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Influence of co-administration of oral insulin and docosahexaenoic acid in mice

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Abstract

Insulin and docosahexaenoic acid are both present in human milk. The aim of this study was to examine the effect of co-administration of oral insulin and DHA in mice. Immediately after weaning, Balb C mice were divided into four groups of seven mice each for a period of 4 weeks. Group 1 received a chow diet only. Group 2 received a chow diet and also was given human insulin (1 unit/mL of drinking water) without docosahexaenoic acid. Group 3 received a chow diet supplemented with docosahexaenoic acid (500 mg/kg/day in the chow) and no insulin. Group 4 received a chow diet and supplementation with both human insulin and docosahexaenoic acid. At 28 days, fasting blood levels of glucose, insulin, lipids, lipid peroxidation analysis, docosahexaenoic acid plasma levels, and docosahexaenoic acid content in red blood cells were determined. We found that glucose levels were lower in the group that was supplemented with insulin only (group 2, 61.4 mg/dL ± 2.8,mean ± SD) and in the group that was supplemented with DHA only (group 3, 61.1 mg/dL ± 2.0) compared to controls (group 1, 71 mg/dL ± 6.9, $P < 0.0001$). Supplementation of both insulin and docosahexaenoic acid (group 4) resulted in significantly lower glucose levels (56.4 mg/dL ± 2.6) compared to those in groups 2 and 3 ($P < 0.01$). No significant differences were found in lipid profile or lipid peroxidation between the groups. We conclude that adding insulin or docosahexaenoic acid to the diet of weaned Balb C mice reduces glucose blood levels. Supplementation with both substances has a synergistic effect. The presence of insulin and docosahexaenoic acid in human milk may be the cause for reduced glucose levels in breast-fed infants, in addition to the known effects of DHA on insulin sensitivity.

Keywords: Oral insulin; Docosahexaenoic acid; Human milk

1. Introduction

Insulin is present in human breast milk but is absent from infant formula [1]. Insulin can interact with the intestinal mucosa, both systemically and when given orally, since insulin receptors are found both on the apical and basolateral membranes [2]. The effects of oral insulin on gut maturation and mucosal enzyme expression have been demonstrated in numerous animal models [3–6]. In addition, the importance of systemic insulin deprivation for intestinal integrity has been demonstrated in humans [7].

Insulin is a macromolecule with poor bioavailability (<1%) on oral administration [8,9]. This has been attributed to the extensive hydrolysis of the protein by proteolytic enzymes within the gastrointestinal tract and to poor membrane permeability because of high molecular weight [9]. To overcome problems of absorption, the use of protease inhibitors and enhancers has been suggested. Enhancers can augment the intestinal absorption by reacting with the cell membrane of the intestinal wall [10,11].

Essential long chain polyunsaturated fatty acids (LCPUFA) such as eicosapentaenoic acid (20:5 ω3, EPA)

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and docosahexaenoic acid (20:6 ω3, DHA) are present in breast milk, and are found in high levels in brain lipids and retinal photoreceptors' cell phospholipids. Both EPA and DHA strongly enhance insulin absorption and induce hypoglycemia after colonic and rectal administration [12,13]. Since this class of enhancers has the advantage of being endogenous compounds present in human skin lipids and bio-membranes, the proposed mechanism is by altering-membrane permeability and by increasing the motional freedom or fluidity of membrane phospholipids [12–14]. Breast-feeding increases LCPUFA levels in skeletal muscle membranes and is associated with lower fasting plasma glucose levels [14,15].

We recently investigated the influence of insulin supplementation of drinking water in Balb C mice [16] and apo E knock-out mice [17], and found that insulin supplementation caused a significant reduction in fasting glucose serum levels. Therefore, the lower serum glucose levels observed during breastfeeding, may be due to increased insulin sensitivity induced by DHA [14], increased insulin absorption, or a combination of both.

Since DHA and insulin are present in human milk, we investigated 1) the effect of DHA on insulin absorption, and 2) the influence of the co-administration of oral insulin and DHA on serum glucose levels.

