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ORIGINAL RESEARCH

A Self-Emulsifying Nanococktail of Pomegranate and Cannabidiol Reduces Cognitive Decline in Mice

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Introduction: Stress and inflammation in the early stages of cognitive decline has spurred interest in the use of antioxidants as prophylactic and therapeutic agents. Several studies have established the neuroprotective capacities of a pomegranate oil nano-formulation (Nano-PSO). In parallel, cannabis-derived cannabidiol (CBD) prevented social recognition deficits and reduced neuroinflammation in mice. This work assessed the impact of Nano-PSO:CBD on memory and learning.

Methods: A battery of hemp extracts was screened for their effects on acetylcholine esterase inhibition and neuronal protection activities. The best-performing extract in the in-vitro assays was nano-formulated via self-emulsifying system with PSO and administered to aged mice and mice with MK-801-induced cognitive decline, which were then subjected to a Morris water maze test and Y-maze test, respectively.

Results: Aged mice chronically fed Nano-PSO:CBD performed with a 22–31% shorter escape latency ($p \leq 0.05$), 23% shorter distance swam and increased average speed as compared to aged control mice. Mice pretreated with Nano-PSO:CBD prior to MK-801 had a significantly higher percentage of entries into the novel arm of the Y-maze compared to untreated controls.

Discussion: PSO nano-formulated with CBD had a salutary effect on memory, learning and spatial recognition and should be further studied in a clinical context.

Keywords: cognition, nanoformulation, CBD, pomegranate seed oil, nano-omega 5, brain protect, punicic acid

Introduction

Advances in clinical medicine and the improved accessibility to healthcare services have been instrumental in the significant extension in life expectancy seen in recent decades. In turn, the prevalence of age-related conditions is on the rise. Specifically, the absolute global number of individuals affected by cognitive decline almost tripled within 30 years, estimated to have risen from 20.2 million in 1990 to 57.4 million cases in 2019.[1,2] This has been mirrored by a marked increase in dementia-related expenditures across the globe, estimated at over 1 trillion USD in 2019.[3] Given these trends, alongside the mitigative and marginally and inconsistently effective therapeutic interventions,[4] many research efforts focus on development of methods to delay onset of and treat cognitive impairment.

Oxidative damage to the brain and subsequent neuronal death, are implicated in the early stages of cognitive decline in the elderly and in patients with chronic central nervous system diseases.[5–8] In turn, antioxidant-rich diets have been associated with higher cognitive performance and slower rates of cognitive decline.[9–11] This understanding spurred the testing of an array of nutraceuticals with established antioxidant activity, to treat age-related diseases.[12–15] Among these, pomegranate seed oil (PSO) prevented neuronal death, reduced inflammatory and oxidative signatures and accelerated learning processes in several neurological disease animal models.[16] These findings were corroborated by the measured increase in brain antioxidant capacity and decrease in lipoperoxidation and protein oxidation in obese rats fed daily with PSO for 12 weeks.[17] A brain-targeted PSO supplement prepared in a nano-formulation (Nano-PSO), conferred a neuroprotective impact in the hippocampus, which postponed disease manifestation and deterioration in a genetic prion disease mouse model.[18] Similarly, its long-term administration to animal models of neurodegeneration delayed

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mitochondrial damage and disease exacerbation, prevented aberrant protein aggregation, lipid oxidation and cognitive decline and extended survival.[19,20] Similarly, the product attenuated traumatic brain injury-induced memory loss and behavioral decline in mice.[21] In a first-in-human, randomized, double blind, crossover clinical trial, three months of treatment with Nano PSO led to a significant and sustained improvement in verbal learning performance and had a positive effect on visuospatial memory in multiple sclerosis patients.[22]

