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Polyphenolics enhance red blood cell resistance to oxidative stress: in vitro and in vivo[1]

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Abstract

In this study we investigated the potential antioxidant properties of blueberry polyphenolics in vitro and vivo, using red blood cell (RBC) resistance to reactive oxygen species (ROS) as the model. In vitro incubation with anthocyanins or hydroxycinnamic acids (HCA) (0.5 and 0.05 mg/ml) was found to enhance significantly RBC resistance to H_2O_2 (100 μM) induced ROS production. This protection was also observed in vivo following oral supplementation to rats at 100 mg/ml. However, only anthocyanins were found to afford protection at a significant level, this at 6 and 24 h post supplementation. This protection was not consistent with the measured plasma levels of anthocyanins. Indeed, plasma polyphenolic concentrations were highest after 1 h, declining considerably after 6 h and not detected after 24 h. The difference in absorption between anthocyanins and HCA is likely to have contributed to the observed difference in their abilities to afford protection to RBC. This protection represents a positive role following dietary consumption of polyphenolics from blueberries, against ROS formation within RBC in vivo.

Keywords: Polyphenolic; Anthocyanin; Plasma; Absorption; Oxidative stress; Red blood cell

1. Introduction

Many of the flavonoids found in fruits and vegetables have sugar residues bound to their structures. Initially it was thought that only flavonoid aglycones (without bound sugars) were able to pass through the gut wall, and that enzymes capable of cleaving the β-glycosidic bonds were not secreted into the gut or the intestinal wall. Contrary to this, numerous studies have clearly shown that flavonoid glycosides can indeed enter into the circulation intact [1–3]. With the inherent difficulty in being able to directly examine methods of absorption in vivo, application of cell model systems have provided the most useful information to date. In this regard a recent study by Gee et al. [4] suggested that quercetin glucosides are capable of interacting with the sodium dependent intestinal glucose/galactose transporter in the mucosal epithelium, which may facilitate their absorption into the circulation via the small intestine.

Of particular interest in our lab are the beneficial actions of anthocyanins, common components in certain grapes, wine, and berries, the absorption of which has previously been reported [5–7]. A number of beneficial actions elicited by anthocyanins include maintenance of normal vascular permeability [8], vasoprotective and anti-inflammatory properties [9], and anticancer activity [10]. Reports from our lab have also shown that old rats supplemented with a blueberry (*Vaccinium* spp.) extract for 8 weeks (pelletized with an AIN-93 defined diet) exhibited less deficits in neuronal signal transduction (e.g., striatal dopamine release and GTPase activity, and calcium clearance from hippocampal synaptosomes) and cognitive behavior impairment compared to untreated animals [11,12]. As such, we became interested in further examining the potential beneficial roles that blueberry polyphenolics may be eliciting that could contribute to the enhancement in cognitive performance.

In this regard, in addition to the aforementioned beneficial action of polyphenolics, numerous studies also report

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their capacity to reduce general markers of oxidative stress (OS) [13–17] including protection of red blood cells (RBC) against free radicals [18,19]. We believe that this may be important as changes in whole red blood cell structural conformation may compromise effective blood flow, and oxygen uptake and release [20]. During normal aging, oxygen and glucose consumption progressively decrease and these changes are accompanied by reduced cerebral blood flow (CBF), which could be responsible in part for the cognitive impairments observed in aging [21]. Thus, the aim of this study was to identify first if different families of polyphenolics isolated from blueberries could protect RBC from reactive oxygen species (ROS) in vitro, and subsequently whether or not these polyphenolics could be absorbed into the circulation and promote similar protection in vivo.

2. Materials and methods

2.1. Animals

Twenty 6 month old male Fisher 344 rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing approx. 300 g were individually housed in stainless steel mesh suspended cages, provided food and water ad libitum, and maintained on a 12 h light/dark cycle. All animals were utilized in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, USPHS, Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Care and Use Committee of our Center.

