ONLINE SUPPLEMENTAL MATERIAL

Supplementation with oil rich in eicosapentaenoic acid, but not in docosahexaenoic acid, improves global cognitive function in healthy, young adults: results from randomized controlled trials

Authors: Michael J. Patan, David O. Kennedy, Cathrine Husberg, Svein-Olaf Hustvedt, Philip C. Calder, Julie Khan, Joanne Forster, Philippa A. Jackson

1. Supplementary Method

1.1. Treatment Delivery

Regarding optimal digestion and absorption of n-3 PUFAs, evidence is emerging to support the importance of specific formulations of the interventions themselves, so that maximal delivery of DHA and EPA to tissues is ensured (1-3). Additionally, the time at which the supplement is consumed has also been identified as important (4-6) with recent data from our own research center supporting consumption of n-3 PUFAs at night time (7). Additionally, due to the resistance of all long chain PUFA to intestinal lipase, participants were also instructed to take their capsules with a glass of water at their usual bed time, so that the fatty acids should be present in the intestines at the time of peak digestion and absorption the following morning (4, 7).

1.2. Cognitive task descriptions

1.2.1. Stimuli Presentation

Prior to the start of the Cognitive Demand Battery participants were presented with fifteen randomly selected photographic images to remember. Presentation was at a rate of 1 picture every 3 seconds, with a stimulus duration of one second. Following this, participants were presented sequentially with 15 words selected at random from a large bank of words derived from the MRC Psycholinguistic Database (8) and matched for word length, frequency,

familiarity and concreteness. Stimulus duration was one second, with an inter-stimulus duration of one second.

1.2.2. Immediate Word Recall

Immediately after the presentation of the words participants were given 60 seconds to write down as many of the 15 words that they were presented with during the stimulus presentation period. Outcomes are accuracy (% correct), errors (number).

1.2.3. Delayed Word Recall

After completing all other tasks participants were once again given 60 seconds to write down as many of the 15 words that they were presented with during the stimulus presentation period. Outcomes are accuracy (% correct), errors (number).

1.2.4. Delayed Picture Recognition

Thirty pictures, comprising the 15 pictures presented during the stimuli presentation period plus 15 distractor pictures were presented, with the participant making a yes/no response indicating whether the picture was in the original set. Outcomes are accuracy (% correct), reaction time of correct responses (msecs).

1.2.5. Delayed Word Recognition

Thirty words, comprising the 15 words presented during the stimuli presentation period plus 15 distractor words were presented, with the participant making a yes/no response indicating whether the word was in the original set. Outcomes are accuracy (% correct), reaction time of correct responses (msecs).

1.2.6. Verbal Fluency

Participants were presented with a letter on a sheet of paper (F, A or S) and were given 60 seconds to write down as many words as they could, beginning with that letter. Outcomes are total number of permitted words, with names (proper nouns) and perseverations (e.g. ask, asked, asks) discounted from the total score.

1.2.7. Simple Reaction Time

An upwards pointing arrow was displayed on the screen 50 times with a randomly varying inter-stimulus interval of between 1 and 3 seconds. Participants responded with a single button press as quickly as they could as soon as they saw the arrow appear. Outcomes are overall mean reaction time (msec).

1.2.8. Stroop Task

In this computerized version of the classic task 50 words describing one of four colors ('RED', 'YELLOW', 'GREEN', 'BLUE') were presented in different colored fonts in the center of a computer screen. The participant needed to press one of four colored response buttons in order to identify the font color (e.g. if the word 'GREEN' was presented in a blue font, the correct response would be to respond with the blue button). The presented words were either 'congruent' (word and font are the same color) or 'incongruent' (word and font are different colors) and were presented in a random order. Outcomes are reaction time of correct responses (msec), and for accuracy (% correct).

1.2.9. Numeric Working Memory (NWM)

Five random digits between 1-9 were presented sequentially. Participants are required to try and hold these five numbers in their memories. Once the five stimuli have been presented the participant is then presented with 30 "probe" digits between 1-9 (15 correct targets and 15 distractors). For each of these probe digits the participant must indicate whether or not it was one of the original five digits presented within the original series by pressing a 'yes' or 'no'

response. This procedure was then repeated three times. Outcomes are overall accuracy (% correct) and mean reaction time for correct responses (msec).