A growing body of evidence has also flagged the cannabis plant as a promising nutraceutical for various states of cognitive dysfunction. In particular, long-term oral treatment with cannabidiol (CBD), a central, non-psychoactive phytocompound in the plant, prevented social recognition deficits and reduced neuroinflammation in the APP/PS1 Alzheimer's disease (AD) mouse model.[23] In a rat AD model, intraperitoneal administration of CBD imparted an anti-inflammatory effect, prevented neuronal loss and stimulated hippocampal neurogenesis.[24] In parallel, inhaled CBD triggered durable improvements in global network connectivity in aging mice.[25] In line with these findings, oral CBD pretreatment blunted development of positive psychotic symptoms and verbal learning and memory deficits in healthy volunteers later intravenously administered Δ9-tetrahydrocannabinol (Δ9-THC), the central psychomimetic compound of the cannabis plant.[26] Similarly, verbal memory performance was higher among recreational users of cannabis containing high CBD levels as compared to those using products with lower CBD concentrations.[27] Its prophylactic potential was demonstrated by pretreatment of a rat Parkinson's disease model with CBD, which prevented reserpine-induced cognition and motor deficits.[28] Other works support the proposed entourage effect, in which synergistic interactions between other phytochemicals in whole-plant extracts augmented the therapeutic potential of CBD, likely due to simultaneous modulation of multiple targets. Five weeks of concomitant intraperitoneal CBD and THC treatment had an enhanced pro-cognitive effect and prevented learning impairments in young APP/PS1 mice as compared to treatment with CBD or THC alone.[29] These were mirrored by significant reductions in astrogliosis, microgliosis and proinflammatory cytokine expression and by modified plaque composition. The CBD-THC formulation also proved more effective than the vehicle control in rescuing memory impairment in aged APP/PS1 mice, and induced an upregulation in markers of synaptic plasticity and function.[30]

The present study aimed to explore the effects of nano-formulation of PSO together with a CBD-enriched cannabis extract, on oxidative stress-challenged neuroblastoma cells and on memory and learning processes in both aged mice and MK801 mice model with impaired cognition. The nano-cocktail was generated following extensive screening of a battery of cannabis extracts and their combination at various ratios with PSO. The screening was conducted in vitro specifically for acetylcholine esterase inhibition and neuronal protection to match the most effective extract for cognition decline inhibition, demonstrating the differences between extracts, enhancing the entourage effect of CBD extracts, and emphasizing the importance of in-vitro bioactivity assays to match extracts for specific medical indication.

Materials and Methods

Cell Culture and Treatment

SH-SY5Y human neuroblastoma cell line was obtained from American Type Culture Collection (ATCC, Beit Ha'emek, Israel). Cells were maintained in DMEM:F12 supplemented with 15% fetal bovine serum, and 1% penicillin/streptomycin, at 37 °C, in a humidified atmosphere containing 5% CO_2/95% air. Cells were seeded in triplicates in a 96 well plate, and differentiated by the addition of fresh retinoic acid (10 mm) every 48 h for 8 days as was previously described by Shaltiel Karyo et al.[31] Differentiated neurons were then pretreated with PSO:CBD (12:1), for 24 h before addition of 300mM hydrogen peroxide (H_2O_2) for an additional 24 h.

Nano-PSO:CBD Formulations

PSO (Seekwell, Tel Aviv, Israel) was formulated alone (300 mg/mL) or with CBD extract containing CBD (273.4 mg/mL pomegranate + 26.6 mg/mL CBD extract) by mixing the components together with surfactants over a magnetic stirrer to generate a self-emulsifying drug delivery system (SEDDS). The PSO:hemp extract ratio was selected based on preliminary in vitro calibration studies. In the in vitro studies, the vehicle (ethanol) prepared without PSO and CBD extract, served as a negative control. The formulation spontaneously formed oil-in-water nano emulsions when introduced into an aqueous phase under gentle mixing, and remained stable as an emulsion for at least 12 weeks under accelerated conditions of 40 °C.

Particle size was measured using a Zetasizer Nano ZS particle characterization system (Malvern Panalytical, Malvern, United Kingdom) and was found to be 217 nm. The nano formulation was stored at room temperature for up to 1 week.

