strong affinity for phospholipidic bilayers. Kaneko *et al.*[44] have suggested that the effectiveness of protection by phenolic antioxidants against cytotoxicity depended primarily on their incorporation rate into cells and on their orientation in biomembranes. Furthermore, the antioxidant properties of the hepatoprotective flavonoid silybin were suggested to be determined, partially at least, by its location at the cellular membrane–lipid/water interface.[45] These studies further support the observations that polyphenols bind to RBC surfaces to act as major surface antioxidants and membrane stabilizers, capable of acting in concert with plasma antioxidants to enhance defense mechanisms against oxidant toxicity.

It is accepted that even after hefty consumption of fruits and vegetables rich in polyphenols, only small amounts of polyphenols (up to 7 μmol/L[8]) can be detected in plasma. However, since we have shown that polyphenols avidly bind to RBC, we suggest that TOSC of whole blood should be considered as the sum of the antioxidants of erythrocytes (mainly catalase), of other blood cells, of plasma antioxidants (albumin, LMWA, etc.) and of the polyphenols from supplemented nutrients, which can accumulate on cell surfaces.[39,40,46]

Polyphenols might exert their beneficial effects mainly in the oral cavity, downstream in the GI tract and later on, in the circulation. Soon after their consumption, polyphenols can rapidly interact with oral saliva, dentures, oral epithelium, the gingival crevice, with oral microbiota and in inflamed sites, and also with whole blood extravasated from injured blood vessels and with blood leukocytes. In recent observations (to be published) RBC were also shown to synergize strongly with washed platelets, lymphocytes, salivary antioxidants and with plasma to enhance TOSC. Therefore, it might also be postulated that following destruction of RBC in the spleen, higher levels of polyphenols might be accumulated in this organ. Studies along this line are now in progress.

The possible beneficial role of supplemented polyphenol antioxidants as potential reducers of risks of cardiovascular diseases, cancers, neurodegenerative diseases[47] and also locally in the oral cavity and in the GI tract,[48,49] was studied in human trials. However, the mechanisms by which polyphenols exert their beneficial effect *in vivo* is still debatable. Polyphenols can act as chelating agents for transitional metals, as antioxidants, as antiapoptotic, antiaging, anticarcinogenic, anti-inflammatory and anti-angiogenic agents, and as anticell proliferating agents. These beneficial effects of polyphenols are also related to their molecular mechanisms of action on intracellular signaling pathways (e.g. nuclear factor kappa B and mitogen-activated protein kinases).[50]

In conclusion, our study offers a new look at the role played by RBC, platelets and lymphocytes (to be published) and by polyphenols as scavengers of ROS. RBC and platelets[51] are normally laden with potent intracellular antioxidants (mainly catalase) and capable of acting in concert with LMWA, albumin, plasma and with small amounts of free polyphenols which enhance the TOSC of whole blood. Although the total amounts of free polyphenols found in plasma are in the micromolar range, these might still be effective when combined with erythrocytes and other blood cells. It is hypothesized that over time, RBC in the circulation can constantly and cumulatively be complexed with polyphenols to further endow upon such complexes higher TOSC. The analytical methods used in the current study are well adapted to quantify TOSC in whole blood. It is also proposed that the protective role of polyphenols, either in the oral cavity, in the GI tract or in the circulation is probably due to the ability of mammalian cells and of microorganisms to attach polyphenols upon themselves and to enhance their TOSC. This might be one of the mechanisms by which human RBC play a pivotal role in the distribution and bioavailability of circulating polyphenols, which contribute to the defense against injury induced by ROS in various clinical disorders. Therefore, *in vivo* studies should be performed in order to further clarify and confirm these promising findings, which support the phenomenon whereby RBC and other blood cells can form stable complexes with polyphenols. This novel approach can interpret the low polyphenol plasma concentrations detected in plasma following their consumption and at the same time their beneficial role against ROS in humans. Our results suggest that TOSC should be determined in whole blood and not exclusively in plasma or serum as customary performed in clinical settings. This implies that the data presented in the literature regarding the quantifications of TOSC in different clinical conditions should be re-evaluated.

Author contributions: EK designed, performed and contributed to data analysis and interpretation of results, and was also responsible for writing of the paper and compilation of the data presented in the manuscript. IG was involved in the conception and design, interpretation of the data and in the drafting-revising process of the manuscript. IG is also the developer of the novel luminescence assay used in the study, and its use to quantify TOSC in whole blood and isolated whole cells. RK was involved in the conception and design, and in the drafting-revising process of the manuscript.

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