1.2.10. Task Difficulty visual analogue scale

Participants rated how difficult they found the task they had just completed by making a mark on a line representing 0-100% with the end points labelled “not at all” (left hand end; 0) and “very much so” (right hand end; 100).

1.2.11. Word List Learning & Recall

Participants were presented sequentially with 15 words selected at random from a large bank of words derived from the MRC Psycholinguistic Database and matched for word length, frequency, familiarity and concreteness. Stimulus duration was one second, with an inter-stimulus duration of one second. Following this, 30 words, comprising the 15 words presented during the stimuli presentation period plus 15 distractor words were presented, with the participant making a yes/no response indicating whether the word was in the original set or not. Task outcomes were accuracy (% correct) and reaction time for correct responses (msec). This sequence was repeated 5 times with the same 15 ‘target words’ but different 15 ‘decoy words’ every sequence to ensure maximum retention of the 15 ‘words to be remembered’.

A total displacement score was calculated as the sum of the percentage of errors on the five learning trials and a learning index was calculated as the average relative difference in performance between trials (9). This was calculated by subtracting the error percentage of word recognition during learning trial 1 (A) from the error percentage of word recognition during learning trial 2 (B) and then dividing this value by the error percentage of learning trial 1 (A). This same calculation was then made for; trial 2 (B) subtract trial 3 (C) divide error percentage of trial 2 (B); trial 3 (C) subtract trial 4 (D) divide error percentage of trial 3

(C) and trial 4 (D) subtract trial 5 (E) divide error percentage of trial 4 (D). The summed values of these calculations were then divided by 4 to generate a learning score for each participant. These calculations were completed for each of the testing visits. These calculations are visualized below:

$$\frac{\left(\frac{A-B}{A}+\frac{B-C}{B}+\frac{C-D}{C}+\frac{D-E}{D}\right)}{4}$$

Supp Figure 1. Formula used to create the learning index scores where the letters A-E indicate one of the five learning trials.

During the recall phase participants were given 60 seconds to write down as many of the 15 words that they were presented with during the learning phase the night prior to their study visit. Outcomes are number of words correct and number of errors.

1.2.12. Computerized Location Learning & Recall

Participants were shown a 5x5 grid containing 10 pictures of objects and asked to remember the location of the objects as accurately as possible. The presentation duration was 15 seconds. They were then shown an empty grid and asked to relocate the objects to the correct location shown to them previously. There was no time limit for responding. This was repeated five times during the learning phase. For each of the five learning trials, a displacement score was calculated as the sum of the errors made for each object (calculated by counting the number of cells the object had to be moved both horizontally and vertically in order to be in the correct location). A total displacement score was calculated as the sum of the displacement scores on the five learning trials. A learning index was also calculated using

the same formula outlined in **Supp Figure 1** as the average relative difference in performance between trials (9).

During the recall phase, participants were again asked to place the objects in the correct location on the empty grid as presented during the learning phase with no further prompting. The delayed trial was scored for displacement, and a delayed displacement score was then calculated as the difference between displacement score on the final learning trial and the delayed trial.

1.2.13. Digit Vigilance

A fixed number appeared on the right of the screen and a series of changing numbers appeared on the left side of the screen. Participants were required to respond when the number on the left matched the number on the right. Task outcomes were accuracy (%), reaction time to correct responses (msec) and number of false alarms. This timed task lasted for five minutes.

1.2.14. Peg & Ball

Two configurations were shown on the screen. In each there was three colored balls (blue, green, red) on one of 3 pegs. The configuration at the top of the screen was the goal configuration and participants needed to arrange the balls on the starting configuration (shown in the center of the screen) to match the position of balls in the goal configuration. They needed to do this in the least number of moves possible with difficulty increasing as the task progressed. Task outcomes were number of errors, average thinking time (msec) and speed of performance (msec). Five stimuli at each of the three levels (3, 4 and 5 moves) were completed.

