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Incorporation of Omega-3 Fatty Acid and Synthesis of their Metabolites in Muscle Tissue in Mice Fed with Flaxseed and Perilla-Enriched Diet

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ABSTRACT

Polyunsaturated fatty acids omega 3 and omega 6 are believed to have beneficial physiological effects on the human body. The present study aimed to evaluate the incorporation and synthesis of omega-3 fatty acid and their metabolites in muscle tissue in mice fed with a flaxseed and perilla-enriched diet. The animals were fed a diet supplemented with flaxseed and perilla meals for fifty-six days and control animals received a commercial diet. Mice were sacrificed on the 7th, 28th and 56th days and the muscle tissue was collected. The FA concentration was analyzed by gas chromatography. The levels of alpha-Linolenic Acid (LNA) and their metabolites, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) increased as a function of feeding length in the groups given the supplemented diet. LNA was incorporated into muscle tissue and conversion into Very Long Chain Polyunsaturated Fatty Acids (VLC-PUFA) was observed. The muscle tissue of animals fed with enriched diets presented a high sum of *n*-3 FA and high concentrations of LNA, EPA and DHA, as compared to mice fed commercial diet. The intake of flaxseed and perilla-meal enriched diets by mice may result in the deposition of LNA and its metabolites EPA and DHA into muscle tissue.

Keywords: Incorporation, Omega-3, Omega-6, Supplementation, Mice

1. INTRODUCTION

Alpha Linolenic Acid (LNA, 18:3n-3) and Linoleic Acid (LA, 18:2n-6) are strictly Essential Fatty Acids (EFA). Because they are not produced by mammals, including human beings, they must be obtained through the diet or by supplementation (Perini *et al*., 2010). Once consumed, they are metabolized by the same sequential desaturation and elongation enzyme systems, which results in the production of Long Chain Polyunsaturated Fatty Acids (LC-PUFAs) of the n-3 and n-6 series. LA is found in high concentrations in various vegetable oils, such as soy, sunflower and corn oils. Flaxseed (*Linum usitatissimum*) and perilla (*Perilla frutescens*) are the richest known sources of alpha-Linolenic Acid (LNA, 18:3n-3), a precursor of the long chain n-3 PUFA series (Roberfroid, 2002; Asif, 2011).

The conversion of LNA and LA into their longer chain homologues occurs by a combination of subsequent reactions involving Δ6 desaturation of LNA to 18:4n-3 and of LA to 18:3n-6, followed by elongation and Δ5 desaturation to Eicosapentaenoic Acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6). Final elongation and Δ4 desaturation produces Docosapentaenoic Acid (DPA,

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22:5n-6) and Docosahexaenoic Acid (DHA, 22:6n-3) (Kim, 2007). This conversion takes place in the endoplasmic reticulum, especially in liver cells (MacDonald-Wicks and Garg, 2007).

AA is important for fetal growth and the metabolism of prostaglandins (Innis, 2007). Among the LC-PUFA, EPA and DHA have received much attention from the scientific community because of their positive role in human health (Wurtman, 2008). Numerous health benefits have contributed to the popularity of n-3 PUFA. In general, increased dietary *n*-3 PUFA intake promotes retina and brain development in infants; in adults, *n*-3 PUFA consumption has been reported to reduce the risk of cardiovascular disease, obesity, diabetes, inflammation, cancer and to improve immunity (Calder *et al*., 2009; Ratnayake and Galli, 2009). Deckelbaum *et al*. (2006) reported that increasing tissue levels of *n*-3 PUFA exerts beneficial physiological effects by influencing cell membrane fluidity, membrane-bound receptors, signaling molecules and gene expression.

The dietary level of n-3 FA may be changed by the concomitant intake of other types of fats and n-6 and n-3 FA are known to compete for elongation and desaturation. This competition for Δ6 desaturase affects the incorporation of n-3 LC-PUFA metabolites by tissues and thus the impact of n-3 LC-PUFA on chronic diseases (James *et al*., 2000).

