Neurochemical differences in learning and memory paradigms among rats supplemented with anthocyanin-rich blueberry diets and exposed to acute doses of ^{56}Fe particles

Shibu M. Poullose¹, Bernard M. Rabin², Donna F. Bielinski¹, Megan E. Kelly¹, Marshall G. Miller¹, Nopporn Thanthaeng¹, and Barbara Shukitt-Hale^{1,*}

1. USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111, U.S.A.
2. Department of Psychology, UMBC, Baltimore, MD 21250, U.S.A.

CORRESPONDENCE:

Barbara Shukitt-Hale, Neuroscience and Aging Laboratory

USDA HNRCA at Tufts University

711 Washington Street, Boston, MA 02111, USA

Phone: 617-556-3118

Fax: 617-556-3299

Email: barbara.shukitthale@ars.usda.gov

ABSTRACT

The protective effects of anthocyanin-rich blueberries (BB) on brain health are well documented and are particularly important under conditions of high oxidative stress, which can lead to “accelerated aging.” One such scenario is exposure to space radiation, consisting of high-energy and -charge particles (HZE), which are known to cause cognitive dysfunction and deleterious neurochemical alterations. We recently tested the behavioral and neurochemical effects of acute exposure to HZE particles such as ^{56}Fe , within 24–48 h after exposure, and found that radiation primarily affects memory and not learning. Importantly, we observed that specific brain regions failed to upregulate antioxidant and anti-inflammatory mechanisms in response to this insult. To further examine these endogenous response mechanisms, we have supplemented young rats with diets rich in BB, which are known to contain high amounts of antioxidant-phytochemicals, prior to irradiation. Exposure to ^{56}Fe caused significant neurochemical changes in hippocampus and frontal cortex, the two critical regions of the brain involved in cognitive function. BB supplementation significantly attenuated protein carbonylation, which was significantly increased by exposure to ^{56}Fe in the hippocampus and frontal cortex. Moreover, BB supplementation significantly reduced radiation-induced elevations in NADPH-oxidoreductase-2 (NOX2) and cyclooxygenase-2 (COX-2), and upregulated nuclear factor erythroid 2-related factor 2 (Nrf2) in the hippocampus and frontal cortex. Overall results indicate that ^{56}Fe particles may induce their toxic effects on hippocampus and frontal cortex by reactive oxygen species (ROS) overload, which can cause alterations in the neuronal environment, eventually leading to hippocampal neuronal death and subsequent impairment of cognitive function. Blueberry supplementation provides an effective preventative measure to reduce the ROS load on the CNS in an event of acute HZE exposure.

Key Words: Learning, Memory, Inflammation, hippocampus, Radiation, blueberries.

INTRODUCTION

Recent discoveries from NASA's Mars Exploratory Program have offered new insights and raised scientific questions regarding challenges for future crewed missions, including exposure to cosmic rays, which are composed of particles of high-energy and -charge (HZE particles), protons, and alpha particles (Hellweg and Baumstark-Khan, 2007). Exposure to HZE particles at NASA-relevant doses (>30 cGy) is known to cause detrimental effects in animals, affecting both physiological and cognitive function, and may pose a risk to the crew in exploratory Mars missions (Hellweg and Baumstark-Khan, 2007; Rabin et al., 2015). Among the variety of HZE particles in space, ^{56}Fe particles have been well studied and shown to cause alterations in the redox status and bioenergetics of the central nervous system, leading to an overproduction of oxidants, which has been implicated in an array of health conditions, including neurodegenerative diseases, cancer, cardiovascular diseases, diabetic complications and physiological aging (Brieger et al., 2012; Ungvari et al., 2010). Moreover, studies using animal models have revealed that ^{56}Fe exposure can cause neuroinflammation, defects in neurogenesis, and cognitive impairment (Britten et al., 2012; Poulouse et al., 2014; Rabin et al., 2015; Rola et al., 2004).