2.2. Sample collection

The various polyphenolic families from wild blueberries (lowbush) were isolated using the procedure reported by [22]. Anthocyanin and hydroxycinnamic acids (HCA) extracted from skin and flesh respectively, were dissolved in 1 ml distilled water at 100 mg/ml. These were then administered to the rats under isofluorane anesthesia by stomach intubation. Food had been withheld 24 h prior to experimentation. Rats were subsequently anesthetized using pentobarbitol (150 mg/kg) and blood samples collected by cardiac puncture. Samples were centrifuged at 2500 rpm for 10 min at 4°C and the plasma collected and stored at −80°C for subsequent analysis. This procedure was performed in rats 1 h, 6 h and 24 h post supplementation.

2.3. Dichlorofluorescein assay

RBC were isolated and washed twice with normal saline (human whole blood was used for in vitro experiments as it was more accessible). Automated analysis of RBC susceptibility to an OS insult was performed as previously reported in our lab [23]. This assay uses a non-fluorescent probe, 2′,7′-diacetyldichlorofluorescein (DCFH-DA) that is able to penetrate into the intracellular matrix, that becomes oxidized by ROS to the fluorescent dichlorofluorescein (DCF). Briefly an aliquot of RBC (25 μl) was made up to a final volume of 2 ml in normal phosphate buffered saline (PBS) pH 7.4. A 25 μl aliquot of this mixture diluted 100-fold and the cell number counted. 2×10^7 cells were diluted in 500 μl PBS to which 500 μl DCFH-DA (200 μM) was added and the samples incubated at 37°C for 30 min. Samples were then centrifuged and washed twice with PBS and finally reconstituted in 1 ml PBS. 50 μl aliquots of samples were plated into a 96-well plate to which 50 μl of 200 μM hydrogen peroxide (H_2O_2) was then added. Fluorescent measurements were made with excitation and emission filters set at 485 ± 10 nm and 530 ± 12.5 nm respectively. All initial fluorescence values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage increase in fluorescence calculated using the following formula $[(Ft_{30}-Ft_0)/Ft_0\times100)]$; where Ft_0 and Ft_{30} are the fluorescence intensities at 0 and 30 min.

2.4. Polyphenolic identification

Flavonoid extraction from rat plasma was performed using established procedures reported by Miyazawa et al. [7]. In brief, 3M Empore extraction disk cartridges were preconditioned with methanol (10 ml), followed by 1.5 mol/l formic acid (aq) (10 ml). Plasma (1 ml) diluted with 0.2 ml 0.44 mol/l TFA (trifluoroacetic acid) was added to the cartridge and subsequently washed with 5 ml each of 1.5 mol/l formic acid (aq), dichloromethane and benzene. Flavonoids were finally recovered in 1.5 mol/l formic acid dissolved in methanol, dried and reconstituted in 200 μl mobile phase A. Percentage recovery of extracts were $>90\%$.

2.5. HPLC analysis of rat plasma polyphenolics

Chromatographic analysis was performed on a HP1100 series HPLC (Hewlett Packard, Palo Alto, CA) equipped with a quaternary HPLC pump, column heater, diode array detector, and HP Chemstation for data collection and manipulation. Chromatographic separation was performed at 30°C using a HP Zorbax SB-C18 (stable bond) rapid resolution column (150 mm×4.6 mm I.D.; particle size 3.5 μm, 80 Å) fitted with a Zorbax SB-C18 analytical guard column (12.5 mm×4.6 mm; particle size 5 μm, 300 Å). Aliquots (200 μl) of each extraction were injected for analysis using a Bioanalytical Systems Sample Sentinel autosampler (Model MF-9069) (West Lafayette, IN, USA) maintained at 4°C. Normal phase separation of anthocyanins was achieved using a binary gradient program. Mobile phase A was 25 mM sodium acetate in water, mobile phase B was 25 mM sodium acetate in methanol, each adjusted to pH 1.5 with trichloroacetic

acid. The gradient cycle consisted of a linear increase in mobile phase B from 0% to 24% (0.48%/min), 24% to 38% (0.23%/min) and a 10 min isocratic run (100% B). At the end of each run (120 min), the program returned to the initial conditions and the system was re-equilibrated for 10 min. The various known compounds were characterized by chromatographic comparison with available authentic standards. Total anthocyanins and HCA were expressed as cyanidin-3-glucoside (C-3-G) and gallic acid (GA) equivalents respectively.