Studies have shown that the distribution of EFA is modulated by dietary intake and that food manipulation can directly influence property regulations, such as the formation of eicosanoids (Wallace *et al*., 2003). Eicosanoid acids are oxygenated metabolites of EFA originated from AA and EPA. They are components of prostaglandins, leukotrienes, thromboxanes and prostacyclins, which participate in many infectious processes. Moreover, they are also involved in the reproductive function, platelet formation and regulation of blood pressure, gastric acid secretion and a variety of other processes important to human health (Bagga *et al*., 2003).

ALA, EPA and DHA are believed to have beneficial physiological functions and, therefore, are important to understand the metabolism of LNA in mammals, which grants the interest in the metabolism of PUFA. Thus, experimental studies are sorely needed, so the aim of this work was evaluated the levels of incorporation in muscle tissue of mice fed with flaxseed and perila-enriched diet for 56 days.

2. MATERIALS AND METHODS

2.1. Animals and Experimental Design

Ninety four-week-old male Swiss mice were provided by the central animal house of the State University of Maringa. A 12-h dark/12-h light cycle and a room temperature of 23 ± 1°C were maintained. The animals received food and water *ad libitum*. After acclimatization for 7 days, 30 animals were randomly assigned to each of three groups according to the experimental diet: Control Group (CG), Flaxseed Group (FG) or Perilla Group (PG) and were kept in polycarbonate cages. In order to evaluate the incorporation of fatty acid omega-3 in muscle tissue, ten mice of each group were sacrificed on the 7th, 28th and 56th days and paw muscle tissue was removed and kept in polyethylene bags in N_2 atmosphere at-18°C until analysis. At the beginning of each analysis, the tissue samples were allowed to equilibrate to room temperature, diced and homogenized. The level of fatty acids in the tissue was evaluated. This study was approved and conducted according to the guidelines of the Ethics Committee for Animal Research of State University of Maringa (No. 007-2011).

2.2. Diets

Experimental diets were designed according to the NRC (1995). The experimental diets were nutritionally complete (isocaloric, isoproteic and normolipidic). The basic diet composition is shown in **Table 1**. Enriched diets were pelleted and prepared in bulk, separated in daily portions, vacuum packed and stored at 4°C for a maximum period of 1 week to prevent fatty acid oxidation. Total lipids and fatty acid composition of total lipids of freshly prepared diets were monitored.

2.3. Total Lipids and Fatty Acid Composition

Total Lipids (TL) in tissues and feed were determined according to Bligh and Dyer (1959). Fatty Acid Methyl Esters (FAME) were prepared by methylation of total lipids following Joseph and Ackman (1992). The methyl esters were separated by gas chromatography using a Varian 3300 (USA) gas chromatograph fitted with a flame ionization detector and a fused-silica CP-select CB-Fame capillary column (100 m×0.25 mm i.d., 0.25 μm cyanopropyl CP-7420) operated at a detector temperature of 240°C and injection port temperature of 240°C. The column temperature was maintained at 165°C for 12 min and programmed to rise from 165 to 185°C at 40°C/min for 15 min and from 180 to 240 °C at 15°C/min in 18 min.

Table 1. Composition, total lipid content and fatty acid composition of the experimental diets

Ingredients (% w/w)	Control diet	Flaxseed meal-enriched diet	Perilla meal-enriched diet
Flaxseed meal	----	10	----
Perilla meal	----	----	7
Soybean meal	38.74	32.09	28.4
Wheat meal	11.48	9.63	31.58
Ground corn	42.71	40.78	23.54
Dicalcium phosphate	20.35	2.7	3.07
Sodium chloride	0.3	0.3	0.3
Premix	4.5	4.5	4.5
Composition	Means±SD	Means ± SD	Means ± SD
Total Lipids (%)	6.5 ± 1.15	6.2±0.4	6.8 ± 1.4
Fatty acid (mg/g total lipids)[a]			
16:00	127.8±0.23[a]	83.2±2.8[c]	101.8±1.67[b]
17:00	0.7±0.02	0.6±0.3	nd
18:00	26.5±0.12[a]	33.5±1.6[a]	16.9±0.26[b]
18:1n-9	211.5±0.69[a]	181.0±2.3[c]	152.4±2.05[b]
18:1n-7	9.7±0.03	18.6±1.8	9.5±0.11
18:2n-6 (LA)	422.7±1.75[a]	254.1±2.2[b]	265.2±1.38[b]
18:3n-6	0.2±0.29	0.8±0.2	nd
18:3n-3 (LNA)	34.5±0.08[a]	260.2±2.5[b]	310.7±1.80[b]
20:00	2.9±0.01[a]	7.6±1.3[c]	1.7±0.01[b]
20:1n-9	3.4±0.02[a]	0.4±0.1[c]	2.5±0.07[b]
MUFA	222.7±0.36[a]	201.2±2.1[a]	136.1±1.06[b]
SFA	157.9±0.21[a]	124.3±1.0[b]	120.3±0.95[b]
PUFA	464.0±0.87[a]	514.2±0.8[b]	577.3±0.97[b]
n-6	422.9±1.75[a]	254.1±1.9[b]	266.6±0.99[b]
n-3	34.5±0.08[a]	260±1.7[b]	310.7±1.80[b]
PUFA/SFA	2.9±0.05	4.1±0.4	8.3±0.02
n-6/*n*-3	12.2±0.03[a]	0.9±0.3[b]	0.9±0.05[b]