Numerous reports from our laboratory and others' have indicated that diets rich in antioxidants, such as those found in fruits and vegetables, can prevent the neurochemical and behavioral changes that occur during aging (Elks et al., 2011; Joseph et al., 2009; Miller and Shukitt-Hale, 2012; Poulouse et al., 2012; Poulouse et al., 2016). Recently, we have reported that young rats, exposed to 1.5 Gy or 2.5 Gy of 1 GeV/n high-energy ^{56}Fe particles, showed altered gene expression in the hippocampus, a region of the brain important for memory, within 36 hours after irradiation (Shukitt-Hale et al., 2013). Feeding the animals with 2% BB or 2% strawberry diets prior to irradiation produced an up-regulation of some protective stress signal genes such as IKK ϵ (an apoptotic regulator gene and an up-regulator of the inflammatory/oxidative stress signal, NF- κ B) and MMK3 (also an apoptotic regulator gene) (Shukitt-Hale et al., 2013). We have also reported that feeding animals diets supplemented with antioxidant-rich berries, specifically blueberries and strawberries, can counter the deleterious effects of ^{56}Fe , both short-term and long-term, by reducing oxidative stress and inflammation (Poulouse et al., 2014; Shukitt-Hale et al., 2007). While radiation increased the incidence of

neuronal damage through oxidative stress and inflammation, the animals which were fed with berry diets not only had a better cognitive outcome but also showed improved neuroprotection through improved protective signaling and sequestration of damaged or misfolded proteins into inclusion bodies.

Previously we have reported that memory-related changes in cognitive performance can occur within 24-48 hours following exposure to HZE particles, independent of learning (Rabin et al., 2015). That study enabled us to verify the acute effects of ^{56}Fe on learning without testing for memory (i.e., whether the subject remembered the task) or test for effects on memory without testing for learning (i.e., whether the subject learned the task). While deficits have been reported in the performance of some cognitive tasks within this time frame, there have been no studies of acute effects to distinguish the effect of HZE on memory and learning independently, nor of the potential protection against the effects of acute HZE exposure offered by antioxidant-rich diets. The current study investigated the protective effects of blueberry (BB) supplementation prior to exposure to acute levels of HZE particles (^{56}Fe) and consequent neurochemical alterations using established learning and memory paradigms. We used a “learning” and “memory” paradigm, wherein irradiation precedes conditioning in the “learning” condition and conditioning occurs prior to irradiation in the “memory” condition (Rabin et al., 2015). Therefore, given the accumulating evidence of the deleterious effects of radiation on the CNS and of the protective effects afforded by antioxidant-rich berry diets, in the current study we examined the acute effects of ^{56}Fe particle exposure, especially radiation-induced oxidative damage and the expression of pro-inflammatory mediators, in the hippocampus and frontal cortex of adult rats, and the benefits of berry diets in preventing those effects.

METHODS AND MATERIALS

Animals

Sixty male Sprague-Dawley rats (Taconic Biosciences Inc., Germantown, NY) of approximately 2 months of age and weighing 175–200 g were maintained on a 12:12 h light/dark cycle with *ad libitum* access to food and water. The animals were shipped by truck to the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL) Long Island, NY for irradiation. All research protocols were approved by the Institutional Animal Care and

Use Committees of BNL, the University of Maryland Baltimore County (UMBC), and the Human Nutrition Research Center on Aging at Tufts University (HNRCA).

Diet

Animals were maintained on either blueberry (2%) or control diets (n=5/group) at UMBC for 4 weeks prior to irradiation. The details of the preparation of the diet have been published previously (Poulose et al., 2014). Briefly, freeze-dried blueberry powder, supplied by the U.S. Highbush Blueberry Council (Folsom, CA), was added to the NIH-31 rodent chow. This 2% diet for animals is approximately equivalent to a human serving of one cup of fresh berries/day. Corn was added to the control diet (NIH-31) to compensate for the addition of BB to the experimental diet.