1.2.15. Cognitive Demand Battery tasks

The following tasks were repeated four times in the order of: Serial three subtraction, Serial seven subtraction, Rapid Visual Information Task, 'Mental Fatigue' Visual Analogue Scale and 'Alertness' Visual Analogue Scale. Previously, this battery has been successfully used to investigate the effects of various nutritional interventions on cognitive and mental fatigue during periods of sustained cognitive processing (10-14).

1.2.15.1. Serial Threes Subtraction Task

Participants were required to count backwards in threes from a given number as quickly and as accurately as possible using the number keys to enter each response. A random starting number between 800 and 999 is presented on the computer screen, which is cleared by the entry of the first response. In the case of an incorrect response, subsequent responses are then scored as correct in relation to the previous incorrect number. The task is scored for number of total responses and the number of errors.

1.2.15.2. Serial Sevens Subtraction Task

Same task as outlined above but subtract seven instead.

1.2.15.3. Serial Seventeens Subtraction Task

Same task as outlined above but subtract seventeen instead.

1.2.15.4. Rapid Visual Information Task (RVIP)

The participant was required to monitor a continuous series of digits for targets of three consecutive odd or three consecutive even digits. The digits were presented at the rate of 100 per minute and the participant responded to the detection of a target string by pressing the response button as quickly as possible. The task was continuous and lasted for 5 minutes, with 8 correct target strings being presented each minute. Outcomes are percentage of target

strings correctly detected (% correct), average reaction time for correct detections (msec) and number of false alarms.

1.2.15.5. ‘Mental Fatigue’ Visual Analogue Scale

Participants rated their current subjective ‘mental fatigue’ state by making a mark on a line representing 0-100% with the end points labelled “not at all” (left hand end; 0) and “very much so” (right hand end; 100). Higher scores represented higher levels of mental fatigue.

1.2.15.6. ‘Alertness’ Visual Analogue Scale

Participants rated their current subjective ‘Alertness’ by making a mark on a line representing 0-100% with the end points labelled “not at all” (left hand end; 0) and “very much so” (right hand end; 100). Higher scores represented higher levels of alertness.

1.3. Profile of Mood States (POMS; (15))

The 65 item POMS provided scales of tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue-inertia and confusion-bewilderment. A total “mood disturbance” score was also computed via subtracting the vigor-activity score from the sum of tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia and confusion-bewilderment.

1.4. Procedure

All study visits took place at Northumbria University’s Brain, Performance and Nutrition Research Centre (BPNRC). Potential participants attended the site for an initial screening visit. The principal investigator or designee discussed with each participant the nature of the trial, its requirements and restrictions in line with the participant information sheet previously given to the participant. Following informed consent eligible participants underwent training on the computerized cognitive tasks. The training session followed standard operating procedures to decrease the chance of learning effects during the main trials. This entailed the

participants completing three shortened versions of the tasks to gain familiarity, followed by two full length versions of the tasks. This results in participants achieving their highest scores in one of the full-length versions of the tasks once they are completely familiar with said task. Once this session was completed to the required standard the participant was then eligible to be enrolled and randomized into the trial.

Before the baseline, week 13 (for memory consolidation subgroup) and week 26 assessments participants were asked to avoid alcohol and refrain from intake of 'over the counter' medications for 24 hours and caffeine for 18 hours. Participants were contacted to remind them of the requirements prior to each visit. On the morning of the baseline testing visit, participants were requested to eat their usual breakfast or no breakfast if they usually skipped breakfast at least 1 hour prior to arrival at the laboratory (but to avoid any caffeinated products). Adherence to this abstinence was ensured via completion of the case report form (CRF) prior to the participant completing the cognitive tasks, mood measures and blood samples. After completion of the CRF participants then completed all cognitive tasks as well as completing the POMS questionnaire which took approximately 60 minutes to complete (see **Figure 1** for schematic depicting the assessment schedules for the cognitive, NIRS and memory consolidation assessments,). Participants were then provided with the first batch of capsules (3 bottles of 100 capsules each) and given a diary in which to record their daily consumption of the capsules along with any adverse events and concomitant medications, should there be any throughout the supplementation period.

