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Differential protection among fractionated blueberry polyphenolic families against DA-, A β ₄₂ and LPS-induced decrements in Ca²⁺ buffering in primary hippocampal cells

James A. Joseph^{*,1}, Barbara Shukitt-Hale¹, Gregory J. Brewer², Karen A. Weikel¹, Wilhelmina Kalt³, and Derek R. Fisher¹

¹USDA-HNRC, Boston, MA 02111

²So. IL Univ Sch of Med, Springfield IL 62794

³Agriculture & Agri-Food Canada, Nova Scotia, B4N 1J5, Canada

Abstract

It has been postulated that at least part of the loss of cognitive function in aging may be the result of deficits in Ca²⁺ recovery (CAR) and increased oxidative/inflammatory (OX/INF) stress signaling. However, previous research showed that aged animals supplemented with blueberry (BB) extract, showed fewer deficits in CAR, as well as motor and cognitive functional deficits. A recent subsequent experiment has shown that DA- or A β ₄₂-induced deficits in CAR in primary hippocampal neuronal cells (HNC) were antagonized by BB extract, and (OX/INF) signaling was reduced. Present experiments assessed the most effective BB polyphenol fraction that could protect against OX/INF-induced deficits in CAR, ROS generation, or viability. HNCs treated with BB extract, BB fractions (e.g., proanthocyanidin, PAC), or control medium were exposed to dopamine (DA, 0.1mM), amyloid beta (A β ₄₂, 25 μ M) or lipopolysaccharide (LPS, 1 μ g/ml). Results indicated that the degree of protection against deficits in CAR varied as a function of the stressor and was generally greater against A β ₄₂ and LPS than DA. The whole BB, anthocyanin (ANTH) and pre-C18 fractions offered the greatest protection, while chlorogenic acid offered the lowest protection. Protective capabilities of the various fractions against ROS depended upon the stressor, where the BB extract and the combined PAC (high and low m.w.) fraction offered the best protection against LPS and A β ₄₂ but were less effective against DA-induced ROS. The high and low m.w. PACs and the ANTH fractions enhanced ROS production regardless of the stressor used and this reflected increased activation of stress signals (e.g., P38 MAPK). The viability data indicated that the whole BB and combined PAC fraction showed greater protective effects against the stressors than the more fractionated polyphenolic components. Thus, these results suggest that, except for a few instances, the lesser the polyphenolic fractionation the greater the effects, especially with respect to prevention of ROS and stress signal generation, and viability.

Keywords

calcium recovery; blueberry fractions; anthocyanins; proanthocyanidins; reactive oxygen species

Introduction

Our research has shown that supplementation with fruits and vegetables rich in polyphenolics is beneficial in both forestalling and reversing the deleterious effects of aging on neuronal communication and behavior (1). For example, in a previous experiment (2) we found that dietary supplementation (for 8 weeks) of blueberry (BB) extracts in the rodent diets was effective in reversing age-related deficits in neuronal and behavioral (motor and cognitive) function in aged (19 mo) Fischer 344 (F344) rats. We also observed that BB diet reversed age-related dysregulation in Ca_{45} buffering capacity and decreased reactive oxygen species. Similarly, buffering was decreased in dopamine (DA)- or (amyloid beta, $\text{A}\beta_{42}$)-exposed cultured primary hippocampal neuronal cells (HNC), and BB pre-treatment of the cells prevented these deficits.

The beneficial properties of the BB have been postulated to be due to the polyphenolic makeup of the fruit (3). All plants, including fruit or vegetable bearing plants, synthesize a vast array of chemical compounds that are not necessarily involved in the plant's metabolism. These secondary properties, of course, would include their antioxidant and anti-inflammatory (INF) properties.

The question remains, however, as to which polyphenolic “family” might be responsible for the beneficial BB effects. It appears that some of these positive benefits may be derived from flavonoids, possibly from the anthocyanin flavonoids, which are responsible for the colors in fruits such as berry fruits (e.g., strawberries, blueberries). Anthocyanins have been shown to have potent antioxidant/anti-INF activities (4–5), as well as to inhibit lipid peroxidation and the INF mediators COX-1 and -2 (6–7). These fruits also contain high levels of proanthocyanidins that have antioxidant effects similar to those of anthocyanins (8). Indeed, there is a long history of studies which have described the potent antioxidant activities of numerous flavonoids. As examples, flavonoids have been reported to inhibit lipid peroxidation in several biological systems including: mitochondria and microsomes (9–10), as well as erythrocytes (11–12) and liver (13). They appear to be potent inhibitors of both NADPH and CCl_4 -induced lipid peroxidation (14) and readily chelate iron (15). Thus, the present study was carried out to determine the most effective BB polyphenolic component that would alter DA- or $\text{A}\beta_{42}$ -induced deficits in calcium buffering, activation of ROS, increases in signals and decreases in viability in primary hippocampal cells. Note that the fraction and whole BB concentrations utilized in this study are based on previous cell studies (16–17) wherein the concentrations were chosen based on the effects on Recovery in the calcium imaging parameter. In vivo levels of many of these compounds in plasma and brain are sparse. Our major purpose of this experiment was to determine the mechanistic effects of these fractions on calcium buffering through their putative protective properties with respect to stress signaling.