Quantitation of Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity was measured using a modified 96-well microplate assay[32] based on Ellman's method. Acetylcholinesterase was incubated for 15 min with 5.5'-dithio-bis-[2-nitrobenzoic acid] (DTNB) as well as with 20 L/mL of different cannabis extracts containing 10% ethanol. Absorbance was then measured at 412 nm in Tecan microplate reader, and values were used as blank. Thereafter, the enzymatic reaction was initiated by the addition of acetylthiocholine iodide (ATCI) and the hydrolysis of acetylthiocholine was monitored by reading the absorbance after 15 min. Positive control wells were treated with 250 nM tacrine. All reactions were tested in three biological replicates at three technical repeats. Percent inhibition was calculated as follows: % Inhibition = E-S /E * 100, where E is the activity of the enzyme without extract and S is the activity of enzyme with the extract.

Quantitation of Neuroprotection

Approximately 24 h after cells were treated with cannabis extracts, H_2O_2 was added and samples were incubated for an additional 24 h. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.5mg/mL for 1 h at 37 °C. Thereafter, wells were treated with DMSO (150 µL/well) and plates were placed on a shaker for 30 min. OD570 was measured using a Tecan GENios microplate reader. The OD of the untreated control samples was referred as 100% viability, H_2O_2 without treatment was referred as the negative control.

Mouse Models

All animals experiments were performed in compliance with Council Directive No. 2010/63/UE, and French decree No. 2013–118. Experiments were also approved by the ethics committee for animal experimentation of Porsolt (CE060).

Young (6-weeks-old) and aged (17–18 months) male C57BL6J mice (Janvier Labs, France) were housed on wood litter in groups of 6, with free access to food and water throughout the study. The animal house was maintained under artificial lighting (12 h) in a controlled ambient temperature (22 ± 2 °C), with relative humidity between 30–70%. Aged animals were acclimatized to laboratory conditions for 8 weeks and young animals for 5–7 days before the beginning of the behavioural evaluation. In the age-related cognitive decline experiment, Nano-PSO, Nano-PSO:CBD or vehicle (saline), were administered by oral gavage for 7 weeks to 6–16 animals/group, with the last dose administered 24 h before learning and memory retention were assessed using the Morris water maze test. In the toxin-induced cognitive decline experiment, Nano-PSO, Nano-PSO:CBD or vehicle were administered by oral gavage for 29 days to 12 aged mice/group, with the last dose administered 60 min before cognitive deficit was assessed using the Y-maze test. Thirty minutes before the Y-maze test, 0.1 mg/kg (+)-MK-801 hydrogen maleate was administered i.p. to each mouse.

Morris Water Maze

To assess spatial memory, the Morris water maze test was performed as previously described.[33] During the acquisition phase, mice were trained over 9 sessions (Days 1–5 and Days 8–11). Each training session consisted of four trials in the Morris Maze, performed at 15-min intervals. Immediately before the first trial on Day 1, mice were placed on the platform for 30s. After that, for each trial, each mouse was placed in the maze, at one of the two starting points equidistant from the platform, and allowed to swim freely in the maze for 60s. If the mouse found the escape platform, it was left on it for 30s before being returned to a resting cage. If the mouse did not find the platform within 60s, the mouse was removed from the water and placed on the platform for 30s before being transferred to the resting cage. Escape latency, the distance swam to reach the hidden platform and swim speed were recorded.

A probe test was performed on Day 12. During the probe test, the platform was removed from the maze and mice were placed in a novel start point opposite the platform, and allowed to swim freely in the maze for 60s. The distance swam, time spent in each quadrant, virtual latency, proximity and number of platform crossings were measured. All trials were video-recorded and animal behavior was analyzed using a video-tracking system (Panlab: SMART).