2.6. Statistical analysis

Results are expressed as mean ± S.D. Statistical analyses were performed using one- or two-way ANOVA where applicable, using Systat (SPSS, Chicago, IL).

3. Results

3.1. In vitro

In vitro investigations into the protective effects of blueberry anthocyanins and HCA at reducing H_2O_2 mediated ROS production in RBC are shown in Fig. 1. Results showed that anthocyanins reduced RBC susceptibility to ROS formation as measured by inhibition of DCF fluorescence in both a time- and concentration-dependent manner ($P < 0.01$ and $P < 0.001$). However, HCA was only effective at the 0.5 mg/ml concentration. Post-hoc analyses identified that anthocyanins incubated with RBC for 6 h and 24 h at 0.5 or 0.05 mg/ml afforded significant protection as compared with non-supplemented RBC.

3.2. In vivo

When anthocyanins and HCA were provided by oral supplementation to rats at 100 mg/ml, a significant increase in plasma levels were observed at 1 h and 6 h (Fig. 2). However, no detectable levels were measured 24 h following supplementation, of either polyphenolic family. Maximum concentration under the experimental conditions was observed at 1 h; 0.64 μmol/l C-3-G equivalents for anthocyanins and 0.21 μmol/l GA equivalents in the case of HCA. With respect to peak identification, the following 14 anthocyanins were observed in plasma, as determined from retention time and spectra matches with available and/or reported anthocyanins previously identified in blueberries [24], these included delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-galactoside, delphinidin-3-arabinoside, cyanidin-3-glucoside, petunidin-3-galactoside, petunidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, malvidin-3-galactoside, malvidin-3-glucoside, malvidin-3-arabinoside, cyanidin and malvidin (chromatogram not shown). Unfortunately, with the exception of chlorogenic acid reliable characterization of the HCA within blueberries has not been reported and peak assignment fell outside the scope of this present study. As such, only chlorogenic acid can be assigned to one of the observed peaks in the plasma extract from HCA supplemented rats (chromatogram not shown). With respect to the ensuing in vivo protection against increased intracellular ROS production in RBC,

