Published in final edited form as:

Exp Neurol. 2013 January ; 239: 183–191. doi:10.1016/j.expneurol.2012.09.019.

Neuroprotective effect of Pycnogenol® following traumatic brain injury

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Abstract

Traumatic brain injury (TBI) involves primary and secondary injury cascades that underlie delayed neuronal dysfunction and death. Oxidative stress is one of the most celebrated secondary injury mechanisms. A close relationship exists between levels of oxidative stress and the pathogenesis of TBI. However, other cascades, such as an increase in proinflammatory cytokines, also play important roles in the overall response to the trauma. Pharmacologic intervention, in order to be successful, requires a multifaceted approach. Naturally occurring flavonoids are unique in possessing not only tremendous free radical scavenging properties but also the ability to modulate cellular homeostasis leading to a reduction in inflammation and cell toxicity. This study evaluated the therapeutic role of Pycnogenol (PYC) a patented combinational bioflavonoid. Young adult Sprague-Dawley rats were subjected to a unilateral moderate cortical contusion and treated post injury with PYC or vehicle. At either 48 or 96h post trauma, the animals were killed and the cortex and hippocampus analyzed for changes in enzymatic and non-enzymatic oxidative stress markers. In addition, possible changes in both pre and post synaptic proteins (synapsin-1, PSD-95, drebrin, synapse associated protein 97) were analyzed. Finally, a separate cohort of animals were used to evaluate two proinflammatory cytokines (IL-6, TNF- α). Following the trauma there was a significant increase in oxidative stress in both the injured cortex and the ipsilateral hippocampus. Animals treated with PYC significantly ameliorated levels of protein carbonyls, lipid peroxidation, and protein nitration. The PYC treatment also significantly reduced the loss of key pre and post synaptic proteins with some levels in the hippocampus of PYC treated animals not significantly different from sham operated controls. Although levels of the proinflammatory cytokines were significantly elevated in both injury groups, the cohort treated with PYC showed a significant reduction compared to vehicle treated controls. These results are the first to show a neuroprotective effect of PYC following TBI. They also suggest that the diverse effects of bioflavonoids may provide a unique avenue for possible therapeutic intervention following head trauma.

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Keywords

bioflavonoids; head injury; natural compounds; neuroinflammation; oxidative stress; synaptic proteins; TBI; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in healthy populations of all ages. It is estimated that approximately 1.7 million individuals in the United States will suffer TBI this year, with a major percentage requiring hospitalization (Faul et al., 2010). A financial burden is associated with TBI due to increased specialized care, long term rehabilitation and lost financial income. Primary and secondary injury cascades resulting in delayed neuronal dysfunction, synapse loss, and cell death are commonly associated with TBI (Hovda et al., 1991; Kawamata et al., 1995; McIntosh et al., 1996; Baldwin et al., 1997; Xiong et al., 1997; Lenzlinger et al., 2001; Scheff et al., 2005; Hall et al., 2010). Most researchers adhere to the idea that if these secondary injury cascades can be interrupted, either through pharmacologic or even dietary intervention, there will be a more favorable outcome, leading to decreased cognitive and behavioral dysfunction. Although the exact time frame for these secondary injury cascades varies, it is generally thought they occur within the first few hours to days after an insult.

As part of the secondary injury cascade, there are significant increases in oxidative stress (Ansari et al., 2008c; 2008b), ionic imbalances, ATP depletion, excitotoxicity, and proteolysis (Sullivan et al., 1998; Biegon et al., 2004). A close relationship exists between the degree of oxidative stress and the pathogenesis of TBI (Shao et al., 2006). Enhanced reactive oxygen/nitrogen species (ROS/RNS) causes oxidative/nitrosative stress in TBI leading to damage in lipids, proteins, and nucleic acids (Ansari et al., 2004; Ozdemir et al., 2005; Solaroglu et al., 2005; Ansari et al., 2006). These TBI-related cascades have been implicated in cytoskeletal damage, mitochondrial dysfunction and altered signal transduction (Hall et al., 2004; Bayir et al., 2007; Singh et al., 2007). Clinical trials in humans following TBI have not been shown to be very effective. One of the primary reasons is that most trials focus on a single aspect of the cascade. It is now clear that some type of pharmacologic intervention with a multifaceted approach, addressing multiple secondary injury targets in a complimentary way, will be necessary (Margulies and Hicks 2009).