Y-Maze Spontaneous Alternation Test

Short-term memory was assessed using as Y-maze, as previously described.[34] The Y-maze consisted of three identical arms constructed of grey opaque Plexiglas (35×8×14cm) placed at 120° angles to each other. During the exposure phase, mice were placed at the end of the start arm and were allowed to explore two arms of the maze for 5 min. Access to the third arm of the maze (novel arm) was blocked by an opaque door. Next, the mice were transferred to their home cage for 2 min. During the testing trial, mice were placed into the start arm and were allowed to explore all three arms of the maze for 5 min. Each session was video-recorded and animal behavior was analyzed using a video-tracking system (EthoVision, Noldus). The number of entries (visits), time spent and distance moved in each arm were measured as a measure for spatial short-term memory. The total distance travelled during the exposure session served as a measure of locomotor activity.

Statistical Analysis

In the in vitro studies, outcomes were compared using the unpaired Student's *t*-test. For the Morris water maze experiment, data between groups were compared using two-way ANOVA with groups and sessions as repeated factor, followed by Dunnett's multiple comparisons test. For the Y-maze experiment, data between groups were compared using one-way ANOVA with groups as repeated factor followed by Dunnett's test.

All in vivo statistical calculations were performed using commercial software (Microsoft Excel® or GraphPad Prism® Version 7.03) and verified test by test according to Porsolt's standardized internal procedures. All differences were considered statistically significant when the null hypothesis could be rejected at a risk α of less than 0.05.

Results

Neuroprotective Benefits of Nano-PSO and CBD extract

In order to select the best CBD extract, the bio-functionality of a battery of CBD extracts was evaluated by in-vitro assays. Relative AChE inhibition activity by CBD was measured in vitro and its neuroprotective effect was assessed by measuring cell viability in neurons treated with CBD prior to hydrogen peroxide exposure. AChE activity was inhibited by up to 65% (Figure 1A) and neuronal death was prevented by up to 100% (Figure 1B), depending on the specific extract applied. The extract that had both the most marked neuroprotective effect and acetylcholine inhibition effect was selected to be combined with PSO.

Selection of the cannabis extract to be tested in the in vivo studies was made based on both in vitro studies.

Nano-PSO:CBD Enhances Spatial Learning Processes and Locomotor Activity in Aged Mice

To assess the potential effects of Nano-PSO:CBD on spatial learning and memory retention, the performance of aged mice in the Morris water maze was monitored. Young control mice learned to locate the hidden platform as the 11-day acquisition period progressed, as shown by the marked reductions in mean escape latency (Day 1: 46.1 s vs Day 11: 13.6 s) (Figure 2A) and distance swam (Day 1: 700.0 cm vs Day 11: 194.6 cm) over time (Figure 2B). The average speed remained stable throughout the acquisition sessions (Figure 2C). During the probe trial performed on Day 12, the mice spent 41.6% of their time and 41.1% of the total distance swam in the platform quadrant, suggesting retention of the learned position of the platform.

Aged control mice displayed age-related spatial learning deficits, as evidenced by a flatter learning curve (Day 1: 48.6 s vs Day 11: 38.4 s mean escape latency, and Day 1: 578.1 cm vs Day 11: 418.9 cm mean distance swam) and significantly higher mean escape latencies (Figure 2A) and mean distance swam (Figure 2B) to reach the hidden platform as compared to young control mice. In addition, their average speed was significantly slower in almost all acquisition sessions (Figure 2C). When compared to young mice, the aged mice spent 33% less time ($p<0.01$) and covered 32% less distance in the platform quadrant during the probe test.