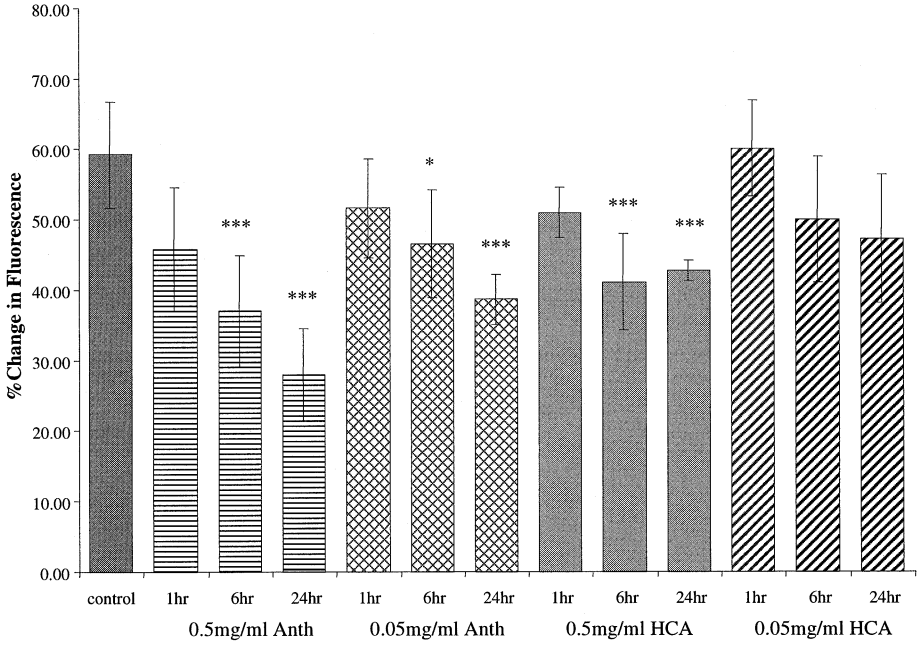

Fig. 1. In vitro protective effects of polyphenolic families (anthocyanins and hydroxycinnamic acids) against H_2O_2 (100 μM) induced reactive oxygen species generation in red blood cells incubated at different doses and times. *,***Significant differences from H_2O_2 treated control red blood cells at $P < 0.05$ and $P < 0.001$ respectively. Each value represents the mean ± S.D. of eight separate measurements.

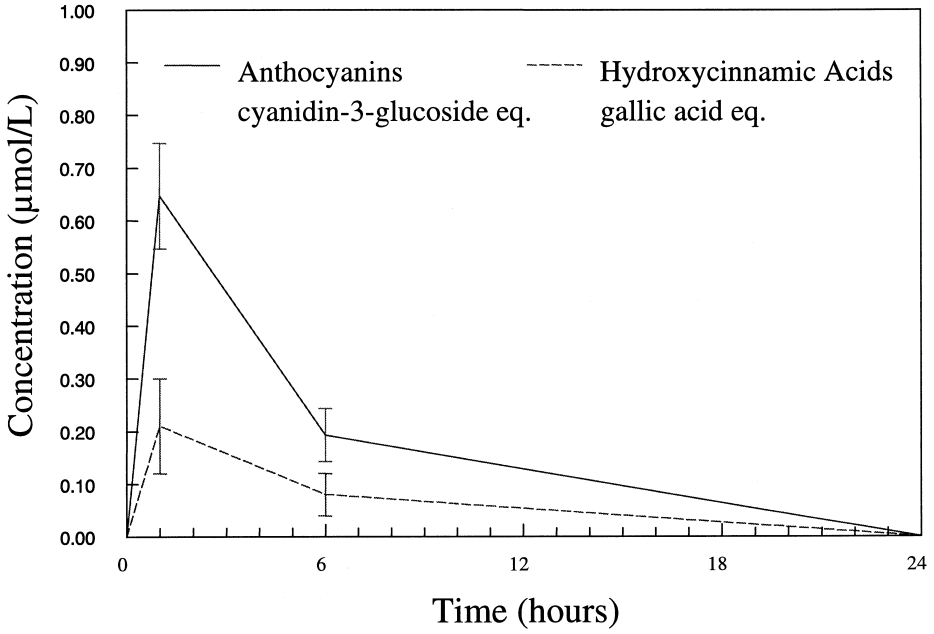

Fig. 2. Total concentration (µmol/l) of anthocyanins (cyanidin-3-glucoside equivalents) and hydroxycinnamic acids (gallic acid equivalents) in plasma isolated from rats at different time points following oral supplementation of 100 mg.

only anthocyanin supplementation was found to exhibit any significant effects (Fig. 3). Here, RBC analyzed at 6 h and 24 h were protected against H_2O_2 induced ROS production ($P < 0.001$ in each case).

4. Discussion

We have shown here that both anthocyanins and HCA isolated from blueberries were able to ameliorate the experimentally induced formation of ROS in RBC using an in vitro model. This finding in itself would suggest a potential beneficial role that may arise following dietary consumption of blueberries. Indeed a large number of studies have reported various beneficial physiological effects that polyphenolics may elicit as shown by the use of in vitro model systems. Some examples of these include: modulation of cell signaling [25,26]; altered proliferation and cytotoxicity in cancer cell lines [10,27,28]; protection of DNA integrity [26,29–31]; alterations of immune and inflammatory responses [32–35]; and modifications in cytokine production [36–39]. As such, it becomes of para-

