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Effects of fish oil supplementation on spatial memory in rats with pilocarpine-induced epilepsy assessed using the Morris Water Maze test

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ABSTRACT

Objective. Patients with temporal lobe epilepsy (TLE) are at high risk of experiencing cognitive impairment. Such dysfunction is also observed in an animal model of TLE, the rat model of pilocarpine-induced epilepsy.

Methods. We investigated the effects of fish oil supplementation on spatial memory in rats with pilocarpine-induced epilepsy using the Morris Water Maze (MWM) test.

Results. Although rats with pilocarpine-induced epilepsy treated with fish oil learned the platform location significantly faster by Day 7 of the acquisition phase, spatial memory performance of these rats was unaffected by fish oil supplementation during probe trials.

Significance. Our study provides insights into the importance of considering nutraceutical strategies for enhancing cognitive abilities in patients with TLE.

Key words: temporal lobe epilepsy, pilocarpine-induced model of epilepsy, cognitive impairment, fish oil supplementation, Morris Water Mazer, spatial learning and memory.

Temporal lobe epilepsy (TLE), frequently associated with hippocampal sclerosis, constitutes approximately 65% of all cases of drug-resistant focal epilepsy [1, 2]. It has been proposed that neuronal loss mainly within the hippocampus, amygdala, and entorhinal cortex, which are brain structures directly involved with memory, leads to cognitive impairment in patients with TLE [1-4].

The pilocarpine-induced model of epilepsy has been widely used to study TLE because it reproduces most of the histopathological and behavioural features seen in human TLE, including hippocampal neurodegeneration [5-7]. This is consistent with previous studies showing evidence of deficits in hippocampal-dependent memory tasks in pilocarpine-treated rats [8-13].

Fish oil supplementation has been shown to play an important role in maintaining brain health [14-16], particularly among those in whom the level of such fatty acids is significantly decreased, as in patients with epilepsy [17, 18]. Furthermore, evidence from clinical and animal studies demonstrated that fish

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oil supplementation was effective in controlling and reducing seizure frequency and duration [19]. Neuroprotective effects of omega-3 fatty acids against epilepsy-induced hippocampal damage [20] as well as oxidative stress [21, 22] were also reported in previous studies.

Supplementation with fish oil has been shown to exert beneficial effects on cognitive performance although findings vary considerably across different studies [23, 24]. One approach to explore whether fish oil supplementation positively affects cognitive abilities is by studying the effect of fish oil in an animal model with some degree of memory impairment. Therefore, in this study, we aimed to investigate whether daily supplementation with fish oil protects against hippocampal-dependent memory impairment in rats with pilocarpine-induced epilepsy.

Methods

Animals

Eighty-nine male Wistar rats (4-5 weeks) were obtained from Experimental Models Development Center (CEDEME) of UNIFESP. Animals were maintained in a standard 12:12 h light/dark cycle (lights on at 7:00 a.m.) with free access to food and water. All experimental procedures were approved by the Ethical Committee on Animal Use of UNIFESP (CEUA – 188439) and were carried out in accordance with the Ethical and Practical Principles of the Use of Laboratory Animals.

Epilepsy induction model

Epilepsy was induced according to Turski *et al*. [25]. Briefly, in order to minimize the peripheral cholinergic effects of pilocarpine, rats were first injected with methylscopolamine (1 mg/kg, s.c - Sigma, MO, USA). After 30 minutes, *status epilepticus* (SE) was induced by a single injection of pilocarpine hydrochloride (350 mg/kg, i.p - Sigma, MO, USA), while control rats received a similar volume of sterile saline 0.9% (Day 0 of the experimental protocol) (*figure 1*). To terminate or limit behavioural seizures, diazepam (10 mg/kg – Cristalia, Compaz) was administered subcutaneously three hours after the onset of SE. Next, rats followed the natural course of the pilocarpine model of epilepsy: acute, silent and chronic periods [26].

