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Abstract

Polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are major maternal dietary supplements due to their positive benefits on neurological tissue growth during the first 12 weeks of gestation. Previous studies show that EPA and DHA inhibit muscle formation but promote adipogenesis. However, no research has addressed the question whether high intake of EPA and DHA affects brown fat development during gestation. The objective of this study was to measure the effect of EPA and DHA supplement on brown adipogenesis and potential pathways related to mitochondrial biosynthesis using fibroblasts as in vitro model. Using Oil-Red-O staining and PCR testing, lipid droplet formation and tested six genes were examined and PGC1α presented statistically significant difference from the control group when treated with PUFAs. Results indicated that PGC1α gene expression can be to be alternated by EPA and DHA treatment. Mitochondrial biosynthesis can potentially be promoted by increased PGC1α gene expression. However, the lipid droplets accumulated in the PUFAs treated group show an unknown mechanism of the n-3 PUFA on adipogenesis that needs to be revealed.

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Introduction and Literature Review

In the United States, childhood and adolescent obesity has been on the rise for years and has nearly tripled since the 1970s. The CDC reports data taken from 2015-2016 shows that nearly 1 in 5 school age children and young people (6 to 19 years) in the United States has obesity. There is increasing evidence that infants exposed to obesity-induced diabetes in utero have an increased incidence of childhood obesity and diabetes [1; 2]. Understanding the mechanism of the relationship between maternal and infant obesity is becoming an urgent task in the study of childhood obesity.

Epidemiological and experimental studies have shown that food substrates, such as fatty acids, supplied to the fetus during pregnancy and to the newborn immediately after birth, can have long-term health effects on the development of metabolic diseases. These diseases include cardiovascular diseases, Type 2 diabetes, hypertension, and obesity [3; 4]. Growing bodies of experimental studies indicate that reducing the risk of a variety of obesity-related diseases is strongly linked to an increase in the dietary supplementation and consumption of n-3 fatty acids [5]. While a substantial number of studies have delineated many differences between the biological effects of saturated versus polyunsaturated fatty acids, less is known about the long-chain n-3 fatty acids commonly present in certain fish oils, such as eicosapentaenoic acid (EPA, 20:5,n-3) and docosahexaenoic acid (DHA, 22:6,n−3) [5]. Fish oil components, particularly two key biological regulators, EPA and DHA, appear to have the ability to modulate both cellular metabolic functions and gene expression. Based on outcomes from series of studies, the synthesis of EPA and DHA from their 18:3 precursor α-linoleic acid is relatively inefficient, so meeting the body need of n-3 fatty acids depends to a significant degree on the direct delivery of EPA and DHA with diet particularly from marine or industrial sources, such as fish oils [6].

Clinical research also showed that EPA and DHA supplementation during pregnancy accumulates in fetal tissues and causes a longer gestation.

Our previous studies showed that EPA and DHA inhibit muscle formation but promote adipogenesis. However, no research has addressed the question whether high intake of EPA and DHA affects brown fat development during gestation. Brown adipose tissue (BAT) is an essential target in obesity prevention as well as treatment due to the ability to utilize fatty acids and glucose to generate heat by non-shivering thermogenesis. Most brown adipocytes originate from precursor cells in the embryonic mesoderm that express skeletal muscle marker genes and have similar mitochondrial proteomes with muscle [4; 5]. In most eukaryotes, mitochondria are primary organelles that response for energy metabolism which derived from the breakdown of carbohydrates and fatty acids. It was reported that the n-3 PUFAs could cause higher oxidation levels of mitochondrial fatty acids in the myocardium [7; 8; 9; 10]. We hypothesize that EPA and DHA treatment impacts the brown adipogenesis of BAT precursor cells via metabolic changes in mitochondria. The objective of the current study is to measure the effect of maternal EPA and DHA supplement on brown adipogenesis and potential pathways related to mitochondrial biosynthesis using fibroblasts as in vitro model.