Naturally occurring flavonoids possess neuroprotective properties in part due to their free radical scavenging properties and their ability to modulate intracellular signals (Mercer et al., 2005; Vauzour et al., 2007; Spencer 2008). These flavonoids are found in fruits, vegetables and plant-derived beverages, and may have important roles as dietary components via cytoprotective actions in many organs (Paganga et al., 1999; Youdim and Joseph 2001). Flavonoids can act as a vasodilator (Duarte et al., 1993), anti-carcinogenic, anti-inflammatory, antibacterial, immune-stimulating anti-allergic, and antiviral compounds (Brown 1980). Pycnogenol® (PYC) is a patented combination of bioflavonoids extracted from the bark of the French maritime pine, *pinus maritima*. Major constituents of PYC are polyphenols, specifically mono- and oligomeric units of caffeic acid, ferulic acid, catechin, epicatechin, and taxifolin. Caffeic acid, when given intra peritoneal to rodents following either focal ischemia or cryo brain injury (Jatana et al., 2006; Zhou et al., 2006; Zhang et al., 2007), is neuroprotective suggesting access to the brain. Several different flavonoids have been shown to protect neurons in a wide array of animal models of neurological disease (Robert et al., 2001; Yan et al., 2001; Abd El Mohsen et al., 2002; El Mohsen et al., 2006; Mandel et al., 2006). Many different flavonoids have been shown to cross the blood brain barrier (Vauzour et al., 2008).

PYC has been shown to prevent neurotoxicity and apoptotic cell death in the presence of stressors (Kobayashi et al., 2000; Peng et al., 2002). Concomitantly, PYC protects against lipid peroxidation, and pro-oxidants and peroxynitrites (Kobuchi et al., 1999; Packer et al., 1999; Nardini et al., 2000). We have previously reported the protective effect of PYC as a potent antioxidant to the acrolein induced cytotoxicity in human neuroblastoma (SH-SY5Y) cells (Ansari et al., 2008a).

In the present set of experiments we tested whether or not PYC, when given immediately after an experimental model of TBI, can alter secondary injury cascades.

Materials and Methods

Young adult male Sprague-Dawley rats (n = 65, 275–300g; Harlan Labs, Indianapolis, IN) were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water. All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. The animals were randomly assigned to one of three groups: Sham (n = 19), TBI+Vehicle (n = 23), TBI+PYC (n = 23).

Cortical contusions were carried out under isoflurane anesthesia (2%) as previously described (Ansari et al., 2008b). All injuries were produced using a pneumatic controlled cortical impact device (TBI 0310; Precision Systems and Instrumentation, Fairfax Station, VA) with a hard stop Bimba cylinder (Bimba Manufacturing, Monee, IL). The beveled impactor tip size was 5 mm. The depth of the impact was set at 2.0mm with a velocity of 3.5m/sec and a dwell time of 500 msec. The animals were maintained at 37°C. Following injury, animals were treated either with PYC (100mg/kg) or vehicle (6% dimethylsulfoxide in physiological saline). Animals were treated with three i.p. injections at 15m, 3h, and 6h following the injury. Dose and timing were decided based on preliminary experiments. Animals were allowed to survive for 48h or 96h post injury.

Tissue Processing

Enzymatic and oxidative stress assays—Analysis was carried out as previously described (Ansari et al., 2008b). Briefly, animals (n = 8/group) were rapidly killed with CO₂ and the brain removed at 48h post surgery. An 11 mm punch of the cerebral cortex was obtained from both the ipsilateral (IP) injury site and corresponding region in the contralateral (CON) hemisphere. At the time of dissection, none of the samples were compromised by the presence of surface blood products. The entire hippocampus, both ipsilateral and contralateral to the injury, was assayed separately. Tissue samples were stored at –80°C until used for analysis. Tissue samples were lysed using an ultrasonic cell disruptor (Microson Farmingdale, NY) on ice in 2.0 ml 0.1M, PBS (pH 7.4) containing 10 mM HEPES, 2.0 mM EDTA, 2.0 mM EGTA, 0.6 mM MgSO₄, 4.6 mM KCl, pepstatin A, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at 1000g for 10 min/4°C to remove cell debris and the collected supernatant was centrifuged at 15,000g for 10 min/4°C. Post mitochondrial supernatants (PMS) were used for enzymatic and non-enzymatic analyses. Biochemical assays were carried out in 96-well plates and analyzed with a SpectraMaxR microplate reader (Molecular Devices, Sunnyvale, CA). Total protein concentrations were determined using the Pierce BCA method (Sigma, St. Louis, MO).

