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Cognitive assessment of Pycnogenol therapy following traumatic brain injury

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Abstract

We have previously shown that pycnogenol (PYC) increases antioxidants, decreases oxidative stress, suppresses neuroinflammation and enhances synaptic plasticity following traumatic brain injury (TBI). Here, we investigate the effects of PYC on cognitive function following a controlled cortical impact (CCI). Adult Sprague-Dawley rats received a CCI injury followed by an intraperitoneal injection of PYC (50 or 100 mg/kg). Seven days post trauma, subjects were evaluated in a Morris water maze (MWM) and evaluated for changes in lesion volume. Some animals were evaluated at 48h for hippocampal Fluoro-jade B (FJB) staining. The highest dose of PYC therapy significantly reduced lesion volume, with no improvement in MWM compared to vehicle controls. PYC failed to reduce the total number of FJB positive neurons in the hippocampus. These results suggest that the reduction of oxidative stress and neuroinflammation are not the key components of the secondary injury that contribute to cognitive deficits following TBI.

Keywords

bioflavonoids; head injury; natural compounds; cortical contusion; water maze; recovery of function

1. INTRODUCTION

Traumatic brain injury (TBI) is a global health problem that is financially crippling. In the United States alone, it is estimated that approximately 1.7 million individuals will suffer from some form of TBI [14]. Following the initial trauma, a secondary injury cascade begins leading to the loss of brain connectivity, neuronal death, and reduced cognitive function. Because of the complexity surrounding the many components of the secondary injury cascade, it is now recognized that a multifaceted or combinational therapeutic approach is necessary [26]. A new field using complementary and alternative medical therapeutic

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Conflict of interest

All authors declare no conflict of interest.

approaches appears to be very promising following TBI [18]. An increasing number of natural compounds, with multifaceted pharmacological effects, may provide essential neuroprotection and increased cellular health following TBI to promote greater recovery [6, 10, 23, 36, 50, 52–54].

Pycnogenol® (PYC) is a patented combinational bioflavonoid extracted from the French maritime pine, *Pinus maritima*, which has well documented antioxidant and anti-inflammatory properties [25, 33, 38]. One of the mechanisms behind PYC's ability to suppress inflammation is its ability to inhibit the NF-κB and AP-1 pathway, which suppresses the activation of microglia [13, 15]. In addition, PYC modulates nitric oxide (NO) production through the suppression of inducible nitric oxide synthase (iNOS), a key enzyme of NO [13]. Several studies have documented PYC's ability to inhibit apoptosis [22, 34, 48, 49].

We have previously shown that PYC is effective in significantly reducing components of the secondary injury cascade including, oxidative stress, neuroinflammation, loss of synaptic proteins and synaptic dysfunction in both the cortex and hippocampus [4, 31, 42]. It is unclear whether PYC-related changes in the secondary injury cascade translate into true neuroprotection and improvement in cognitive ability. The purpose of the present study was to investigate if post trauma therapy with PYC can offset injury-related cognitive dysfunction and protect neurons in the cortex and hippocampus.

2. Materials and methods

2.1 Animal model

Adult male Sprague-Dawley rats (n = 38, 275–300 g; 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. Cortical contusions were carried out under isoflurane anesthesia (2%) as previously described [5]. Briefly, following a midline incision, a 6 mm diameter craniotomy was made lateral to midline and midway between bregma and lambda. The skull disk was removed without disturbing the dura. The exposed brain was then contused. 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) and a 5 mm beveled impactor tip. The depth of the impact was set at 2.0 mm with a velocity of 3.5 m/sec and a dwell time of 500 msec. After the impact, the craniotomy site was sealed with an 8 mm disc formed from clear polyester and MASCOT adhesive. Following injury, animals were treated with PYC (generously provided by Horphag Research, Hoboken, NJ) (50 mg or 100 mg/kg) or vehicle (6% dimethyl sulfoxide in physiological saline). Animals were treated with PYC or vehicle with three i.p. injections (15 min, 3 h, 6 h) after the injury as previously described using the same batch of PYC [42]. Sham operated animals were subjected to a craniotomy and three i.p. injections.

