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Dietary docosahexaenoic acid alters pregnant rat reproductive tissue prostaglandin and matrix metalloproteinase production

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Abstract

Shortened gestation is a major cause of infant mortality and morbidity. Evidence from both human and animal studies suggests that essential fatty acids of the n-6 and n-3 series play important and modifiable roles in gestational duration. We examined the influence of linolenic acid (LnA) vs. docosahexaenoic acid (DHA) on rat reproductive tissue prostaglandin (PG) and matrix metalloproteinase (MMP) indices of gestational duration. By varying the oil source of the diet, AIN-93G diets were constructed to provide either 0.7 energy % (en%) LnA, the current US intake of n-3 fatty acids, or 0.7 en% DHA. In addition, enhanced levels of 2.0 en% LnA or 2.0 e% DHA diets were also constructed. All diets contained approximately 6.0 en% linoleic acid (LA), the current US intake of LA. Four groups of 10 female rats were time-mated and fed the respective diets from conception through Day 20 of gestation. Day 20 uterus and placenta DHA were significantly increased by 160–180% by the 0.7 en% DHA diet, and by 250–350% by the 2.0 en% DHA diets in comparison to 0.7 en% LnA diet. DHA diets also significantly reduced uterus and placenta arachidonic acid content. Day 20 placenta and uterus PGE_2 and placenta $PGF_{2\alpha}$ production rates were significantly reduced by 27–47% in the 0.7 en% DHA group in comparison to 0.7 en% LnA. Increasing LnA to 2.0 en% was without effect. Providing DHA at the enhanced 2.0 en% did not significantly enhance the suppression of PG production. Placenta active MMP-2 and active MMP-9 (gelatinase) production was suppressed significantly by 30–43% in the 0.7 en% DHA group in comparison to the 0.7 en% LnA group, and 2.0 en% DHA did not enhance this suppression. Placenta collagenase activity comprising the sum of MMP-1, MMP-8 and MMP-13 was also suppressed by 60% in the 0.7 en% DHA diet group with no additional effect with 2.0 en% DHA provision. These results suggest that substituting DHA for LnA even at the current US n-3 fatty acid intake of 0.7 en% is effective in suppressing indices of premature delivery and shortened gestation. Increasing LnA intake by 3-fold to 2.0 en% is not effective. The form of dietary n-3 fatty acid, DHA vs. LnA, appears to be more important than the amount.

Keywords: n-3/n-6 ratio; Docosahexaenoic acid (DHA); Pregnancy; Prostaglandins; Matrix metalloproteinases (MMPs)

1. Introduction

Birth weight and gestational age at birth are critical determinants of infant mortality and morbidity, and in the United States, preterm birth resulting in low birth weight comprises 6–10% of all births — approximately 300,000 per year [1,2]. Evidence from both human and animal studies suggests that essential fatty acids of the n-6 and n-3 series play important and modifiable roles in gestational duration [3]. Consumption of preformed n-3 long-chain polyunsaturated fatty acids (n-3 LC PUFA) has been shown to increase gestational duration and to decrease the incidence of premature birth in human studies [3]. In most of these human studies (referenced in [3]), the dose of n-3 LC PUFA was appreciable, approximately 2.0–3.0 g day^{-1}, or about 1.0–1.5% of calories. However, a recent study has shown that daily consumption of an additional 100 mg of docosahexaenoic acid (DHA) during the last trimester of pregnancy significantly increased gestational duration by 6 days [4]. The mechanisms proposed by which n-3 LC PUFA may prolong gestation have focused on prostaglandin (PG) E_2 and $PGF_{2\alpha}$ production since they mediate cervical maturation and uterine contractions [3]. In both preterm and term delivery, PG production increases at labor [5], and this increase is mediated by cyclooxygenase-2 (COX-2) expression [6]. Our studies in pregnant women [7] have also shown that women delivering prematurely had markedly

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elevated arachidonic acid (AA) and linoleic acid (LA) pools in both erythrocyte membranes and plasma phospholipids and increased erythrocyte membranes n-6 docosapentaenoic acid (n-6 DPA). The high n-6 fatty acid status of pregnant women who delivered prematurely could be due to low dietary n-3 intakes, an interpretation supported by the increased erythrocyte n-6 DPA content. Alternatively, high dietary intake of AA or preferential incorporation of n-6 PUFA could account for these findings. Decreased tissue n-3 PUFA may lead to an impairment of n-3 PUFA modulation of the AA cascade.