Materials and Methods

Cell culture:

NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO2 atmosphere. When cells confluency reached 90%, cells in the control group wells (Con, n = 6) were induced to brown adipocyte differentiation switching to the differentiation medium 1 (DM1) containing 10% FBS, 1% penicillin-streptomycin, 170nM insulin, 1uM Dexamethasone, 0.5mM IBMX, and 1nM 3,3',5-Triiodo-L-thyronine sodium salt (T3), while 50μM EPA and 50μM DHA were added to DM1 in the fatty acid treatment group (FA, n = 6) for 3 days. Then Con cells were introduced DM2 which only contained 10% FBS, 170nM insulin, and 1nM T3. The DM2 in the FA group contained 50μM EPA and 50μM DHA. DM2 with or without fatty acids was changed every 24 hr for 3 days.

Oil-Red-O staining:

Oil-Red-O staining was used to identify mature adipocytes. Cells will be fixed in 10% formalin (or 4% paraformaldehyde) in PBS for 10 min at room temperature. Fixed cells were stained with the Oil-Red working solution for 7 min and rinsed with PBS to remove the excessive Oil-Red dye. The presence of Oil-Red O dye in adipocytes was further quantified by measuring the optical absorbance at 520 nm.

Real-time PCR:

Gene expression related to brown adipogenesis, mitochondrial biosynthesis, and peroxisome biosynthesis were measured by quantitative real-time PCR method. Total mRNA was extracted from cells with the TRIzol reagent (Fisher, Pittsburgh, PA). The concentration of total RNA was

assessed by Nanodrop OneC (Thermo Scientific, Waltham, MA), and quality was examined in the absorption ratio of OD260nm/OD280nm. The cDNA was synthesized from the RNA with iScript cDNA synthesis kit (Bio-Rad, Richmond, CA). Real-time PCR was carried out by using SYBR Green Supermix (Bio-Rad, Richmond, CA) on CFX Connect Real-Time PCR Detection System (Bio-Rad, Richmond, CA). The oligonucleotide primers used were designed with NCBI database and Primer Quest (IDT.com). The primers (Table1) were designed to target the genes: uncoupling protein 1 (UCP1), PR/SET domain 16 (PRDM16), iodothyronine deiodinase 2 (DIO2), peroxisome proliferator-activated receptor alpha (PPARα), carnitine palmitoyltransferase 1beta (CPT1β), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). Each reaction yielded amplicons from 80 to 250 bp. PCR conditions were as follows: 30 s at 95°C, 30 s at 55°C, and 40 s at 72°C for 40 cycles. After amplification, a melting curve (0.01 °C/s) was used to confirm product purity, and the PCR products were electrophoresed to verify the targeted sizes. Results will be expressed relative to β-actin. Data were analyzed using the ΔΔCT method, and 18S gene was the reference gene.

Statistical analyses:

Differences between groups will be assessed for significance by the unpaired t-test with the assumption of equal variances, and arithmetic means ± SEM was reported. Statistical significance will be considered as $P \leqslant 0.05$.

Results

Lipid droplets accumulation:

The Oil-Red-O staining showed lipid droplets accumulation in pre-adipocytes differentiated from 3T3 fibroblasts (Fig. 1. A). The quantitative data showed that the red dye accumulated more (20.04 ± 6.95%, $P < 0.05$) in the FA group than Con cells (Fig. 1. B).

Figure 1.

