Randomized clinical trial in children to evaluate the effect of consuming retinol-fortified sugar

Protocol (July 2025)

SUPPLEMENTARY MATERIAL (SM)

-Methods:

Double-blind, randomized controlled trial (RCT) design with parallel groups: intervention
The intervention consisted of a daily dose per child of 13 g of white sugar commonly consumed in the country (<10% of the total daily caloric value of the RIEN by age and sex), refined and fortified (**AFR**) with water-dispersible retinol palmitate (23.5 mcg ER/g) (**Fig. S1**), produced by a national sugar manufacturer (its potency was confirmed prior to the start of the intervention through independent chemical analysis and biological testing), and distributed by a city food bank (**BA**), to be included as a culinary ingredient in the daily food preparations served at various mealtimes in educational institutions for three months in 2025, following dehelmintization of the participating children and the first collection of blood samples with informed consent (**IC**) signed by the parents; furthermore, at the start of the intervention, verification was conducted to ensure the absence of clinical signs of chronic (permanent or frequent) infection and other chronic diseases that could affect vitamin A metabolism.

Packaging and Masking of Sugar

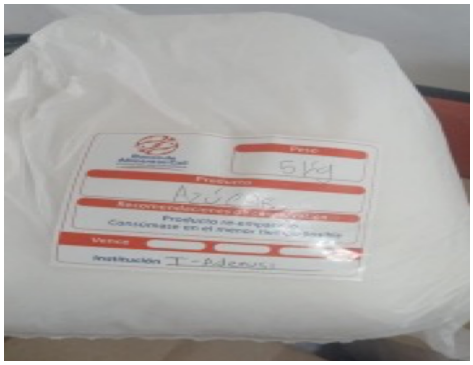
Figure S1. Packaging design and standardized quantity of sugar distributed to each child and educational institution (fortified or unfortified), and organization of distribution at the Food Bank (product blinding [label and packaging bag] and standardized spoon)



Label designed for the distribution of the two types of sugar (total of 15 kg every two weeks per educational institution, both fortified and unfortified) in educational institutions



Spoon for standardizing sugar portions in educational institutions and at home per child per day



Packaging (3 packages of 5 kg each) per school



Sugar packaging at the Food Bank

EE: Educational Institution. Photos of the research team

Participants and Recruitment

The invitation to the children's caregivers came from their teacher, who had previously been presented with the research proposal. Following the randomization of the educational institutions to assign them to each group in the RCT (control group—GCON—or intervention group—GINT)— the project presentation at each institution—which met the requirements established by the researchers—was conducted by the supervising nutritionist (**ND-S**) of the field team, who was accompanied by the nutritionist from each institution (**ND-EE**), also a member of the field team, to whom the children from each selected institutions were assigned. To encourage participation in the RCT among administrators, teachers, and primary caregivers of the children benefiting from the BA, various in-person, virtual, and telephone strategies were used: - in-person meetings led by the RCT coordinating nutritionist to address questions and concerns from administrators and teachers at institutions that provided daily meals to their students; -various videos recorded by the research team to be shared via the smartphones of the primary caregivers of all enrolled children who met the inclusion criteria; -posters, handouts, and motivational talks by the field team's nutritionists during parent or primary caregiver meetings regarding the importance of daily vitamin A intake for children's health and proper handwashing.

Prior to the signing of the informed consent form (CI) by the caregivers of the children who agreed to participate, all those interested in the project were also informed that participation in the RCT would be free of charge. The inclusion and exclusion criteria were explained, and it was clarified that the children could withdraw from the RCT at any time without any consequences or costs to themselves or the institution.

Variables, RCT Outcomes, Techniques and Procedures, Intervention, and Blinding

Anthropometric measurements: For weight, a Tanita InnerScan Dual RD-907 Body Composition scale was used, with a capacity of 180 kg and a sensitivity of 100 g; height was measured using a SECA 213 height rod, with a capacity of 200 cm and a sensitivity of 1 mm. Arm circumference (MUAC) was measured using a fiberglass measuring tape with a range of 150 cm and a resolution of 1 mm. All equipment was calibrated and provided by the University's Exercise Physiology Laboratory. Z-scores were classified according to WHO criteria.

Serum retinol (SR) and CRP: The blood sample was divided into two tubes containing sodium heparin for the determination of CRP and retinol and was stored in a refrigerator at a temperature between 4°C and 8°C during the collection process and transport to the clinical laboratory for analysis. The plasma sample was protected from light during separation and storage and transported in liquid nitrogen to the analytical laboratory, where it was processed by high-performance liquid chromatography (HPLC) using Shimadzu UFC Prominence HPLC and VP 20 chromatographs. This methodology was used to identify, separate, purify, and quantify vitamin A. During the procedure, the plasma was diluted 1:1 with a solution of retinol acetate in ethanol. This solvent precipitated the proteins, releasing the retinol, which was then extracted with hexane; the resulting extract was evaporated using nitrogen gas, and the residue was suspended in methanol. Finally, the retinol concentration was correlated with the area under the curve for retinol and with that for the acetate, which was used as an internal control.