Methods

Cell Maintenance and Treatment

NeuroPure™ E18 primary rat hippocampal cells were plated and grown in Neurobasal Medium according to the procedures of Gene Therapy Systems (San Diego, CA) and treated as described by Joseph and colleagues (16). Briefly, cells were allowed to differentiate for 4 to 5 days at 37°C before being tested. The primary treatment was carried out with frozen whole Tifblue BB (*Vaccinium virgatum*) (as a positive control) prepared as described in previous experiments (e.g., (18)). Briefly, BBs were homogenized in water (2:1 w/v) for 3 min. The homogenate was centrifuged at $13,000 \times g$ for 15 min at 4°C. The supernatant was collected, lyophilized, and freeze-dried extract was prepared in media for application to the cells. Additional groups of cells were pre-treated with BB fractions derived from wild

blueberry juice made from *Vaccinium angustifolium* Aiton (see below) and incubated for 45 min at 37°C. The fraction concentrations reflected the percentage of the amount that was contained in the whole BB fraction (at 0.5mg/ml) based on phenolic level (see BB fraction section below). Secondary treatments included: dopamine (DA, 0.1mM, 2hrs), A β ₄₂ (25 μ M, 24hrs) or LPS (1 μ g/ml, 4hrs). Following the incubations, cells were evaluated for alterations in calcium parameters, ROS generation and viability.

BB fractions

Polyphenolic fractions were obtained using a modified version of a procedure initially reported by Kader and colleagues (19). A commercially prepared wild blueberry juice was derived from whole ripe frozen blueberries (*Vaccinium angustifolium* Aiton) (PRE-C18) by Van Dyk's Health Juice Products Ltd. (Caledonia, NS, Canada). Berries were thawed, pressed, filtered, and pasteurized. One kilogram of blueberry fruit produced approximately 735 mL of single-strength juice. The anthocyanin content of whole blueberries is approximately 13.96 mg cyanidin 3 glucoside eq per g dry weight while the commercial juice was 8.87 mg cyanidin 3 glucoside eq per g dry weight (or 64%). The concentrations of the fractions utilized were determined as a function of their phenolic levels in whole BB as described in Kalt and colleagues (20). This study provided a breakdown of the proportions of major phenolic groups in BB. The juice was applied to an activated C18 column (Waters Ltd, Mississauga, ON), washed with water to remove sugars and organic acids (POST-C18), then washed with methanol (Fisher Scientific, Mississauga, ON) to elute the anthocyanins/proanthocyanidins (PAC). The post-C18 material was lyophilized, then re-dissolved in a minimum volume of water. The aqueous solution was extracted 5 times with a 2 $\times$ volume of ethyl acetate. The aqueous portion was rotovapped to remove traces of ethyl acetate (Fisher Scientific), then methanol (or ethanol) was added to bring the final solution to 25% methanol. This was applied to a Sephadex LH20 (Sigma, St. Louis, MO) column. The column was washed with 50% methanol, which eluted anthocyanins (ANTH). The LH-20 column was then washed with 70% acetone (Fisher Scientific) to elute the high molecular weight proanthocyanidins (HMW). The ethyl acetate fraction from the extraction step was rotovapped to dryness, then resolubilized in a minimum volume of 25% methanol. This was applied to a second LH20 column. The column was washed with 50% methanol to remove chlorogenic acid (CA) and other hydroxycinnamates, then with 70% acetone to remove low molecular weight proanthocyanidins (LMW). Chlorogenic acid used in this study was purchased from Sigma. All fractions of interest were dried and resolubilized accordingly. The concentrations of the various fractions that were used to treat the cells were determined by their phenolic levels in the whole BB. These fraction concentrations are shown in Table 1.

Ca²⁺ Imaging

Calcium imaging was carried out as previously described by Joseph and colleagues (21). Figure 1 shows typical Baseline, Response (to 30 mM KCl, > 30%), and Recovery (CAR) in control hippocampal cells. Note that for this experiment Baselines and Responses under the various conditions did not differ. Thus, only the % Recovery is reported.

Viability

Viability was assessed using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes Eugene, OR). Cells were stained for 30 minutes with calcein AM, which stains the live cells green, and ethidium homodimer-1 (EthD-1), which stains the dead cells red. Fluorescent images of the cells were then captured with a Nikon TE2000U Inverted Microscope. The numbers of live and dead cells were then counted for each image, and percent viability was determined.

ROS generation

ROS production was assessed using an Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Invitrogen Eugene, OR). Cells were stained for 30 minutes using carboxy-y-H₂ DCFDA and counterstained with Hoechst 33342. Fluorescent images of the cells were then captured with a Nikon TE2000U Inverted Microscope. The mean green value of each cell was then measured by circling each individual cell and normalizing for the background.

Immunofluorescence

Cellular changes in the level of activation of a subset of stress signals (Jun kinase, JNK; nuclear factor kappa B, NFκB; P38 mitogen activated protein kinase, P38 MAPK) and a protective signal (MAPK) in the hippocampal control or fraction-supplemented cells exposed to DA under the various conditions were analyzed by fluorescence immunocytochemistry. Briefly, the differentiated hippocampal cells in 96-well plates were treated with various stressors with or without the fractions as indicated. After treatments, cells were fixed for 15 min in 100% methanol at -200°C. The fixative was then removed and the cells were washed twice in 4°C PBS (5 min/wash). The primary antibody diluted in blocking solution was then applied to the wells, and the cells were incubated for 1 hr at room temperature in a humid chamber, then washed 4 times in PBS. The fluorescence-labeled secondary antibody was added to the wells, and the cells were incubated for 1 hr at room temperature as before. The cells were then washed 4 times with PBS. The cells were mounted with 95% glycerol in PBS and images were captured with a Nikon Eclipse TE200U inverted fluorescence microscope coupled to a digital CCD camera (Hamamatsu Photonics). The levels of fluorescence intensity, for each individual cell less that of the background, per treatment were analyzed and averaged with Simple PCI software (Compix, Inc. Mars, PA). Note that only DA was used for these determinations, since as seen below, there was greater variation among the fractions with DA used as the stressor than with LPS or Aβ₄₂. The following antibodies were used: phospho P38 MAPK (Cell Signaling, rabbit polyclonal); phospho JNK (Cell Signaling, rabbit polyclonal); phospho NFκB (Abcam, rabbit polyclonal); and phospho MAPK, which detects erk1 and erk2 (Cell Signaling, mouse monoclonal). Negative controls for each antibody were also run by omitting either the primary or secondary antibodies.

