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Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults☆

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Abstract

Although antioxidant systems help control the level of reactive oxygen species they may be overwhelmed during periods of oxidative stress. Evidence suggests that oxidative stress components as well as inflammatory mediators may be involved in the pathogenesis of vascular disorders, where localized markers of oxidative damage have been found. In this regard we investigated the putative antioxidant and anti-inflammatory effects of blueberry and cranberry anthocyanins and hydroxycinnamic acids against H_2O_2 and TNFα induced damage to human microvascular endothelial cells. Polyphenols from both berries were able to localize into endothelial cells subsequently reducing endothelial cells vulnerability to increased oxidative stress at both the membrane and cytosol level. Furthermore, berry polyphenols also reduced TNFα induced up-regulation of various inflammatory mediators (IL-8, MCP-1 and ICAM-1) involved in the recruitment of leukocytes to sites of damage or inflammation along the endothelium. In conclusion, polyphenols isolated from both blueberry and cranberry were able to afford protection to endothelial cells against stressor induced up-regulation of oxidative and inflammatory insults. This may have beneficial actions against the initiation and development of vascular diseases and be a contributing factor in the reduction of age-related deficits in neurological impairments previously reported by us.

Keywords: Endothelial cells; Polyphenolics; Free radicals; Oxidative stress; Blueberry; Cranberry

1. Introduction

An alteration in the redox state has been suggested to contribute in vascular dysfunction and ultimately the initiation and/or progression of vascular disorders such as atherosclerosis and even neurodegeneration that may result from the dysfunction of the blood brain barrier (BBB). Important advances in our understanding of the pathogenesis of these vascular disorders and in the genetics that mediate the relationship between risk factors and disease have opened new areas of research. One such area is that of reducing vascular susceptibility to oxidative and inflammatory insults. While vascular endothelial cells are constantly in contact with reactive oxygen species (ROS) generated by neutrophils and monocytes and must be especially well equipped to resist their toxic effects and generate appropriate physiological responses. Despite the importance of certain ROS in the physiopathology of the vascular endothelium, the mechanisms regulating the oxidative response of endothelial cells (EC) lining blood vessels and also those that constitute the BBB, are poorly understood.

One possible process by which ROS could become available to elicit such deleterious actions is from activated monocytes and neutrophils. Molecular mechanisms involved in their recruitment, via the actions of different chemoattractants secreted by EC, appear to depend on a host of complex processes. These include an alteration of the cell redox status [1], responses to inflammatory insults i.e. tumor necrosis factor (TNFα), [2] and ischemic episodes [3,4]. The activated EC further increase their expression of cytokines such as TNFα; chemokines/chemoattractants involved in attracting different leukocytes, such as interleukin-8 (IL-8) for neutrophils and monocyte chemoattractant protein-1 (MCP-1) for monocytes, to sites of inflammation, and adhesion molecules such as intercellular adhesion mol-

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ecule-1 (ICAM-1) involved in binding of leukocytes to the EC surface.

This localized accumulation of leukocytes and subsequent adhesion to the vascular endothelium together with high cholesterol levels in the arterial intima, play a key role in early artherogenesis, as well as in plaque rupture in advanced atherosclerotic lesions [5]. Moreover, leukocyte migration through the BBB, subsequently results in the activation of glial cells, further enhancing levels of ROS and cytokines [1], ultimately enhancing brain tissue damage contributing to neurological dysfunctions. In light of this, it appears essential that a means to maintain the EC integrity against these deleterious effects be identified. In this respect numerous in vitro studies having shown the antioxidative and anti-inflammatory properties of polyphenols towards vascular cells [6–9]. With this in mind, we decided to examine both the oxidative and anti-inflammatory properties of the two major polyphenolic families, namely anthocyanins and hydroxycinnamic acids (HCA) present in blueberry and cranberry fruits. The former of which has previously been shown by us to improve age-related deficits in neurological functions known to be sensitive to the deleterious actions induced by OS and inflammatory insults.