Postmitochondrial supernatants (Ansari et al., 2006) were used to estimate the following enzymatic and nonenzymatic activity: *Glutathione (GSH)*: was assessed in the reaction mixture containing 0.1M sodium phosphate buffer (PBS), 5mM EDTA, 10 µl α -phthaldehyde (1mg/ml), and 10 µl sample. For the estimation of GSSG, samples were incubated first with 0.04 M *N*-ethylmaleimide for 30 min and then reaction mixture prepared

as 0.1 N NaOH, 5mM EDTA, 10 μ l *o*-phthalaldehyde (1mg/ml), and 10 μ l of sample. After incubation for 15 min at room temperature, fluorescence at emission 420 nm was recorded by excitation at 350 nm. *Glutathione Peroxidase (GPx)*: activity was measured with H₂O₂ breakdown in the reaction mixture consisted of PBS (pH 7.0), 1mM EDTA, 1mM sodium azide, 1mM GSH, 0.2 mM NADPH, 0.25 mM H₂O₂, and PMS. *Glutathione Reductase (GR)*: activity was measured in the assay mixture consisted of PBS (pH 7.6), NADPH, EDTA, 1mM GSSG, and PMS. *Glucose-6-Phosphate Dehydrogenase (G-6-PD)*: activity was measured in the reaction mixture consisted of 50mM tris-HCl buffer (pH 7.6), 0.1mM NADP, 0.8mM glucose-6-phosphate, 8mM MgCl₂ and 0.03 ml of PMS. *Glutathione-S-Transferase (GST)*: activity was measured in the reaction mixture consisted of PBS (pH 6.5), GSH, 1mM CDNB, and 0.03 ml PMS. In total volume of 0.3ml, absorbance changes were recorded at 340 nm. *Superoxide Dismutase (SOD)*: activity SOD was measured for the epinephrine oxidation reaction and changes in absorbance recorded at 480 nm. *Catalase (CAT)*: activity was assayed by monitoring H₂O₂ breakdown at 240 nm. The activity of each enzyme was calculated by using molar extinction coefficient of their reaction product.

Thiobarbituric Acid Reactive Substance (TBARS), marker of total oxidative damage, was measured as previously described (Ansari et al., 2008c; 2008b). Briefly, two sets of samples were simultaneously incubated at 37 ± 1 °C and 0 °C for 60 min. After 1 h of incubation, 0.2 ml of 10% trichloroacetic acid and 0.4 ml of 0.67% TBA was added and reaction mixture centrifuged at $3500 \times g$ for 15 min. The collected supernatant was heated in boiling water bath for 10 min, absorbance was recorded at 535 nm and values were calculated by using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. *Protein carbonyls (PC)*, *3-nitrotyrosine (3-NT)*, and *4-hydroxynonenal (4-HNE)*: assayed as previously described (Ansari et al., 2008c; 2008b). For PC, first samples were derivatized with 2,4-dinitrophenyl hydrazine and neutralized with 2 M Tris in 30% glycerol. For 3-NT, and 4-HNE samples were incubated for 20 minutes at room temperature with 12% SDS and Laemmli buffer. Each sample (250 ng) was loaded into a well on a nitrocellulose membrane in a slot-blot apparatus under vacuum. Following application of primary antibodies (PC,1:100; 3-NT,1:2000 and 4-HNE 1:5000) for 1h, alkaline phosphatase secondary antibody (at 1:8000) for 1h and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image (PC NIH Image). The numerical values of the blots were exported into Microsoft Office Excel. Changes were determined by first comparing the IP hemisphere to the CON hemisphere, thus using each animal as its own control, and subsequently converted into percent as compared to sham operated animals.

Synaptic proteins—Levels of several synaptic marker proteins: synapsin-I, SAP97, PSD95, and drebrin were evaluated at 96h post surgery by Western-blot method as previously described (Ansari et al., 2006; Ansari et al., 2008c) (Sham n = 6; Injured n = 10/ group). Synaptic proteins are significantly reduced at this time post TBI (Ansari et al., 2008c; 2008b). Briefly, the tissues were homogenized in the lysis buffer and centrifuged at $13,000 \times g$ for 10 min. Supernatants, (50 μ g protein) were normalized for protein concentration in 25 μ l (loading buffer) and loaded with the appropriate marker on gradient gels, followed by transfer to nitrocellulose membrane using a semi-dry transfer system (Bio-Rad). Following application of primary antibodies for synapsin-I, GAP-43, PSD-95, and SAP-97 at a concentration of 1:1,000 for the overnight at 4°C. Beta-actin was also simultaneously probed as a loading control. The blot was then washed three times in tris-buffered saline/Tween and incubated for 1 h with alkaline phosphatase conjugated secondary antibodies in a 1:8000 dilution. The blots were developed with Sigma Fast™ BCIP/NBT tablets (Sigma, St. Louis, MO). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh-compatible NIH Image). The numerical values of the blots were exported into Microsoft Office Excel. Changes were determined by first comparing the IP hemisphere to the CON hemisphere,

thus using each animal as its own control, and subsequently converted into percent as compared to sham operated animals.