Fish oil treatment

The rats were randomly distributed into six different groups:

- 1) control rats treated daily with vehicle (CV, *n*= 13);
- 2) control rats treated daily with 85 mg/kg fish oil (CFO85, *n*=10);
- 3) control rats treated daily with 510 mg/kg fish oil (CFO510, *n*=15);
- 4) rats with epilepsy treated daily with vehicle (EV, *n*=21);
- 5) rats with epilepsy treated daily with 85 mg/kg fish oil (EFO85, *n*=17);
- 6) and rats with epilepsy treated daily with 510 mg/kg fish oil (EFO510, *n*=13).

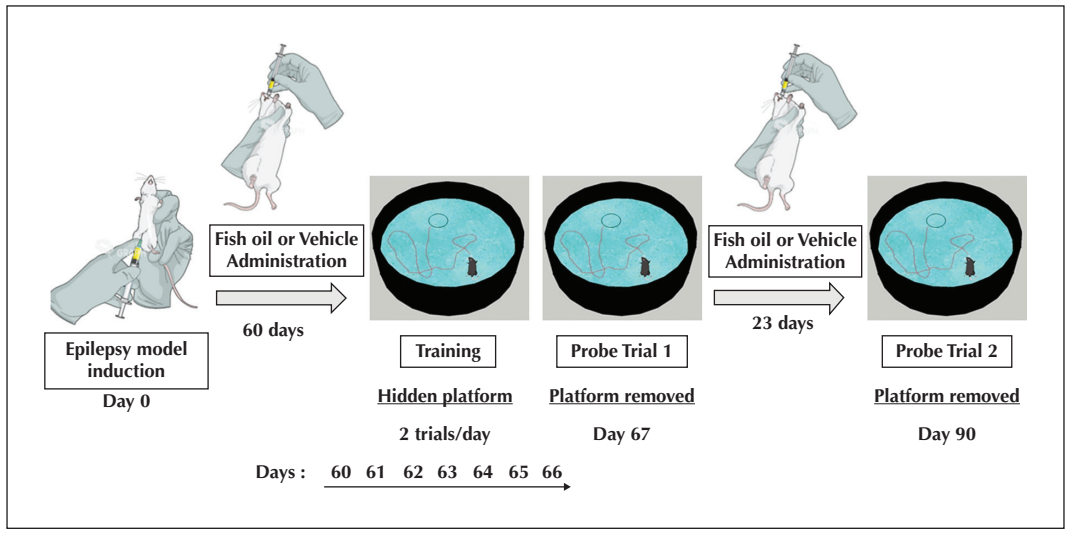
Fish oil capsules (1g; PROEPA®), which contain 120 mg/g DHA and 180 mg/g EPA, were dissolved in distilled water containing 0.009% Cremophor (Sigma ®) to produce a solution with a final concentration of 21.25 mg/mL fish oil (*i.e.*, 3.82 mg/mL EPA and 2.55 mg/mL DHA). The vehicle solution consisted of 0.009% Cremophor in distilled water and was administered at the same dose volume as treatment solution. Fish oil or vehicle administration was initiated by Day 0 of the experimental protocol (*figure 1*). Each group was treated orally by gavage for 90 consecutive days. Body weights were recorded three times weekly and individual doses were adjusted to maintain the dosing regimen of 1 mL of fish oil or vehicle solution per 250 g of body weight (*i.e.*, 4 mL/kg).

The 85 and 510-mg/kg fish oil doses that were used in this study correspond to a daily dosage of approximately one and six capsules of fish oil (each capsule contains 1 g fish oil) for a 70 kg human, respectively. The dose extrapolation from human to rat was based on allometric scaling, frequently used in experimental research [27]. The dose of 85 mg/kg fish oil was used in previous studies by our group [22, 28, 29].

Morris water maze test

Sixty days after the induction of epilepsy, the Morris water maze (MWM) task was performed. The pool consisted of a black-painted, circular tank (diameter of 200 cm, height of 50 cm) with a 9-cm diameter platform that was placed in the southeast quadrant. The platform was positioned 1 cm underneath the water surface. A variety of extra-maze visual cues was placed on testing room walls to aid navigation. During the acquisition phase (training), each animal received two trials per day for seven consecutive days. A trial consisted of an animal being carefully placed into the pool facing the wall in one of four possible starting locations: north (N), northwest (NW), west (W), and southwest (SW).