2. Materials and methods

2.1. Polyphenolic isolation

Polyphenolic fractions were obtained using a modified version of a procedure initially reported by Oszmainski, et al. [10]. Briefly, 15g berries were homogenized in 50 ml of acetone:methanol:water:formic acid (40:40:20:0.1 v/v/v/v). To obtain the fractions, 3 ml of crude extract was dried and resolubilized in water, applied to an activated C18 column, washed with water, then 15% methanol in water and then finally methanol acidified with formic acid. The 15% methanol fraction eluted the phenolic acids, and the acidified methanol eluted the anthocyanins and other components of interest. The fraction containing the anthocyanins was dried and resolubilized in 50% methanol in water and applied to a Sephadex LH20 column. The column was washed with 70% methanol acidified with 10% formic acid, which eluted anthocyanins and flavonols. The LH-20 column was then washed with 70% acetone to elute the tannins or procyanidins. The methanolic (anthocyanins & flavonols) fraction from the LH20 column was dried and resolubilized in 5% formic acid in water and applied to the second C18 column. This column was washed with 5% formic acid, followed by ethyl acetate and then 10% formic acid in methanol. The ethyl acetate eluted the flavonols and the acidified methanol eluted the anthocyanins. All fractions of interest were dried, and resolubilized accordingly.

2.2. Cell culture

Human microvascular endothelial cells (HMVEC) were purchased from Cell Applications (San Diego, CA.), and were maintained in a humidified atmosphere of 95% air, 5% CO_2, in plated cultures at 37°C in CADMEC™ Complete Growth medium (CGM) (Cell Applications, San Diego, CA.). The culture medium was replaced every 2 days until the cells attained confluence (90%) and were subcultured every 4 days using calcium and magnesium free PBS, 0.25% trypsin, and 0.2% EDTA.

2.3. Dichlorofluorescein assay

Automated analysis of HMVEC susceptibility to an OS insult was performed as previously reported [6]. Results were expressed as percentage increase in fluorescence calculated using the following formula $((Ft_{30}\text{-}Ft_0)/Ft_0*100))$; where Ft_0 and Ft_{30} are the fluorescence intensities at 0 and 30 mins. DCF fluorescence in control cells in the absence of an oxidative stressor was not found to differ significantly from unsupplemented control cells in the absence of an oxidative stressor.

2.4. Fluorescent measurement of lipid peroxidation using cis-Parinaric acid

cis-Parinaric acid (cPnA) is a membrane fatty acid that contains a four conjugated double-bond system, which upon excitation cause the molecule to fluoresce, any damage to these double bonds i.e through oxidative damage, results in fluorescence decline. cis-Parinaric acid prepared in DMSO was incorporated into HMVEC using the same experimental procedures reported by Dubey and co-workers [11] with some modifications. Briefly, HMVEC previously pre-supplemented with anthocyanins (0.1–0.01mg/ml, for 2 hr at 37°C) were washed and then dislodged by trypsinization, washed twice with CGM. Cells were diluted to 1.5 x 10^6 cells/ml and then incubated with cPnA (final concentration 5μg/ml, DMSO content was less than 0.01% of the final volume) in Kreb's Ringer buffer at 37°C in the dark for 45 mins. Cells were then washed twice with Kreb's buffer and the protective effects afforded to HMVEC by anthocyanins against 100μM H_2O_2. Changes in cPnA fluorescence (excitation 324nm, emission 413nm, band widths 7nm) were measured over a 2 hr period. Background decrease in fluorescence was corrected for by subtracting fluorescence decline of control cells in the absence of inducer. Results are expressed as percentage inhibition of fluorescence changes observed in control non-supplemented cells 2 hr following addition of inducer.