Data Analysis

Recovery was analyzed by Kruskal-Wallis one-way analyses of variance (ANOVA) and Mann Whitney U tests. *Viability* and *ROS generation* were analyzed by ANOVA and Fisher's LSD post-hoc tests. Alterations in phospho (p)MAPK, pNFκB, pP38 MAPK, and pJNK were assessed via ANOVA and Fisher's LSD post hoc tests.

Results

Ca²⁺ Imaging

Figure 2A shows the differences in the values of Recovery in whole BB, fraction pre-treated, or control cells exposed to DA (control vs DA, $p < 0.001$). In cells pretreated with the whole BB extract, LMW, ANTH, or PRE-C18, the effects of DA on recovery were antagonized (BB vs BB + DA; LMW vs LWM + DA; ANTH vs ANTH + DA; PRE-C18 vs PRE-C18 + DA, all $p > 0.05$). However, as can be seen from Figure 2A, it appeared that in the cells pre-treated with PAC, HMW, POST-C18, or CA (PAC vs PAC + DA, $p < 0.001$; HMW vs HMW + DA, $p < 0.01$; POST-C18 vs POST + DA, $p < 0.0001$; CA vs CA + DA, $p < 0.001$), the fractions offered only reduced effects on CAR. Interestingly, though, all of the fractions except for the POST-C18 and the chlorogenic acid fractions showed some protection against DA, since comparisons with the DA alone condition differed from all the

remaining treatments (BB, PAC, HMW, LMW ANTH, or PRE-C18 vs DA under all these conditions showed increases in Recovery, $p < 0.05$).

As shown in Figure 2B, and as seen previously, Recovery was reduced in the $A\beta_{42}$ -exposed cells (control vs $A\beta_{42}$, $p < 0.001$). However, unlike the effects seen with DA, a greater number of the fractions were effective in antagonizing the effects of $A\beta_{42}$ (e.g., PAC vs $A\beta_{42}$ + PAC, $p > 0.05$). The only fraction that failed to protect CAR from $A\beta_{42}$ was CA (CA vs CA + $A\beta_{42}$, $p < 0.001$).

Similar effects were seen with respect to LPS treatment (Figure 2C) where pretreatment of the cells with the BB fractions was effective in antagonizing the effects of LPS on CAR. The only fractions that failed to protect CAR from LPS were PRE-C18 (PRE-C18 vs PRE-C18 + LPS, $p < 0.039$) and CA (e.g., CA vs CA + LPS, $p < 0.01$).

Viability

Overall, it appeared that some of the fractions may have lowered the viability of the cells in the absence of the stressor (e.g., control vs: LMW, PRE-C18, or POST-C18, $p < 0.011$), while BB, PAC, HMW, ANTH, and CA had no significant effect on viability (control vs: BB, PAC, HMW, ANTH or CA, $p > 0.05$, Figure 3). As was seen above with respect to Recovery, DA also reduced viability in the hippocampal cells (control vs DA, $p < 0.0001$, Figure 3A). It also appeared that neither LMW nor ANTH offered protection against DA induced decreases in viability (LMW vs LMW + DA, $p < 0.024$; ANTH vs ANTH + DA, $p < 0.001$). However, it did appear that although the pre-C18 fraction lowered the viability of the cells in the absence of DA, no further reductions were seen in the PRE-C18 pre-treated cells that were further treated with DA ($p > 0.05$). In fact, the BB + DA cells did not differ from the PRE-C18 cells ($p > 0.05$). Interestingly, CA, while not protecting against DA-induced deficits in CAR, did protect against the DA-induced deficits in viability (CA vs CA + DA, $p > 0.05$). In fact, only the BB + DA, PAC + DA, and HMW + DA treatments differed from the DA alone condition (e.g., DA vs HMW + DA, $p < 0.004$, Figure 3A).

It also appeared that several of the fractions were able to prevent the decreases in viability induced by $A\beta_{42}$, most notably BB, HMW, LMW, PRE- and POST-C18 (e.g., all comparisons of fractions with the $A\beta_{42}$ + fractions, $p > 0.05$), while ANTH, PAC, and CA were not (PAC vs PAC + $A\beta_{42}$, $p < 0.0001$; ANTH vs ANTH + $A\beta_{42}$, $p < 0.001$; CA vs CA + $A\beta_{42}$, $p < 0.02$) (Figure 3B).

Additionally, as seen with DA and $A\beta_{42}$, LPS reduced the viability of the hippocampal cells; however, almost none of the fractions were effective in preventing the LPS-induced deficits, except for the LMW, POST-C18, and the CA fractions (e.g., LMW, POST-C18, or CA vs LMW, POST-C18 or CA + LPS, $p > 0.05$) (Figure 3C).