2. Methods and materials

After weaning, 28 Balb C mice were divided into four groups of seven mice each. Group 1 (ins−, DHA−) received regular drinking water and was fed a chow diet. Group 2 (ins+, DHA−) received a chow diet and supplementation with human insulin (Humulin R, Eli Lilly, Indianapolis, IN; 1 unit/mL of drinking water). Group 3 (ins−, DHA+) received a chow diet containing 500 mg/kg/day of DHA (DHA-supplemented chow from Materna Laboratories, Kibbutz Maabarot, Israel) and regular water with no insulin. Group 4 (INS, DHA + Group 4 (INS+, DHA+) received a chow diet containing 500 mg/kg/day of DHA and supplementation with human insulin (1 unit/mL of drinking water). The study conformed to the "Guide for the Care and Use of Laboratory Animals" published in the United States by the National Institutes of Health.

Weight was determined at baseline and once per week. After 28 days the mice were sacrificed, and fasting serum levels of lipids, insulin, glucose, lipid peroxidation, and red blood cell (RBC) fatty acid content were determined.

Blood glucose was determined using a glucometer (Elite, Kyoto Daiichi Kagaku Co., Kyoto, Japan) and an enzymatic kit (Roche Diagnostics, Mannheim, Germany). Serum insulin concentrations were determined by radioimmunoassay with a commercial kit (Bio Data, Sorin, Italy).

Serum total cholesterol (TC), high density lipoprotein cholesterol (HDL), and triacylglycerol (TG) were measured by enzymatic spectrophotometric determination [18,19]. Direct measurement of HDL was performed using polyethylene glycol-modified enzymes as previously described [20].

2.1. Serum lipid peroxidation

To determine serum lipid peroxidation, serum samples were incubated for 2 hours in the absence or presence of 100 mmol/L of 2,2′-Azobis 2-amidinopropane hydrochloride (AAPH, Wako Chemical Industries Ltd, Japan) for 2 hours at 37°C. AAPH is a water-soluble azo compound that thermally decomposes to produce peroxyl radicals at a constant rate. Plasma lipid peroxide content was determined using the thiobarbituric reactive substance assay [21].

2.2. Measurement of polyunsaturated fatty acid in red blood cells

After an overnight fast, venous blood was obtained into EDTA. After centrifugation for 10 minutes at 500 × *g*, the plasma was removed and the red blood cells will be washed with isotonic saline (×3). The packed cells were diluted with an equal volume of saline and mixed thoroughly. Two aliquots (200 μL) of the suspension were stored under nitrogen. To the 200 μL of erythrocyte suspension, 2 mL of methanol benzene 4:1 (v/v) containing 0.5 g/L butylated hydroxytoluene was added. Then 200 μL of acetyl chloride was added slowly and the sample was heated at 80°C in a heating block.

When the tube was cool, 5 mL of aqueous 6% potassium carbonate was added and centrifuged at 750 × *g*. The upper layer of benzene transferred and evaporated under nitrogen. The dry residue was mixed in approximately 100 μL of hexane.

The fatty acids were separated and measured by gas chromatography (model 5890; Hewlett Packard, Wilmington, DE), with helium used as the carrier gas, and a two-step temperature program that separates all the spectrum of polyunsaturated fatty acid (PUFA) [22].

2.3. Statistical analysis

Differences between groups were evaluated using nonparametric Kruskal-Wallis test and the Mann-Whitney test when indicated. Graphic presentation is provided by box plots.

3. Results

Weight and blood glucose levels were similar in all study groups at study entry (Table 1).

At day 28, fasting blood glucose was significantly lower in groups supplemented with either insulin (group 2) or DHA (group 3) compared to controls (Fig. 1). Supplementation of both insulin and DHA (group 4) resulted in a further significant decrease in glucose levels compared to

Table 1
Weight and blood glucose at study entry

	Group 1 $N = 7$	Group 2 $N = 7$	Group 3 $N = 7$	Group 4 $N = 7$
Weight, day 0 (g)	11 (8–12)*	11 (6–12)	12 (11–13)	10 (10–12)
Glucose, day 0 (mmol/L)	7.21 (6.49–7.82)	7.21 (6.22–8.32)	7.16 (6.49–8.82)	7.05 (6.12–7.88)

Data given as median (range).
* $P > 0.05$ (nonsignificant) for all items.

those in groups 2 and 3 (Fig. 1). There were no statistically significant differences in blood glucose levels between groups 2 and 3.