PUFA = Polyunsaturated Fatty Acid, MUFA = Monounsaturated Fatty Acid, SFA = Saturated Fatty Acid, *n*-6 = Omega- 6 fatty acid, *n*-3 = Omega- 3 fatty acid. Results expressed as mean ± standard deviation for analysis in nine replicates. N = 10. Means followed by different letters in the same line are significantly different (P <0.05) by Tukey's test and test t. Control diet *versus* flaxseed and perilla meal-enriched diets. nd = not detected.

The ultra-pure gas flows were: 1.4 mL.min^{1} carrier gas (hydrogen), 30 mL.min^{-1} make-up gas (nitrogen), 300 mL.min^{-1} synthetic air and 30 mL.min^{-1} hydrogen flame gas, split injection, 1:100 ratio (injection in triplicate). Retention times and peak area % values were automatically computed by a Varian 4290 integrator. For the identification of FA, FA retention times were compared to those of standard methyl esters (Sigma, USA).

2.4. Quantification of Fatty Acid Methyl Esters (FAME)

The concentration of FA in mg.g^{-1} of total lipids in paw muscle tissue was measured against tricosanoic acid methyl ester (23:0) from Sigma (USA) as an internal standard, as described by Joseph and Ackman (1992) and Visentainer (2012). The following formula was used to calculate the concentrations: FA (mg g^{-1} TL) = (A_X x W_{IS} x CF_X)/(A_{IS} x W_X x CF_{AE}), where TL is total lipids, Ax is the peak area of fatty acids, A_{IS} is the peak area of the Internal Standard (IS) tricosanoic acid methyl ester (23:0), W_{IS} is the weight (mg) of IS added to the sample (in mg), W_X is the sample weight (in mg), CF_X is the theoretical correction factor and CF_{EA} is the correction factor of methyl ester for fatty acids.

2.5. Statistics

The mean values were statistically compared by Tukey's test and test t at 5% with one-way ANOVA. Data were processed using Statistica 7.0 software.

3. RESULTS

No significant differences in body weight were observed between the diet groups in the 56 days of experiment. The mean body weight values were: CG 47.58±4.27 g, FG 42.10±4.18 g and PG 46.09±5.2 g.

The TL and FA composition of the diets are presented in **Table 1**. The TL values were 6.5% for control diet, 6.2% for flaxseed meal-enriched diet and 6.8% for perilla meal-enriched diet.

The quantitative analysis of FA in the diets and paw muscle tissue are expressed in mg of FA per gram of total lipid (mg.g^{-1} TL) and are presented in **Table 1 and 2**, respectively. The major FA in both experimental diets were palmitic acid (16:0) and oleic acid (18:1*n*-9). The control diet had the highest amount of linoleic acid (422.73 mg.g^{-1} TL) and the flaxseed and perilla meal-enriched diets had the highest amounts of alpha-linolenic acid, 260.2 mg.g^{-1} TL and 310.7 mg.g^{-1} TL, respectively. These values were significantly different (P <0.05).