2.2 Morris Water Maze (MWM)

A total of 28 animals were used in these experiments: Sham + vehicle (n = 7); TBI+ vehicle (n = 7); TBI+low PYC (n = 7); TBI+high PYC (n = 7). Seven days following the injury, animals were acquisition trained in a Morris Water Maze (MWM) as previously described [43]. Briefly, animals were trained to locate a 13.5 cm in diameter circular black plastic platform in a featureless black pool 127 cm (diameter) × 56 cm (height). Nontoxic black powdered tempera paint was added to the water (23–25°C) to obscure the goal platform located 1 cm below the water surface. A video camera recorded swimming during each trial. Each recording was processed by a Videomex V system (Columbus Instruments, Columbus, OH). The maze was divided conceptually into four quadrants, and the hidden platform was always located in the SE quadrant, approximately 30 cm from the pool wall.

Animals were given five consecutive days of testing with four trials each day and a five minute intertrial interval. For each trial, rats were placed in the pool facing the perimeter of the tank and allowed to search for the platform. If unable to find the platform within the allotted time (120 sec), they were guided to it and remained on it for 10 sec before returned to a holding cage. Rats were started from one of the four different quadrants on each trial with the starting location randomized across trials. Latency and path length to find the platform were recorded with the Videomex system and used to measure performance on each trial. After the final trial on day 5, the submerged platform was removed and each animal was given a 30 sec probe test. The percent time the animal swam in the maze quadrant that previously contained the platform was computed.

2.3 Cortical Tissue Sparing

Cortical damage was assessed blindly with respect to treatment group using an unbiased estimate of tissue sparing as a measure of change in injury volume [51]. Briefly, after MWM testing (day 12), animals were overdosed with Fatal-Plus (Med-Vet International, Mettawa, IL) and transcardially perfused with 4% paraformaldehyde. Brains were cryoprotected and coronal sections (50 μm) cut with a freezing microtome. Twelve equidistant sections throughout the anterior-posterior extent of the damaged hemisphere were stained with cresyl violet and subjected to morphological analysis (Scion Image 4.0.2, Frederick, MD). Quantitative determination of the volume of cortical tissue sparing used the Cavalieri method [28]. On each section, the total cortical area was determined for the entire hemisphere independently. Both the ipsilateral and contralateral hemispheres were evaluated. The amount (percent) of damage (spared tissue) is calculated by dividing the volume of the cortex ipsilateral to the injury site by the cortical volume of the same region in the contralateral (uninjured) hemisphere. In this regard, each animal serves as its own control and histological artifacts such as shrinkage or swelling of tissue that might occur during tissue processing are negated. All quantitative results are reported as mean percent tissue sparing.

2.4 Fluoro-Jade B (FJB)

Assessment of degenerating neurons in the hippocampus was determined using FJB as previously described [3]. Only animals treated with the high dose of PYC (100 mg/kg) were compared to the TBI+ vehicle cohort. Briefly, 48h post trauma, rats (TBI+vehicle n = 5; TBI

+high PYC n = 5) were overdosed with Fatal-Plus and the brains processed as above. Twelve equidistant sections throughout the hippocampus, with a variable starting location, were stained for FJB (HistoChem Inc., Jefferson, AR) according to the method of Schmued [45, 46]. The total number of FJB-positive neurons was determined blindly with respect to treatment group using unbiased stereology with well described anatomical boundaries [2]. The sections were examined with an Olympus BX50 microscope using blue (450–490 nm) excitation light. Counts were limited to the dorsal and ventral leaf of the dentate gyrus granule cell layer and the CA3 region.

2.5 Statistics

Both the escape latency and path length MWM data on acquisition days 1 and 5 separately and also tissue sparing were evaluated for possible differences using a one-way analysis of variance (ANOVA) and the Fisher-Hayter [17] post hoc test. Group means were graphed ± SD. Possible differences in group FJB staining used a Mann-Whitney U-test. Significance for all statistical comparison was set at $p < 0.05$.