Neuroinflammation—Levels of tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) were assayed at 48h post trauma in both cortex and hippocampus using the MSD multi-array assay system for rodent cytokines (L453AKA, L453BHA; Meso Scale Discovery, Gaithersburg, MD) according to kit directions.

Statistical analysis

Differences in enzymatic and nonenzymatic activity and estimations of protein levels are reported as means $\pm$ standard deviation. Differences between group means were evaluated with a one-way analysis of variance coupled with a Fisher's PLSD post hoc test when warranted (StatView 5.0; SAS Institute). For significance, alpha was set at 0.05.

Results

Antioxidant: Cortex 48h post trauma

The levels of the antioxidant GSH showed a significant treatment effect [$F(2,21) = 6.074$, $p < 0.01$] with animals sustaining TBI showing a significant decline compared to sham operated controls [$t(22) = 3.145$, $p < 0.005$] (Fig. 1). Animals treated post injury with PYC, showed an increase in GSH levels but this difference was not significantly greater than vehicle treated animals ($p > 0.10$). The levels of GSSG significantly increased as a result of the injury [$F(2,21) = 55.384$; $p < 0.0001$], an indication of increased oxidative burden. Animals treated with PYC showed a significant decrease ($p < 0.001$) indicative of a decline in oxidants. The GSH/GSSG ratio was consequently significantly decreased in the cortex [$F(2,21) = 289.422$; $p < 0.0001$] with the PYC animals showing significant improvement ($p < 0.001$). Levels of other antioxidant enzymes were also evaluated and showed significant treatment effects. Although GPx [$F(2,21) = 8.103$; $p < 0.005$] was significantly decreased by the injury, treatment with PYC was significantly greater ($p < 0.05$) compared to vehicle treated animals. GR [$F(2,21) = 3.549$; $p < 0.05$] also showed significant improvement compared to vehicle treated animals ($p < 0.05$). GST [$F(2,21) = 2.679$; $p > 0.5$], which is used for detoxification, showed no difference between PYC and sham animals, and no significant improvement compared to vehicle controls. A significant change was observed for G-6-PD [$F(2,21) = 3.514$; $p < 0.05$] with PYC significantly better than vehicle treated controls ($p < 0.05$). Both CAT [$F(2,21) = 3.472$, $p < 0.05$] and SOD [$F(2,21) = 8.629$, $p < 0.005$] showed a significant injury effect with the PYC treated animals significantly better ($p < 0.05$) than the vehicle cohort.

Oxidants: Cortex 48h post trauma

As expected, levels of TBARS were significantly elevated following the injury [$F(2,21) = 73.147$; $p < 0.0001$] (Fig. 2). The greatest increase was observed in the vehicle treated injured group, which was significantly elevated compared to the PYC treated cohort ($p < 0.0001$). Although the PYC treated group showed decreased levels of TBARS, it was still significantly elevated compared to sham operates ($p < 0.001$). Levels of protein oxidation, as measured by PC, were increased significantly as a result of the injury [$F(2,21) = 47.057$; $p < 0.0001$]. PYC treated animals showed a significant reduction ($p < 0.001$) compared to the vehicle control group. Protein nitration, as measured by 3-NT, was significantly elevated 48h after injury [$F(2,21) = 10.307$; $p < 0.001$] with PYC animals manifesting levels equivalent to sham operated controls ($p > 0.5$). Lipid peroxidation, as measured by 4-HNE, showed a significant injury effect [$F(2,21) = 41.402$; $p < 0.0001$]. Animals treated with PYC were significantly better than vehicle treated subjects ($p < 0.005$) but remained significantly elevated compared to sham operates ($p < 0.0001$).

Antioxidant: Hippocampus 48h post trauma

Antioxidant levels in the hippocampus were significantly decreased at 48h following TBI (Fig. 3). Levels of GSH showed a significant decline [$F(2,21) = 31.542$; $p < 0.0001$] similar to that observed in the IP cortex. Although PYC significantly improved levels compared to vehicle ($p < 0.0005$), these levels were still significantly worse than sham operates ($p < 0.001$). Changes in both GSSG and the ratio of GSH/GSSG in the IP hippocampus were almost identical to that observed in the cortex after TBI. Although GSSG levels, in animals treated with PYC, were still significantly elevated ($p < 0.0001$) at 48h, they were significantly lower ($p < 0.0001$) than vehicle treated animals. The ratio of GSH/GSSR was significantly increased ($p < 0.01$) in PYC compared to vehicle treated subjects. Injury-related levels of GPx were also significantly lower [$F(2,21) = 6.521$; $p < 0.01$] with both PYC and vehicle treated animals showing similar levels that were not significantly different ($p > 0.1$) from each other. Hippocampal levels of GR were significantly reduced following injury [$F(2,21) = 4.903$; $p < 0.02$]. PYC significantly improved GR levels compared to the vehicle treated group ($p < 0.05$). The hippocampal levels of GST were different compared to the other enzymes. While there was not a significant injury effect [$F(2,21) = 3.926$; $p < 0.05$] the PYC treatment elevated the levels in the hippocampus. Surprisingly, injury-related levels of G-6-PD remained relatively stable [$F(2,21) = 2.571$; $p > 0.1$]. PYC significantly elevated levels of CAT in the hippocampus compared to vehicle treated animals ($p < 0.05$) and also elevated the levels of SOD ($p < 0.05$).