2.5. TNF-α induced inflammatory responses

Under these conditions, HMVEC were pre-supplemented with polyphenols (0.1mg/ml) for 2 hrs in a humidified

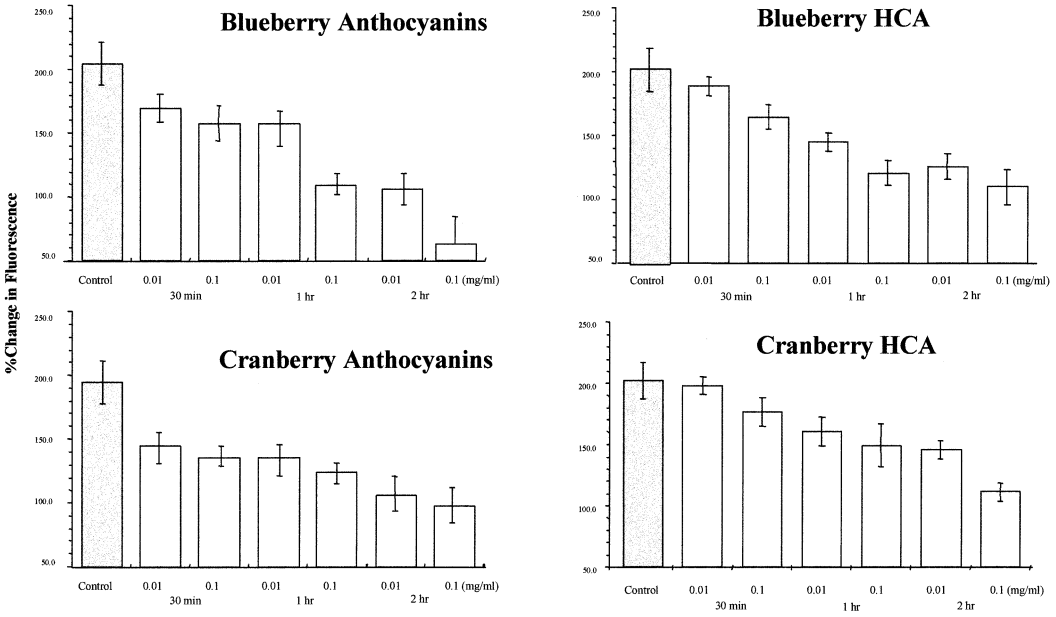

Fig. 1. Effect of pre-supplementing endothelial cells with blueberry or cranberry anthocyanins and HCA (0.01–0.1mg/ml) at different durations (30 min-2 hr) on intracellular reactive oxygen species induced damage, as assessed by the fluorescent marker dichlorofluorescein.

atmosphere at 37°C, washed with sterile Hank's balanced salt solution (HBSS) and then incubated with TNF-α (10ng/ml) for a further 6 hrs in a humidified atmosphere at 37°C. Following incubation, supernatants were collected, centrifuged to remove any residual cell debris and then stored at −80°C for subsequent analysis. Supernatants were allowed to thaw in ice and the concentrations of inflammatory markers, namely intercellular adhesion molecule (ICAM-1), monocyte chemoattractant protein (MCP-1) and IL-8 analyzed using commercially prepared ELISA kits (VENDORS), and the concentrations calculated based upon the appropriate standard curves.

2.6. Cell viability: MTT method

Human microvascular endothelial cells were seeded onto 96-well plates at a density of 10^3 cells/well. Polyphenols were incubated at 0.01–0.1mg/ml with HMVEC for 2 hr. The solutions were removed, and the effects of H_2O_2 at 100μM and TNF-α at 20ng/ml investigated using the MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) method as previously described [6]. Cell viability of control and polyphenolic supplemented cells in the absence of inducers did not differ significantly (data not shown).

2.7. Statistical analysis

Results are expressed as mean ± SD. Statistical analyses were performed using two-way ANOVA. *Post-hoc* comparisons were performed using Student's *t*-test using Systat (SPSS, Inc., Chicago, IL).