Release points were randomized in each trial. The animal was allowed to swim freely for 120 seconds or until it reached the platform where it was allowed to



■ **Figure 1.** Schematic diagram of the experimental protocol used in this study. On Day 0, rats were subjected to systemic administration of pilocarpine (or saline). Fish oil or vehicle administration was also initiated by Day 0. Starting by Day 60, experimental and control rats received hidden platform training for seven consecutive days (two trials/day). After the platform was removed from the pool, epileptic and control rats were submitted to two probe trials; Probe Trial 1 was performed 24 hours following the last day of training (*i.e.*, Day 67) and Probe Trial 2 was performed on Day 90.

rest for 10 seconds. If the animal failed to reach the platform within 120 seconds, it was gently guided to the platform by the experimenter and allowed to rest for 10 seconds. Latency, which is the time elapsed before the animal reaches the platform, was recorded by a video tracking system (Noldus, Wageningen, The Netherlands). Spatial learning was measured by daily performances, which were calculated by averaging the latencies (seconds) across the two trials for each rat each day.

On Day 8, the hidden platform was removed from the pool and the animals were subjected to a probe trial of 120 seconds. The time spent in each of the four quadrants as well as the time spent in the quadrant that previously housed the platform was measured in order to evaluate the strength and accuracy of the spatial memory. Finally, 30 days following the first day of training (*i.e.*, after 90 days of treatment), the animals were subjected to an additional probe trial. A diagram of the experimental protocol is illustrated in *figure 1*.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Statistica Academic (Statistica, version 13, TIBCO Software, Palo Alto, CA). Mean values of latency over the days of training (dependent variable) were compared by two-way repeated measures analysis of variance (RM ANOVA), with group and treatment as categorical factors. Mean values of the time spent by the animals in the area (dependent variable) where the platform was previously located as well as the time spent by the animals in each quadrant (dependent variable) were compared by two-way ANOVA, with group and treatment as categorical factors. Fisher's least significant difference (LSD) post hoc analysis was performed when group dependence was detected by ANOVA. A p value of < 0.05 was considered statistically significant. In all figures, p values of <0.05, 0.01, and 0.001 are represented as *, **, and ***, respectively (GraphPad Software, version 7, La Jolla, CA).

Results

Spatial learning was assessed by averaging escape latencies (seconds) across the two trials for each rat each day. We found that group and training day had significant effects on the mean values of latency ($F(1, 83) = 241.967$, $p < 0.0001$; and $F(6, 498) = 61.506$, $p < 0.0001$; respectively). However, no significant treatment effect was detected ($F(2, 83) = 0.384$, $p = 0.6820$). Significant interactions were observed between group and treatment ($F(2, 83) = 4.601$, $p = 0.0127$), between group and training day ($F(6, 498) = 14.866$, $p < 0.0001$), and between training day and treatment ($F(12, 498) = 2.224$, $p = 0.0098$). As expected, post hoc testing confirmed