ROS generation

Figure 4 shows that neither whole BB extract, PAC, PRE-C18 or POST-C18 enhanced ROS in the absence of a stressor (e.g., control vs: BB, PAC, PRE-C18, or POST-C18, $p > 0.05$). Conversely, HMW, LMW, ANTH, and CA increased ROS in the absence of stressor (control vs: HMW, LMW, ANTH, or CA, $p < 0.011$). The data also indicate that there were differences among the levels of protection offered by the fractions that were dependent upon the stressor. As shown in Figure 4A, only the whole BB extract and the CA fraction were effective in preventing ROS via DA stimulation (DA vs BB + DA or CA + DA, $p < 0.0001$). However, if the cells treated with the extracts are compared to cells treated only with DA + the extract, then PAC vs PAC + DA and PRE-C18 vs PRE-C18 + DA ($p > 0.05$) are also effective in this regard. The results also indicated that HMW, LMW, ANTH and POST-C18 produced a synergistic effect with DA such that the ROS production was **greater** than

that seen in the absence of these fractions (DA vs DA + HMW, $p < 0.01$; DA vs DA + ANTH, DA + LMW, $p < 0.0001$, Figure 4A).

In the case of $A\beta_{42}$, BB and PAC decreased $A\beta_{42}$ -enhanced ROS ($A\beta_{42}$ vs BB + $A\beta_{42}$ or PAC + $A\beta_{42}$, $p < 0.0001$). However, similar to the findings seen with DA, there was a significant synergistic effect between $A\beta_{42}$ and the remainder of the fractions in increasing ROS ($A\beta_{42}$ vs: $A\beta_{42}$ + HMW, LMW, ANTH or CA, $p < 0.001$; $A\beta_{42}$ vs POST-C18 + $A\beta_{42}$, $p < 0.012$) where ROS were enhanced to a **greater extent** than that seen with $A\beta_{42}$ alone (Figure 4B). Interestingly, unlike the findings concerning DA or $A\beta_{42}$, Figure 4C shows that there were no synergistic increases with LPS and the various fractions as compared to the LPS alone condition (all LPS vs: LPS + ANTH, LMW, or CA comparisons, $p > 0.05$). Additionally, all fractions + LPS (BB, PAC HMW, LMW, ANTH, PRE-C18, CA), except for the POST-C18 fraction, showed ROS greater than their respective fraction pre-treatment condition. However in comparisons between the various fraction treatment in the presence of LPS vs. with LPS alone, the results showed that there was some lowering of the LPS-induced increases in ROS [BB (LPS vs BB + LPS, $p < 0.001$), HMW (LPS vs HMW + LPS, $p < 0.018$) PRE-C-18 (LPS vs LPS + PRE-C-18, $p < 0.027$); POST-C18 (LPS vs POST-C18 + LPS, $p < 0.0001$)].

Immunofluorescence

Phospho (p)MAPK—In the absence of DA, BB and ANTH significantly raised pMAPK over that seen in control cells (control vs BB, $p < 0.003$; control vs ANTH, $p < 0.0001$) (Figure 5A). DA also significantly raised pMAPK ($p < 0.0001$). Each of the fractions except for ANTH significantly increased pMAPK as compared to their respective non-DA-pre-treated, fraction-treated cells (ANTH vs ANTH + DA, $p > 0.05$), but none of the fractions increased pMAPK to the level seen with DA alone.

pJNK—In the absence of DA, neither BB nor any of the fractions increased pJNK to a value greater than that seen in control cells (all comparisons $p > 0.05$ with control). However, in the presence of DA, BB treatment significantly reduced pJNK to a level significantly lower than that seen with any of the other fractions (BB + DA vs: PAC + DA, HMW + DA, LMW + DA, ANTH + DA, PRE-C18 + DA, POST-C18 + DA, all comparisons $p < 0.0001$) (Figure 5B).

pP38 MAPK—In the absence of DA, all of the fractions significantly increased pP38 MAPK (control vs: PAC, HMW, LMW, ANTH, PRE- and POST-C18, all comparisons $p < 0.009$, Figure 5C), except for BB (control vs BB, $p > 0.05$). In the presence of DA, BB significantly lowered pP38 MAPK (DA vs BB + DA, $p < 0.0001$). The effects of the other fractions were mixed, with the HMW (DA vs HMW + DA, $p < 0.002$), LMW (DA + LMW + DA, $p < 0.012$), ANTH (DA vs ANTH + DA, $p < 0.0001$) and the PRE-C18 (DA vs PRE-C18 + DA, $p < 0.0001$) fractions showing decreases in pP38 MAPK. Conversely, cells treated with DA + PAC or DA + POST-C18 fractions showed similar increases in pP38MAPK to that seen in the DA-treated cells all comparisons $p > 0.05$, Figure 5C).

pNFkB—DA significantly increased NFkB as compared to control ($p < 0.001$). In the absence of DA, only one of the fractions significantly increased pNFkB (control vs: HMW, $p < 0.0001$, Figure 5D). In the presence of DA, BB significantly lowered pNFkB (DA vs BB + DA, $p < 0.0001$). The effects of the other fractions were mixed, with the HMW (DA vs HMW + DA, $p < 0.0001$), PRE-C18 (DA vs PRE-C18 + DA, $p < 0.0001$), and ANTH (DA vs ANTH + DA, $p < 0.05$) fractions showing decreases in pNFkB. Conversely, cells treated with DA + PAC, LMW, and POST-C18 fractions showed similar increases in pNFkB to that seen in cells treated with DA alone (all comparisons $p > 0.05$, Figure 5D). Note also that the

LMW, ANTH, and POST fractions had higher activity in pNF κ B with DA than its respective fraction-treated control (e.g. ANTH vs ANTH + DA, $p < 0.01$).