Serum levels of insulin were significantly elevated in the groups that were supplemented with insulin (groups 2 and 4) compared to those not supplemented with insulin (Fig. 2). There was no statistically significant difference in serum insulin levels between groups 4 and 2 (insulin supplemented), or between groups 1 and 3 (no added insulin).

The supplementation of oral insulin, oral DHA, or both had no significant effect on fatty acid content in RBC (Table 2).

The DHA content in RBC was significantly higher in the groups that were supplemented with DHA (groups 3 and 4) compared to those not given DHA (groups 1 and 2). There was no statistically significant difference between group 3 and group 4 (the latter also supplemented with insulin) (Fig. 3).

Weight at 28 days was similar in all study groups. Fasting lipid profile including TC, TG, and HDL, as well as lipid peroxides, were similar in all study groups (Table 3).

4. Discussion

The main finding of our study is that the co-administration of oral insulin and DHA to weaned Balb C mice reduces fasting glucose serum levels more than levels observed when only one substrate is used for supplementation. These effects were accompanied by significant elevation of serum insulin and DHA in the groups supplemented with these substrates respectively.

Several animal studies have demonstrated a local effect of insulin on the intestinal mucosa and on blood glucose levels in the suckling period [5,6,23–25]. We recently dem-

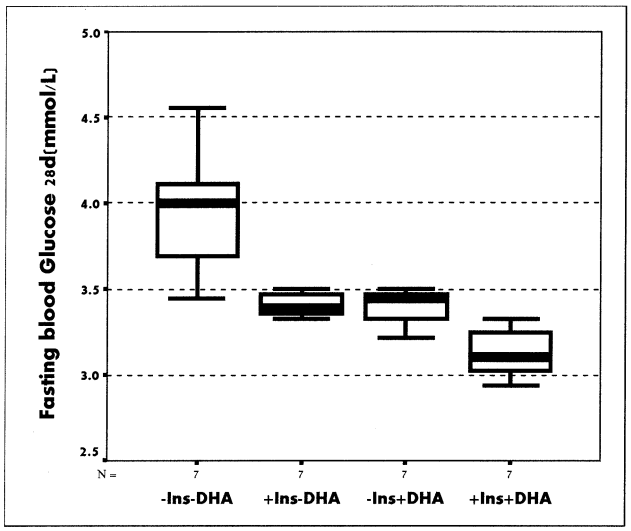

Fig. 1. Blood glucose levels at study end. Balb C mice were divided into four groups of seven mice each. Group 1 (ins−, DHA−) received a chow diet and regular drinking water. Group 2 (ins+, DHA−) received a chow diet and human insulin supplementation (1 unit/mL of drinking water). Group 3 (ins−, DHA+) received a chow diet containing 500 mg/kg/day of DHA and regular water with no insulin. Group 4 (ins+, DHA+) received a chow diet containing 500 mg/kg/day of DHA and supplementation with human insulin (1 unit/mL of drinking water). Fasting blood glucose was determined on day 28. Values are given as median and range. DHA = docosaexaenoic acid; INS = insulin. *$P < 0.01$ between group 1 and all other groups; **$P < 0.01$ between group 4 and groups 2 and 3.