3. Results

3.1 Morris water maze

All four groups showed improvement in ability to locate the submerged platform over the five days of acquisition training (Fig. 1A–B). A one-way ANOVA showed that there were no group differences on the first day of acquisition training for both the escape latency [$F(3,24) = 1.096$, $p > 0.05$] and for path length traveled [$F(3,24) = 0.600$, $p > 0.05$]. On day 5 of acquisition training, the analysis revealed a significant difference between groups for both escape latency [$F(3,24) = 5.469$, $p < 0.005$] and path length traveled [$F(3,24) = 4.665$, $p < 0.01$]. Post hoc analysis showed that the Sham+vehicle treatment group performed significantly better than all other groups ($p < 0.05$) for both escape latency and path length traveled, and that TBI+ high PYC group was not significantly different from the TBI +vehicle group ($p > 0.05$) (See insets in Figures 1A–B). The vehicle treated TBI group performed significantly better than the TBI+low PYC group in escape latency ($p < 0.05$) but not total path length. Mean percent search time during the probe test was significantly different [$F(3,24) = 24.679$; $p < 0.0001$] (Fig. 1C). Post hoc analysis showed that the Sham treated group spent significantly more time in the correct quadrant ($p < 0.001$). The injured groups were not significantly different from each other ($p > 0.05$).

3.2 Tissue sparing following TBI

All injured animals showed an obvious loss of cortical tissue ipsilateral to the cortical impact. An ANOVA revealed a significant group difference in cortical tissue sparing [$F(2,18) = 4.245$, $p < 0.05$] (Fig. 2A–B). Post hoc analysis showed that the TBI+high PYC group had significantly more spared cortical tissue than the low PYC or vehicle treated animals ($p < 0.05$). The TBI−low PYC group was not significantly different from the vehicle treated animals ($p > 0.05$).

3.3 FJB staining following TBI

FJB staining was evaluated in the hippocampal formation 2 days after a cortical contusion. All injured animals had FJB-positive neurons in the ipsilateral hippocampus in both the dentate gyrus (Fig. 2C) and CA3 regions. Only animals treated with the high dose of PYC were evaluated based upon the cortical tissue sparing results. A Mann-Whitney U test failed to demonstrate a significant difference for either the granule cell layer ($p > 0.05$) or the CA3 region ($p > 0.05$) (Fig. 2D).

4. Discussion

PYC has previously been shown to reduce oxidative stress, spare hippocampal synaptic proteins, and protect hippocampal synaptic function [4, 31, 42]. In the present study, two different doses of PYC were used to assess cognitive function. Neither dose of PYC significantly improved MWM performance compared to the TBI+vehicle treated controls. These same animals were subsequently analyzed for possible cortical tissue sparing as a measure of neuroprotection. The high PYC treated group showed increased tissue sparing compared to the vehicle treated animals. Animals treated with the low dose of PYC showed no improvement. Because the MWM is considered to be a hippocampal dependent task and the high dose indicated cortical tissue sparing, neuroprotection in the hippocampus was explored with FJB staining. The analysis failed to demonstrate a PYC-related neuroprotection in these hippocampal neuronal layers.

The present results do not support our previous post-trauma PYC studies demonstrating significant alterations of some aspects of the secondary injury cascade following a moderate TBI. We previously reported that an i.p. injection sequence, identical to that used in this study, significantly decreased oxidative stress, neuroinflammation, and spared synaptic proteins in both the cortex and hippocampus within 96h of the injury [42]. The same type of cortical contusion injury paradigm was used with the same parameters, the same batch of PYC, and the same strain of adult rat. Although there was some cortical neuroprotection, it did not appear to be comparable to the levels observed for spared synaptic proteins [42]. In the previous investigation, the assessment of synaptic proteins utilized the penumbra as opposed to the cortex directly below the injury location. There are several instances where a natural compound has been shown to significantly reduce secondary injury cascades but fail to improve cognitive performance in the MWM [1, 11, 21]. Somewhat surprising is the fact that caffeic acid, which is one of the main components of PYC, has been shown to significantly reduce lesion volume and improve cognition when administered post-trauma [56]. Previous studies using an extended treatment with PYC have reported significant enhancement in cognition in both animals [20, 24] and humans [8, 9, 39].