Oxidants: Hippocampus 48h post trauma

The levels of TBARS dramatically increased in the IP hippocampus [$F(2,21) = 26.875$; $p < 0.0001$] following the injury (Fig. 2). Treatment with PYC significantly lowered the level of TBARS ($p < 0.005$) compared to the vehicle treated cohort. Protein carbonyls were also significantly elevated [$F(2,21) = 20.366$; $p < 0.0001$]. Animals treated with PYC displayed a significant reduction in PC ($p < 0.001$) compared to the vehicle group and were not significantly different from sham operates ($p > 0.1$). Levels of 3-NT were also significantly elevated following the injury [$F(2,21) = 6.038$; $p < .01$] with the vehicle treated group showing the most significant increase ($p < 0.005$). PYC-treated animals were found not to be significantly different from sham controls ($p > 0.1$). Evaluation of 4-HNE in the hippocampus showed a significant main effect [$F(2,21) = 24.853$; $p < 0.0001$] with both PYC and vehicle treated groups significantly elevated compared to sham operates. The PYC-treated cohort was significantly improved ($p < 0.001$) compared to the vehicle treated group.

Synaptic proteins: Cortex 96h post trauma

Several different synaptic proteins were evaluated in the IP and CON cortex. Western blot analysis revealed a decline in both pre and post synaptic proteins in the IP cortex in injured groups compared to sham (Fig. 4A). Levels of synapsin-1 revealed a significant main effect [$F(2,23) = 28.541$; $p < 0.0001$] with the vehicle treated animals showing a significant loss ($p < 0.0001$) (Fig. 5). Animals treated with PYC showed significantly greater levels compared to vehicle treated ($p < 0.0001$) but were significantly different from sham operated controls ($p < 0.05$). Levels of the post synaptic protein drebrin also showed a significant main effect [$F(2,23) = 19.671$; $p < 0.0001$] with both the PYC ($p < 0.005$) and vehicle treated cohort ($p < 0.0001$) showing significant declines. The PYC treated animals were significantly better than vehicle treated animals ($p < 0.005$). Analysis revealed that levels of PSD-95 were also affected by the injury [$F(2,23) = 50.989$; $p < 0.0001$] with the PYC treated subjects significantly better than the vehicle treated ($p < 0.0001$) but significantly different from sham operates ($p < 0.05$). Results for SAP-97 were similar to those for PSD-95 [$F(2,23) = 30.960$; $p < 0.0001$] with PYC significantly better than vehicle treated subjects ($p < 0.0001$) and significantly different from sham operates ($p < 0.05$).

Synaptic proteins: Hippocampus 96h post trauma

Synaptic proteins in the IP hippocampus were also altered by the cortical contusion (Fig. 4B). Western blot analysis revealed that synapsin-1 was significantly affected at 96h [$F(2,23) = 14.137$; $p < 0.0001$]. Vehicle treated animals showed a 30% decline ($p < 0.0001$) compared to sham operates (Fig. 5). The cohort treated with PYC was not significantly different from controls ($p > 0.05$) and was significantly higher than vehicle treated ($p < 0.0051$). Levels of drebrin were effected to a lesser degree than synapsin-1 but significantly altered following the injury [$F(2,23) = 9.942$; $p < 0.001$]. Vehicle treated animals showed a significant decline of 30% ($p < 0.005$) while PYC animals were not significantly different ($p > 0.05$) from sham operates. Levels of the synaptic protein PSD-95 was significantly altered [$F(2,23) = 19.725$; $p < 0.0001$] with vehicle treated animals showing a 30% loss ($p < 0.0001$) and PYC treated animals (12% decline) also significantly different from sham ($p < 0.05$), but significantly higher than vehicle treated animals ($p < 0.0005$). SAP-97 mirrored the results of PSD-95 with a significant main effect [$F(2,23) = 36.297$; $p < 0.0001$]. Vehicle treated animals showing the greatest loss (50%) ($p < 0.0001$) and significantly different from the PYC treated cohort ($p < 0.0001$), which was not significantly different from sham ($p > 0.05$).

