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Study design and protocol overview

The ICONIC (Investigating Cognition and Ocular Nutrition In Children) trial is a pilot study assessing the effects of lutein, zeaxanthin, and *meso*-zeaxanthin supplementation on cognitive development and visual performance in children.

Study participants: recruitment and screening

Children in academic year groups 2, 3, and 4 (usually aged 7-11 years) are recruited from local primary schools in the Dublin area. All schools in the Greater Dublin area are invited to participate in the study due to the high enrolment rate in childhood education within the chosen age group (Marie-Helene, 2019). This class group was selected because (I) it represents the expected sensitive period during which the prefrontal cortex, responsible for higher-order cognitive functions, begins to develop; (II) this age cohort has been the focus of previous cross-sectional studies on cognitive functions and MPOD; (III) participants are required to remain in primary school settings throughout the study duration, to avoid introducing variables arising from the complex demands of secondary schools; and (IV) they are old enough to undergo fundus autofluorescence for MPOD imaging reliably. Additionally, this study employed various recruitment strategies to raise awareness; it publicised the call for volunteers through advertising campaigns aimed at the general public, engaged in educational events for local community groups and school liaison heads, utilised email lists sourced from the CERI recruitment database, and liaised with national and local teaching societies in Dublin. Parents/guardians who expressed interest in volunteering for their children received detailed information about the trial, including its purpose, intervention methods, and duration. In accordance with the study's inclusion and exclusion criteria, a structured screening questionnaire was used to assess eligibility. Flowchart of participants in the ICONIC trial is provided in **supplementary Fig. 1**.

Eligibility criteria

Participant inclusion criteria comprise:

- Healthy children aged 7-11 years at visit 1 of either sex and any race, who will not complete primary school before study completion;
- Best-corrected distance visual acuity (BCVA) of 0.2 logarithms of the minimum angle of resolution (logMAR) in both eyes at baseline visit;
- Ability to understand all study requirements and give written informed consent; from parents or guardians approved by the Ethics Committee (EC) and Institutional Review Board (IRB). Assent was sought from children before study inclusion;
- Ability to comply and attend the required study follow-up visits accompanied by a parent or legal guardian and adhere to trial protocol;
- Ability to chew and swallow the study supplements

Participant exclusion criteria include:

- Any cognitive impairment and/or learning disability; identified disability that would make testing challenging (e.g. ADHD, sensory processing disorder);
- Have previously or is currently taking dietary supplements, including multivitamins without lutein at least within one month or multivitamins with >1mg of lutein at least within six months of the baseline visit;
- Any ocular health abnormality from slit lamp examination or fundus assessments;
- Photosensitive epilepsy;
- Active or a history of chronic ocular or systemic condition (e.g. diabetes mellitus, phenolketonuria or any other paediatric metabolic disorder, moderate to severe blepharitis, allergic conjunctivitis, peripheral ulcerative keratitis, scleritis) likely to impair assessments;
- History of detached placenta;
- History of head surgery or concussion or traumatic brain injury or brain tumour;
- Been prescribed any medication affecting the pupil or accommodation;

- History of any condition which in the investigator's opinion, may put the participant at increased risk, confound study data, or interfere significantly with the participant's participation in the study.

Consent and assent procedures

Parents and participants received an information leaflet (designed in a child-appropriate format for the participant) prior to the baseline visit to ensure they were fully informed about the study. During the baseline visit, the study was explained in detail, covering the procedures, duration, potential adverse events, and the commitment required. They had the opportunity to ask questions about the study. Parental consent and child assent were obtained before any study activities commenced, in accordance with the National Consent Policy of Ireland (Document reference QPSD-D-O26-1, 2013v-16). Parents were asked to sign and date the informed consent form, while children were asked to sign or mark the assent form as appropriate. All signatures were collected in the presence of the investigator, who also signed and dated the informed consent form prior to starting the assessments. Children and/or parents of children who were unable to understand the verbal explanations or the written information were not enrolled in the study. Parents and participants were informed that they could withdraw consent at any time and without consequence. Participation in the study was entirely voluntary, and any participant who discontinued supplement intake remained enrolled for cognitive assessments at subsequent visits, although this was not mandatory. It was not anticipated that withdrawal from this study would impact the continued safety of the participant. A copy of the written informed consent was provided to the participants. Child assent was obtained within the framework of Children First: National Guidance for the Protection and Welfare of Children (DCYA, 2011)[6].

Study Procedures Description of study procedures

Summary of study assessment and timelines are provided in **supplementary Table 1**.

Demographics

At the baseline visit, all parents/ legal guardians are required to complete a demographic and health questionnaire on behalf of their child. Demographic information includes contact details (name, date of birth, contact(s), address, email, education, ethnicity, race, socio-economic status (defined by Irish state support for schools with 'Delivering Equality of opportunity In School' (DEIS) status, identifying socioeconomically disadvantaged and advantaged schools)[8], height and weight (for BMI calculation), iris colour, health history, ocular history, family medical and smoking history (history and frequency). To be eligible for the trial, all participants must satisfy all the inclusion and exclusion criteria defined.

Pertinent family and personal medical and ocular histories are recorded at baseline to ensure that all trial participants are in good general and ocular health. This was to prevent recruiting participants who are intolerant to focused bright light and allergens in the product composition of the study interventions.