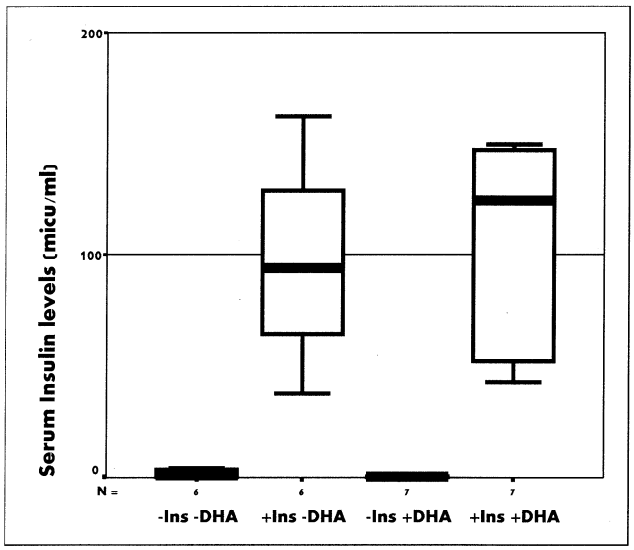

Fig. 2. Serum insulin levels at study end. Balb C mice were divided into four groups of seven mice each. Group 1 (ins−, DHA−) received a chow diet and regular drinking water. Group 2 (ins+, DHA−) received a chow diet and human insulin supplementation (1 unit/mL of drinking water). Group 3 (ins−, DHA+) received a chow diet containing 500 mg/kg/day of DHA and regular water with no insulin. Group 4 (ins+, DHA+) received a chow diet containing 500 mg/kg/day of DHA and supplementation with human insulin (1 unit/mL of drinking water). Serum insulin levels were determined on day 28. Values are given as median with range. DHA = docosaexaenoic acid; INS = insulin. * $P < 0.01$ between group 1 and groups 2 and 4; ** $P < 0.01$ between group 3 and groups 2 and 4.

Table 2
Polyunsaturated fatty acid measurements in red blood cells

	Group 1 $N = 7$	Group 2 $N = 7$	Group 3 $N = 7$	Group 4 $N = 7$
Linoleic acid (pmol/10e6RBC)	44 (42–49)*	50 (47–63)	57 (53–63)	54 (49–59)
Arachidonic acid (pmol/10e6RBC)	56 (55–62)	66 (60–90)	62 (48–72)	60 (55–66)
Eicosapentaenoic acid (pmol/10e6RBC)	8 (7–10)	9 (8–11)	9 (8–11)	9 (8–9)

* $P > 0.05$ (nonsignificant) for all items.

onstrated that the absorption of insulin is not limited to the suckling period, and we were also able to show that oral supplementation resulted in a dose-dependent increase in serum insulin concentration [17], supporting previous observations demonstrating that insulin is absorbed by the intestine of mature animals [9].

The gastrointestinal tract mainly degrades insulin. Therefore, the bioavailability of insulin may be augmented by the use of absorption enhancers that usually react with the cell membrane of the intestinal wall [10].

The use of free fatty acids as penetration enhancers has been reported for intestinal drug delivery systems [13,26]. Specifically, it has been proposed that DHA increases the permeability of phospholipids vesicle [27], suggesting that DHA present in human breast milk may be an enhancer for the absorption of insulin. The similar insulin serum levels in the group supplemented only with insulin and the group supplemented with both DHA and insulin suggests that in our animal model DHA did not enhance insulin absorption. However, orally administered insulin is absorbed mainly through the portal vein, and portal insulin levels may be several times higher than those found in the systemic circulation [28]. Since we did not measure portal insulin serum levels, the possibility that DHA serves as an enhancer to insulin absorption cannot be ruled out. Furthermore, previous studies demonstrating that DHA is an enhancer to insulin absorption [12,13] used water oil water emulsion containing insulin and DHA incorporated together as an enteral carrier, whereas we incorporated DHA into the chow diet. Nonetheless, although our preparation may be less efficient, we were able to observe significant elevation of DHA in RBC using our method of supplementation.