Following TBI, there is a significant reduction in the total number of neurons in the hippocampal CA3 region [3, 7]. This neuronal loss results in a transient loss of synaptic contacts and synaptic strength eventually recovers [30]. We have previously shown that PYC, when administered following TBI, significantly enhances synaptic efficacy in the injured hippocampal CA3-CA1 pathway [31] suggesting a positive role for PYC. The hippocampus is well known for its plasticity [41] and previously shown to support synaptogenesis in the denervated CA1 region following TBI [44]. Our previous studies

indicated that PYC treatment might accelerate synaptogenesis [31] while failing to totally protect the vulnerable CA3 neurons as evidenced by the FJB staining. It is puzzling why PYC failed to facilitate the MWM acquisition task. Unlike many motor skills that show a transient deficit following TBI and spontaneously recover, deficits in the MWM have been shown to be long lasting [32]. It may also be the case that increased hippocampal plasticity in the PYC treated animals, as evidenced by enhanced levels of synaptic proteins [4, 42], may not have had time to properly reorganize. The benefits of PYC therapy may require an extended recovery time such as 30 days. Alternatively, it is well known that injury-induced axon sprouting can lead to detrimental consequences such as spasticity, epilepsy, pain, or no discernible behavioral change [12, 16, 19, 27, 29, 35, 37, 40, 47, 55].

The failure to observe a beneficial effect of PYC in the MWM maze task may have a much greater implication. These results may indicate that the simple suppression of neuroinflammation and oxidative stress may not be enough to improve cognitive ability in this model of TBI. Because PYC has been shown to suppress NO through the inhibition of iNOS and also the NF-kB/AP-1 pathway, these pathways may be only partial players in the secondary injury cascade following TBI. Future experiments would have to probe whether or not PYC may be effective with a less severe injury.

Acknowledgments

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Abbreviations

ANOVA	analysis of variance
CCI	controlled cortical impact
FJB	fluoro-jade B
iNOS	inducible nitric oxide synthase
MWM	Morris water maze
NO	nitric oxide
PYC	pycnogenol
TBI	traumatic brain injury

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Highlights

- A combinational bioflavonoid, pycnogenol, was used as a therapy enhance cognition following brain injury
- Pycnogenol significantly reduced TBI-related cortical injury volume
- Fluoro-jade B staining failed to demonstrate hippocampal neuroprotection
- Pycnogenol administered post-injury did not improve Morris water maze acquisition