Participants also reported to BPNRC during Week 13 to collect the second batch of capsules (3 bottles of 100 capsules each). Participants also brought with them their diary, which was replaced with a new diary to complete between week 13-26 and any remaining unused treatment capsules, so that treatment compliance could be calculated. Participants within the memory consolidation subgroup completed additional assessments at week 13 (**Figure 1C**)

The week 26 assessment was identical to the baseline assessment in all aspects apart from collecting in the subject diaries, all remaining treatments, completion of a treatment guess questionnaire and finally a full debrief once all assessments were completed. During both the baseline and week 26 visits participants were also required to provide a 6 mL venous blood sample to determine blood fatty acid profile. Finally, a full debrief was given once all assessments had been completed. An outline of the baseline and week 26 study assessments is given in **Figure 1**.

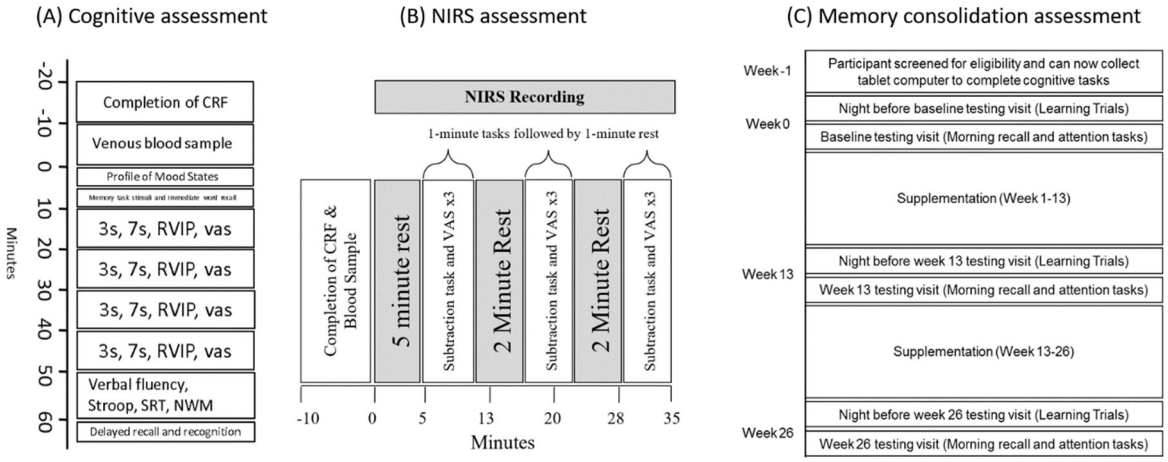

Figure 1. Schematics showing the procedures for the (A) cognitive trial assessment schedule, (B) NIRS assessment schedule (C) memory consolidation schedule. CRF, Case Report Form; 3s, Serial 3 subtractions; 7s, Serial 7 subtractions; RVIP, Rapid visual information processing; VAS, visual analogue scales; SRT, Simple reaction time; NWM, Numeric working memory.

1.5. Data Cleaning Procedures

Before each analysis was conducted, the data sets were cleaned following the same procedures. These procedures included removing anomalous results and outliers from the raw data. Box plots were generated for each outcome variable to identify potential outliers. These boxplots present five sample statistics - the minimum, the lower quartile, the median, the upper quartile and the maximum. SPSS has a two-stage flagging process. Values which are

between one and a half and three box lengths from either end are denoted by open circles and are interpreted as outliers. Values which are more than three box lengths from either end of the box are denoted by asterisks and interpreted as extreme values. Once any identified outliers had been removed, residual values were calculated and histograms produced to view the spread and distribution of the data. If any values were seen to be separate from the spread and distribution from the dataset then these values were also removed. For RT outcomes specifically, extremely low values (<0.1 msec) were highlighted as potential anticipatory responses and removed if not already flagged as outliers. Once these processes had been completed for each outcome variable the analysis commenced.

1.6. Linear mixed model descriptions

All data were analyzed with SPSS (version 25; IBM Corp) using the MIXED procedure. Full descriptions of the factors included within each model are outlined in the sections below.