Nano-PSO had no significant effect on acquisition or probing parameters in aged mice. However, when formulated together with CBD extract, significant decreases in mean escape latency were measured on Day 3 (−31%, $p < 0.001$),

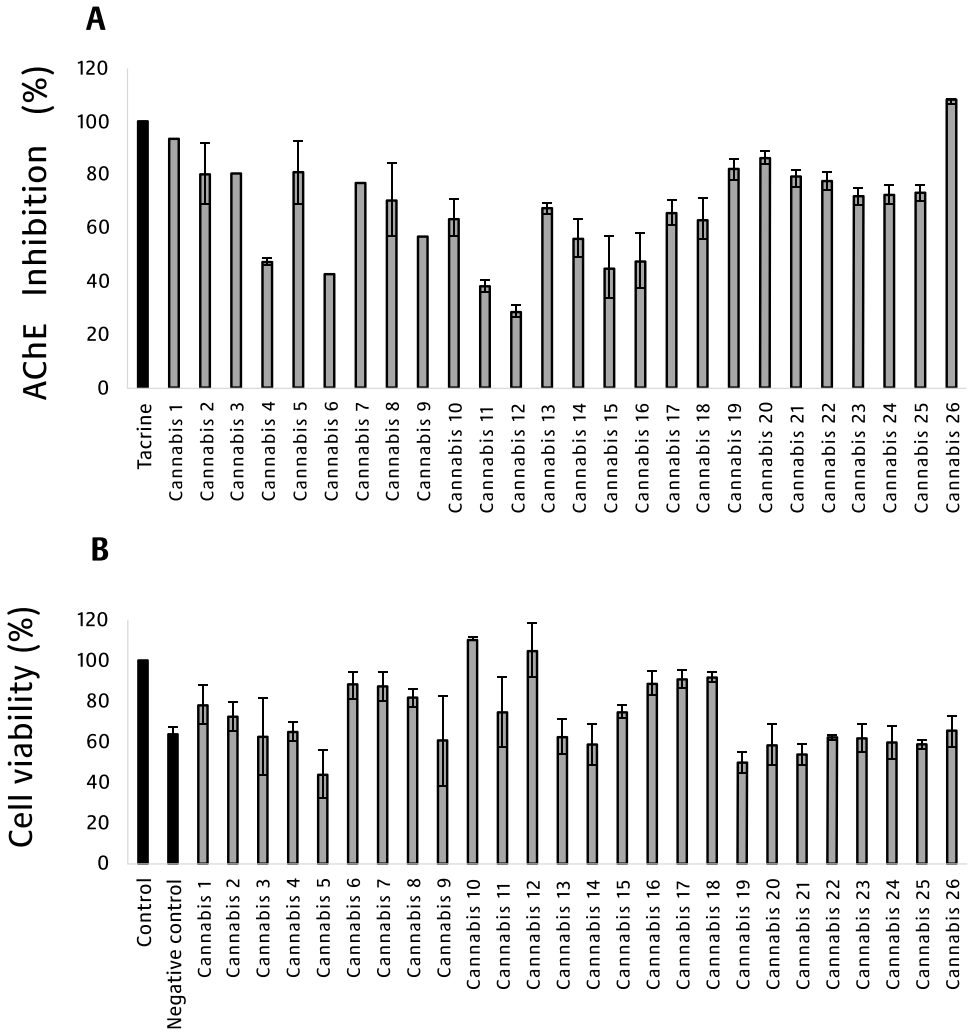

Figure 1 CBD extracts inhibit acetylcholine esterase activity and impart a neuroprotective effect. **(A)** A panel of 26 CBD extracts was incubated with acetylcholinesterase for 15 min, after which, the enzyme activity was measured at 412 nm using the Ellman's method. All reactions were tested in triplicates. Percent inhibition was calculated as follows: % Inhibition = E-S /E * 100, where E is the activity of the enzyme without extract and S is the activity of enzyme with the extract. Tacrine (250 nM) served as a positive control. **(B)** SH-SY5Y human neuroblastoma cell line were incubated with CBD extracts for 24 h and then treated with H_2O_2 for an additional 24 h. Cell viability was measured using the colorimetric MTT method.

Day 4 (−22%, $p < 0.05$), Day 8 (−28%, $p < 0.05$) and Day 10 (−29%, $p < 0.05$) as compared to aged control mice (Figure 2A). In addition, it significantly decreased distance swam to reach the hidden platform on Day 3 (−23%, $p < 0.05$) (Figure 2B) and significantly increased average speed on Days 1–4 and Days 10–11 ($p < 0.05$) (Figure 2C). The combined treatment did not significantly affect retention, although it was higher than that expected by chance (31.8%, $p = 0.0697$ for the time spent and 31.8%, $p < 0.05$ for the distance swam).