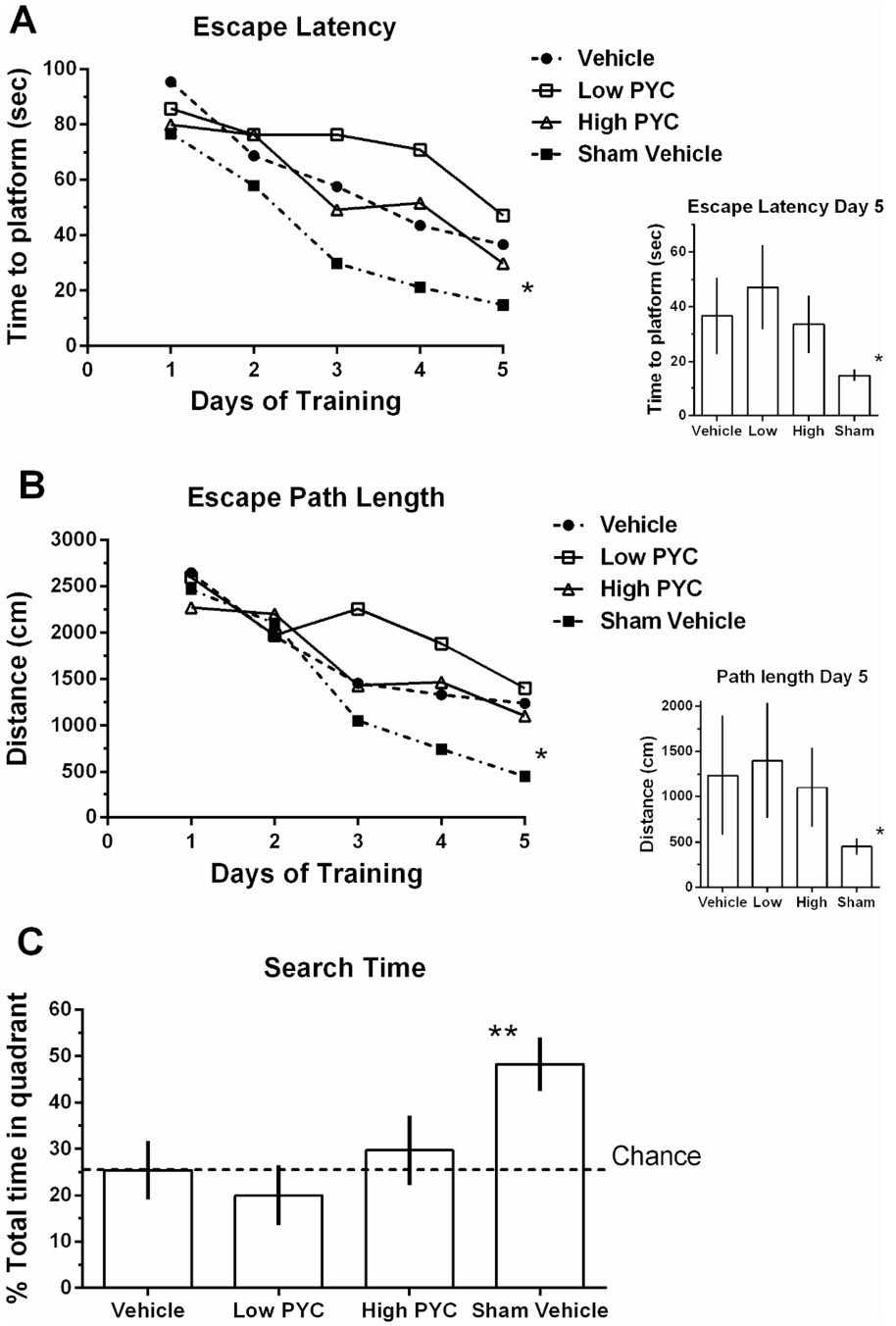

Figure 1.

Beginning seven days post injury, animals were trained to locate a submerged platform in a standard Morris water maze. (**A**) The time to locate the submerged platform (Escape Latency) was averaged over the four trials on each day of training. Inset shows the different groups' performance on day 5. (**B**) Total distance required to find the submerged platform (Escape Path Length) was averaged over the four trials on each day of training. The inset shows the different groups' performance on day 5. (**C**) Following the final acquisition trial on day five of training in the Morris water maze, animals were tested for spatial memory

during a probe trial. Horizontal dashed line represents chance performance. Points represent group means (n=7/group) * $p < 0.05$ ** 0.01 Sham vehicle compared to all other groups. Bars represent group mean ± SD.