Remodeling of reproductive tissue occurs as parturition approaches by breakdown of extracellular connective tissue components. Cervix, uterus, placenta, and fetal membrane structure changes at parturition via this remodeling process [3]. Matrix metalloproteinases (MMPs) are a family of enzymes that mediate the breakdown of the extracellular matrix of connective tissue [8]. Production of MMPs and tissue inhibitors of metalloproteinases (TIMPs) mediates extracellular matrix breakdown [8]. MMP regulation occurs by enzyme expression (pro-MMP), activation of pro to active forms by proteolytic cleavage, and inhibition of enzyme activity by binding to TIMP [9]. MMP expression is up-regulated by inflammatory mediators including PGE_2 [10] and cytokines [8,9]. Pro-MMP are proteolytically activated to MMP forms in the extracellular matrix [9]. Urokinase plasminogen activator (uPA) is a key agent in the proteolytic activation of MMPs [8,9,11] by proteolytic cleavage of plasminogen to plasmin, which subsequently cleaves pro-MMPs to active MMPs. TIMP production is also stimulated by PGE, most likely through cytokine production [12]. Increases in amniotic total MMP-9 have been linked to membrane rupture in both term and preterm delivery [13,14].

Human diets in the United States provide 6–7% of calories as LA and only 0.7% of calories from n-3 PUFA, with 90% of this provided by linolenic acid (LnA) [15]. Several studies indicate that humans are not efficient in converting LnA to the functional n-3 LC PUFA such as DHA and eicosapentaenoic acid (EPA) [16,17]. The reasons for this inefficient conversion may be competition between the n-6 (LA) and n-3 (LnA) fatty acids for the Δ-5 and Δ-6 desaturase enzyme conversions [15,16], or suppression of Δ-5 and Δ-6 expression by high PUFA [18,19]. Thus, this study was designed to examine reproductive tissue PG and MMP production in rats fed with n-3 diets provided at the current US % calorie intake either as LnA or as DHA, and at an enhanced intake, while maintaining relatively constant dietary LA reflective of current US intakes [15].

2. Materials and methods

2.1. Animals

Animal procedures were approved by the Animal Care and Use Committee of the Colorado State University. Forty female Sprague–Dawley rats (VAF-virus antibody free), 176–200 g, were obtained from Charles River Laboratories (Wilmington, MA). Rats were individually housed at 37°C with a 12-h light/dark cycle. Animals were fed ad libitum with Harlan Teklad (Madison, WI) stock rodent diet with free access to water. After 1 week, animals were time-mated with proven breeder Sprague–Dawley VAF males (Charles River Laboratories), with mating established by the presence of a cervical plug 2 h after the females were placed in the males' cages [20]. Following mating, animals were assigned to one of four experimental diets, which were provided ad libitum until Day 20 of gestation.

2.2. Diets

Rats were fed ad libitum with one of four modified American Institute of Nutrition AIN-93G diets [21] from the day of mating until Day 20 of gestation (Table 1). The four diets differed in oil composition and fatty acid content only (Table 1). The fat component of all diets was 7% by weight. The n-3 and n-6 polyunsaturated ratio and the LA, LnA and DHA content of each of the diets was achieved by using mixtures of olive, corn, flaxseed and DHASCO (Martek, Columbia, MD) oils (Table 2). All oils were analyzed by gas chromatography (GC) to establish their fatty acid composition. Diet components were obtained from Dyets (Bethlehem, PA) and DHASCO oil from Martek. Rat food intakes were measured at Day 8 and Day 16 of feeding, and rat weights were measured at Day 20 of gestation. Reagents and materials for gel electrophoresis and ELISA assays were obtained from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA), Chemicon International (Temecula, CA), Bio-Rad Laboratories (Hercules, CA) and Calbiochem-Oncogene Research Products (Boston, MA).