Nano-PSO:CBD Enhances Spatial Working Memory and Locomotor Activity in Mice with Cognitive Decline

To assess the potential effects of Nano-PSO:CBD on spatial working memory, mice with MK-801-induced cognitive decline were monitored in a standard Y-maze. As expected, naive mice (who did not receive MK-801) displayed higher exploration of the novel arm than of the familiar and departure arms, reflecting spatial recognition. The percentage of time spent in each arm was similar (Figure 3). Conversely, mice with cognitive decline showed significantly fewer entries into the novel arm as compared to controls (31.8% versus 41.7%, $p < 0.01$), with a same tendency for the percentage of total distance moved (36.2% versus 42.2%, $p = 0.1414$). These deficits were prevented by pre-treating mice with Nano-PSO:

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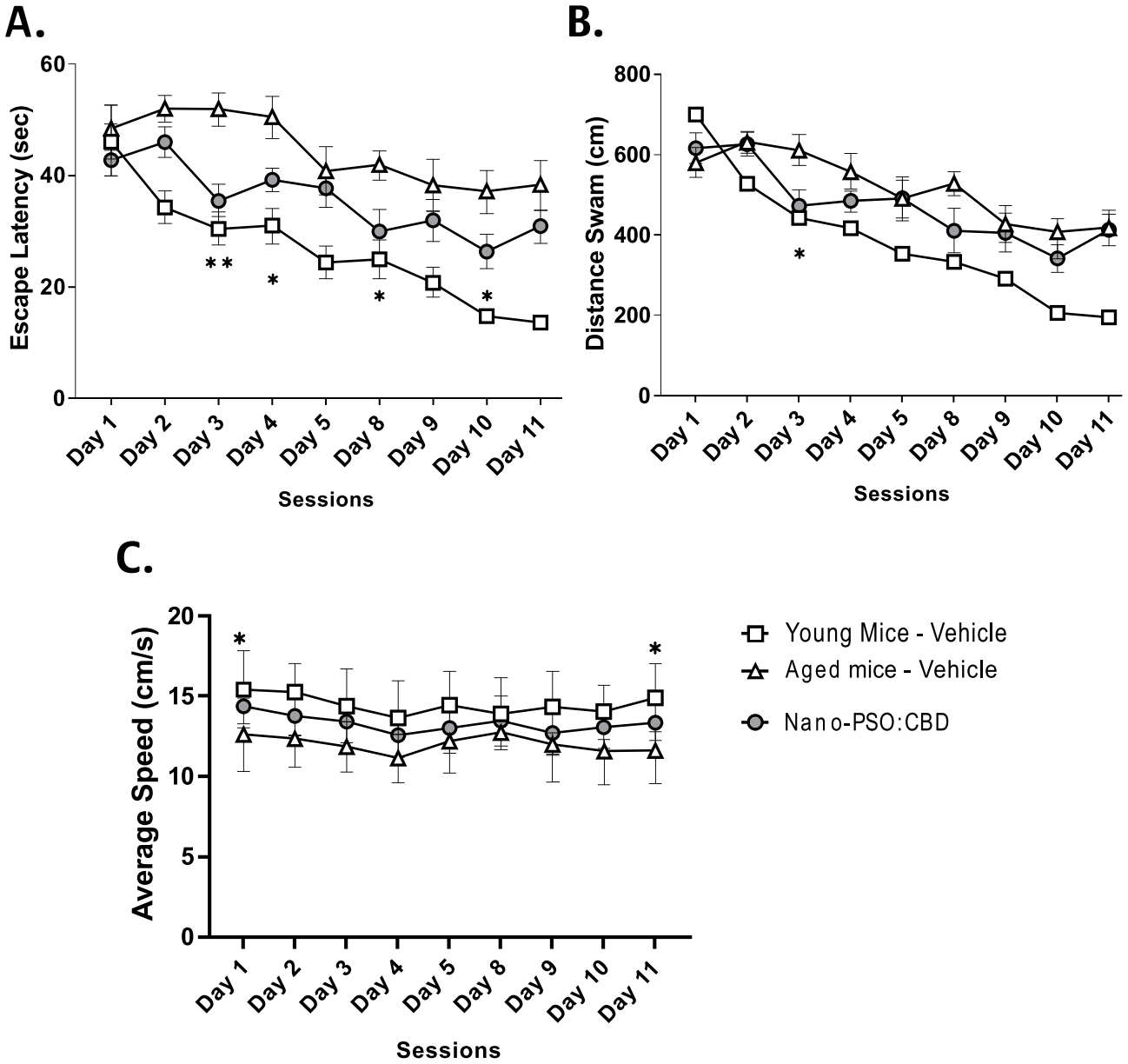