Cognitive assessment using the Cambridge Neuropsychological Test Automated Battery (CANTAB)

Participants were tested in a room with ambient lighting and a quiet environment. The official CANTAB protocol is followed in the administration of all tests. The selected battery of tests includes:

- (A) Motor Screening (MOT) Task: is a training task to familiarise the participants with the touch screen. This task aids in assessing movement, comprehension, and vision.
- (B) Stop Signal Task (SST): This response suppression task is designed to assess inhibitory control. A white ring on the task screen for the SST alerts the participant, followed by the display of a visual stimulus (an arrow) within the ring after a fixed 500ms delay, pointing in a direction that is randomly assigned to be either a Go or a No-Go stimulus. Participants must quickly press one of the two buttons on the screen corresponding to the direction of the arrow (Go Stimulus) and to inhibit their response whenever an auditory signal (a beep: No-Go stimulus) is heard. The trial consists of a practice block of 16 trials, allowing participants to rehearse their responses to the Go stimulus. It incorporates an adaptive staircase to adjust the stop signal delay, enabling the task to adapt to the participant's ability and converge towards a 50% success rate. The trial includes a 1000ms inter-stimulus interval. The outcome measure will be the stop signal time, which estimates the participant's ability to successfully suppress their response 50% of the time. This indicates the time before all responses become ballistic movements, at which point participants can no longer stop their responses.

- (C) The Multitasking Test (MTT): A response suppression task is administered to assess the ability to ignore task-irrelevant stimuli and manage conflicting inputs. Each participant is presented with two virtual buttons on either side of the touchscreen. An informative cue (direction, side) and test stimulus (an arrow) positioned above either button (right or left) will indicate which button to select. Participants must press the button quickly to indicate the location of the arrow corresponding to the presented cues throughout the trial. The trial consists of 3 series of consistent tasks (single cue task) and inconsistent tasks (intermixed cues), each with a practice block to help participants become accustomed to the task. In the congruent trial, the arrow will be on the right side pointing right, while in the incongruent trial, the arrow will be on the right side pointing left. The trials are randomly allocated, comprising 50% switch trials, 25% congruent trials, and 25% incongruent trials. Outcome measures include reaction latencies, errors, incongruency, and multitasking cost (mean latency of single task blocks subtracted from mean latency during intermixed task blocks). Given the processing of conflicting information during this multitasking test, outcomes will measure indices of alerting (low-level attentional capture), orienting (mid-level spatial selective attention), and conflict (high-level selection or control).
- (D) Paired Associate Learning (PAL) Task: A spatial associative learning task is administered to assess visual memory. Each mode of the task consists of several stages, which the participant must complete in order, starting from a 4-box level to an 8-box level, with 4 attempts at any given stage. Participants will view boxes displayed on the screen that open in a randomised order, with two or more revealing a pattern. They are instructed to remember the positions of the patterns as they appear. Afterwards, the patterns shown in the boxes are then displayed in the middle of the screen, one at a time, and the participant is tasked with accurately selecting the box where the pattern was originally displayed. There will be 2 pattern practice stages, with scores generated from the subsequent stages. Outcomes are based on the number of times the participant chose the incorrect box, adjusted for the estimated number of errors made on attempts not reached, as well as the number of times the correct box was selected across all assessed stages. A lower value indicates better performance.
- (E) Reaction Time (RTI) task: Reaction time assesses processing speed independently of significant attentional or memory demands. Participants are instructed to hold down a response button located at the bottom of the screen until a yellow spot appears in one of five possible locations above the button. Participants must then release the button and press the exact location of the yellow spot as it appears on the screen before returning to the bottom button. The practice block of 10 trials will be completed first, followed by 30 trials assessed for outcome. Reaction time will be the median time (measured in milliseconds) taken for the participant to release the response button and correctly press the location where the target stimulus was presented.
- (F) Spatial Span (SSP) Task: A computerised version of the CORSI block tapping test (Milner, 1971) is administered to assess working memory. Participants view nine white boxes presented in a fixed location on the screen. The boxes briefly change colour, one by one, in a predetermined sequence, followed by a tone at the end of the sequence. Participants must remember the sequence as the boxes change colour and are asked to point to the boxes in the same order after hearing the tone. When touched, the squares light up to confirm that the device has detected the response. The task consists of two practice blocks followed by eight assessed trials. The trial begins with a two-box sequence and progresses to a nine-box sequence (one more span length) for each iteration. The outcome is the highest span length (the longest sequence) that the participant correctly recalled before the task was terminated. Total errors (the number of times an incorrect box was selected that was not next in the sequence) and total usage errors (the number of times an incorrect box was selected that was not in the presented sequence) will be assessed. The task is conducted only in the forward condition.

Statistical Considerations -sample size calculation

The primary outcome is the change in MPOV over the course of the trial. Previous studies reporting consecutive dosing derived a percentage change in MPOV of 60-65% following supplementation with lutein, zeaxanthin and meso-zeaxanthin compared to placebo[1,2]. The effect size estimate was derived from Power et al.[2] where a 12-month carotenoid intervention in adults yielded a large increase in MPOV (Cohen's $d=1.27-1.73$). To account for greater variability in paediatric populations, this effect was attenuated by 40%, resulting in a conservative adjusted effect size ($f = 0.35$ for mixed-design ANOVA). Assuming a two-tailed $\alpha = 0.05$, power $(1-\beta) = 0.80$, a correlation of 0.3 among repeated measures, 2 active arms, 5 repeated

measurements, a minimum sample size of $N = 44$. We assumed this moderate effect size (Cohen's guidelines [3]) to address the study aims and estimated that these effects would allow us to distinguish between the daily and alternate-day supplementation regimens with 22 participants per group. Anticipating an attrition rate of approximately 25% (due to potential withdrawals, spoiled data, or supplement intake discontinuation, which is commonly associated with intervention trials of this nature) (Crichton et al., 2012), we aimed to recruit at least 64 participants (32 per group). This power analysis was conducted using G*Power software [5].

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