Neuroinflammation: Cortex and hippocampus 48h post trauma

Following the injury there was a significant increase in levels of IL-6 [$F(2,12) = 69.362$, $p < 0.0001$] and TNF- α [$F(2,12) = 151.036$, $p < 0.0001$] in the cortex (Fig. 6). Post hoc testing showed that vehicle treated and PYC treated animals were significantly elevated compared to sham operated controls ($p < 0.0001$). Animals treated with PYC showed significantly lower level of both IL-6 ($p < 0.001$) and TNF- α ($p < 0.0001$) compared to the vehicle cohort. Levels of markers of neuroinflammation in the hippocampus were similar to those in the cortex with a significant injury affect for IL-6 [$F(2,12) = 28.267$, $p < 0.0001$] and TNF- α [$F(2,12) = 161.836$, $p < 0.0001$]. Further analysis revealed that the two injury groups were significantly elevated compared to sham operates for both markers ($p < 0.0001$). Treatment with PYC demonstrated a significant decrease in levels of IL-6 ($p < 0.001$) and TNF- α ($p < 0.05$) compared to vehicle treated subjects.

Discussion

Several different flavonoids and polyphenols have been used as a therapeutic intervention following experimental TBI suggesting that such compounds may be useful clinically. When adult animals have been fed curcumin both pre and post TBI injury (Wu et al., 2006; Sharma et al., 2009) or (–)-epigallocatechin-3-gallate (Itoh et al., 2011) there was a significant beneficial effect including a decrease in oxidative stress and an increase cellular energy homeostasis. These compounds also influenced cognitive performance. More importantly, multiple studies have shown that adult animals treated only post trauma with natural compounds also have significant beneficial effects including a reduction in neuroinflammation (Chen et al., 2008; Laird et al., 2010; Chen et al., 2012; Lee et al., 2012), reduction in edema (Ates et al., 2007; Laird et al., 2010; Chen et al., 2012), increased cell survival (Ates et al., 2007; Chen et al., 2008; Chen et al., 2012; Lee et al., 2012) and enhanced neuronal/cognitive function (Di Giovanni et al., 2005; Schultke et al., 2005; Chen et al., 2008; Laird et al., 2010; Singleton et al., 2010; Itoh et al., 2011).

This study is the first to report possible *in vivo* neuroprotective effects of PYC following a cortical contusion injury. Animals administered PYC within 15 minutes following TBI show reduced levels of oxidative stress, increased levels of key synaptic proteins, and reduced levels of neuroinflammation. Oxidative stress is a key component of the secondary injury cascade following TBI. The brain is highly sensitive to oxidative stress because of its high

content of poly unsaturated fatty acids, which are vulnerable to free radical attack and lipid peroxidation (LPO) (Hall et al., 2010). Aldehydic products of LPO, such as 4-HNE, are of interest because of their involvement in oxidative stress (Lovell et al., 1997; Liu et al., 2003; Engle et al., 2004). Enzymatic and non-enzymatic antioxidants play an important role in protecting the brain against oxidative damage (Ozturk et al., 2005). CAT and GPx, acting in concert with SOD, constitute the major defense enzymes against superoxide radicals (Islekel et al., 1999; DeKosky et al., 2004; Dringen et al., 2005). We have previously reported time-dependent changes in oxidative stress in both the hippocampus and cortex in an animal model of TBI (Ansari et al., 2008c; 2008b).

In the present study, all animals that received a cortical contusion showed a decline in the primary antioxidant GSH and increased levels of oxidative stress compared to sham operated controls at 48h post injury. Previous studies from our laboratory investigated the early temporal sequence of the balance between oxidants and antioxidants as part of the secondary injury cascade. The maximum reported changes occurred between 24 and 48h following an injury identical to that reported here (Ansari et al., 2008c; 2008b). Glutathione is one of the primary antioxidants in the CNS and participates in the management of oxidative stress by donating a reducing equivalent. In the process it is oxidized to form GSSG. The enzyme GR is responsible for reducing GSSG back to GSH where it can become active. A significant reduction in the amount of GR would severely limit the effectiveness of GSH. Normally glutathione exists in the reduced form as GSH. Reduced levels of GSH and elevated levels of GSSG are indicators of oxidative stress and/or nitrosative burden in the system. In vehicle treated animals the levels of GR were significantly reduced post trauma and augmented in the PYC cohort.