Radiation

The rats were irradiated at the NSRL at BNL. For the exposures, the rats were placed in well-ventilated clear plastic tubes, and their heads were located in the center of the beam. Their bodies were shielded with overlapping tungsten bricks. Rats were exposed to 25 cGy of ^{56}Fe particles (600 MeV/n) at a nominal dose rate of 25cGy/min. The doses were selected based on previously reported studies (Poulose et al., 2014; Rabin et al., 2015), which indicated that exposure to these doses of ^{56}Fe reliably disrupted cognitive performance. The dose rate was designed to minimize the amount of time the rats were subject to restraint. Dosimetry was provided by the NSRL staff using parallel plate ionizing chambers. Animals serving as non-irradiated controls were taken to the NSRL and subjected to a similar duration of restraint but were not exposed.

Memory and learning task

Animals were randomized into either a “learning” group, which were irradiated prior to conditioning, or a “memory” group, which were irradiated between conditioning and testing (for more information see (Rabin et al., 2015)). Cognitive behavior was examined using the novel object recognition task, which is a measure of recognition learning and memory (Rabin et al., 2015). Subjects were tested in an open field (93x93 cm) with stimulus objects that vary in shape

and color. On the conditioning day, two identical (familiar) stimulus objects are placed in symmetrical locations in the open field. The rat is allowed to explore the stimuli until it accumulates 25–30s total object exploration (i.e., exploration of either object) or until 15 min have passed. On the test day, 24 h later, the rat is placed back in the field with one familiar and one novel object and allowed to explore both stimuli until it has accumulated 25–30s of object exploration on either object or until 15 min have passed. Intact rats spend more time exploring the novel object, while old rats and rats that have impaired recognition memory do not. Only the subjects ($n = 5-7$) that were euthanized at BNL are included in the present report in order to provide a better understanding of the relationship between HZE particle-induced changes in neuronal function and behavior. For both experimental designs, subjects that did not meet the criterion of 25–30s total exploration time on either conditioning or test day were eliminated from the specific test to minimize the effects of different amounts of exploration time on subsequent performance.

To measure the effects of exposure to HZE particles on memory, one set of rats was trained on the task immediately prior to irradiation, which occurred 2–4 h after conditioning. Twenty-four hours after conditioning (approximately 18 h after exposure) the ability of the rat to recall the familiar object was tested by placing the subject in the arena with one familiar and one novel object. To measure the effect of exposure on learning, a second set of rats was irradiated prior to conditioning. Twenty-four hours after irradiation, the rat was placed in the arena with two identical objects (conditioning day). Twenty-four hours after conditioning (approximately 48 h after exposure) the ability of the rat to recall the familiar object was tested. Because conditioning (learning) occurs prior to irradiation in the memory paradigm, if the acute effect of exposure to HZE particles disrupts memory (recall of the familiar object), the rat should spend equal amounts of time with both the familiar and novel object. Conversely, disruption of cognitive performance in the learning paradigm, where irradiation occurs prior to conditioning, is demonstrated by a failure of the subject to spend more time with the novel object, indicating an effect of irradiation on learning.

Sample collection

The animals that were in the memory group were euthanized via decapitation 22 hours post irradiation, while those in the learning group were euthanized 48 hours post irradiation. Half the

brain was post-fixed and used for immunohistochemistry. The hippocampus and frontal cortex were dissected from the other half of the brain and snap frozen for the protein carbonylation assay and western blot analyses.

Protein carbonylation

To determine the effect of ^{56}Fe on oxidative damaged proteins in cognitively-critical regions of the brain, accumulation of protein carbonyl was measured using 2,4-Dinitrophenylhydrazine (DNPH) protein carbonyl assay kits (Abcam) according to the manufacturer's suggested protocols (n=5/group). Samples were run in triplicate. Carbonyl protein contents were determined using the formula:

$$C = [(OD\ 375\ \text{nm})/6.364] \times (100) \text{ nmol/well}$$

$$CP = \text{nmol carbonyl per mg protein} = (C/P) \times 1000 \times D,$$

where 6.364 is the extinction coefficient using the enclosed 96 well plate in mM (= $22\ \text{mM}^{-1}\ \text{cm}^{-1} \times 0.2893\ \text{cm}$ path length in well), **C** is the Carbonyl in the sample well (nmol), **P** is the protein from standard curve $\times 20 = \mu\text{g/well}$, **D** is the dilution or concentration step applied to sample, **1000** is the factor to convert μg to mg.