3. Results

3.1. Intracellular antioxidant activity

Following pre-supplementation with either BB or CB anthocyanins or HCA, there were marked reductions in DCF susceptibility to H_2O_2 mediated increases in its fluorescence, indicating reduced intracellular ROS production, as compared with H_2O_2 mediated increases in the fluorescence of non-supplemented control cells (Fig. 1). Maximum inhibition for all treatments was observed with supplementation at 0.1 mg/ml for 2 hr ($P < 0.001$ in all cases). Protection by all 4 fractions at 0.1mg/ml was also found to be significant with respect to incubation time ($P < 0.05$ in all cases) but not at 0.01mg/ml. A significant dose response effect was also observed following supplementation at 0.1mg/ml as compared with 0.01mg/ml ($P < 0.05$) with all treatments except in the case CB anthocyanins. Overall however, no significant differences were observed between the efficacy of BB and CB anthocyanins or that between BB and CB HCA. Yet overall, anthocyanins did appear to be slightly more protective than HCA, although this was just outside the limit of significance ($P < 0.056$). DCF fluorescence in control cells in the absence of an OS was not found to differ significantly during the analysis period. Neither fraction from BB or CB were found to quench fluorescence in the absence of oxidative stressors.

3.2. Membrane lipid peroxidation

Protection of membrane fatty acids was assessed by the inhibition of OS induced decrease in cis-PnA fluorescence

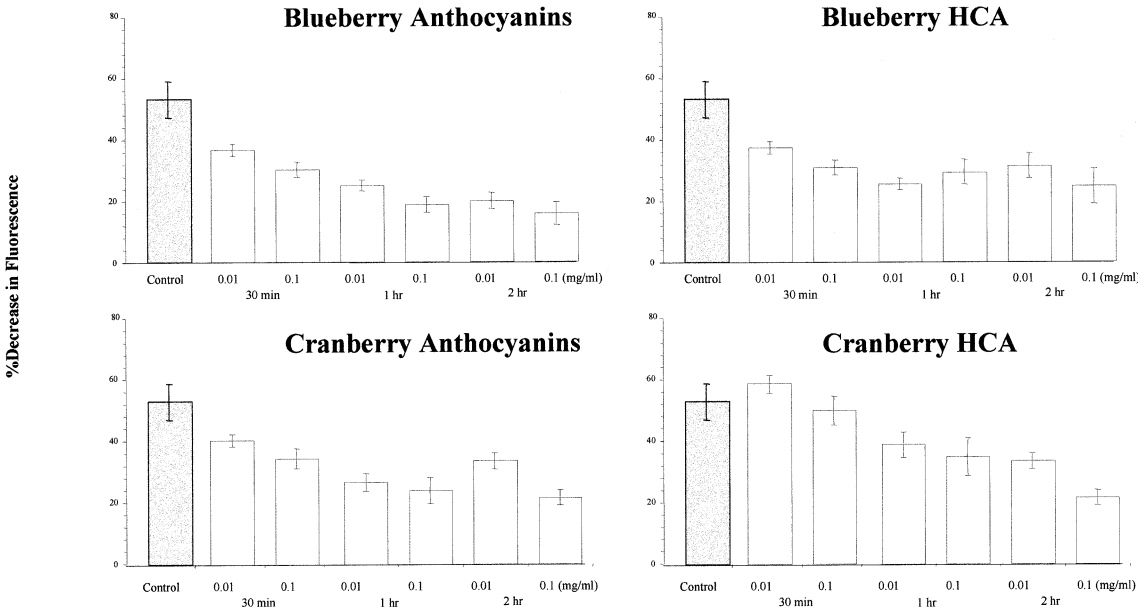

Fig. 2. Incubation of endothelial cells with anthocyanins or HCA (0.01–0.1mg/ml) at different time points, protects membrane fatty acids from oxidation, as measured by the fluorescent membrane fatty acid probe; cis-parinaric acid.