1.6.1. COMPASS task models

The COMPASS task data were analyzed using the same linear mixed model procedure described previously with treatment (DHA-rich, EPA-rich, Placebo) appearing as a fixed factor in the models and respective pre-dose values were entered into each model as a covariate. Age was also added as a covariate in the model for Stroop accuracy and years spent in education was also added as a covariate for the word recognition task accuracy model.

1.6.2. Cognitive Demand Battery models

The data were analyzed using the same linear mixed model procedure described previously with all models using an identity covariance matrix. The fixed factors appearing in all models were treatment (DHA-rich, EPA-rich, Placebo) and repetition (1-4). Subject was also added into all models as a random factor and respective pre-dose values were entered into each

model as a covariate. Age was also added as a covariate in the model for serial 3 subtraction, serial 7 subtraction and VAS ratings. Years spent in education was added as a covariate in the model for serial 3 subtraction, serial 7 subtraction and RVIP.

1.6.3. Cognitive Domain Data

As the current study aimed to build upon previous findings (16) the same cognitive domains that were calculated and analyzed by the researchers previously were also analyzed within the current study wherever possible. This included measures of memory and attention. Additionally, measures of global cognition were also included representing both global speed and global accuracy to capture the overall performance on all tasks. Calculation of these cognitive domains involved transforming outcomes from the individual tasks into z scores and clustering these z scores into their respective cognitive domains. The calculations for each cognitive domain are outlined below.

1.6.3.1. Attention Domain models

The data were analyzed using the same linear mixed model procedure described previously. The only fixed factor appearing in both models was treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were also entered into both models as a covariate.

1.6.3.2. Memory Domain models

The data were analyzed using the same linear mixed model procedure described previously. The only fixed factor appearing in the models were treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were entered into each model as a covariate and age was entered as a covariate for both speed and accuracy of memory models.

1.6.3.3. Global Cognition Domain models

The data were analyzed using the same linear mixed model procedure described previously. The only fixed factor appearing in both models was treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were also entered into both models as a covariate.

1.6.4. Subjective Mood (POMS) models

The subjective mood data consisted of scores for tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue-inertia, confusion-bewilderment and total mood disturbance. The data were analyzed using the same linear mixed model procedure described previously. The only fixed factor appearing in the models was treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were also entered into the model as a covariate.

1.6.5. NIRS models

Fixed effects appearing in the resting NIRS models were treatment (DHA-rich, EPA-rich, Placebo) and hemisphere (left, right) whilst the fixed effects appearing in the active NIRS models consisted of treatment (DHA-rich, EPA-rich, Placebo), hemisphere (left, right), task (3s, 7s, 17s) and task randomization order (1 – 6). Participant was also added into all models as a random factor and respective baseline values were entered as a covariate.

1.6.6. NIRS serial subtraction models

The fixed effects appearing in both models were treatment (DHA-rich, EPA-rich, Placebo), task (3s, 7s, 17s) and task randomization order (1 – 6). Subject was also added into all models as a random factor and respective pre-dose values were entered as a covariate.

1.6.7. Subjective Task Difficulty models

The fixed effects appearing in both models were treatment (DHA-rich, EPA-rich, Placebo), task (3s, 7s, 17s) and task randomization order (1 – 6). Subject was also added into all models as a random factor and respective pre-dose values were entered as a covariate.

1.6.8. Efficiency Index models

The fixed effects appearing in both models were; treatment (DHA-rich, EPA-rich, Placebo), hemisphere (left, right), task (3s, 7s, 17s) and task randomization order (1 – 6). Subject was also added into all models as a random factor and respective pre-dose values were entered as a covariate.

1.6.9. Memory consolidation models

The fixed factors appearing in all models were treatment (DHA-rich, EPA-rich, Placebo) and visit (week 13 or week 26). Respective pre-dose values and age were entered into each model as a covariate. Subject was also entered as a random factor in the model for delayed word recall, computerized location learning, computerized location recall, simple reaction time, digit vigilance and peg & ball.