NRF2-ARE regulation

To explore the common mechanisms linking neuronal adaptation to oxidative stress, inflammation, and autophagy regulation in aging, and to investigate the role of the Nrf2 pathway, total fractions of hippocampus and frontal cortex (n=5/group) were prepared and analyzed using western blots according to Poulou et al., 2016 (Poulou et al., 2016).

NRF2-IHC staining

Immunohistochemical staining for NRF2 expression in hippocampus was performed as previously described (Poulou et al., 2014). In brief, half of the brain was post-fixed in 4% paraformaldehyde (PFA) and sectioned at $30\ \mu\text{m}$ thickness using a cryostat microtome (Leica Microsystems, Nussloch, Germany) and the serial sections were collected on 24-well plates in 0.1M PBS. Every 5th hippocampal section was selected for 3, 3' diaminobenzidine (DAB; Vector labs, Burlingame, CA) histological staining according to manufacturer's protocol. The

sections were stained using avidin-biotin complex (ABC) Vectastain kits and DAB (Vector labs, Burlingame, CA), counterstained with haematoxyline-eosine, and mounted using Permount (Sigma-Aldrich, St. Louis, MO).

Data analysis

The quantitative data from the biochemical measurements were analyzed by one-way analyses of variance (ANOVA) using SYSTAT software (Systat Software, Inc.), and post hoc comparisons between the groups were performed using Fisher's Least Significant Difference. The behavioral data were analyzed using the NCSS11 statistical package. Results were considered statistically significant if the observed significance level was $p \leq 0.05$. Sample size for the neurochemical analysis was 5 rats/treatment. In order to facilitate comparison between the acute behavioral and neurochemical effects of exposure to ^{56}Fe particles, the behavioral analysis presented in this report is based only on the same animals which were subsequently subjected to neurochemical analysis (an additional 10 animals were irradiated and shipped to UMBC for subsequent behavioral testing). Sample size for the neurochemical analysis was 5-10 rats/group. These sample sizes were derived from the g*power (v. 3.1) program which allows for the calculation of the number of subjects needed to obtain significance (i.e., reject the null hypothesis).

RESULTS

Protein carbonyl content

Exposure to ^{56}Fe radiation produced a significant increase in the carbonyl content in the hippocampus ($F_{1,71}=29.57$, $P<0.001$) and frontal cortex ($F_{1,71}=50.96$, $P<0.001$; Fig 1). Overall, supplementation with BB significantly reduced the carbonyl content in hippocampus ($F_{1,71}=6.55$, $P<0.05$) but not in frontal cortex ($F_{1,71}=0.132$, $P=0.72$). However, there was a significant interaction between radiation and BB supplementation, both in the hippocampus ($F_{1,71}=5.05$, $P=0.028$) and frontal cortex ($F_{1,71}=11.36$, $P<0.01$). Group-wise comparisons between learning and memory groups indicated no significant differences in the hippocampus ($F_{1,71}=3.34$, $P=0.072$) or frontal cortex ($F_{1,71}=1.55$, $P=0.217$).

Post hoc comparisons indicated a significant increase in hippocampal carbonyl content with radiation exposure, both in the learning ($P<0.001$) and memory groups ($P<0.01$), compared to their respective controls (Fig 1). Memory groups had significantly lower carbonyl content in the hippocampus than animals in the learning group ($P<0.05$). Supplementation with BB diet prior to

irradiation significantly reduced the oxidative protein carbonyl accumulation only in the learning group ($P<0.01$) and not in the memory group ($P=0.155$), compared to their respective irradiated controls. As with the hippocampus, post hoc comparisons in frontal cortex also indicated that radiation significantly increased the carbonyl content in both the learning ($P<0.001$) and memory groups ($P<0.001$), compared to controls (Fig 1). However, unlike in the hippocampus, there were no significant differences between learning and memory groups in frontal cortex ($P=0.203$). Supplementation with BB diet prior to irradiation significantly reduced the oxidative protein carbonyl accumulation only in the learning group ($P<0.05$), compared to their respective irradiated controls. Non-irradiated animals consuming the BB diet were not significantly different than control animals in carbonyl content, indicating a possible threshold limit for carbonyl protein measurements, which primarily increases under high oxidative stress conditions in young animals.