Discussion

Early findings suggested that there was significant neuronal loss and other morphological changes that occur in the aging brain (22). However, additional studies indicated that the age-related changes are much more subtle and involve calcium dysregulation (23–25). While these age-associated changes appear to be dependent upon the particular neurons involved, they all involve various changes in calcium homeostasis and alterations in calcium regulation, with hippocampal and cortical neurons showing the greatest alterations (26–27). As indicated in the Introduction, however, all of the changes involve some decline in calcium buffering. It has been postulated that these changes are the result of oxidative stress (28).

Indeed, results of the present paper would support these findings, since DA, A β_{42} , and LPS all reduced calcium buffering in the hippocampal cells. However, it appeared that, overall, the protective effects of the various fractions on calcium buffering were dependent upon the particular stressor to which they were exposed. Generally, the whole BB extract and pre-C18 fraction were the most effective in protecting CAR among the stressors, especially with regard to DA and A β_{42} , while CA offered the least protection. These findings suggest that it may be the synergistic effects among the various polyphenolic families in the least fractionated forms of the blueberry (e.g., BB and PRE-C18) that provided the most generalized protection against DA and A β_{42} . However, note that there were differences between the BB extract and the PRE-C18 fraction in protecting CAR against LPS. It appeared that CAR was somewhat impaired when LPS was applied to the PRE-C18 treated cells. This was not seen with the BB extract-treated cells, where CAR decreases were not seen with LPS. These differences were also reflected in the assessments of Viability where it appeared that the PRE-C18 fraction lowered viability in the absence of the stressors and a further lowering was seen with LPS treatment. Again these alterations in viability were not seen in the cells treated with the BB extract, although there were no differences in viability in DA- or A β_{42} -treated cells that were pre-treated with the BB extract or the PRE-C18 fraction. There was also some indication that the PRE-C18 fraction was not as protective against increases in ROS with DA- or A β_{42} . These differences could be the result of differences in concentrations between the two pre-treatments (BB or PRE-C18), since the concentrations utilized were based upon the phenolic levels of the compounds in the whole BB. The water soluble BB extract was utilized at 500 μ g/ml while the PRE-C18 fraction utilized was one-half that of the BB extract (250 μ g/ml). These differences could also reflect source variations, since the BB extract was derived from frozen whole Tifblue cultivated BBs, while the PRE-C18 fraction was derived from wild blueberry juice. The types of phenolics in the two fractions may differ, making direct comparisons between BB and PRE-C18 difficult. However, since we have previous data using the Tifblue BBs it was necessary to add this condition as a positive control. Additionally, the *Vaccinium angustifolium* berries used to produce the juice included literally many hundreds, and possibly thousands, of genotypes harvested from the semi-cultivated wild stands. This complex mixture would therefore represent the average phenolic composition for the species. This is in contrast to cultivated blueberries (*Vaccinium virgatum* was used in this study), whose commercial juice may be produced from far fewer genotypes and, for that reason, may be subject to genotypic variability within the species.

Among the fractions, PAC, the high molecular weight (HMW) proanthocyanidins, and POST-C18 fractions were not effective against DA in protecting CAR. However, except for CA which was not effective with any of the stressors, all of the fractions were significantly

more protective when LPS or A β ₄₂ was used as the stressor. In fact, overall, fewer differences in protection among the fractions were observed with LPS or A β ₄₂ than that seen with DA.

DA oxidation may be involved in the neuronal toxicity in neurodegenerative diseases such as Parkinson disease, where DA is easily oxidized to form DA quinones and other ROS species such as 3,4-dihydroxyphenylacetaldehyde or 3,4-dihydroxyphenylethyleneglycolaldehyde (DOPEGAL), and may be responsible for neuronal loss in Parkinson disease (29). One could speculate that reductions of ROS via nutritional supplementation with BBs may reduce DA toxicity. In this respect, in the present experiments when ROS was assessed among the various fractions, the data showed that even in the absence of the stressors (DA, LPS or A β ₄₂) some of the fractions raised rather than lowered ROS. These included the HMW, LMW, ANTH and CA. These findings support previous research which has shown that plant polyphenols can act as potent pro-oxidants. For example, research has indicated that resveratrol (30), flavonoids in general (31), tannins (32) and curcumin (32) can increase ROS.

While it has been suggested that the pro-oxidant effect of plant polyphenols may be an artifact (33) of in vitro methods, it is clear from the present paper that not all of the fractions, at least at the levels assessed here, increase ROS. It is possible that higher concentrations of these fractions may induce ROS increases. These include PAC, POST-C18 and to some extent the PRE-C18 fractions, in addition to the whole BB. As alluded to above these findings suggest that even in vitro the less fractionated that the fruit extract is, the less likely that ROS generation is seen in the absence of the stressors.

What is even more important is that when one considers the use of polyphenolic fractions as antioxidants in the presence of the various stressors, the pro-oxidant effects of the treatments may be even greater than that seen with the stressors alone. In the case of A β ₄₂ only the whole BB and the PAC treatments reduced the A β ₄₂-induced ROS effects; virtually all of the other fractions acted synergistically with the A β ₄₂, while HMW, LMW and ANTH fractions all acted synergistically with DA to increase ROS. Similar pro-oxidant synergistic effects have been reported previously with plant polyphenols (34–36).

