Research article

Cognitive assessment of pycnogenol therapy following 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.

ARTICLE INFO

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 48 h 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.

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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 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/s and a dwell time of 500 ms. 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) X 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 s), they were guided to it and remained on it for 10 s 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 s 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, 48 h 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