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Sub-chronic (13-week) oral toxicity study, preceded by an in utero exposure phase and genotoxicity studies with fish source phosphatidylserine in rats

Y. Lifshitz*, L. Levi, I. Eyal, T. Cohen, S. Tessler

Nutrition R&D, Enzymotec, Migdal HaEmeq, Israel

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ABSTRACT

The safety of fish phosphatidylserine (PS) conjugated to DHA (InCog™) was examined in a series of toxicology studies as first step to support future use in infants and general population using in vitro genotoxicity tests and in a sub-chronic toxicity study with an in-utero exposure phase. PS is a major lipid in the cell membrane, active in various membrane-mediated processes. PS-DHA, present in human milk, has been suggested to be important for early brain development. Rats were exposed to diets containing 1.5%, 3% or 4.5% InCog or two control diets. Parental (F_0) animals were fed throughout mating, gestation and lactation. Subsequently, a subchronic, 13-week study was conducted on the F_1 animals followed by 4 weeks of recovery.

The genotoxicity tests showed no mutagenicity potential. No significant toxicological findings were found in the F_0 rats or the F_1 pups. In the 13-weeks study, an increase in the presence of renal minimal-mild multifocal corticomedullary mineralization was noted in nine females of the high-dose group. This change was not associated with any inflammatory or degenerative changes in the kidneys. The no-observed-adverse-effect level (NOAEL) in the present study was placed at 3% in the diet (mid-dose group), equivalent to an overall intake of at least 2.1 g InCog/kg bw/day in the F_1 generation.

1. Introduction

Nutrition during early infancy is critical in supporting both optimal physical and mental development. Approximately 60% of the total energy intake of an infant during the first year is utilized by the brain, and much of this energy is used to synthesize neuronal membranes. Milk fat is a critical component of the dietary energy supplied to healthy breastfed infants, providing between 45% and 55% of total energy. The content of milk fat in human milk changes with the stages of lactation, time of day and within feedings (Koletzko et al., 2011). Human milk lipids are composed of 98% triglycerides, small amounts of mono- and diacylglycerols, non-esterified FA, cholesterol and phospholipids (PLs).

PLs contain hydrophobic tails and a hydrophilic head, enabling them to form lipid bilayers and become the main lipid building blocks of the cell membrane. The different phospholipid structures include phosphatidic acid, phosphatidylethanolamine (PE), phosphatidylcholine (lecithin; PC), phosphatidylserine (PS) and phosphoinositides (PI, PIP, PIP2 and PIP3) (Parletta et al., 2013). PS is primarily located in the inner leaflet of the cell membranes, where it is involved in a number of cellular activities related to enzymatic functions and signal transduction processes. Furthermore, the negative surface charge of the inner leaflet of the plasma membrane, contributed in part by the negatively charged PS, determines the targeting of proteins containing polycationic motifs. While in most tissues PS is not a major constituent of cell membranes, human brain and other neuronal cell membranes are highly enriched with PS. Nerve cells, in particular, depend on healthy membrane function for normal neuro-transmitter metabolism and neuronal signal transmission. The PS levels in these tissues ensure the membrane structure and proper function. Furthermore, maintaining brain PS levels has been associated with normal and efficient signal transduction processes, efficient glucose consumption, and other biological pathways that are crucial to ensuring normal and healthy cognitive and mental functions (McDaniel et al., 2003; Mozzi et al., 2003; Pepeu et al., 1996; Vance and Steenbergen, 2005).

* Corresponding author. P.O. Box 6, Migdal HaEmeq 2310001, Israel.
E-mail address: yaell@enzymotec.com (Y. Lifshitz).

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Cell membranes are made up of a PL bilayer with spatial arrangements that are dependent on the fatty acids associated with the PLs.

In the brain, the most abundant unsaturated fatty acids conjugated to PLs are docosahexaenoic acid (DHA, 22:6n-3), arachidonic acid (AA, 20:4n-6) and oleic acid (18:1n-9). Neuronal survival appears to be linked to the amount of PS-DHA in the brain. In human brains, a high priority is placed on ensuring that the levels of PS-DHA are maintained (Parletta et al., 2013). Several publications related to PS-DHA absorption, transport and metabolic fate have supported the rationale behind its important role. It has previously been shown in animal models that long chain polyunsaturated fatty acids (LCPUFA) conjugated to phospholipids is delivered more efficiently to the developing brain cortex and other neuronal tissues than LCPUFA conjugated to Triacylglycerol (TG) (Thies et al., 1992, 1994; Wijendran et al., 2002). A recent report proposed that fish PS modified to contain DHA in the sn-1 position may be the most effective form for DHA delivery to the brain (Takahashi and Inoue, 2012). In a recent study (Vaisman and Pelled, 2009), the effect of different carriers on the delivery of omega-3 LCPUFAs to middle-aged rats was examined. A diet supplemented with PS-omega-3 FAs, containing mainly DHA and Eicosapentaenoic acid (EPA), was compared to a control unsupplemented diet, as well as to diets containing soybean-derived PS (soy PS), fish oil (containing TG-DHA) or a mixture of fish oil with soy-PS. The only significant increase (approximately 40%) in the brain DHA levels over the control diet group was observed following PS-omega-3 consumption. This increase exceeded the levels achieved by providing similar amounts of dietary DHA with or without soy-PS and was higher than the increase recently reported for TG-DHA supplementation in rats (Barcelo-Coblijn et al., 2003). Moreover, although PS-DHA was found to significantly increase the DHA concentrations in the cerebral cortex compared with the control group, there were no differences between the groups in the other eight fatty acids examined. This fact emphasizes the specific importance of the PS-DHA conjugate compared to other phospholipid entities.