However, when the parameters of calcium buffering and ROS are considered together, except possibly for CA treatment in the presence of LPS or A β ₄₂, there appears to be little relationship between ROS generation in the presence or absence of the stressor and the ability of the various fractions to mitigate the effects of the stressors on calcium buffering. As mentioned above, most of the fractions were protective, at least under the LPS or A β ₄₂ treatment conditions. These dichotomies suggest that ROS effects may not be reflective of the beneficial effects of the fractions on calcium buffering. It may be that their protective effects on calcium buffering involve alterations in downstream stress signals rather than direct quenching effects on ROS. We have shown in a previous study that the BB extract decreases several stress signals such as calcium response element binding protein (CREB), protein kinase C γ (PKC γ), and P38 MAPK, among other stress mediators that were enhanced by DA application to hippocampal cells (16). In the present experiment, DA, BB, ANTH and PRE-C18 significantly raised pMAPK over that seen in control cells, and each of the fractions (except for ANTH) increased pMAPK in the presence of DA.

BB reductions in pJNK in the presence of DA were greater than those of the other fractions, while PAC and POST-C18 increased pJNK in the presence of DA. Although BB and all the fractions increased pNF κ B, in the absence of DA only the whole BB reduced this parameter to a value lower than the control value. Similar findings were seen with DA and BB treatment with respect to pP38 MAPK where DA alone slightly increased this stress signal

but BB prevented the DA-induced increases in pP38 MAPK to a greater extent than any of the fractions. Thus, some of the differences between BB extract and the PRE-C18 fraction that were discussed above are also reflected in stress signal assessments, where DA-induced increases in JNK was greater with PRE-C18 pre-treatment than with the BB extract. However, the findings with respect to the other stress signals and pMAPK were similar.

However, despite these differences, taken together these data suggest that the major protective effects of the whole BB extract and to some extent the PRE-C18 fraction involve the reductions of DA-induced increases in stress signal activation (pP38 MAPK, and pNFkB). Additional evidence for this hypothesis regarding the various berry extract and fraction pre-treatments can be seen when the viability and ROS parameters are compared. In some cases there were parallels between the two parameters (e.g., ANTH increases in ROS under stressor conditions with corresponding decreases in viability, LMW under DA and LPS conditions with decreases in viability, and PAC with decreases in viability and increases in ROS with A β ₄₂ and LPS) but in other cases there was a dichotomy between the two dependent measures (e.g., HMW increases in ROS with A β ₄₂ and DA with no decreases in viability; increases in ROS with PRE-C18 and POST-C18 treatments with no decreases in viability with A β ₄₂), suggesting alternative forms of protection that may have protected CAR and also prevented losses of viability.

When viability was assessed in the hippocampal cells in the absence of the stressors, some of the fractions decreased viability (LMW, PRE- and POST-C18). One of the most interesting findings concerning viability is that, as with ROS, there seemed to be a dichotomy between the effects of some of the fractions on viability (e.g., CA) and the level of protection provided by these fractions on hippocampal cell calcium buffering (CAR). For example, in the presence of DA there were differences among the fractions regarding the level of protection of CAR. However, there were fewer differences in the efficacy of the fractions when A β ₄₂ or LPS were used as stressors. This was not seen with viability where there were differences among the fractions regardless of the particular stressor utilized. This would suggest that assessments of viability may not be related to changes in CAR, and the cell, while showing decreases in viability, may still function with respect to CAR as a normal cell. This dichotomous condition is similar to that seen in normal aging where viability does not seem to explain losses in CAR. For example, it has been suggested that the degree of disruption of calcium buffering may not alter cell function or even viability, but it does make the cell more vulnerable to other stressors (37). As mentioned above, in the present study, chlorogenic acid was not protective against CAR decreases induced by any stressor, but offered some protection against viability loss with these stressors. Clearly, however, these relationships are complex and are stressor/fraction/parameter dependent.

Additionally, previous mechanistic studies have also shown that, in addition to their potent antioxidant/anti-inflammatory effects, the beneficial properties of fruit polyphenols such as those found in BB might occur through alterations in stress signaling mediators such as extracellular signal regulated kinase (ERK), protein kinase C (PKC), cyclic AMP response element binding protein (CREB), and nuclear factor kappa B (NFkB). Indeed, our research has, thus far, shown that the BB protection against A β ₄₂- or DA- induced decrements in intracellular calcium clearance following oxotremorine-induced depolarization in M1 muscarinic receptor (MACHR)-transfected COS-7 cells or neonatal hippocampal neurons involved reductions in phosphorylated MAPK, PKC γ , and phosphorylated CREB (17). Similar findings, have also been seen with BB treatment in primary hippocampal cells (16). The results showed that BB pre-treatment prevented the deficits in calcium buffering, normalized cyclic CREB and PKC γ associated with ROS signaling, and increased expression of protective ERK. Thus, these findings and those of previous studies suggest that the primary mechanisms involved in the beneficial effects of the berries may involve

alterations in stress signals. However, since the concentrations utilized here may have been higher than those that occur in vivo, the effects of the treatments will have to be assessed further in whole animal models. Nevertheless, the findings here suggest that one of the mechanisms involved in the beneficial effects of BB may involve reductions in stress signals.