Expression of NOX2

Elevated NOX2 contributes to the generation of intracellular ROS, and exposure to ^{56}Fe irradiation significantly increased NOX2 levels in both the hippocampus ($F_{1,39}=32.50$, $P<0.001$) and frontal cortex ($F_{1,39}=20.62$, $P<0.001$; Fig 2). Overall, the BB diet significantly attenuated NOX2 levels within both the hippocampus ($F_{1,39}=24.26$, $P<0.001$) and frontal cortex ($F_{1,39}=7.40$, $P<0.05$). A significant effect between the learning and memory groups was also observed for frontal cortex ($F_{1,39}=4.87$, $P<0.05$), but not for hippocampus ($F_{1,39}=2.77$, $P=0.104$).

In the hippocampus, post hoc comparisons between the treatment groups indicated a significant effect of radiation on NOX2 in both learning ($P=0.034$) and memory ($P=0.00$), compared to non-irradiated controls (Fig 2). Exposure to ^{56}Fe also resulted in a significant difference between learning and memory, both in the hippocampus ($P=0.017$) and frontal cortex ($P=0.031$). BB supplementation prior to ^{56}Fe exposure significantly reduced NOX2 levels in the hippocampus in both the learning ($P=0.02$) and memory groups ($P<0.001$), compared to their respective irradiated controls. Supplementation with BB in non-irradiated animals significantly reduced NOX2 in the learning group ($P=0.046$) compared to controls. In the frontal cortex, interestingly, there was a significant difference between the learning and memory groups among non-irradiated controls ($P<0.01$), irradiated controls ($P<0.05$), and BB-supplemented irradiated animals ($P<0.0$) (Fig 2). Additionally, there was a significant effect of radiation ($P<0.001$) and

BB supplementation ($P<0.01$) in the learning group, but not in the memory group, when NOX2 levels were compared to those of non-irradiated controls.

Expression of COX2

Cyclooxygenase-2 is the primary isoform responsible for prostanoid production in acute and chronic inflammatory conditions. Exposure to ^{56}Fe irradiation caused an overall significant increase in the levels of COX-2 in hippocampus ($F_{1,32}=90.13$, $P<0.001$) as well as frontal cortex ($F_{1,32}=143.04$, $P<0.001$; Fig 3). BB supplementation had an overall significant effect in reducing the levels of COX-2 both in hippocampus ($F_{1,32}=68.89$, $P<0.001$) and frontal cortex ($F_{1,32}=26.03$, $P<0.001$). A significant interaction was noticed between radiation and BB supplementation only in frontal cortex ($F_{1,32}=4.33$, $P<0.05$).

Post hoc comparisons indicated a significant increase in the expression of COX2 in both learning and memory irradiated groups ($P<0.05$), compared to their respective controls, both in the hippocampus and frontal cortex (Fig 3). Supplementation with BB significantly reduced levels of COX2 in the hippocampus of non-irradiated animals ($P<0.05$), compared to controls, in both the learning and memory groups. Supplementation with BB prior to ^{56}Fe irradiation significantly reduced the levels of COX2 ($P<0.05$), both in the hippocampus and frontal cortex.

Expression of Nrf2

Radiation significantly increased the expression of Nrf2 in hippocampus ($F_{1,39}=0.6.28$, $P<0.05$) but not in frontal cortex ($F_{1,39}=0.73$, $P=0.399$; Fig 4). Supplementation with BB significantly increased levels of Nrf2 in the hippocampus ($F_{1,39}=1057.04$, $P<0.001$) but not in frontal cortex ($F_{1,39}=2.84$, $P=0.100$). There was also a significant interaction between radiation and BB supplementation, both in the hippocampus ($F_{1,39}=149.11$, $P<0.001$) and frontal cortex ($F_{1,39}=52.46$, $P<0.001$). The overall effect between learning and memory was also significantly different in hippocampus ($F_{1,39}=9.37$, $P<0.01$).