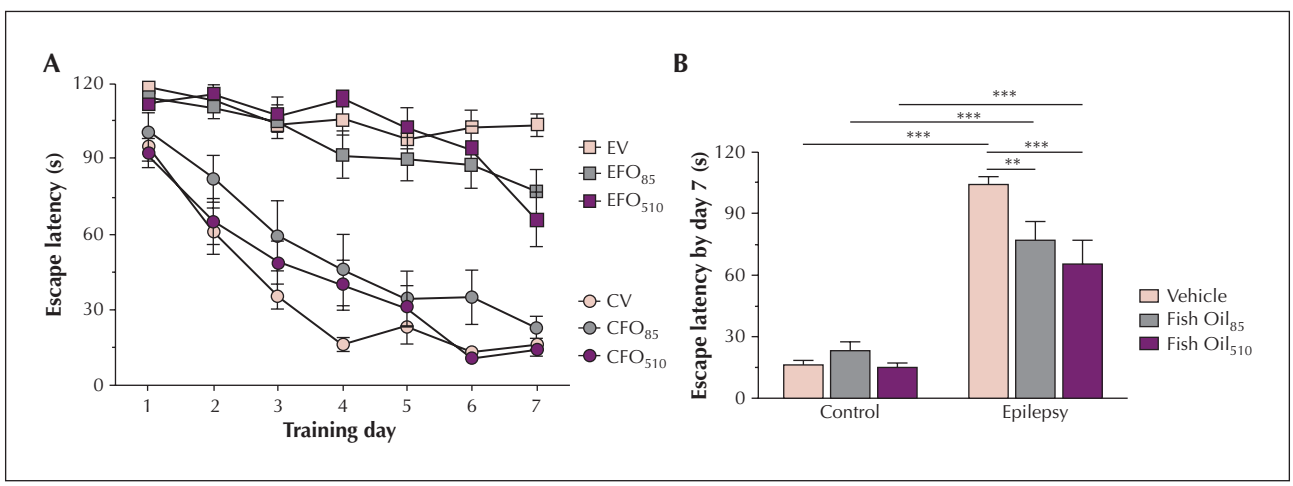
that the latency to reach the platform was significantly increased for vehicle-treated epileptic rats compared with vehicle-treated control rats through training Days 1-7 (Day 1, $p = 0.0144$; Day 2-7, $p < 0.0001$) (*figure 2A*). In addition, rats with epilepsy treated with fish oil reached the platform in a significantly shorter time on Day 7 compared with vehicle-treated experimental rats (EV *vs* EFO85, $p = 0.0029$; EV *vs* EFO510, $p < 0.0001$) (*figure 2B*).

To assess spatial memory at the end of the acquisition phase, a probe trial was administered. We found that group ($F(1, 83) = 52.545$, $p < 0.0001$), but not treatment ($F(2, 83) = 0.794$, $p = 0.4550$), had significant effects on the mean values of time spent in the target zone (*i.e.*, a virtual area centred on the former location of the platform). However, no significant interaction between group and treatment was detected ($F(2, 83) = 1.366$, $p = 0.2606$). Post hoc comparisons showed that vehicle-treated experimental rats spent a significantly shorter time in the target zone compared with vehicle-treated control rats ($p < 0.0001$) as well as fish oil-treated experimental rats compared with their fish oil-treated control peers (CFO85 *vs* EFO85, $p = 0.0055$; CFO510 *vs* EFO510, $p < 0.0001$) (*figure 3A*).