Food intake (FI): To assess the semiquantitative frequency of consumption of supplements and foods (**FSCSA**) that are sources of vitamin A (ER) and carotenoids, a list of 52 frequently consumed foods with these characteristics was used. This list was derived from analysis using the Multiple Iterative Steps Method on the 24-hour dietary recalls (**MMPI-R24H**) and from sources consulted regarding food composition, both at the E institution and at home. The

daily schedules of each childcare center were taken into account by meal times (verified through visits to the childcare center at different times of the day), as well as reports provided by caregivers regarding FI upon returning home in the afternoon, on weekends, and during school breaks or when the children were absent. Information from the home was collected from caregivers by telephone using the list of supplements and foods, and was verified in person when caregivers attended meetings or training sessions at the EE. *To control for repetition or exhaustion bias among caregivers, the order of the foods surveyed was changed during the three data collection rounds*. At the institution, the presence of foods on the list was verified in the various meal preparations throughout the day corresponding to the date of data collection for each FSCSA.

Blinding: Two nutritionists (ND-S and ND-C) and the research statistician were the only people who knew which early institution was receiving AFR; however, none of them had personal contact with the children participating in the clinical trial. ND-S was also responsible for packaging, labeling, and delivering the usual amount of sugar allocated to each institution (3 packages of 5 kg each = 15 kg every two weeks) to the cook on a biweekly basis (Figure S1). Both nutritionists also visited all the institutions monthly, without prior notice, to observe the handling and storage of the sugar, the standardization of the daily sugar portion per child, the utensils used at each institution, and the types of preparations specified in the recipes. The primary point of contact was the cook at each institution. Neither teachers nor administrators, nor cooks, nor caregivers, nor the children participating in the RTC knew which sugar was fortified with water-dispersible retinol palmitate (STD Vitamin A CWS Sugar Premix) (Figure S1). The ND-EE monitored the standardization of the amount of sugar used in the EE and at home through visits during key meal preparation times (Figure S1).

The ND-S, in turn, served as the direct point of contact with the BA, the biweekly food supplier to the institutions; furthermore, the BA stored the AFR to protect it from sunlight and heat and for biweekly distribution to each EE and the child's home when warranted (Figure S1); during school breaks and times of social crisis that prevented the child from attending the institution, the ND-S delivered the sugar (fortified or unfortified) allocated to the child to each household. She also served as the direct point of contact with the principals of the institutions to schedule and authorize each RTC activity and to submit interim administrative progress reports to them.

The University provided each ND-EE and the ND-S with a portable scale for weighing food, a measuring spoon to standardize sugar portions at each institution and in each household, and a laptop to continuously enter data into the web application designed for the project by an engineer.

Data Analysis and Software

Various physical (paper-based) and digital methods were used to collect information at the EE and in the households. Forms were designed and printed for use during caregiver meetings at the EE and were later entered into the corresponding module of the web application developed specifically for the RCT. The analysis was a comprehensive process of data capture and preprocessing, in which the data were downloaded directly from the web application's structured databases and subjected to cleaning and standardization protocols by the statistician. At this stage, multi-source databases were integrated (based on the child's unique, anonymized ID), where each entry in the food consumption database, for example, was standardized to ensure consistency in the reported portion sizes and the chemical composition of the food; Furthermore, micronutrient levels were standardized, allowing subsequent statistical analysis to identify potential variations directly attributable to the AFR intervention. This preemptive validation process was essential for mitigating measurement errors and biases and ensuring that statistical deviations in intake reflected the AFR intervention rather than inconsistencies in the collection or conversion of food content units (portions) to nutrients and calories.

According to the WHO, the Z-scores of the anthropometric indicators used in this RCT allowed for the normalization of biological growth against a standard reference population and eliminated variance due to age and sex. Furthermore, in clinical nutritional practice, parameters such as nutritional biomarkers often follow log-normal distributions due to mechanisms of absorption and biological satiety; therefore, limiting the analysis to conventional measures of central tendency could distort the results if extreme values were present. On the other hand, in multivariate nutritional studies, it is common for a single intervention to generate simultaneous changes in multiple parameters; while this generates a large amount of useful data, it also increases the statistical probability of finding significant results by chance when multiple independent comparisons are made between subgroups or types of nutrients. To minimize the risk of false positives (Type I errors), a strict control system was implemented for the multiple comparisons inherent in this RCT. To mitigate this phenomenon, all resulting p-values were corrected using the Benjamini-Hochberg method to control the false discovery rate. Unlike the more conservative and restrictive Bonferroni adjustment, the Benjamini-Hochberg approach is preferred in RCTs because it offers a better balance between Type I error control and statistical power, as

it more optimally controls the false discovery rate.

Ethical Considerations and Oversight for Data Quality Control

The information obtained during the study will be stored by the principal investigator at the University (sponsor) in Excel databases for at least three years and will remain in the web application for as long as the research remains active. To prevent data manipulation, each ND-EE, who was responsible for each EE, could access the data from the various modules of the web application corresponding to their records assigned by the engineer using their password, and only with their authorization or upon request from the ND-S to perform quality control on the entered data, as well as by the ND-C. No member of the field team could access data on children from other EEs, since each ND-EE had a different password assigned by the engineer to access that data. Upon completion of each scheduled data collection session for a specific module of the application (e.g., Health), the engineer would lock it to prevent any subsequent manipulation of the data by the field team. After data entry, the statistician, together with the engineer, verified the data's quality and plausibility and removed from the databases any child who voluntarily withdrew from the project in order to update the databases for each module. With this information, the ND-C held virtual meetings with the field team to review and correct errors based on the original records in the field team's paper forms. If doubts persisted, the ND-EE were required to verify the information with the caregivers and cooks at the EEs. These supervision meetings were held every two weeks and led by the field team supervisor.