(Fig. 2). Exposure of HMVEC to H_2O_2 for 2 hr resulted in maximal cPnA fluorescence declines of 56% in non-supplemented cells. However, these declines were protected against when cells were pre-supplemented with BB or CB anthocyanins and HCA. Protection was found to be significant with respect to incubation time for all fractions at 0.1mg/ml ($P < 0.05$) except with BB HCA. Similar to intracellular activity, maximum inhibition for all treatments was observed with supplementation at 0.1mg/ml for 2 hr ($P < 0.001$ in all cases). Interestingly, though a protection was found to be significant with respect to incubation time, no dose response effects were observed between supplementation at 0.01 and 0.1mg/ml for any treatment. Furthermore no significant differences were observed between the efficacy of BB and CB anthocyanins or that between BB and CB HCA.

3.3. TNFα induced in increases in inflammatory mediators

Following exposure to TNFα, unsupplemented endothelial cells displayed significant increases in the release of IL-8, MCP-1 and ICAM-1 into the cell media. However, cells pre-supplemented with either BB or CB anthocyanins or HCA appeared to be less susceptible to this activation (Fig. 3 A-C). In the case of IL-8 and ICAM-1, all families from both berries provided significant protection as compared to non-supplemented cells ($P < 0.001$ in each case). However, in the case of MCP-1, anthocyanins from both berries but not HCA were protective against its up-regulation by TNFα ($P < 0.01$ for both BB and CB). Overall, no significant treatment effects were observed between BB and CB against the up-regulation of these different inflammatory mediators.

3.4. Cell viability

Control cells not incubated with any inducers were not found have displayed significant declines in viability during the 24 hr incubation period. Viability of EC pre-incubated with either anthocyanins or HCA, in the absence of any inducer were not found to differ from control cells (data not shown). Following incubation (24 hr) with H_2O_2, and TNFα, EC not pre-supplemented with polyphenols exhibited significant declines in viability, 58 and 79% respectively (Table 1.). In contrast, EC pre-incubated with either BB or CB anthocyanins or HCA were afforded significant protection against H_2O_2 and TNFα ($P < 0.001$ in all cases), except CB HCA which was not found to be protective at the concentration tested.

4. Discussion

It has been suggested that OS contributes in the pathogenesis of vascular disorders [12] where localized markers of oxidative damage have been found. Acting in concert with this, is the production of pro-inflammatory cytokines, also known to play a significant role in or during the manifestation of these disorders. Increased production of pro-inflammatory cytokines such as TNFα and IL-1β, in the absence of an inflammatory stimulus is a frequent phenomenon that occurs as we age [13]. Increased circulating levels of these biochemical markers have been found to be particularly prevalent in patients with cardiovascular disorders