Figure 2 Nano-PSO:CBD enhanced spatial learning and locomotor activity in aged mice. Young (6-weeks-old) and aged (17–18 months) male C57BL6J mice were treated with Nano-PSO:CBD or vehicle by oral gavage for 7 weeks, with the last dose administered 24 h before mice were subjected to the Morris water maze test. (**A**) Escape latency (sec), (**B**) distance swam (cm) and (**C**) average speed (cm/s) in the maze are presented. Intergroup comparisons were conducted using a two-way analysis of variance followed by the Dunnett's test * = $p < 0.05$; ** = $p < 0.01$ when comparing Aged mice - Pomegranate/CBD with Aged mice – Vehicle.

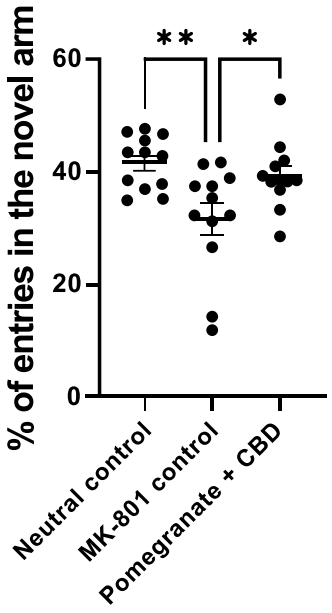

Figure 3 Nano-PSO:CBD enhanced spatial working memory and locomotor activity in mice with cognitive decline. Male C57BL6J mice (12/group) were treated with Nano-PSO:CBD or vehicle (neutral) by oral gavage for 29 days, with the last dose administered 60 min before cognitive deficit was assessed using the Y-maze test. Thirty minutes before the Y-maze test, 0.1 mg/kg (+)-MK-801 hydrogen maleate was intraperitoneally administered to each mouse. Shown are percent entries to novel arm during a 5-min test period. Intergroup comparisons were conducted using a one-way analysis of variance followed by the Dunnett's test * = $p < 0.05$ when comparing Pomegranate/CBD with MK-801 control ** = $p < 0.01$ when comparing MK-801 control with neutral control.

CBD, as demonstrated by a significantly higher percentage of entries into the novel arm (39.3% versus 31.8%, $p < 0.05$), with similar trend for the percentage of total distance moved (41.1% versus 36.2%, $p = 0.2405$) as compared with MK-801-treated controls. The effects were significantly higher than chance level (39.3% and 41.1%, $p < 0.01$, respectively).