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Abbreviations used

CAR	calcium recovery
ROS	reactive oxygen species
MACHRs	muscarinic acetylcholine receptors
OS	oxidative stress
INF	inflammation
HNC	hippocampal neuronal cells
DA	dopamine
Aβ	amyloid beta
BB	blueberry
NFκB	nuclear factor kappa B
JNK	jun kinase
MAPK	p38 mitogen activated protein kinase
ERK	extracellular signal regulated kinase
CREB	Cyclic AMP Response Element Binding Protein
PKC	Protein Kinase C
LPS	lipopolysaccharide
ANTH	anthocyanin
PAC	proanthocyanidin
HMW	high molecular weight PAC
LMW	low molecular weight PAC
CA	chlorogenic acid
PRE	PRE-C18
POST	POST-C18

Literature Cited

1. Lau FC, Shukitt-Hale B, Joseph JA. The beneficial effects of fruit polyphenols on brain aging. *Neurobiol Aging*. 2005; 26 Suppl 1:128–132. [PubMed: 16194581]
2. Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ, Bickford PC. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral

- deficits with blueberry, spinach, or strawberry dietary supplementation. *J Neurosci*. 1999; 19(18): 8114–8121. [PubMed: 10479711]
3. Lau FC, Joseph JA, McDonald JE, Kalt W. Attenuation of iNOS and COX2 by blueberry polyphenols is mediated through the suppression of NF-[kappa]B activation. *J Funct Foods*. 2009; 1(3):274–283.
 4. Wang H, Nair MG, Strasburg GM, Chang YC, Booren AM, Gray JI, DeWitt DL. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J Nat Prod*. 1999; 62(2):294–296. [PubMed: 10075763]
 5. Seeram NP, Momin RA, Nair MG, Bourquin LD. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine*. 2001; 8(5):362–369. [PubMed: 11695879]
 6. Seeram NP, Nair MG. Inhibition of lipid peroxidation and structure-activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *J Agric Food Chem*. 2002; 50(19): 5308–5312. [PubMed: 12207466]
 7. Seeram NP, Zhang Y, Nair MG. Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. *Nutr Cancer*. 2003; 46(1):101–106. [PubMed: 12925310]
 8. Pan MH, Ho CT. Chemopreventive effects of natural dietary compounds on cancer development. *Chem Soc Rev*. 2008; 37(11):2558–2574. [PubMed: 18949126]
 9. Bindoli A, Cavallini L, Siliprandi N. Inhibitory action of silymarin of lipid peroxide formation in rat liver mitochondria and microsomes. *Biochem Pharmacol*. 1977; 26(24):2405–2409. [PubMed: 597328]
 10. Cavallini L, Bindoli A, Siliprandi N. Comparative evaluation of antiperoxidative action of silymarin and other flavonoids. *Pharmacol Res Commun*. 1978; 10(2):133–136. [PubMed: 652840]
 11. Sorata Y, Takahama U, Kimura M. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Biochim Biophys Acta*. 1984; 799(3): 313–317. [PubMed: 6733152]
 12. Maridonneau-Parini I, Braquet P, Garay RP. Heterogeneous effect of flavonoids on K⁺ loss and lipid peroxidation induced by oxygen-free radicals in human red cells. *Pharmacol Res Commun*. 1986; 18(1):61–72. [PubMed: 3006093]
 13. Kimura Y, Okuda H, Tani T, Arichi S. Studies on *Scutellariae radix*. VI. Effects of flavanone compounds on lipid peroxidation in rat liver. *Chem Pharm Bull (Tokyo)*. 1982; 30(5):1792–1795. [PubMed: 7116513]
 14. Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol*. 1989; 38(11):1763–1769. [PubMed: 2735934]
 15. Ratty AK, Das NP. Effects of flavonoids on nonenzymatic lipid peroxidation: structure-activity relationship. *Biochem Med Metab Biol*. 1988; 39(1):69–79. [PubMed: 3355718]
 16. Joseph JA, Carey A, Brewer GJ, Lau FC, Fisher DR. Dopamine and abeta-induced stress signaling and decrements in Ca²⁺ buffering in primary neonatal hippocampal cells are antagonized by blueberry extract. *J Alzheimers Dis*. 2007; 11(4):433–446. [PubMed: 17656822]
 17. Joseph JA, Fisher DR, Bielinski D. Blueberry extract alters oxidative stress-mediated signaling in COS-7 cells transfected with selectively vulnerable muscarinic receptor subtypes. *J Alzheimers Dis*. 2006; 9(1):35–42. [PubMed: 16627932]
 18. Youdim KA, Shukitt-Hale B, Martin A, Wang H, Denisova N, Bickford PC, Joseph JA. Short-term dietary supplementation of blueberry polyphenolics: beneficial effects on aging brain performance and peripheral tissue function. *Nutritional Neuroscience*. 2000; 3:383–397.
 19. Kader F, Rovel B, Girardin M, Metche M. Fractionation and identification of the phenolic compounds of Highbush blueberries (*Vaccinium corymbosum*, L.). *Food Chemistry*. 1996; 55(1): 35–40.
 20. Kalt W, MacKinnon S, McDonald J, Vinqvist M, Craft C, Howell A. Phenolics of *Vaccinium* berries and other fruit crops. *J Sci Food Agric*. 2008; 88(1):68–76.