DHA concentrations in the central nervous system accumulate during late gestation and fetal development through early childhood and are affected by the diet during early development (Green and Yavin, 1996; Makrides et al., 1994; Yavin et al., 2001). The effects of LCPUFA status and dietary intake (particularly DHA) have been increasingly evaluated through the use of measures of early cognitive development (Cheatham et al., 2006; Colombo et al., 2013). DHA was demonstrated to be safe for use by infants and adults (Lien, 2009; Lucas et al., 1999) and is recommended by EFSA as a nutrient that should be added to all infant formulas (EFSA NDA Panel (EFSA Panel on Dietetic Products, 2014)).

Several studies have reported the levels of PLs and more specifically of DHA conjugated to PLs in mature human milk and have shown a wide range of concentrations (0.8–4.34% w/w of total fatty acid on PLs) (Clark and Hundrieser, 1993; Garcia et al., 2011; Sala-Vila et al., 2005; Zou et al., 2012). The available published data describe the fatty acid composition of the PL fractions well, but are limited regarding the specific levels of PS-DHA. Nonetheless, the data show that DHA conjugated to PS is available in human milk throughout lactation (Bitman et al., 1984; Morrison and Smith, 1967; Wang et al., 2000). Present in human milk and instrumental to early brain development, PS-DHA appears to be an essential ingredient for early nutrition. Its supplementation in infant formula at levels equivalent to that of human milk might be beneficial.

InCog™ is the trade name for PS conjugated to DHA from fish sources for human nutrition in general and for infant (i.e., infant formula) and child nutrition more specifically. As a first step to support safety evaluation of PS-DHA intended for use for infants' nutrition, the potential genotoxicity of InCog was tested in a bacterial reverse mutation assay and a micronucleus assay in cultured human lymphocytes. In addition, we conducted a toxicity study including an in utero exposure phase in which the parental animals F_0 received InCog in their diet throughout mating, gestation and lactation until weaning of the F1 rats. Following that phase, a sub-chronic study was conducted with the F_1 rats, who received InCog in their diet for 13 weeks.

2. Materials and methods

2.1. Materials

InCog is a phosphatidylserine derived from fish phospholipids manufactured by Enzymotec Ltd. The primary component of the unique composition consists of a glycerophosphate skeleton conjugated to 2 fatty acids and to L-serine *via* a phosphodiester linkage. The test ingredient used in the current study comprised 81% phospholipids of which phosphatidylserine comprised 49%. The fatty acid profile reflects the fish lecithin source, comprising approximately 15% DHA and 7.5% EPA. The InCog specifications are detailed in Table 1.

The reference control diet in the subchronic toxicity study was composed of a mixture of two oils. The first was soy lecithin (SOLEC™FS-B) containing 67% w/w total PL, with 30% of the total PL being phosphatidylcholine (PC). By comparison, InCog contains 80% w/w PL, and 54% of the total PL was PS. The second oil was DHA algal oil extracted from a marine microalgal source (DHASCO™). Both oils are approved for use in infant formulas.

2.2. Genotoxicity

2.2.1. Bacterial reverse mutation assay

InCog was examined for its possible mutagenic activity in the bacterial reverse mutation test using the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 and the tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*, in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix). The study was conducted in accordance to the OECD guideline for Testing of Chemicals no. 471, Genetic Toxicology: Bacterial Reverse Mutation Test, Paris, 21 July 1997. The assay has been described in detail by Ames et al. and by Maron and Ames (Ames et al., 1975; Maron and Ames, 1983).

Ethanol was used as vehicle for the test substance. Because the test substance could not be dissolved in an appropriate solvent,

Table 1
Phospholipid and fatty acid composition of InCog mixed into the RM3 diets.

	% by weight (g/100 g)
Phosphatidylserine (PS, % of total phospholipids)	43.6
Total phospholipids	81.4
Fatty acid	
Caprylic acid (C8:0)	0.5
Myristic acid (C14:0)	0.8
Palmitic acid (C16:0)	11.4
Palmitoleic acid (C16:1 n-7)	1.4
Stearic acid (C18:0)	2.6
Oleic acid (C18:1 n-9)	3.5
Vaccenic acid (C18:1:n11)	1.1
Linoleic acid (C18:2 n-6)	1.1
Octadecatetraenoic acid (C18:4n3)	0.5
Eicosenoic acid (C20:1n9)	0.4
Arachidonic acid (C20:4 n-6)	0.5
Eicosapentaenoic acid (C20:5 n-3; EPA)	7.5
Docosahexanoic acid (C22:6 n-3; DHA)	15.0
Nervonic acid (C24:1n9)	0.5