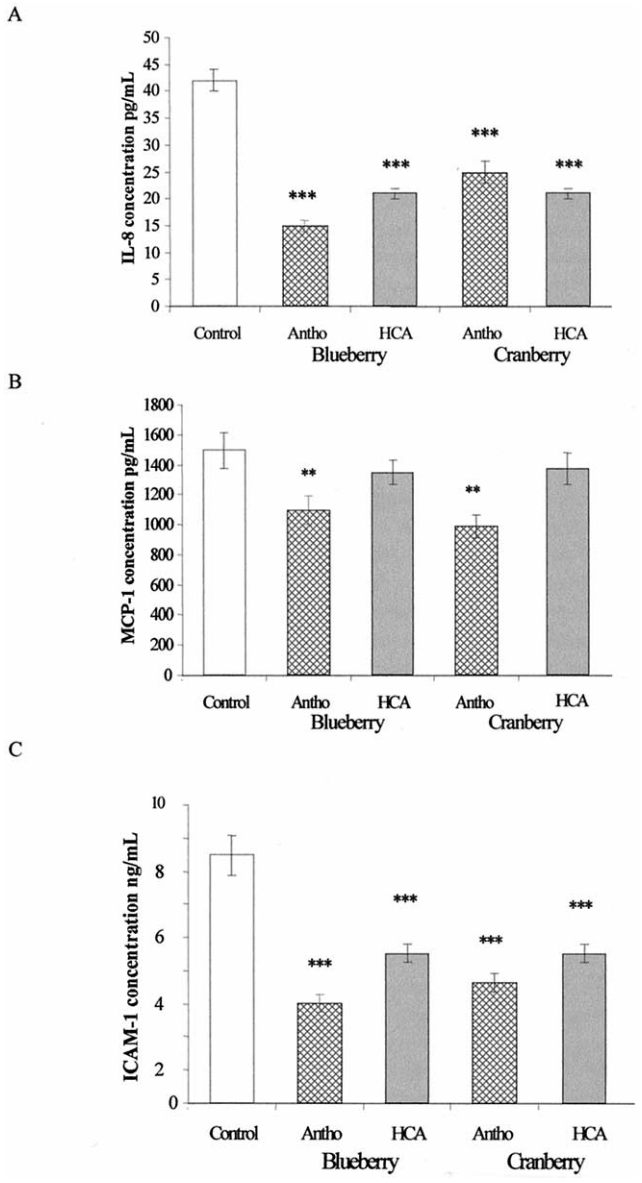

Fig. 3. (A-C) The effect of polyphenolics (0.1mg/ml) on TNFα (10ng/ml) induced IL-8 (A), MCP-1 (B) and ICAM-1 (C) expression in human microvascular endothelial cells.

and dyslipidemia [14,15], and patients exhibiting signs of AD [13,16].

Here we report that both anthocyanins and HCA are protective within different cellular compartments of EC against ROS and inflammatory insults. These findings are in agreement with previous studies examining the protective capacities of polyphenols, involving inhibition of inflammatory mediators and eicosanoids [8,17], and reduction of free radicals and markers of OS [6,7,9]. The previous polyphenols found to be protective included; procyanidins in the form of Pycnogenol [18], flavonoids such as quercetin and catechin [7], and also anthocyanins [6]. Furthermore, ROS mediated regulation of adhesion molecule expression has also been shown to be down-regulated by flavonoids resulting in improved endothelial function [19–21].

Table 1
Beneficial effects of berry anthocyanins and HCA supplementation (0.1 mg/ml for 2 hrs) on the percentage viable cells remaining following exposure to different oxidative stressors.

	Inducer	
	H_2O_2 (100 μM)	TNFα (10 ng/mL)
Control	42.0 ± 3.4	21.2 ± 4.8
Blueberry		
Anthocyanins	71.2 ± 5.5c	57.2 ± 5.0
HCA	59.3 ± 4.3	39.3 ± 9.9
Cranberry		
Anthocyanins	74.2 ± 3.3	62.2 ± 5.0
HCA	49.3 ± 6.3	47.3 ± 6.9

* represents significant difference from control at $P < 0.05$. Values in the presence of each individual inducer not sharing a common subscript differ significantly from each other at $P < 0.01$.