The beneficial effects of PYC may extend beyond its ability to augment the antioxidant defenses. Flavonoids have been reported to interact with a variety of different protein kinases and lipid kinase signaling cascades (Gamet-Payraastre et al., 1999; Schroeter et al., 2001; Williams et al., 2004; Spencer 2007). Numerous protein kinase cascades are known to be activated following TBI that play important roles in neuroprotection (Noshita et al., 2002; Otani et al., 2002; Neary 2005). Among those that appear to have a particularly significant participation are mitogen-activated protein kinases (MAPKs) which include the extracellular signal-regulated protein kinase (ERK), c-Jun NH(2)-terminal kinase (JNK), and p38 pathways. When ERK is phosphorylated it plays an important role in cell survival (Seger and Krebs 1995). Protein kinase B (Akt), a member of the protein kinase A (PKA) family, and also glycogen synthase kinase (GSK) have also been well studied following neurotrauma (Noshita et al., 2002; Endo et al., 2006; Shapira et al., 2007; Shein et al., 2007; Carloni et al., 2010; Hu et al., 2010). Akt is an important signaling mediator involved in cell survival by inhibiting JNK which is involved in apoptosis and also a modulator of synaptic activity. Recent studies have shown that flavonoids activate Akt and inhibit several signal-regulating kinases related to apoptosis in cortical neurons (Vauzour et al., 2007). Flavonoids are also known to alter the levels of GSK resulting in significant cell survival (Azevedo et al., 2010; Johnson et al., 2011; Parajuli et al., 2011; de la Torre et al., 2012).

Neuroinflammation is part of the secondary injury cascade and has both a detrimental effect on brain cells and also plays a role in brain edema. Antioxidants have been reported to modulate the production of reactive oxygen species and inflammatory molecules such as TNF- β and IL-6 that result from oxidative stress. Flavonoids can modulate both oxidative stress and levels of proinflammatory cytokines (Sharma et al., 2007; Ansari et al., 2008a; Vauzour et al., 2008). In the current experiments, two proinflammatory cytokines, TNF- α and IL-6 were evaluated. Both of these cytokines are markers of neuroinflammation (Morganti-Kossmann et al., 2002). Treatment with PYC significantly reduced the levels of neuroinflammation in both the cortex and hippocampus following TBI, supporting previous

work demonstrating the effect of PYC on the expression of proinflammatory cytokines (Cho et al., 2001). Surprisingly, even though PYC was given within 15 min post injury, there remained a significant elevation in the neuroinflammatory response. Similar results following TBI have been observed using a variety of different anti-inflammatory agents (Atkins et al., 2007; Lloyd et al., 2008; Lee et al., 2012). It is well known that the inflammatory response is one of the early events following TBI and it may have taken the PYC too long to be incorporated using an i.p. route. Future studies may need to employ an intravenous administration protocol. Since PYC did have some beneficial effects it clearly has crossed the blood brain barrier, which has previously been shown to be breached soon after the injury (Baldwin et al., 1996).

The most important beneficial effects of PYC were observed in the protection of the different synaptic proteins in both the cortex and hippocampus. This is the first report that bioflavonoids can protect key synaptic proteins following a traumatic brain injury. In both the injured cortex and underlying hippocampal formation, pre and post synaptic proteins were significantly elevated compared to vehicle treated controls. Previous work from our laboratory has shown a significant decline in these proteins in both regions by 96h post injury. While the cortex still showed a significant change in the PYC treated animals, only the PSD-95 levels were significantly different in the hippocampus of this cohort. Clearly PYC has exerted a significant amount of neuroprotection. Further experimentation will be necessary to clarify the physiological consequences of the hippocampal protection. We have previously described significant hippocampal plasticity and electrophysiological changes following this type of injury (Scheff et al., 2005; Norris and Scheff 2009).

In conclusion, the current set of studies demonstrates the substantial beneficial effects of treatment with the combinational bioflavonoid PYC following moderate TBI. This natural compound significantly altered some of the secondary injury cascades such as reducing oxidative stress in both the cortex and hippocampus. Treatment with this compound protected key synaptic proteins and reduced levels of neuroinflammation in these same two regions of the brain. Left unanswered is whether or not PYC can provide significant neuroprotection when delayed following the injury. It is also unclear whether or not the current level and route of administration of PYC provides the maximal neuroprotective benefit. These questions clearly await future experimentation. The present results support the idea that a combinational bioflavonoid can offer significant neuroprotection following acute brain trauma. The mechanism of action of PYC appears to be more complex than that of a simple antioxidant.

Acknowledgments

This work was supported by National Institute of Health Grant R21NS66117 and Kentucky Spinal Cord Brain Injury Trust 8-15A. PycnogenolR was a very generous gift from Horphag Research Inc., Hoboken, NJ.