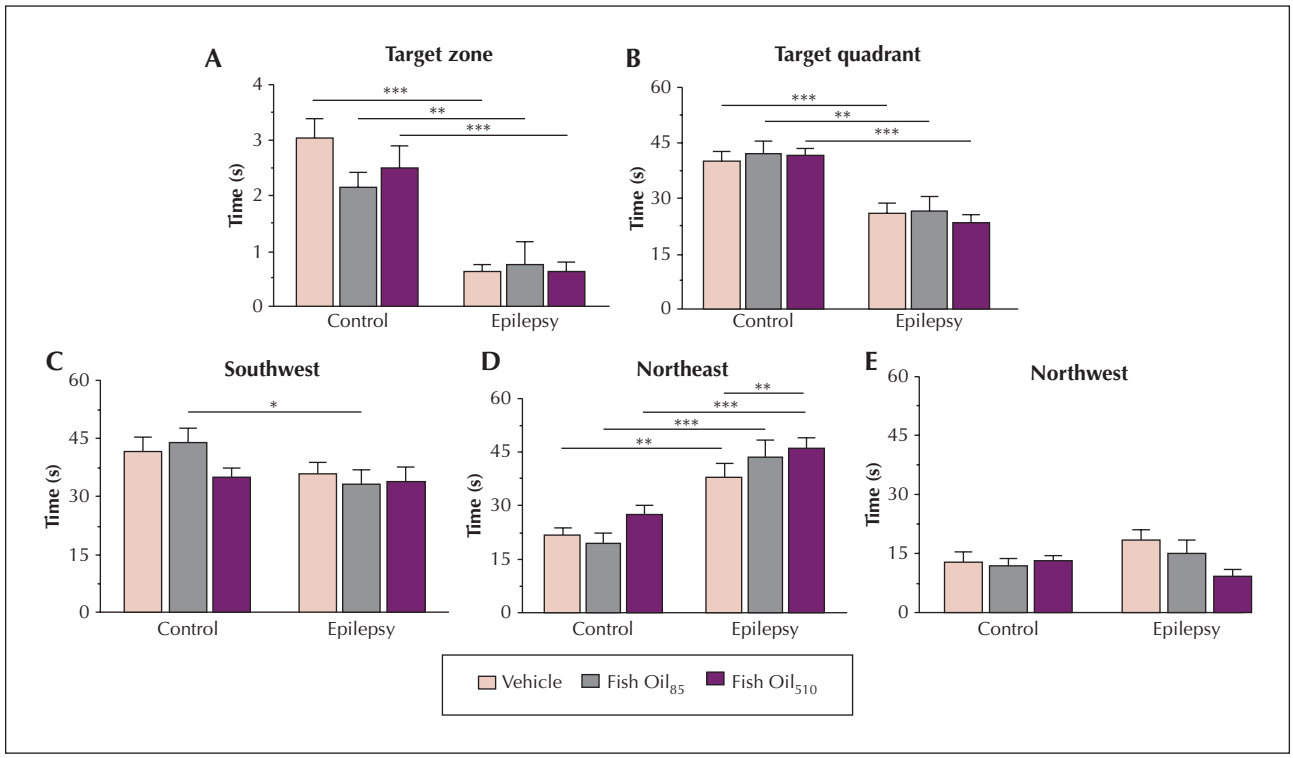
Spatial memory during the probe trial was assessed by evaluating the time spent in the target quadrant (*i.e.*, the virtual quadrant that previously housed the platform). We found that group ($F(1, 83) = 40.898$, $p < 0.0001$), but not treatment ($F(2, 83) = 0.272$, $p = 0.7624$), had significant effects on the mean values of time spent in the southeast quadrant. However, no significant interaction between group and treatment was detected ($F(2, 83) = 0.269$, $p = 0.7645$). Post hoc tests showed that vehicle-treated experimental rats spent a significantly shorter time in the target quadrant compared with vehicle-treated control rats ($p = 0.0008$) as well as fish oil-treated experimental rats compared with their fish oil-treated control peers (CFO85 *vs* EFO85, $p = 0.0010$; CFO510 *vs* EFO510, $p < 0.0001$) (*figure 3B*).

The time spent by the animals in the non-target quadrants of the pool was also evaluated. Analysis of the time spent in the SW quadrant revealed a significant dependency on group ($F(1, 83) = 4.162$, $p = 0.0445$), but not on treatment ($F(2, 83) = 1.164$, $p = 0.3172$). No significant interaction between group and treatment was detected ($F(2, 83) = 0.938$, $p = 0.3954$). Post hoc tests identified a significant difference in this measure only between rats with epilepsy and control rats treated with 85 mg/kg fish oil ($p = 0.0411$) (*figure 3C*).

Next, we quantified the time that the animals spent in the northeast (NE) quadrant. We found that both group ($F(1, 83) = 41.090$, $p < 0.0001$) and treatment $F(2, 83) = 3.356$, $p = 0.0396$) had significant effects on the mean values of this measure. However, we found no



■ **Figure 2.** Improved performance of fish oil-treated epileptic rats in escaping from the water. (A) From Day 1 to Day 7 of the acquisition phase, both vehicle- and fish oil-treated control rats, as well as fish oil-treated experimental rats, progressively learnt the location of the platform. Vehicle-treated epileptic rats, however, continuously struggled to find the hidden platform. (B) By Day 7, rats with epilepsy treated with 85 mg/kg and 510 mg/kg fish oil showed a 1.3-fold and 1.5-fold decrease in escape latency compared to vehicle-treated experimental rats, respectively. Data are presented as mean ± SEM. Statistical significance is expressed as ** and *** for $p < 0.01$ and $p < 0.001$, respectively; two-way ANOVA followed by Fisher's LSD post hoc comparisons. CV: control animals treated with vehicle; CFO85: control animals treated with 85 mg/kg fish oil; CFO510: control animals treated with 510 mg/kg fish oil; EV: animals with epilepsy treated with vehicle; EFO85: animals with epilepsy treated with 85 mg/kg fish oil; EFO510: animals with epilepsy treated with 510 mg/kg fish oil.