The LA level in tissue was higher in mice fed the control diet than the flaxseed and perilla meal-enriched diets due to its higher concentration of LA. The LA metabolites were 20:3*n*-6, 20:2*n*-6 and AA. AA was found in lower quantities in FG and PG, when compared with CG. However, the levels in PG significantly decreased (P <0.05) from the 7th (14.2 mg.g^{-1} TL) to the 56th day (5.0 mg.g^{-1} TL).

The highest level of LNA was found in muscle tissue (FG and PG) with a significant increase from the 7th day (41.9 mg.g^{-1} TL and 57.9 mg.g^{-1} TL) to the 28th day (60.4 mg.g^{-1} TL and 82.3 mg.g^{-1} TL) (P <0.05), remaining on this level until the 56th day (58.9 mg.g^{-1} TL and 85.2 mg.g^{-1} TL), respectively (**Table 2**).

Table 2. Total lipids content and fatty acid composition of mice muscle tissue

		Flaxseed group				Perilla group				
Composition	Control group Mean	7 days SD	28 days Mean	56 days Mean	Mean	7 days SD Variation	28 days Mean	56 days Mean	Mean	SD Variation
Total Lipids (%)	3.73	0.5	4.4^{a}	3.7^{b}	3.1^{b}	0.4-0.5	4.6	2.7	3.1	0.07-1.28
Fatty acids (mg FA/g TL)										
12:00	1.0	0.2	1.5^{a}	0.6^{b}	0.6^{b}	0.1-0.3	2.80	1.1	0.6	0.01-0.1
14:00	9.2	1.1	11.7^{a}	8.6^{b}	6.7^{c}	0.6-0.9	11.70	8.5	6.8	0.05-0.6
14:1n-9	8.3	1.2	1.1	1.2	1.2	0.1-0.3	0.90	0.7	0.9	0.04-0.1
15:00	1.5	0.2	1.3	1.2	1.3	0.2-0.3	0.90^{a}	0.9^{a}	1.1^{b}	0.1
15:1n-5	1.7	0.3	1.1	1.2	1.1	0.2-0.3	1.90	1.7	1.7	0.1-0.2
16:00	177.0	6.3	200.8^{a}	187.7^{b}	156.3^{c}	1.1-5.1	149.50	145.6	129.9	2.9-8.2
16:1n-9	5.2	0.3	4.0^{a}	5.5^{b}	5.6^{b}	0.1-0.4	2.90^{b}	3.7^{b}	4.8^{a}	0.1-0.3
16:1n-7	54.2	2.2	49.9	52.0	50.6	0.8-2.6	35.30^{a}	42.9^{b}	50.7^{c}	0.8-3.1
17:00	1.4	0.2	1.3	1.4	1.4	0.1-0.3	1.30	1.4	1.3	0.01-0.1
17:1n-5	1.2	0.2	1.2	1.3	1.3	0.1-0.2	1.20	1.7	1.8	0.1-0.2
18:00	38.9	3.9	36.1	41.8	40.1	1.2-3.3	28.60^{a}	25.9^{b}	21.7^{c}	0.1-2.1
18:1n-9	177.0	6.7	194.0	196.1	188.1	1.9-4.7	169.30	169.2	180.0	1.4-5.3
18:1n-7	30.0	3.4	24.8	28.5	29.1	0.9-3.4	14.40	13.5	14.3	0.3-1.5
18:2n-6 (LA)	239.0	4.6	208.4^{a}	187.1^{b}	176.7^{b}	1.7-3.1	165.00	159.3	168.8	0.9-4.8
18:3n-6	1.0	0.2	0.6	0.6	0.7	0.1-0.2	0.47	nd	nd	0.07
18:3n-3 (LNA)	10.5	0.9	41.9^{a}	60.4^{b}	58.9^{b}	0.8-2.6	57.90^{b}	82.3^{a}	85.2^{a}	0.5-3.4
20:00	0.4	0.1	1.3	1.4	1.4	0.1-0.3	0.50	nd	nd	0.5
20:1n-9	2.4	0.3	2.3^{a}	2.1^{a}	1.7^{b}	0.4-0.6	2.30^{a}	2.2^{a}	2.0^{b}	0.1-1.4
20:2n-6	2.7	0.3	1.5	1.5	1.4	0.4-0.7	1.70	1.1	1.3	0.002-0.1
20:3n-6	3.0	0.4	2.7^{a}	2.5^{b}	2.2^{c}	0.4-0.7	1.90^{ab}	1.7^{bc}	1.1^{c}	0.1-0.3
20:4n-6 (AA)	29.3	2.3	20.5	19.4	19.4	1.4-3.9	14.20^{a}	9.6^{b}	5.0^{b}	0.3-2.2
20:5n-3 (EPA)	0.4	0.1	1.6^{a}	3.1^{b}	3.7^{b}	0.1-0.8	4.10^{ab}	4.8^{a}	3.2^{ab}	0.01-0.1
24:0	3.9	0.3	2.4^{a}	1.1^{b}	1.0^{b}	0.3-0.6	1.70	0.9	nd	0.03-0.1
22:5n-3	4.3	0.4	4.4^{a}	10.1^{b}	13.1^{c}	0.2-0.4	3.20^{ab}	4.8^{a}	4.1^{ab}	0.01-0.1
22:6n-3 (DHA)	10.0	0.2	16.7^{a}	37.4^{b}	54.1^{c}	0.5-1.0	10.70^{bc}	16.4^{a}	15.8^{ab}	0.1-0.3
MUFA	286.0	3.1	279.3^{a}	288.6^{a}	279.4^{b}	1.9-4.7	227.40	235.7	255.5	1.5-7.6
SFA	235.0	3.0	256.4^{a}	243.8^{b}	208.8^{c}	1.8-4.8	235.30^{a}	184.7^{b}	161.7^{b}	1.2-7.1
PUFA	300.0	4.1	297.0^{a}	322.1^{b}	330.2^{b}	1.1-3.3	194.10^{b}	275.6^{a}	280.8^{a}	1.3-7.3
n-6	275.0	4.0	233.7^{a}	211.1^{b}	200.4^{c}	1.3-3.3	178.10	171.5	175.2	1.5-8.7
n-3	25.2	1.5	63.3^{a}	111.0^{b}	129.8^{c}	0.9-2.8	16.00^{b}	103.9^{a}	105.5^{a}	1.3-5.2
PUFA/SFA	1.3	0.3	1.2^{a}	1.3^{a}	1.6^{b}	0.1-0.3	0.80	1.5	1.7	0.004-0.04
n-6/n-3	10.9	0.4	3.7^{a}	1.9^{b}	1.5^{c}	0.3-0.7	11.10^{a}	1.6^{b}	1.7^{b}	0.01-0.1