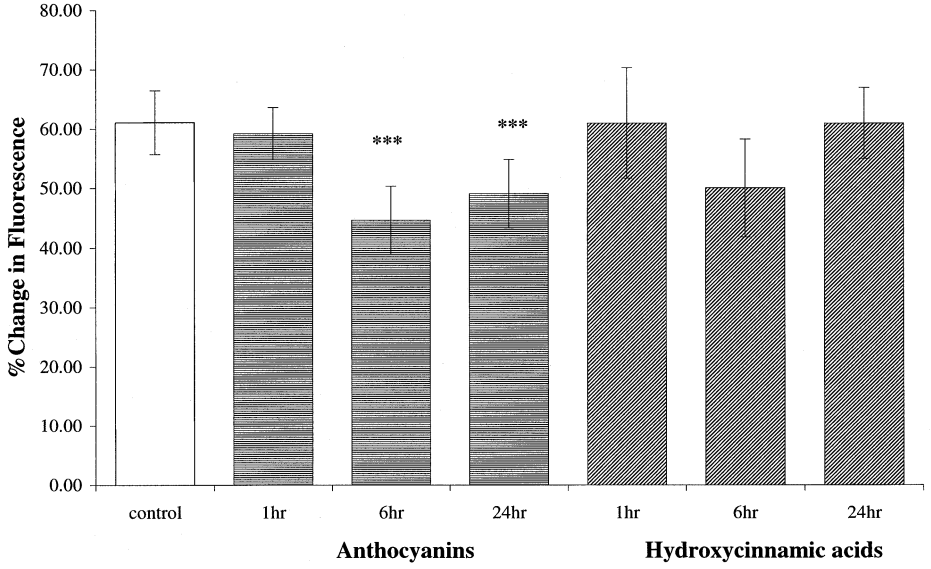

Fig. 3. In vivo protective effects of polyphenolic families (anthocyanins or hydroxycinnamic acids) against H_2O_2 (100 µM) induced reactive oxygen species generation in red blood cells isolated from rats at different time points following oral supplementation of 100 mg. ***Significant difference from H_2O_2 treated control red blood cells at $P < 0.001$. Each value represents the mean ± S.D. three separate measurements.

mount importance to be able to extrapolate potent bioactivities observed in vitro, into a physiological scenario in vivo. In this regard, we have shown in support of our in vitro findings that, following dietary consumption, blueberry polyphenolics are absorbed, and consequently were able to reduce RBC susceptibility to intracellular ROS formation.

It was once considered that only polyphenolics without a bound sugar residue were absorbed into the circulation. This belief has now been challenged, with studies having shown flavonoids glycosides absorbed in vivo [2,40,41]. In addition, from previously established characterizations of the blueberry anthocyanin composition [24], we found that among the various anthocyanins absorbed was cyanidin-3-glucoside. This finding is consistent with previous studies [5–7]. The absorption of anthocyanins similar to those in this study, derived from a commercial crude *Vaccinium myrtillus* extract, called *V. myrtillus* anthocyanin (VMA), has also been reported [5].

Tsuda et al. [6] reported that of the ingested 0.16 mmol C-3-G by rats, less than 0.5 μmol/l was found in the plasma within an hour following supplementation. It is interesting to note that their dosage is approx. 781 mg, approx. 8 times greater than that given to rats in this study. Despite this difference we have found that the total concentration of anthocyanins (expressed as C-3-G equivalents) within 1 h of supplementation is similar to that reported by Tsuda and co-workers. In contrast, Miyazawa and colleagues [7] reported higher plasma levels of C-3-G even though their supplementation regime, at approx. 45 mg and 90 mg, was less than that examined in this study and considerably less than that performed by Tsuda and co-workers. There are a number of possible explanations that may account for these discrepancies. One can speculate that differences in strain and age of animals may have contributed towards absorption potency. In addition, in this study more than one anthocyanin component was used, interactions between which may have aided or restricted the absorption of others. One parallel observation between these studies was the rapid disappearance of anthocyanins from the plasma. In the current study anthocyanin absorption was below 0.2 μmol/l within 6 h of supplementation and not detectable after 24 h.