21. Joseph JA, Fisher DR, Strain J. Muscarinic receptor subtype determines vulnerability to oxidative stress in COS-7 cells. *Free Radic Biol Med*. 2002; 32(2):153–161. [PubMed: 11796204]
22. Murchison D, Griffith WH. Calcium buffering systems and calcium signaling in aged rat basal forebrain neurons. *Aging Cell*. 2007; 6(3):297–305. [PubMed: 17517040]
23. Gibson GE, Peterson C. Calcium and the aging nervous system. *Neurobiol Aging*. 1987; 8(4):329–343. [PubMed: 3306433]
24. Landfield PW. 'Increased calcium-current' hypothesis of brain aging. *Neurobiol Aging*. 1987; 8(4):346–347. [PubMed: 3627350]
25. Khachaturian ZS. Calcium hypothesis of Alzheimer's disease and brain aging. *Ann N Y Acad Sci*. 1994; 747:1–11. [PubMed: 7847664]
26. Toescu EC, Verkhatsky A. The importance of being subtle: small changes in calcium homeostasis control cognitive decline in normal aging. *Aging Cell*. 2007; 6(3):267–273. [PubMed: 17517038]
27. Foster TC. Calcium homeostasis and modulation of synaptic plasticity in the aged brain. *Aging Cell*. 2007; 6(3):319–325. [PubMed: 17517041]
28. Esin MM. Ageing and the brain. *J. Pathol*. 2007; 211:181–187. [PubMed: 17200950]
29. Burke WJ, Li SW, Williams EA, Nonneman R, Zahm DS. 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis. *Brain Res*. 2003; 989(2):205–213. [PubMed: 14556942]
30. de la Lastra CA, Villegas I. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem Soc Trans*. 2007; 35(Pt 5):1156–1160. [PubMed: 17956300]
31. Rahman A, Shahabuddin, Hadi SM, Parish JH. Complexes involving quercetin, DNA and Cu(II). *Carcinogenesis*. 1990; 11(11):2001–2003. [PubMed: 2225332]
32. Hadi SM, Asad SF, Singh S, Ahmad A. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. *IUBMB Life*. 2000; 50(3):167–171. [PubMed: 11142343]
33. Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans*. 2007; 35(Pt 5):1147–1150. [PubMed: 17956298]
34. Bahadorani S, Hilliker AJ. Antioxidants cannot suppress the lethal phenotype of a *Drosophila melanogaster* model of Huntington's disease. *Genome*. 2008; 51(5):392–395. [PubMed: 18438443]
35. Sanchez Y, Amran D, Fernandez C, de Blas E, Aller P. Genistein selectively potentiates arsenic trioxide-induced apoptosis in human leukemia cells via reactive oxygen species generation and activation of reactive oxygen species-inducible protein kinases (p38-MAPK, AMPK). *Int J Cancer*. 2008; 123(5):1205–1214. [PubMed: 18546268]
36. Shamim U, Hanif S, Ullah MF, Azmi AS, Bhat SH, Hadi SM. Plant polyphenols mobilize nuclear copper in human peripheral lymphocytes leading to oxidatively generated DNA breakage: implications for an anticancer mechanism. *Free Radic Res*. 2008; 42(8):764–772. [PubMed: 18661438]
37. Wojda U, Salinska E, Kuznicki J. Calcium ions in neuronal degeneration. *IUBMB Life*. 2008; 60(9):575–590. [PubMed: 18478527]

Figure 1.

This figure shows a typical Baseline (A), peak depolarization following 30 mM KCl (B), and Recovery (C) and in control cells that were not treated with DA, LPS or A β ₄₂.

Figure 2.

Differences in the values of Calcium Recovery in whole BB, fraction pre-treated, or control (Cont) cells exposed to DA (A), A β 42 (B), or LPS (C). **b**= $p < 0.05$ from stressor control; **c**= $p < 0.05$ from own treated control.

Figure 3. Differences in viability in whole BB, fraction pre-treated, or control (Cont) cells exposed to DA (A), Aβ42 (B), or LPS (C). **a**=p<0.05 from control; **b**=p<0.05 from stressor control; **c**=p<0.05 from own treated control.

Figure 4. Differences in ROS in whole BB, fraction pre-treated, or control (Cont) cells exposed to DA (A), A β 42 (B), or LPS (C). **a**= $p < 0.05$ from control; **b**= $p < 0.05$ from stressor control; **c**= $p < 0.05$ from own treated control.

Figure 5.

Cellular changes in the level of activation of a subset of protective (MAPK, Figure 5A) and stress signals (JNK, Figure 5B; P38MAPK, Figure 5C; NfκB, Figure 5D) in whole BB, fraction pre-treated, or control (Cont) cells exposed to DA and analyzed by fluorescence immunocytochemistry. **a**= $p<0.05$ from control; **b**= $p<0.05$ from DA control; **c**= $p<0.05$ from own treated control.

Table 1

Concentrations of the whole BB extract and the various BB fractions used in the hippocampal cells (based upon phenolic levels in the whole BB)

COMPOUND	PHENOLIC LEVEL	CONCENTRATION
BB whole extract	10.15 mg/g	500 µg/ml
PRE-C18	22.70 mg/g	250 µg/ml
POST-C18	329.37 mg/g	17.5 µg/ml
Anthocyanins	363.50 mg/g	15 µg/ml
Proanthocyanidins (PACs)	414.79 mg/g	14 µg/ml
Low molecular weight PACs	1043.37 mg/g	5.5 µg/ml
High molecular weight PACs	492.96 mg/g	11.5 µg/ml
Chlorogenic acid	364.0 mg/g	15 µg/ml