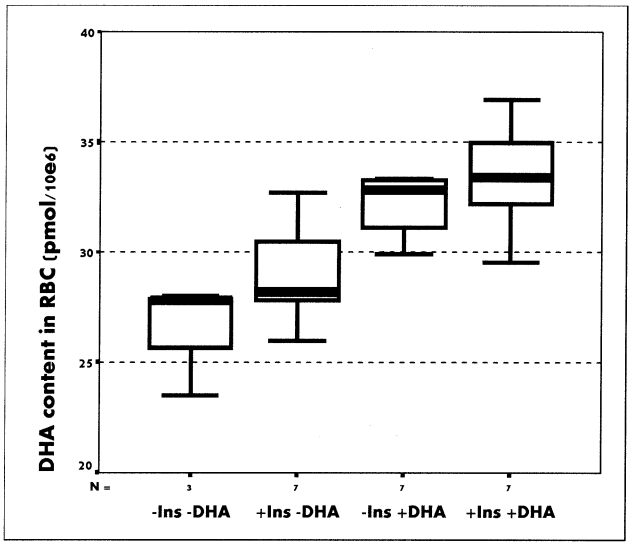

Fig. 3. Docosaexaenoic acid (DHA) content in red blood cells (RBC) at the study end. Balb C mice were divided into four groups of seven mice each. Group 1 (ins−, DHA−) received a chow diet and regular drinking water. Group 2 (ins+, DHA−) received a chow diet and human insulin (INS) supplementation (1 unit/mL of drinking water). Group 3 (ins−, DHA+) received a chow diet containing 500 mg/kg/day of DHA and regular water with no insulin. Group 4 (ins+, DHA+) received a chow diet containing 500 mg/kg/day of DHA and supplementation with human insulin (1 unit/mL of drinking water). DHA content levels were determined at day 28. Values are given as median with range. *$P < 0.01$ between group 1 and groups 3 and 4; **$P < 0.01$ between group 2 and groups 3 and 4.

Previous studies showed that human milk feeding increased LCPUFA, including DHA, in skeletal muscle membrane and erythrocytes, associated with lower fasting plasma glucose. It was also suggested that the reduced glucose levels in breast-fed infants was due to LCPUFA-mediated increased insulin sensitivity [14,15]. The proposed mechanism of DHA affecting insulin sensitivity postulates that the changes in the composition of fatty acids within membrane phospholipids influence insulin action. Indirect support for this hypothesis is the demonstration that decreased insulin sensitivity is associated with decreased concentrations of polyunsaturated fatty acids in skeletal muscle phospholipids [29].

Glucose uptake may be affected by the DHA induced changes in membrane composition. In support of this mechanism of action, it has been demonstrated that LCPUFA in general, and DHA deficiency in particular, resulted in decreased glucose uptake in high energy areas in the brain, accompanied by decreased immunoreactivity of the glucose transporters GLUT 1 and GLUT 3 [30].

In addition, DHA may modify insulin secretion, as suggested in studies in which the supplementation of eicosapentaenoic acid (EPA) and DHA to type 1 and type 2 diabetic rats resulted in improvement in glycosylated hemoglobin only in type 2 diabetes [31].

Our observation of reduced fasting glucose levels after DHA supplementation suggest that DHA induces a reduction in glucose serum levels. Whether this is due to increased insulin sensitivity or to increased cellular uptake

Table 3
Descriptive data of study groups at study end

	Group 1 $N = 7$	Group 2 $N = 7$	Group 3 $N = 7$	Group 4 $N = 7$
Weight (g)	18 (16–19)*	17 (16–19)	18 (17–20)	18 (16–18)
TC (mmol/L)	2.02 (1.86–2.33)	2.02 (1.86–2.48)	2.02 (1.78–2.4)	2.02 (1.55–2.17)
HDL (mmol/L)	1.45 (1.06–1.78)	1.55 (1.47–1.89)	1.55 (1.4–1.81)	1.45 (1.14–1.65)
TG (mmol/L)	1.66 (1.35–2.03)	1.69 (1.22–2.07)	1.83 (1.69–2.37)	1.59 (1.15–1.93)
TBARS (nmol/mL)	80 (58–110)	85 (67–106)	82 (73–104)	72 (71–95)

Data given as median (range).
* $P > 0.05$ (nonsignificant) for all items.
TC = total cholesterol; TG = triacylglycerol; HDL = high density lipoprotein cholesterol; TBARS = thiobarbituric acid reactive substances.

caused by increased glucose transport is yet to be determined. Our results do not support an effect of DHA on insulin secretion, because insulin levels were not influenced by DHA supplementation.

Supplementation with insulin and DHA had a synergistic effect on lowering glucose levels. This suggests that the presence of insulin in human milk may play a role in the hypoglycemic effect of human milk in addition to the LCPUFA-mediated increased insulin action.

In summary, based on our study results, adding insulin or DHA to the diet of weaned Balb C mice reduces glucose blood levels. Supplementation with both has a synergistic effect. The presence of insulin and DHA in human milk may be the cause of reduced glucose levels in breast-fed infants, in addition to the known effects of DHA on insulin sensitivity.

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