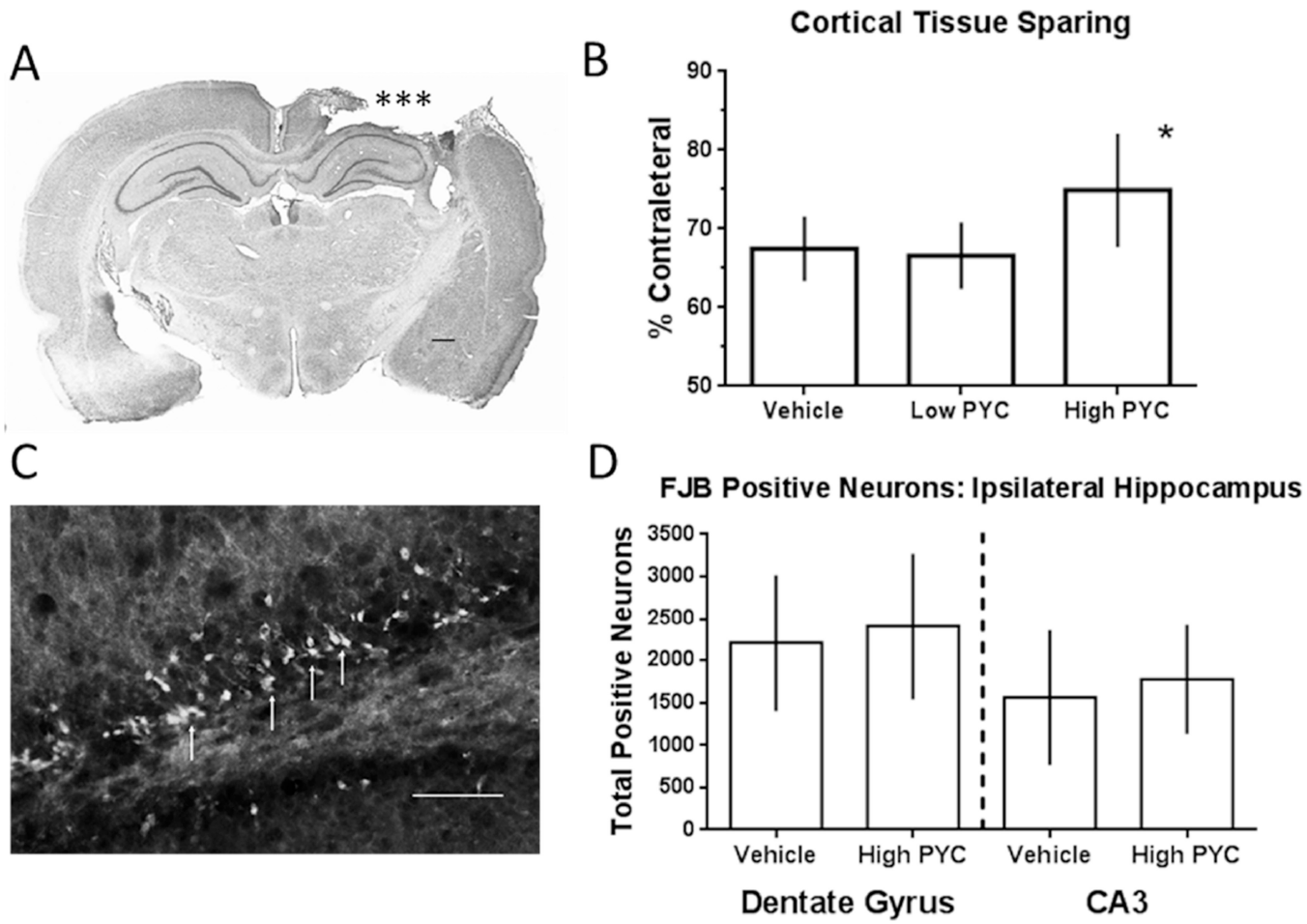

Figure 2.

(**A**) Photomicrograph of a coronal cresyl violate stained section showing a representative lesion resulting from a unilateral cortical impact at 12 days post injury. Tissue loss (***) on the right side resulted from the cortical impact. T (**B**) Changes in injury volume assessed by measuring the total amount of spared tissue using unbiased stereology. The high dose PYC group showed greater tissue sparing. The sham group is not included because there was no injury. (**C**) Photomicrograph of a FJB stained coronal section in the dentate gyrus. The arrows indicate a few of the FJB-positive stained granule cells ipsilateral to the injury. (**D**) Estimates of the total number of FJB positive neurons in two different regions of the hippocampus. There was no significant difference between the vehicle and high-PYC treatment group. * $p < 0.05$ compared to other groups. Calibration bar = 100 μm. Bars represent group mean ± SD.