Abbreviations

Akt	Protein kinase B
CAT	catalase
CON	contralateral
JNK	c-Jun NH(2)-terminal kinase
ERK	extracellular signal-regulated protein kinase
G-6-PD	glucose-6-phosphate

GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSK	glycogen synthase kinase
GSSG	reduced glutathione
GST	glutathione-S-transferase
IL-6	interleukin-6
IP	ipsilateral
LPO	lipid peroxidation
MAPKs	mitogen-activated protein kinases
PC	protein carbonyls
PKA	protein kinase A
PMSF	phenylmethylsulfonylfluoride
PSD-95	post synaptic density 95
PYC	Pycnogenol
RNS	reactive nitrosative species
ROS	reactive oxygen species
SAP-97	synapse associated protein 97
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substance
TNF-α	tumor necrosis factor- α
3-NT	3-nitrotyrosine
4-HNE	4-hydroxynonenal

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Highlights

- We used a natural bioflavonoid to modulate secondary injury cascades following TBI
- The bioflavonoid, Pycnogenol, reduced oxidative in the brain when given post trauma
- Pycnogenol significantly spared key synaptic proteins in the hippocampus and cortex
- Neuroinflammation was significantly reduced in the brain injury with Pycnogenol
- Pycnogenol shows significant potential as a therapeutic intervention following TBI

Fig. 1.

Changes in levels of antioxidants and antioxidant enzymes in the injured cortex at 48h following a unilateral cortical contusion. In both the vehicle treated (Veh-48h) and pynogonol treated (PYC-48h) there was a significant decline in levels of GSH compared to the sham operated (Sham) control group. Levels of several of the important antioxidant enzymes were also significantly decreased. PYC treatment significantly elevated levels of many antioxidants compared to the Veh-48h cohort. Each bar represents the group mean $\pm$ SD. * $p < 0.05$, ** $p < 0.001$ compared to Sham; # $p < 0.05$, ## $p < 0.001$ compared to Veh-48.

Fig. 2.

Changes in the level of oxidative stress in both the cortex and hippocampus at 48h following a unilateral cortical contusion. There was a significant increase in oxidative stress in both brain regions following the injury. PYC treatment significantly ameliorated the oxidative stress compared to the vehicle treated cohort in both the cortex and hippocampus. Each bar represents the group mean $\pm$ SD. * $p < 0.05$, ** $p < 0.001$ compared to Sham; # $p < 0.05$, ## $p < 0.001$ compared to Veh-48.

Fig. 3.

Changes in levels of antioxidants and antioxidant enzymes in the hippocampus ipsilateral to the cortical injury at 48h. In both the vehicle treated (Veh-48h) and pycnogonol treated (PYC-48h) there was a significant decline in levels of GSH compared to the sham operated (Sham) control group. Levels of several of the important antioxidant enzymes were also significantly decreased. PYC treatment significantly elevated levels of many antioxidants compared to the Veh-48h cohort. Each bar represents the group mean $\pm$ SD. * $p < 0.05$, ** $p < 0.001$ compared to Sham; # $p < 0.05$, ## $p < 0.001$ compared to Veh-48.

Fig. 4.

Pre- and post-synaptic proteins in both the ipsilateral cortex (A) and hippocampus (B) were significantly affected at 96h following a unilateral cortical contusion. PYC treatment significantly increased levels of synaptic proteins in both the cortex and hippocampus. Ipsilateral and contralateral samples from eight animals/group were processed for immunoblotting followed by Western-blot. Immunoblots were developed with 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (alkaline phosphatase substrate; SIGMA FAST™ BCIP/NBT tablets) and densities of bands were analyzed using Scion Image. Drebrin, synapse associated protein -97 (SAP-97), PSD-95, and synapsin-I, demonstrated a significant decrease in signal.

Fig. 5.

Levels of both presynaptic (Synapsin-1) and post synaptic (SAP-97, PSD-95, Drebrin) protein markers were quantified in both the cortex (A) and hippocampus (B) following a unilateral cortical contusion. There was a significant injury related decline in cortex at 96h post trauma. Animals treated with PYC showed a significant elevation compared to the Veh-96h cohort. In the hippocampus, levels of synaptic proteins were significantly decreased in the vehicle treated animals. PYC treatment significantly spared the synaptic proteins. Each bar represents the group mean $\pm$ SD. * $p < 0.05$, ** $p < 0.001$ compared to Sham; # $p < 0.05$, ## $p < 0.001$ compared to Veh-96h.

Fig. 6. Pro-inflammatory cytokines are significantly attenuated with PYC treatment in both the cortex and hippocampus. There was a significant elevation in the cytokines (IL-6, TNF- α) following the unilateral cortical contusion. Levels were significantly lower in the PYC treatment group compared to the vehicle treated cohort. Each bar represents the group mean $\pm$ SD. * $p < 0.05$, ** $p < 0.001$ compared to Sham; # $p < 0.05$, ## $p < 0.001$ compared to Vehicle.