■ **Figure 3.** No beneficial effects of fish oil supplementation were detected on memory performance in rats with epilepsy during Probe Trial 1. Both vehicle- and fish oil-treated experimental rats spent a relatively shorter time in the target zone (A) and target quadrant (B). Such behaviour indicates that rats with epilepsy did not remember the location of the platform. The time spent by the animals in the southwest (C), northeast (D), and northwest (E) quadrants were also measured. Data are presented as mean ± SEM. Statistical significance is expressed as *, **, and *** for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; two-way ANOVA followed by Fisher's LSD post hoc comparisons.

significant interaction between group and treatment ($F(2, 83) = 0.781$, $p = 0.4612$). Post hoc tests revealed that vehicle-treated experimental rats spent a significantly longer time in the NE quadrant compared with vehicle-treated control rats ($p = 0.0045$) as well as fish oil-treated experimental rats when compared with their fish oil-treated control peers (CFO85 *vs* EFO85, $p = 0.0002$; CFO510 *vs* EFO510, $p < 0.0001$) (*figure 3D*). We also found a significant difference between vehicle-treated experimental rats and experimental rats treated with 510 mg/kg fish oil with regards to the time they spent in the NE quadrant ($p = 0.0097$) (*figure 3D*). Finally, regarding the time spent in the NW quadrant, we found that neither group ($F(1, 83) = 0.961$, $p = 0.3295$) nor treatment ($F(2, 83) = 2.222$, $p = 0.1147$) had any significant effect on the mean values of this parameter. Also, no significant interaction between group and treatment was observed ($F(2, 83) = 2.745$, $p = 0.0700$) (*figure 3E*).

Our last goal was to evaluate memory consolidation, which was assessed by submitting the animals to an additional probe trial (Day 90). Behavioural findings from Probe Trial 2 were quantitatively similar to those described above for Probe Trial 1. Therefore, for simplicity, the results are summarized in the supplementary material accompanying this report (*supplementary material; figure 1 and tables 1, 2*).

Discussion

Here, we tested the hypothesis that omega-3 fatty acid consumption in the form of fish oil supplementation improves learning and memory abilities in rats with pilocarpine-induced epilepsy. As expected, our MWM behavioural test results showed that vehicle-treated experimental rats exhibited deficits in hippocampal-dependent learning and memory both during the acquisition phase and during the probe trials. Although experimental rats treated with either dosage of fish oil learned the platform location significantly faster by Day 7 of the acquisition phase, spatial memory performance of fish oil-treated experimental

rats during both probe trials was unaffected by fish oil supplementation.

The rat model of chronic epilepsy generated by the systemic administration of pilocarpine has been validated for most neuropathological features of human TLE, including hippocampal neurodegeneration [30, 31]. In fact, neuronal loss is not only restricted to the sclerotic hippocampus, but also affects several extra-hippocampal structures [1, 2]. Because such brain structures are directly involved in the modulation of memory processes, most TLE patients experience some degree of cognitive impairment, which is also found in experimental rats [13, 32]. In agreement with previous studies [9-13], we demonstrated that vehicle-treated experimental rats display profound and long-lasting impairment in spatial navigation as suggested by their inability to accurately identify the platform location. While control rats progressively showed a decrease in escape latency, animals with epilepsy continuously struggled to find the hidden platform. The evidence of spatial navigation impairment in experimental rats is also supported by the fact that such rats spent a relatively shorter time in the target zone and quadrant during both probe trials. In addition, we found that the swimming speed was significantly increased in experimental rats (data not shown). This result is consistent with previous studies showing that rats with pilocarpine-induced epilepsy exhibit attention deficit hyperactivity disorder-like behaviours, such as increased locomotor activity and impulsivity [33, 34]. Together, these cognitive and behavioural abnormalities likely contribute to the impaired performance of epileptic rats in the MWM task.