Post hoc comparisons indicated a significant difference between learning and memory groups in the hippocampus ($P<0.05$) for all four treatment groups (Fig 4). Radiation significantly increased the levels of Nrf2 only in the memory group ($P<0.001$) compared to control. BB supplementation significantly increased the levels of Nrf2 in both of the non-irradiated (learning and memory) groups ($P=0.00$). Supplementation with BB, prior to irradiation, significantly

increased Nrf2 levels in the learning group compared to their irradiated controls ($P < 0.05$). Interestingly, post hoc comparisons in frontal cortex Nrf2 levels yielded significant differences between irradiated and non-irradiated controls in the learning group ($P < 0.001$) but not in the memory group ($P = 0.172$). Supplementation with BB also significantly increased levels of Nrf2 in both of the non-irradiated (learning and memory) groups ($P < 0.05$), compared to their respective non-irradiated controls, but decreased Nrf2 in irradiated animals ($P < 0.001$). Immunohistochemical results support the western data that BB diet substantially increases the expression of Nrf2 in the hippocampus in both the learning and memory groups (Fig 5).

Novel object recognition:

The acute effects of exposure to ^{56}Fe particles on memory (in which irradiation occurred shortly after conditioning but before testing recall) are provided in Fig 6. A one-way ANOVA indicated that the differences between the performance of the four treatment groups were not significant ($F_{3,24} = 1.26$, $P > 0.10$), possibly because of the small sample sizes. However, examination of Fig. 6 does suggest that there were differences in recall between the irradiated animals maintained on the blueberry diet and those maintained on the control diet, with the animals maintained on the blueberry diet spending almost 70% of their time with the novel object compared to the animals maintained on the control diet, which spent only 55% of their time with the novel object. Too few subjects in the learning condition (data not shown) in which conditioning occurred after irradiation met criterion (25-30 sec total exploration time with either object) for the behavioral results to be meaningful.

DISCUSSION

Learning and memory are fundamental processes by which animals adjust to environmental changes, evaluate various sensory signals based on context and experience, and make decisions to generate adaptive behaviors (Gkikas et al., 2014). While learning and memory are closely related concepts, learning involves the formation of new memories whereas memory involves the retention and retrieval of memories (Gkikas et al., 2014). Successful memory formation requires coordinated patterns of neural activity among a distributed network of brain regions such as the hippocampus and related structures within the medial temporal lobe (MTL) and frontal cortex

(Miller et al., 2008). Chronic stress, which can induce atrophy and functional impairments in several key brain areas such as the frontal cortex and hippocampus, plays an important role in memory processing in neurodegenerative diseases like Alzheimer's Disease (Wang et al., 2014). Previous results (Rabin et al., 2015) indicate that the acute effect of exposure to HZE particles is on memory, the retention and retrieval of previously acquired memories, rather than on learning. While the present results, especially for the memory condition, are consistent with the results of our previous experiment, the sample sizes ($n = 5-6$) were not sufficient to obtain significance for the behavioral tests. The factors that may have affected the subjects in the learning condition so that a majority of them did not reach criterion (25-30 sec exploration time) are not known; although it may have been related to the environmental conditions at BNL. However, the available data are consistent with past research showing that maintaining subjects on an antioxidant blueberry diet prevents/ameliorates both the HZE particle-induced disruption of cognitive performance and the changes in neural functioning that mediate the behavioral change. Further, these results confirm a causal relationship between HZE particle-induced changes in neurochemical and behavioral function and oxidative stress in the acute disruption of the consolidation process by which current experiences are converted to long-term memory. Once consolidation has occurred, as would be the case with learning, maintained oxidative stress may be required for the loss of previously acquired memories. Additional research will be necessary to determine the duration of the critical period during which exposure to HZE particles can disrupt memory.