REFERENCES

1. Maki KC, Palacios OM, Buggia MA, Trivedi R, Dicklin MR, Maki CE. Effects of a self–micro-emulsifying delivery system formulation versus a standard ω-3 acid ethyl ester product on the bioavailability of eicosapentaenoic acid and docosahexaenoic acid: a study in healthy men and women in a fasted state. Clinical therapeutics 2018;40(12):2065-76.

2. Qin Y, Nyheim H, Haram EM, Moritz JM, Hustvedt SO. A novel self-micro-emulsifying delivery system (SMEDS) formulation significantly improves the fasting absorption of EPA and DHA from a single dose of an omega-3 ethyl ester concentrate. Lipids in health and disease 2017;16(1):204.

3. West AL, Kindberg GM, Hustvedt SO, Calder PC. A novel self-micro-emulsifying delivery system enhances enrichment of eicosapentaenoic acid and docosahexaenoic acid after single and repeated dosing in healthy adults in a randomized trial. The Journal of nutrition 2018;148(11):1704-15.

4. Bray MS, Young ME. Regulation of fatty acid metabolism by cell autonomous circadian clocks: time to fatten up on information? Journal of Biological Chemistry 2011;286(14):11883-9.

5. Cornélissen G, Galli C, Halberg F, De Meester F, Risé P, Wilczynska-Kwiatek A, Singh RB, Guillaume F. Circadian time structure of fatty acids and vascular monitoring. Journal of Applied Biomedicine 2010;8(2):93-109.

6. Dallmann R, Viola AU, Tarokh L, Cajochen C, Brown SA. The human circadian metabolome. Proceedings of the National Academy of Sciences 2012;109(7):2625-9.

7. Jackson PA, Husberg C, Hustvedt S-O, Calder PC, Khan J, Avery H, Forster J, Kennedy DO. Diurnal rhythm of plasma EPA and DHA in healthy adults. Prostaglandins, Leukotrienes and Essential Fatty Acids 2020:102054.

8. Coltheart M. The MRC psycholinguistic database. The Quarterly Journal of Experimental Psychology Section A 1981;33(4):497-505.

9. Kessels RP, Nys GM, Brands AM, Berg Evd, Zandvoort VMJ. The modified Location Learning Test: Norms for the assessment of spatial memory function in neuropsychological patients. Archives of Clinical Neuropsychology 2006;21(8):841-6.

10. Kennedy DO, Haskell CF, Robertson B, Reay J, Brewster-Maund C, Luedemann J, Maggini S, Ruf M, Zangara A, Scholey AB. Improved cognitive performance and mental fatigue following a multi-vitamin and mineral supplement with added guarana (Paullinia cupana). Appetite 2008;50(2-3):506-13. doi: 10.1016/j.appet.2007.10.007.

11. Kennedy DO, Scholey AB. A glucose-caffeine 'energy drink' ameliorates subjective and performance deficits during prolonged cognitive demand. Appetite 2004;42(3):331-3. doi: 10.1016/j.appet.2004.03.001.

12. Reay JL, Kennedy DO, Scholey AB. Single doses of Panax ginseng (G115) reduce blood glucose levels and improve cognitive performance during sustained mental activity. J Psychopharmacol 2005;19(4):357-65.

13. Reay JL, Kennedy DO, Scholey AB. Effects of Panax ginseng, consumed with and without glucose, on blood glucose levels and cognitive performance during sustained 'mentally demanding' tasks. Journal of Psychopharmacology 2006;20(6):771-81.

14. Scholey AB, French SJ, Morris PJ, Kennedy DO, Milne AL, Haskell CF. Consumption of cocoa flavanols results in acute improvements in mood and cognitive performance during sustained mental effort. Journal of Psychopharmacology 2010;24(10):1505-14. doi: 10.1177/0269881109106923.

15. McNair D, Lorr M, Droppleman L. POMS manual—Profile of mood questionnaire. San Diego: Edits 1992.

16. Stonehouse W, Conlon CA, Podd J, Hill SR, Minihane AM, Haskell C, Kennedy D. DHA supplementation improved both memory and reaction time in healthy young adults: a randomized controlled trial. The American Journal of Clinical Nutrition 2013;97(5):1134-43. doi: 10.3945/ajcn.112.053371.