Total Lipids (TL), Fatty Acids (FA), Linoleic Acid (LA), Alpha-Linolenic Acid (LNA), Monounsaturated Fatty Acids (MUFA); Saturated Fatty Acids (SFA), Polyunsaturated Fatty Acids (PUFA), n-6 (omega- 6), n-3 (omega- 3). Mean values and standard deviations, n = 9. Averages followed by different letters in the same line are significantly different (P <0.05) by Tukey's test and test t. Control group *versus* flaxseed and perilla groups. nd = not detected

LNA metabolites (EPA and DHA) were identified in the three groups, CG, FG and PG. In the FG and PG groups, the synthesis of EPA was significantly higher ($P < 0.05$) than in the CG group. The DHA values were higher ($P < 0.05$) in muscle tissue of mice fed FG and PG rather than CG. The rate of incorporation of DHA increased significantly ($P < 0.05$) on the 7th day (16.7 $mg.g^{-1}$ TL and 10.7 $mg.g^{-1}$ TL), when compared with the levels on the 56th day (54.1 $mg.g^{-1}$ TL and 15.8 $mg.g^{-1}$ TL), respectively.

In relation to omega-6 FA, LA and AA levels were the highest in the CG muscle tissue; the values of LA did not change during perilla meal-enriched diet feeding; however the level of AA decreased during the experiment, specially on the 56th day ($P < 0.05$) (**Table 2**). The reduction of the AA content following LNA dietary supplementation is well documented in the scientific literature (Brenna *et al*., 2009).

The sum of *n*-3 increased throughout the experiment in PG and FG and was higher on the 28th day (103.9 $mg.g^{-1}$ TL and 111.0 $mg.g^{-1}$ TL) and higher than the CG value (25.8 $mg.g^{-1}$ TL) on the 56th day ($P < 0.05$). However, the opposite did not occur with the sum of *n*-6 FA, the quantities of which stayed at the same level as in the tissue of mice fed PG (192.8 to 197.5 $mg.g^{-1}$ TL). The sum of n-6 decreased during FG feeding (233.7 to 200.4 $mg.g^{-1}$ TL) when compared with CG.