Protein carbonyl content in the hippocampus and frontal cortex, a measure of oxidative stress, showed a significant increase with acute exposure to ^{56}Fe radiation compared to that of controls, both in the hippocampus and frontal cortex. Conversely, acute ^{56}Fe irradiation showed no substantial oxidative damage when the subjects were maintained on BB diet prior to irradiation in the learning group. Acute oxidative stress has been shown to induce post-translational changes such as glycation, lipoxidation, and carbonylation which are notably higher in many chronic inflammatory and degenerative diseases, as well as metabolic syndrome and diabetes (Cannizzo et al., 2011; Tanase et al., 2016). Moreover, post-translational protein modification by carbonyl groups has been reported to be associated with aging; such modifications include the conversion of lysine and arginine to aminoadipic acid and glutamic semialdehyde, respectively, tryptophan conversion to kynurenine and oxolactone, and proline to

pyrrolidone and pyrrolidinone, leading to the formation of protein aggregates (Tanase et al., 2016). In many neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases, these insoluble protein aggregates have become pathological hallmarks (Pedersen and Heegaard, 2013; Ross and Poirier, 2004). It can be inferred from the present results that radiation caused the production of excessive amounts of oxidants in the hippocampus as well as frontal cortex, which led to the increased production of carbonyl proteins. The learning group had higher amounts of carbonyl proteins in hippocampus than did the memory group of animals. Although both groups were sacrificed at identical times after testing, the increasing time between sacrifice relative to irradiation in the learning group (48 hrs vs 22 hrs) may allow for more protein carbonylation to be formed and accumulated. The BB diet significantly reduced the oxidative damage in the hippocampus and frontal cortex region, and the antioxidant phytochemicals present in this diet could be the primary protective factor, as demonstrated in our previous studies. Interestingly, the BB-supplemented rats that did not receive radiation were not significantly different than the diet controls, indicating either no effect of BB on basal carbonyl protein accumulation, or a floor effect, where protein carbonylation cannot be any lower under normal physiological conditions.

Nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase-isoform2 (NOX2) is an important source of superoxide for molecular signaling and immune function and a main generator of endogenous free radicals in the brain (Coyoy et al., 2013). However, an excessive amount of superoxide has been shown to cause cellular damage in the CNS, especially during brain injury (Choi et al., 2015). Acute exposure to radiation increased NOX2 expression in the hippocampus of both the learning and the memory groups, while in the frontal cortex NOX2 increased only in the learning group. It was unexpected that radiation had no effect on NOX2 expression in the frontal cortex of the memory group, particularly when the control memory group had higher hippocampal NOX2 expression than the control learning group. Blueberry supplementation was effective in reducing radiation-induced increases in NOX2 in the hippocampus of both the learning and the memory groups. An elevated NOX2 induced by radiation in the memory group in hippocampus could indicate the reason for better memory consolidation in the learning group than in the memory group as observed in other cases (Coyoy et al., 2013). It has been established that NOX2-derived ROS play a critical role in cognitive impairments following chronic stress and also act as a central mechanism for ROS-

induced neuroinflammation (Yasojima et al., 1999). It can be inferred from the results that ^{56}Fe increased production of superoxide-type ROS through NOX2 which, in turn, attenuated the expression of Nrf2 and subsequent AREs. Supplementation with BB, through its ROS-quenching bioactive molecules, reduced the ROS burden through attenuation of NOX2 as well as activation of NRF2.

Besides increasing NOX2 expression, exposure to ^{56}Fe significantly increased the expression of COX2 regardless of brain region in both the learning and the memory groups. Studies have shown that overexpression of COX2 potentiated microglial activation, leading to an overabundance of pro-inflammatory cytokines, nitric oxide and ROS in the brain. Like NOX2, BB supplementation reduced the radiation-induced increase in COX2 expression. These findings are in line with our previous studies where BB polyphenols significantly suppressed LPS-mediated inflammation in BV2 microglial cells and suppressed NF- κ B nuclear translocation (Lau et al., 2009).

