Enhancement of cell viability after treatment with polyunsaturated fatty acids

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HIGHLIGHTS

- Omega-3/-6 fatty acids were postulated to contribute to the development of the infant brain.
- An imbalance of omega-3/-6 fatty acids might provoke risk to develop ADHD.
- We investigated the effects of omega-3/-6 fatty acids on cell viability.
- Measurement and analysis was done *via* real time impedance measurement.
- Special ratio of omega-3/-6 fatty acids enhanced cell viability already at low doses (100 pM).

ARTICLE INFO

ABSTRACT

Attention-deficit/hyperactivity disorder (ADHD) is highly prevalent in children and adolescents and both environmental and genetic factors play major roles. Polyunsaturated fatty acids (PUFAs) are postulated to contribute to the development of the infant brain and an imbalance in these may increase the risk of ADHD. In recent clinical studies, supplementation with PUFAs improved symptoms of ADHD in some cases. Similarly, some beneficial effects were observed with PUFA treatment in neuronal cell cultures. Therefore, in this study, we hypothesized that a specific PUFA combination (available on the market as Equazen[TM] [Vifor Pharma, Switzerland]) along with iron, zinc, or vitamin B5 (vitB5) would produce an additive beneficial effect on the viability of rat pheochromocytoma-12 dopaminergic cells. The specific PUFA combination alone, as well as added to each of the three nutrients, was tested in a dose–response manner. The specific PUFAs significantly improved cell viability, starting at very low doses (100 pM) from 60 h up to 90 h; while the combined treatment with vitB5 and minerals did not provide additional benefit. Our results confirmed the beneficial effect of the specific PUFAs on neuronal cell viability; although supplementation with minerals and vitB5 did not enhance this effect.

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1. Introduction

Dietary polyunsaturated fatty acids (PUFAs), such as omega-3/-6 fatty acids, are not only involved in the development and maturation of neuronal structures, but are essential throughout the entire lifespan for maintaining many normal functions of the brain system [1]. Omega-3/-6 fatty acids exert a broad spectrum of effects, including: acting as facilitators for serotonin, dopamine, and norepinephrine release from neurons; gene transcription regulators; and precursors to pro- and anti-inflammatory chemical components [2]. Converging evidence indicates that PUFA deficiencies or imbalances may also contribute to a range of adult psychiatric and neurologic disorders and to several common and overlapping childhood neurodevelopmental disorders, including dyslexia (specific reading difficulties), dyspraxia, autistic spectrum disorders, and attention-deficit/hyperactivity disorder (ADHD) [3]. ADHD is a common, often life-long, treatable childhood psychiatric disorder, characterized by a pattern of developmentally inappropriate inattention, motor restlessness, and impulsivity [4]. Children diagnosed with ADHD often experience co-morbid symptoms such as excessive thirst, dry skin, brittle nails, dandruff, eczema, and/or allergies, which are also suggestive of a deficiency in PUFAs, especially omega-3 fatty acids [5–8]. Since there is some

evidence that PUFAs can improve neurodevelopmental outcomes, dietary supplementation with omega-3 fatty acids may offer an efficacious treatment for ADHD that addresses not only associated symptoms, but also an underlying causative factor [9].

This study aimed to investigate the effect on cell viability of the specific combination of PUFAs contained in Equazen™ (Vifor Pharma, Switzerland), which is a mixture of omega-3/-6 fatty acids in a 9:3:1 ratio (the omega-3 fatty acids, eicosapentaenoic acid [EPA; 93 mg] and docosahexaenoic acid [DHA; 29 mg], and the omega-6 fatty acid, gamma-linolenic acid [GLA; 10 mg]). For this *in vitro* approach, we selected rat pheochromocytoma (PC12) cells, a clonal cell model for cell replication and differentiation, which grow rapidly and have been widely used as an *in vitro* experimental model to study the effects of various neuroactive compounds. In addition, PC12 cells also contain a number of membrane-bound and cytosolic neuron-associated macromolecules [10], as well as mimicking many features of central dopaminergic neurons, including dopamine production.

All nutrients required by the body interact with each other functionally and metabolically (reviewed in [11]); thus, supplementation with PUFAs alone might not yield maximum benefits if other nutrients are lacking. Indeed, effective PUFA metabolism relies on other nutrients such as vitamins C, B3, and B5, and the minerals zinc (Zn) and magnesium [12]. Clinically, many children with ADHD also have nutritional deficiencies, particularly in Zn [13] and iron (Fe) [14], which could play a crucial role in the effectiveness of PUFAs in this population; therefore, a better understanding of the mechanistic of nutritional supplementation is important. As such, a secondary aim of our study was to investigate any accumulating effects derived from supplementation of PUFAs with micronutrients, namely the minerals Zn, Fe and vitamin B5 (vitB5).