Discussion

The presented studies assessed the neuroprotective effects of a CBD extract nano-formulated with PSO in both in vitro and in vivo settings. Nano-PSO:CBD preserved neuron viability in a highly oxidative environment and significantly reduced AChE activity, which is expected to translate to prolonged neurotransmitter retention in brain synapses. In an in vivo model, chronic administration of Nano-PSO restored the aging-associated reduction in acquisition performance, and when formulated with CBD extract, it significantly improved learning, as demonstrated by shorter escape latency and distance swam in the Morris water maze. Dietary supplementation with Nano-PSO enhanced acquisition performance and average locomotion speed, effects that were further boosted when administered together with CBD extract. In parallel, Nano-PSO:CBD reversed spatial recognition deficits typical of mice with MK-801-induced cognitive decline. Taken together, Nano-PSO:CBD imparted a statistically significant effect on learning processes in mice suffering from impaired cognition. In addition, in view of the absence of a significant beneficial effect of Nano-PSO:CBD on locomotion (data not shown), the observed increase in swimming speed in mice treated with the combination product, suggests an improvement in information processing.

These measured effects are ascribed to the central components of the PSO and CBD extract and their ability to counteract the oxidative and inflammatory insults underlying brain alterations that impair cognitive functioning. Specifically, punicic acid (PA) also known as omega-5 is a highly lipophilic polyunsaturated fatty acid (18:3 n-5), that comprises 74–85% of pomegranate fruit seed. It is a potent antioxidant and anti-inflammatory agent, that has been reported as antidiabetic, anti-obesity, anti-atherosclerotic, antiproliferative, anticarcinogenic and neuroprotective activities, and has been found to be effective in ameliorating neurodegenerative disorders.[35,36] The tested extract was highly enriched in CBD, which has established antioxidant and anti-inflammatory activities that correlate with its accelerative effect on wound healing processes and reparative impact on UV-damaged skin and metabolic and neuronal disturbances.[37–39] Moreover, the endocannabinoid system as a whole is an established player in synaptic transmission and plasticity, and regulator of cytokine release and is altered in most neurological pathologies.[40] Taken together, the array of intracellular, microenvironmental and systemic

pathways reported to be modulated by PA and CBD, orchestrate their effects on locomotion, cognition, mood and perception and reversal of neurodegenerative trends.[36,40] Apart from the direct actions of these two key ingredients, entourage effects imparted by even trace levels of the hundreds of phytochemicals naturally occurring in both the oil and extract, including a wide variety of terpenes, tannins, anthocyanins, fatty acids and polyphenols, were expected.[35,40] For this reason, the CBD extract applied in these experiments was carefully selected following the systematic screening of 27 CBD extracts for their neuroprotective and antioxidant activities.

In the cell assays, the maximal neuroprotective benefits of Nano-PSO:CBD were already achieved at the lowest tested dose. In addition, separate and concomitant applications of the two components yielded identical responses, suggesting that the system was tested at a point of saturation. In the aging mouse model, a specific effect of Nano-PSO:CBD on distinct neuronal networks regulating specific aspects of cognitive performance was noted. In addition, longer treatment periods may be necessary to repair memory-related processes. Further, impairments in data retention vs acquisition capacities may involve higher variability, which would require testing of a larger sample sizes to capture effects of significance.

Conclusions

The presented data demonstrated the neuroprotective effects of Nano-PSO:CBD in a cell culture setting and their translation to improved acquisition, information processing and short-term memory in mice suffering from cognition deficits. The measurable impact was most likely the result of the pleiotropic effects of PA and CBD extract, including naturalization of the oxidative and inflammatory milieu compromising learning-associated neuronal networks. Larger studies of longer durations and integrating additional cognitive tests will be needed to reaffirm these observations. In addition, mechanistic studies of Nano-PSO:CBD activity, with focus on specific neuroinflammatory and neurodegenerative markers in the central nervous system,[41] as well as pharmacokinetics assessment, including its ability to permeate the blood-brain barrier, will enhance the current findings. Furthermore, its potential to ameliorate modifiable risk factors should be longitudinally assessed in young and pre-disease state models. Finally, these observations provide a promising basis for expanding research efforts in humans.

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Disclosure

Avi Palatnik and Oded Shoseyov are shareholders at Tahiro. Professor Oded Shoseyov has a patent pending PCT application at Tahiro. The authors report no other conflicts of interest in this work.

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