2.3. Tissue collection

On Day 20 of gestation, rats were killed using halothane euthanasia, and multiple samples of uterus and placenta, approximately 300 mg, were excised and stored at −70°C. For PG analysis, uterus and placenta samples were used immediately.

2.4. Prostaglandin analysis

Uterus and placenta samples were homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 9 volumes

Table 1
Composition of the AIN-93G diet

Ingredient	g kg^{-1}
Cornstarch	397.5
Casein (high nitrogen)	200.0
Dextrins	132.0
Sucrose	100.0
Oil component	70.0
Fiber (cellulose)	50.0
Mineral mix (AIN-93G)	35.0
Vitamin mix (AIN-93)	10.0
L-Cystine	3.0
Choline bitartrate	2.5

Table 2
Formulation (g oil 100 g^{-1} diet) and fatty acid composition (weight % and en%) of diets

	0.7 en% LnA	2.0 en% LnA	0.7 en% DHA	2.0 en% DHA
(A) Formulation — g of oil 100 g^{-1} diet)				
Soybean oil	4.2	0	0	0
Flaxseed oil	0	1.6	0	0
Corn oil	1.0	4.0	4.0	4.6
Olive oil	1.8	1.4	2.2	0
DHASCO oil	0	0	0.8	2.4
Total	7.0	7.0	7.0	7.0
(B) Fatty acid composition of diets — weight % and en%				
LA	3.14 (6.29)	2.86 (5.71)	2.71 (5.41)	2.82 (5.63)
LnA	0.38 (0.76)	1.04 (2.07)	0.06 (0.11)	0.05 (0.09)
DHA	0	0	0.36 (0.72)	1.07 (2.15)
n-6/n-3 ratio	8.2	2.8	6.6	2.5

Values in parentheses are en%.

of 50 mM potassium phosphate, 84 mM sodium chloride, 5 mM EDTA, pH 7.4, buffer. Homogenates were incubated at 37°C, and at 5 and 10 min, aliquots were removed, PG production stopped by addition of 0.5 volumes of 43 mM aspirin prepared in homogenization buffer and clarified by centrifugation. Supernatant PGE_2 and $PGF_{2\alpha}$ were determined by double-antibody radioimmunoassay [22].

2.5. Fatty acid analysis

Uterus and placenta lipids were extracted in chloroform/methanol (2:1, v/v) and fatty acid methyl esters prepared by BF_3 and methanol treatment. Hexane extracts of fatty acid methyl esters were separated by GC on a 30 m×0.25 mm×0.2 μm film thickness SP-2380 column (Supelco, Supelco Park, PA) using an Agilent (Palo Alto, CA) model 6890 GC equipped with autosampler, Chemstation and flame ionization detection.

2.6. Measurement of pro and active forms of MMP-2 and MMP-9

Frozen uterus and placenta samples were homogenized in 50 mM Tris, 0.138 M sodium chloride, 0.25% Triton X detergent, pH 7. Pro and active forms of MMP-2 and MMP-9 (the gelatinases) were measured by zymography electrophoresis using gelatin-imbedded polyacrylamide gels originally designed for human MMP analysis [23,24] using the NOVEX (Novel Experimental Technology, San Diego, CA) gelatin zymography system. Homogenized aliquots, 5–10 μl, mixed with 10 μl buffer, 0.5 M Tris–HCl (pH 7.0), 20% glycerol, 4% SDS (w/v) and 0.005% bromophenol blue were loaded into each well. Samples underwent electrophoresis on a 10% Tris–glycine acrylamide gel, containing 0.1% gelatin as a substrate, under nonreducing conditions at 125 V for 90 min at room temperature using 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, running buffer. The gels were then removed and washed in renaturing buffer (Triton X-100, 2.5% v/v in water) for 30 min followed by overnight incubation at 37°C while in developing buffer [50 mM Tris, 0.2M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 (w/v), pH 7.6]. Pro and active MMP were visualized by staining the gels with 0.5% (w/v) Coomassie blue R250 in a 40% ethanol, 10% glacial acetic acid solution for 3 h. All gels were then destained in deionized water. One nanogram of pro and active human MMP-2 and nine standards were run on each gel as positive controls (Calbiochem-Oncogene Research Products). All gels were analyzed wet using a densitometer (Molecular Dynamics, Sunnyvale, CA), with associated software. For production of pro and active MMPs, the optical density value of each MMP band was compared to the optical density of the human MMP standard band.