2. Materials and methods

2.1. Cell culture

The PC12 cells used in this study were a generous gift from the laboratory of Dr. Silvia Mandel, Technion Faculty of Medicine, Haifa, Israel (originating from ATCC). Cells were grown in buffered Dulbecco's modified Eagle medium (DMEM) with 4.5 mg/ml glucose (Pan Biotech GmbH, Germany), and supplemented with 10% fetal bovine serum (FBS), 5% horse serum, and 0.3% gentamycin (50 mg/ml) (all from Life Technologies, Switzerland) in a humidified incubator (5% CO_2) at 37 °C.

2.2. Stock solutions for each component

The contents of one Equazen™ capsule (containing 93 mg EPA, 29 mg DHA, and 10 mg GLA) were dissolved in ethanol (9.9 μM stock solution). Ammonium Fe (III) citrate (Sigma–Aldrich, Switzerland, #O9713; 10 μM stock solution) and calcium pantothenate (vitB5; Sigma–Aldrich, Switzerland, #P 5155; 10 μM stock solution) were each dissolved in water; Zn citrate (Sigma–Aldrich, Switzerland, #96494; 10 μM stock solution) was dissolved in warmed water.

2.3. xCELLigence analysis for cell viability

Cell viability was monitored noninvasively, in real-time and label-free, using the xCELLigence Real-Time Cell Analyzer (RTCA; Roche Applied Science) for up to 90 h. Recent publications have confirmed the utility of the xCELLigence system for monitoring viability and cytotoxicity in cell lines [15,16]. For our experiments, the PC12 cell culture medium was changed to be reduced medium for testing viability with different concentrations (10 pM–5 μM) of Zn, vitB5, Fe (III), and the specific PUFA combination (Equazen™). Reduced medium contained: DMEM 4.5 mg/ml glucose (basic medium), 1% FBS, and 150 μM fatty acid-free bovine serum albumin (BSA). Cells were grown for approximately 1 week in the PC12 culture medium containing antibiotics and afterwards diluted to 5×10^5 cells/ml for seeding into a 96-well E-Plate (Roche, Switzerland). After seeding, the cells were treated with different dilutions of Zn, vitB5, Fe (III), and/or PUFAs; each 96-well plate contained four replicates of each concentration. Measurements started immediately after seeding and lasted for about 90 h (4 days) to observe growth patterns of the cells. The RTCA SP xCELLigence software was used for evaluations. All four supplements were tested in at least three independent experiments.

2.4. Statistical analysis using StatView and MATLAB® software

Raw data from the xCELLigence system, presented as cell index (CI) values, were used for statistical analysis by the software program StatView 5.0 (SAS Institute Inc. Cary, NC, USA). The effects of different doses of Zn, vitB5, and Fe (III) compared with PUFA treatment alone at the time-point 60–61 h were assessed using analysis of variance (ANOVA) with a significance level of $p < 0.05$. The demonstrated values were calculated relative to control levels (100%; control was ethanol for Equazen™ and Zn, or water for Fe and vitB5). The first significant time-point of Equazen™-treated cells in comparison with untreated cells was analyzed using a self-written MATLAB® program. For this, the CI data were exported from the xCELLigence software to an Excel file, in which the data format was adapted and saved as a comma separated value file that could be imported into MATLAB® and analyzed. For each measured time-point, outliers were detected and removed. Outliers were defined accordingly: a CI value in each group of replicates that was more than two standard deviations away from the remaining values and was also more than one standard deviation away from the overall plate mean at the same time-point. Comparisons were carried out for each measurement time-point by calculating the mean, standard deviation, and the number of wells used to calculate these two values for the two treatment groups (e.g. controls *versus* PUFAs). Welch's *t*-test was applied and the *p*-value was plotted semi-logarithmically, with an inverted axis against the time scale; thus, the more significant/smaller the *p*-value, the higher the peak on the plot. This allowed time-points where CIs were significantly different between samples and the control to be identified easily; *p*-values <0.01 were considered significant (Supplementary Fig. S1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.11.023.

3. Results

The effect of 10 pM to 5 μM Zn, vitB5, Fe (III), and/or the specific PUFA combination on the viability of PC12 cells was continuously observed for 90 h using the xCELLigence RTCA technique. Incubation of PC12 cells with 100 pM of PUFAs significantly improved cell viability (Fig. 1), which was detectable after 60 h and continued until the end of the observation period (90 h; Supplementary Fig. S1). The significant enhancement of cell viability was noted with up to, but not including, 3 μM of the specific PUFA combination. Compared with untreated cells, we did not detect any significant changes after 60 h in the viability of PC12 cells treated with vitamins or minerals alone (Fig. 1). The administration of all components in a high (100 pM PUFAs, 100 μM vitB5, 500 nM Fe (III), and 1 μM Zn) or low (10 pM PUFAs, 50 μM vitB5, 100 nM Fe (III), and 100 nM Zn) concentration range did not provide significant