The *n*-6/*n*-3 values were lower in muscle tissue in the PG and FG on the 7th day, decreasing significantly ($P < 0.05$) from 28th days until 56th days, when compared to the CG. The average *n*-6/*n*-3 ratio, according to Sugano and Hirahara (2000) and Kris-Etherton *et al*. (2000), depends on diet duration.

4. DISCUSSION

In the present study, Swiss mice were used as experimental animals due to their numerous advantages, such as easy handling and the possibility of working simultaneously with several groups, in addition to their biological similarities with human beings. They are monogastric mammals and present metabolic routes similar to those of human beings (Leon, 2005).

The quantities of LNA, DPA and DHA were higher during all of the FG and PG treatments, as compared to the CG, in contrast to the values of n-6 PUFA LA and AA. This was because FG and PG had a greater concentration of LNA, which is a precursor of *n*-3 PUFA. These results agree with those reported by other authors showing that the inclusion of LNA sources in the diet of mice increased the concentration of the metabolites EPA and DHA in tissue (Tou *et al*., 2011).

The high dietary ratios of LA/LNA decreased the conversion of LNA into VLC-PUFA. Additionally, the ingestion of *n*-6 FA influences the concentration of *n*-3 FA in tissue. There is evidence that the amounts of *n*-3 PUFA can be improved by increasing their ingestion or by decreasing the ingestion of LA or by a combination of both, which is likely to be even more efficient (SanGiovanni and Chew, 2005).

Higher *n*-3 DPA and DHA values were found in the PG and FG due to their higher concentration of their precursor, LNA and its conversion in the liver induced by enzyme activity (Simopoulos, 2003; Martin *et al*., 2006). These higher values contributed to reduce the *n*-6/*n*-3 ratio in relation to the CG values. In addition, diets poor in *n*-3 FA and with high *n*-6/*n*-3 ratios may contribute to the development of many allergic, inflammatory and cardiovascular diseases (Tou *et al*., 2011).

DHA is an important FA in neonatal diet due to its role in the constitution, development and functioning of the head and retina (Esposito *et al*., 2008; Fernandes *et al*., 2011). It is also present in cell membranes and its deficiency is associated with visual impairment and head deformation (Igarashi *et al*., 2007). Muscle tissue showed the highest DHA synthesis during the entire experiment.

Diets with inadequate amounts of LNA cause an imbalance in *n*-3 and *n*-6 FA or deficient desaturation and elongation of FA. These factors influence the *n*-3 PUFA metabolism. In the presence of one or more of these factors, the production of these FA decreases (Moore *et al*., 1990). *n*-6 fatty acid intake influences tissue concentration of *n*-3 PUFA (Brenna *et al*., 2009).

n-3 PUFA-rich diets are important to prevent heart diseases. The presence of LNA in the diet may reduce arrhythmias (Ayalew-Pervanchon *et al*., 2007). Calder (2004) attributes this protective effect to the ability of *n*-3 FA to reduce the plasma triglyceride level, blood pressure and platelet aggregation. *n*-3 FA are incorporated into myocardium cell membranes, potentially diverting the production of n-2 eicosanoids, which derive from arachidonic acid, to *n*-3 FA, which derive from eicosapentaenoic acid and have vasodilating and platelet antiaggregation functions.

Animals treated with flaxseed and perilla meal-enriched diet showed a lesser ability to incorporate Linoleic Acid (LA) and to synthesize Arachidonic Acid (AA). Animals treated with commercial feed did not show the same ability to incorporate and synthesize omega-3 series FA due to the low concentration of LNA.

5. CONCLUSION

The intake of perilla- and flaxseed meal-enriched diets by mice resulted in deposition of LNA and their

metabolites EPA and DHA in muscle tissue. There was a significant increase of *n*-3 fatty acids and consequently a decrease in *n*-6/*n*-3 ratio in the muscle tissue analyzed compared with commercial diet.

6. ACKNOWLEDGMENT

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