The association between DHA deficiency and cognitive dysfunction is well established for a variety of neurological disorders [35-38]. Given the fact that omega-3 fatty acids must be mostly acquired from the diet, fish oil supplementation has been considered a potential nutraceutical strategy to ameliorate cognitive abilities or at least to prevent the progression of cognitive impairment in several animal models [39-41]. Indeed, fish oil supplementation has been demonstrated to enhance spatial learning and memory performance of rodents exposed to the MWM task [42-44]. In the acquisition phase, we found that experimental rats treated with either dosage of fish oil located the platform with a shorter latency time compared to vehicle-treated experimental animals on Day 7. Notably, no beneficial effect was apparent on memory performance of fish oil-treated experimental rats during both probe trials.

It is important to take into consideration several factors when evaluating our findings. From Day 1 to 4 of training, we observed that control rats progressively learned the task since the mean escape latency decreased in all groups (CV, CFO85, and CFO510). At Day 7, it seems that controls needed about the same amount of time to locate the platform as that in the previous day (*i.e.*, their performance was not significantly improved), which suggests that these animals apparently reached a learning plateau (although CFO510 mean escape latency decreased from Day 6 to 7, this reduction was not significant). On the other hand, rats with pilocarpine-induced epilepsy displayed a shallower latency curve than controls. While vehicle-treated experimental rats showed a slight improvement in MWM task performance over days of training, experimental groups treated with fish oil gradually decreased their mean escape latency until they outperformed the experimental group treated with vehicle on Day 7. In fact, EFO510 rats significantly reduced the length of time required for escaping from Day 6 to 7. Considering that days of training play a functional role in learning and memory processes [45], and that escape latency significantly decreased no earlier than the last training day, we hypothesize that training was interrupted prior to optimal learning levels being reached in these animals. This early interruption might have impaired memory retention in experimental rats treated with fish oil as revealed by probe trials. Therefore, long-term memory in fish oil-treated experimental rats may benefit from prolonged training during the acquisition phase.

Our present study also has some limitations. Mainly due to logistical issues (equipment and space availability), we did not perform video-EEG monitoring in rats with pilocarpine-induced epilepsy, which prevented assessment of seizure frequency in these animals (although we emphasize that animals received daily visual inspection and thus we guarantee that all experimental rats included in our study evolved to the chronic phase of the model). Similar to patients with TLE, seizure frequency is highly variable among rats with pilocarpine-induced epilepsy [46-49]. Spontaneous recurrent seizures were found to take place approximately seven days after SE in pilocarpine-treated rats [47], and their frequency was associated with duration of the latent period, in which shorter and longer latent periods were found in rats that experienced high and low numbers of seizures, respectively [46]. In addition, the impact of seizure frequency on neuropathology, behaviour, and cognitive performance is also a matter of debate. For example, Liu and colleagues found that the frequency of seizures was not significantly different between patients with and without brain volume reduction [50]. In addition, neither memory performance nor the degree of hippocampal atrophy was found to differ between patients with frequent and infrequent seizures [51]. Similar results were also reported in rats with pilocarpine-induced epilepsy, wherein hippocampal volume changes were not associated with seizure

frequency [49]. On the other hand, previous studies found an association between seizure frequency and hippocampal volume loss in patients with TLE [52, 53]. A similar association was found between seizure frequency and neuronal loss, as well as between seizure frequency and a variety of behavioural changes (*e.g.*, working memory, sensorimotor gating, and locomotion abnormalities) in animal models of TLE [54, 55]. Given the conflicting findings regarding the role of seizure frequency in the neuropathology and neuropsychology of TLE, future studies will be needed to clarify whether fish oil supplementation has a beneficial effect on the frequency of spontaneous seizures in rats with pilocarpine-induced epilepsy.

In conclusion, the present study sheds light on the possibility of beneficial effects of fish oil supplementation on cognitive performance in rats with pilocarpine-induced epilepsy. Although these findings require further confirmation, this study provides insights into the importance of considering nutraceutical strategies, either alone or in combination with other compounds, to enhance cognitive abilities in patients with TLE as well as with other neurological disorders. ■

Supplementary material.
Supplementary material accompanying the manuscript are available at www.epilepticdisorders.com.

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The authors report no conflicts of interest.

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