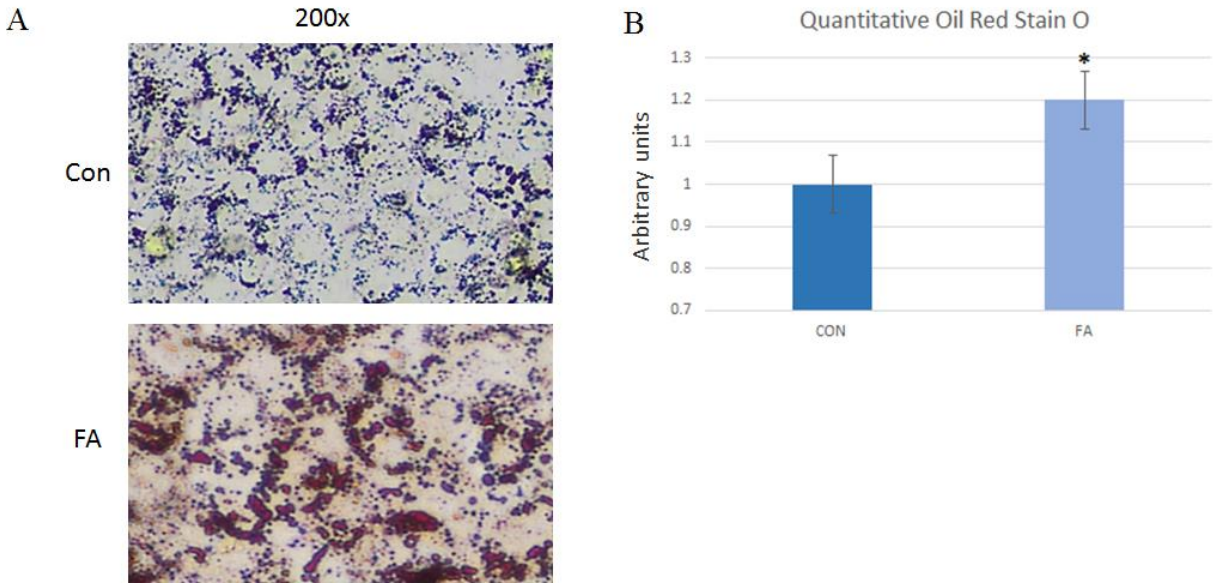

Figure 1. Lipid droplets. A) Representative images of Oil Red O staining of 3T3 fibroblasts after brown adipogenic differentiation. B) Quantitative assessment of Oil Red O staining in FA and CON. Significant differences between the two groups are at the indicated time points. * P < 0.05; n=6. Data was normalized by the total number of cells counted using a hemocytometer in each group.

Adipogenic mRNA expression:

Between the two groups of cells, mRNA expression of adipogenesis was checked (Fig 2). Among the brown and white adipogenic marker genes, the expression of PGC1α was higher (31.81 ± 5.17%, $P < 0.05$). Other gene expression including UCP1, PRDM16, PPARα, and CPT1β remained no significant difference between groups.

Figure 2.

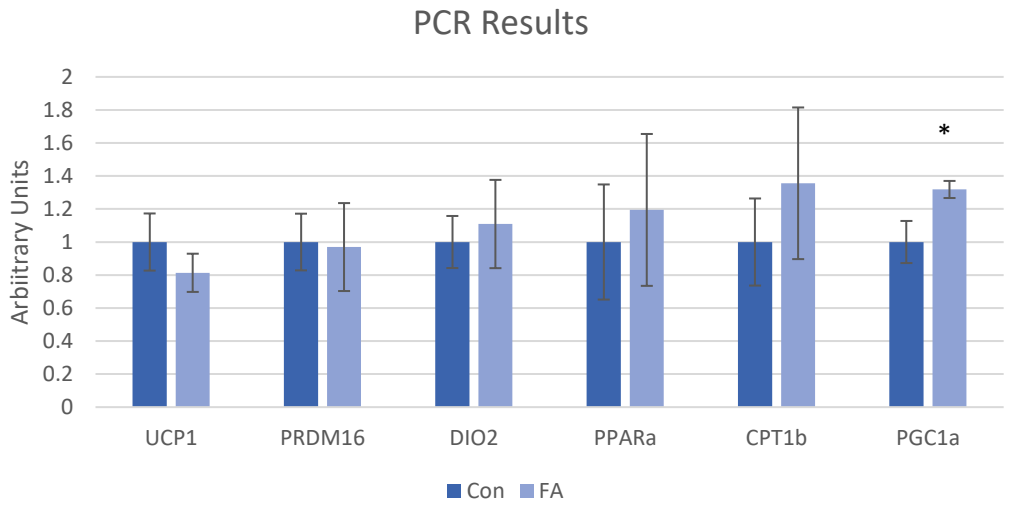

Figure 2. Gene expression analysis by RT-qPCR of key genes related to adipogenesis, mitochondrial biosynthesis and metabolism. Data are expressed as mean + SEM. The relative expressions were calculated in arbitrary units. * $P < 0.05$; n=6.