When we examined the expression of Nrf2, a transcription factor that regulates both basal and inducible expression of various antioxidant and detoxifying enzymes in the CNS (Esteras et al., 2016), acute exposure to radiation significantly increased the hippocampal expression of Nrf2 in the memory group, while having no effect in the frontal cortex regardless of group. This finding suggests that the hippocampus may be more responsive to the elevation of ROS or other potential damages incurred by radiation exposure. Blueberry supplementation significantly enhanced the expression of Nrf2 in both the learning and memory groups compared to the radiation controls, irrespective of their sac times. This finding is similar to our previous study where consumption of fruit rich in phytochemicals increased the expression of Nrf2 in aged animals (Poulose et al., 2016). The elevated expression of Nrf2 may have attenuated the radiation-induced protein carbonyl accumulation in the hippocampus and the frontal cortex of both the learning and memory groups.

In summary, our biochemical analyses demonstrated that acute exposure to ^{56}Fe increased oxidative stress (promoted the formation and accumulation of protein carbonyl and upregulated NOX2) and inflammation (upregulated COX2) in the hippocampus of the memory group more than that in the learning group. It is possible that radiation-induced neuroinflammation and oxidative stress could result in hippocampal neuronal death and ultimately cognitive impairment, as seen in a reduction in the amount of time that the rats in the memory group spent with a new

object in the object recognition test. Even though acute exposure to radiation also increased Nrf2, it is clear that activation of antioxidant response element (ARE) activity alone is not sufficient to counteract the damages induced by radiation exposure. Blueberry supplementation has been consistently effective in suppressing the effects of radiation and improving cognitive function. Therefore, supplementing diets with antioxidant-rich fruits and vegetables, particularly blueberries, may be one practical way to reduce the damage caused by ROS overload and neuroinflammation in the CNS following exposure to HZE radiation outside Earth's magnetosphere.

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FIGURE LEGENDS

Fig 1. Levels of carbonyl protein content in hippocampus and frontal cortex of animals exposed to ^{56}Fe , fed with control and BB-supplemented diets. Quantitative data is expressed as mean (nanomoles/mg of tissue) $\pm$ SEM (n=5/group), with significance “a”= $p<0.05$ from control-fed, non-irradiated animals, “b”= $p<0.05$ from control-fed, irradiated animals, “c” from BB-fed, non-irradiated animals, and “d” from learning or memory.

Fig 2. Levels of pro-oxidant NOX2 proteins in hippocampus and frontal cortex of animals exposed to ^{56}Fe , fed with control and BB-supplemented diets. Quantitative data based on western blots is represented as mean band density $\pm$ SEM (n=5/group), with significance “a”= $p<0.05$ from control-fed, non-irradiated animals, “b”= $p<0.05$ from control-fed, irradiated animals, “c” from BB-fed, non-irradiated animals, and “d” from learning or memory.

Fig 3. Levels of pro-inflammatory COX2 proteins in hippocampus and frontal cortex of animals exposed to ^{56}Fe , fed with control and BB-supplemented diets. Quantitative data based on western blots is represented as mean band density $\pm$ SEM (n=5/group) with significance “a”= $p<0.05$ from control-fed, non-irradiated animals, “b”= $p<0.05$ from control-fed, irradiated animals, “c” from BB-fed, non-irradiated animals, and “d” from learning or memory.

Fig 4. Levels of transcriptional factor Nrf2 proteins in hippocampus and frontal cortex of animals exposed to ^{56}Fe , fed with control and BB-supplemented diets. Quantitative data based on western blots is represented as mean band density $\pm$ SEM (n=5/group) with significance “a”= $p<0.05$ from control-fed, non-irradiated animals, “b”= $p<0.05$ from control-fed, irradiated animals, “c” from BB-fed, non-irradiated animals, and “d” from learning or memory.

Fig 5. Immunohistochemical staining for Nrf2 in hippocampus of learning and memory groups. Hippocampal sections from non-radiated control (C), radiated control (R), non-radiated blueberry (BB) and radiated blueberry (BB-R) were stained using the Vectastain Elite kit with

NovaRed HRP substrate against Nrf2 antibody. Panel A (4x magnification) and Panel B and C (20X magnification) from learning and memory animals respectively.

Fig 6. Novel object recognition test for exposure to ^{56}Fe particles on the memory group of animals. Represented as percent time spent in exploring the novel object by animals maintained on control or blueberry diet with or without radiation.

Fig 1

Fig 2

Fig 3

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