In general, both the anthocyanins and HCA provided equal protection within the cytosol and towards membrane lipid peroxidation. However, anthocyanins were more protective than HCA against inflammatory insults. With respect to overall cell viability, anthocyanins from BB and CB were equally protective although BB HCA's were more protective than those from CB against H_2O_2 induced OS, while the opposite was observed against TNFα. These findings are particularly interesting, since in vitro assessments of antioxidant activity using the ORAC (oxygen radical antioxidant capacity) assay [22], found in the case of BB, that HCA potency was approximately two-fold higher than BB anthocyanins, 12.21 μmol Trolox equivalents/mg extract, compared to 6.32 μmol Trolox eq./mg. The fact that in general the anthocyanins from these fruits were more biologically active than HCA could be due to a culmination of factors. Differences in their cellular localization and concentration could be potential factors. The incorporation of polyphenols into cells has previously been reported [6,23,24] through interaction with certain transporters [25], as well as from a simple passive diffusion event [26]. However, attempts to resolve this issue with respect to the current study were performed (data not shown). Unfortunately, the isolation techniques employed were unable to efficiently identify their cellular distribution and hence could not conclusively support the notion that the different efficacies in cellular protection were related to differences in cellular localization. Besides these possible differences in localization and cellular concentrations, a disparity in their protective effects may also reside in differing potencies at quenching the biological actions of the different types of free radicals generated within the cell. The ORAC assay utilizes the water-soluble lipid radical AAPH and hence may not represent their efficacy to inhibit the cellular damage induced directly and/or indirectly by H_2O_2. One could also argue that the protective effects may in fact have been mediated through interactions with signaling receptors on the cell surface and/or intercellular signaling processes. The con-

trast between the chemical structures of anthocyanins and HCA, could make them more stereologically favorable in being able to interact with certain receptor molecules and/or their respective pathways, such as those that control cell death e.g. stress activated cellular responses and protein kinase signaling cascades, and could also explain why MCP-1 upregulation was more effectively inhibited by anthocyanins, while IL-8 and ICAM-1 were not.

The potential for such properties to be promoted in vivo are strengthened from studies reporting their potential to be absorbed [27–31], hence highlighting their *potential* to promote these reported in actions, in vivo. Of course the development of vascular disorders is one that is progressive and does not result from a single insult. One could argue that the relevance of protection afforded by these various polyphenols following a single insult may not have far reaching implications in the prevention of vascular disease states. It is our belief that consistent consumption of diets rich in polyphenols, can with time afford protection against damage to the vasculature. This has been shown, though in an indirect manner, from in studies investigating the "French Paradox", where a low incidence of artherosclerosis, was suggested to result from consumption of phenolic compounds contained in red wine. For the most part, this protection is often attributed to improved antioxidant capacity in the circulating blood. Moreover, reports from numerous laboratories have identified positive effects of various polyphenolics with respect to inhibition of LDL oxidation [32,33] and expression of inflammatory mediators [34,35], to overall improvement of endothelial function [33,36]. Among the polyphenolic families found in wine products are HCA and anthocyanins [10,37], which are also common in other dietary sources, particularly blueberries and cranberries [38]. Moreover, increased oxidative and inflammatory insults to the BBB vasculature during aging, may contribute to neuronal dysfunction, potentially resulting in cognitive and behavioral performance deficits. In this regard, we and others have reported that polyphenols may protect against these aforementioned decrements [39]. We have shown blueberry in particular to be most efficacious. The current observations thus highlight that one possible mechanism by which BB is able to retard age-related deficits in neurological dysfunctions may be through the anthocyanin component maintaining the integrity of the vasculature.

Despite the beneficial effects observed in the current study, one must be aware of their physiological relevance. With respect to anthocyanin absorption, levels as high as 500μg/L plasma have been reported [27] considerably lower than the concentrations examined in the current study. However, the concentrations reported in the circulation may not take into account the levels of anthocyanins that potentially could have been absorbed into tissues, an observation that has been reported previously [27]. In light of this, the results reported here simply highlight a *potential* role that dietary polyphenols may afford in vivo. Perhaps daily consumption of blueberries or cranberries (which contain 1–4 mg anthocyanins/g fresh weight) may have a "priming effect" on the cells, gradually enhancing their ability to withstand stresses imposed upon it. Nonetheless, this is an area that requires more investigation.

In conclusion, the fact that neither blueberry nor cranberry polyphenolics appeared more potent than the other, indicates that in order to achieve a suitable intake of dietary antioxidants, the consumer is able to diversify as opposed to being limited to a particular source.

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