Table 1. List of primers

Primers	Accession No.	Forward sequence	Reverse sequence
UCP1	NM_009463	CACGGGGACCTACAATGCTT	ACAGTAAATGGCAGGGGACG
PRDM16	NM_027504	AAGGAGGCCGACTTTGGATG	TTTGATGCAGCTCTCCTGGG
PPARα	NM_011144	TGGTGTTCGCAGCTGTTTTG	AGATACGCCCAAATGCACCA
CPT1β	NM_009948	TATAACAGGTGGTTTGACA	CAGAGGTGCCCAATGATG
PGC1α	NM_008904	TCCTCTGACCCCAGAGTCAC	CTTGGTTGGCTTTATGAGGAGG
18S	NR_003278	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Discussion and Conclusion

Long chain fatty acids are known to activate brown adipocytes [11]. In this study, the expression of total six genes were measured: UCP1, PRDM16, PPARα, CPT1β, and PGC1α. UCP1 is known as uncoupling protein 1 and it works to separate oxidative phosphorylation from ATP synthesis with energy dissipated as heat, also referred to as the mitochondrial proton leak and helps to reduce mitochondrial membrane potential in mammalian cells [12]. PRDM16 is a protein coding gene. It has broad expressions in the stomach and thyroid among other tissues [13]. Peroxisome proliferator activated receptor alpha, PPARα, increases the size and number of peroxisomes, which are subcellular organelles found in plant and animal cells and contain enzymes for respiration and for cholesterol and lipid metabolism[14]. Carnitine palmitoyltransferase1β, CPT1β, is a protein coding gene that encodes a protein that is the rate-controlling enzyme of the long-chain fatty acid beta-oxidation pathway in muscle mitochondria[14]. The protein coded by PGC1 α is a transcriptional cofactor that regulates genes involved in energy metabolism[15]. These six genes with EPA and DHA and our results showed that when compared to the control only one genome was significantly different. PGC1α is a transcription cofactor. It functions as a master regulator for many metabolic and physiological processes such as adaptive thermogenesis, glucose and fatty acid metabolism, muscle fiber type, and mitochondrial biogenesis [9; 15]. Overexpression of this transcription coactivator could improve mitochondrial function. It also can help to increase oxidative stress resistance. The observation of this upregulation could be an indicator that fatty acids can increase the speed of the metabolic pathways when introduced to fibroblasts. However, it has also been recognized in recent studies that 3T3 cells are insensitive to both fatty acid and beta-adrenergic agonist stimulation. 3T3 cells are the most commonly used cells because they have a high affinity for

harboring lipids into the cytoplasm when stimulated. The understanding that they are insensitive to the treatment of long chain fatty acids helps to explain the lack of statistical differences between the control group and the treatment group [16]. The results collected are a helpful piece in the equation that is prenatal nutrition. A limitation of this study is that only PCR and staining results could be presented. The results could be fortified by further testing the cell line for oxygen consumption rates, running Western Blot tests, and running PCR testing for thermogenesis, mitochondrial biosynthesis, and protein synthesis. Another limitation was the sample size that survived until final testing. Regardless of limitations and lack of statistical differences between treatment and control, current results indicate that mitochondrial synthesis has the ability to be induced through the introduction of certain long chain fatty acids. This would usually be paired with smaller lipid droplets due to the fact that higher mitochondrial counts allow for more rapid lipid degradation. However, the results of this study show higher lipid concentrations in the cells while also having higher mitochondrial counts. It is for this reason that additional studies are needed to understand the reason behind this discrepancy and to eventual realize the effect of EPA and DHA on adipogenesis in relation to thermogenesis and increase of obesity post-partum when introduced to fibroblasts in-vitro. For further research, it is suggested to use a sturdier cell line that is easily stimulated by fatty acid treatment and to run more diagnostic testing focusing on mitochondrial biosynthesis.

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