Results from these aforementioned studies suggested that following oral supplementation of C-3-G a number of protective actions observed in vivo, including the reduction of serum susceptibility to lipid peroxidation induced by AAPH and Cu^{+} [42], also enhanced protection against hepatic ischemia-reperfusion (I/R) induced damage [43]. In addition to these a further beneficial physiological outcome that can be attributed to the absorption of anthocyanins is the enhanced resistance of RBC to potentially damaging ROS formation following exposure to H_2O_2. In the current study this protection was most beneficial 6 h and 24 h post supplementation, despite anthocyanin concentrations in plasma being maximal at 1 h. One can speculate that during the 24 h period, anthocyanins were able to exert more of a beneficial impact as compared with that after only 1 h. Hydroxycinnamic acids on the other hand, despite being able to enhance RBC resistance to intracellular ROS formation in vitro, were not effective in vivo as compared with anthocyanins. One could attribute this to the lower levels of HCA absorbed in plasma. Moreover, the physiological actions of HCA against H_2O_2 may be different from those of anthocyanins.

Recent findings in our lab have shown using an in vitro cell model [44], that polyphenolics, in this case anthocyanins, were able to localize both within the cell membrane and cytosol of vascular endothelial cells, following supplementation at times as short as 30 min. From this finding, one could argue that these polyphenolics could also be able to localize within the different cellular milieus of the RBC. With this in mind, a number of possible theories can be postulated to explain the mechanism(s) by which these polyphenolics may have afforded protection against ROS formation within RBC. It is possible that the anthocyanins mediated a direct scavenging of H_2O_2, either as it passed through the membrane, or once it was within the cytosol. Indeed, it is also possible that they could be scavenging other ROS induced following H_2O_2 absorption within the RBC that may be contributing to the oxidation of DCFH to the fluorescent DCF. The stimulation some other biological mechanisms utilized by RBC to protect themselves from ROS such as endogenous antioxidant enzyme systems could also be a contributing factor, as has been observed with other antioxidants [45].

A number of previous studies have also reported enhanced protection afforded to RBC by polyphenolics [18,19]. In vitro studies performed by Halder and Bhaduri [18] using black tea polyphenolics found them to be potent inhibitors of phenylhydrazine, Cu^{2+}-ascorbic acid and xanthine/xanthine oxidase induced oxidative damage. Interestingly they reported a positive effect on membrane fluidity but did not investigate if this was a direct interaction of the polyphenolics within the membrane or some other change in the membrane composition/organization. This change can be attributed to a number of factors, such as direct protection of membrane polyunsaturated fatty acids, which control in part the fluidity of the membrane. It is also feasible that the polyphenolics, if localized within the membrane, similar to that reported by us [44], may have directly altered the rigidity of the membrane. This possibility is supported by findings reported by Lenne-Gouverneur and co-workers [45], who found that polyphenolics are able to mediate a fluidizing effect within the membrane.

Other studies by Zhang and co-workers [19], examining Jasmine green tea polyphenolics in vivo, reported enhanced protection afforded RBC from hemolysis in the presence of AAPH. This is interesting as AAPH is likely to initiate damage at the membrane level, as such this finding would suggest that protection may have been con-

ferred at the membrane level. Further studies have also shown that phytochemicals such as eugenol, a terpenoid commonly found in the essential oil from cloves and nutmeg [46], as well as vitamins E and C [47] were able to protect RBC against ROS induced damage. This protection afforded to RBC may have a number of physiological implications with respect to RBC function. In light of our previous studies regarding the beneficial effects of blueberry consumption on aspects of neurological and cognitive function in old rats, maintaining RBC integrity may help with increased cerebral blood flow [20]. Consequently, this may improve the efficiency in provision of oxygen and glucose, essential for efficient brain performance [21].

In conclusion, we have shown that, in addition to the various aforementioned benefits of polyphenolics including that of anthocyanins, their absorption in vivo results in a protection to RBC susceptibility to ROS production. This polyphenolic absorption is likely to increase RBC integrity and functionality. Furthermore, polyphenolics are also likely to provide protection against other ROS in various cell systems. As such, current research is currently under way investigating the beneficial effects of these and other polyphenolic families on the functional integrity of neuronal and vascular cell systems as well as RBC function.

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