2.7. Collagenase activity assay

Analysis of uterus and placenta homogenate Type I collagenase was performed using a Type I Collagenase Activity Assay Kit (Chemicon International). This assay measures the activity of MMP-1, MMP-8 and MMP-13, collectively known as Type 1 collagenases. Duplicate aliquots of uterus and placenta homogenates, and standards generated with MMP-1 positive control, were incubated (37°C, 2 h) with 100 μl of biotinylated collagenase substrate in 96-well microtiter plates. Following incubation, 100 μl of samples and standards were transferred to a separate biotin-binding plate, incubated with streptavidin conjugated with horseradish peroxidase, and optical density measured at 450 nm following substrate addition using an automated plate reader.

2.8. Urokinase plasminogen activator

Analysis of uterus and placenta homogenate uPA was performed with the uPA Activity Assay Kit (Chemicon International). To a 96-well plate, 10 μl of samples and rehydrated uPA positive control standards were added in duplicate. Following the addition of assay buffer, substrate and incubation absorbance was read at 405 nm by a plate reader system.

2.9. Measurement of TIMP-2

Uterus and placenta homogenate tissue inhibitor of MMP-2 (TIMP-2) analysis was conducted by ELISA immunoassay [25] using a TIMP-2 ELISA assay kit (Calbiochem-Oncogene Research Products). Duplicate 100 μl of sample and standards were added to precoated 96-well plates and incubated at 23°C for 2 h. Washed plates were incubated with 100 μl of tetra-methylbenzidine/hydrogen peroxide in 20% dimethylformamide. The absorbance was read at 630 nm using an automated plate reader system.

2.10. Protein measurements

Homogenate cytosolic protein was determined by the Bradford [26] dye binding assay using bovine serum albumin as the standard.

2.11. Statistical analysis

Data are reported as mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS for Windows (SPSS, Chicago, IL). Comparisons between treatment groups were determined using the least significant difference (LSD) method when ANOVA results were statistically significant ($P<.05$). In cases were there was unequal sample variance, ANOVA was performed using $\log_{10}$ transformed data.

3. Results

Growth of rats was normal, and there were no significant differences in the weight of rats, or in food consumption, for the rats fed with the four diets throughout the study (data not shown). Final rat weights at 20 days' gestation were 300–319 g with no significant differences between diet groups. Rat pup numbers averaged 12 per dam, with no significant differences between diets.

3.1. Uterus and placenta fatty acids

The percentage of n-3 and n-6 fatty acid composition of uterine and placental total lipids, LA, DHA, and AA is shown in Table 3. In both placenta and uterus there was no significant difference in LnA and EPA between diet groups. Since the lipids of uterus and placenta are primarily membrane phospholipids, with little triglyceride (data not shown), these n-3 and n-6 fatty acid profiles primarily reflect the membrane phospholipid composition. Increasing dietary LnA from 0.7 energy % (en%) to 2.0 en% did not affect uterus LA, but in the placenta there was a small, but significant, increase in tissue LA of 11%. With the DHA diets there were again minor effects on uterus and placenta LA. DHA-containing diets, particularly the 2.0 en% DHA diet, significantly depressed the percentage of AA in uterus and placenta by 9–38%. However, the 2.0 en% LnA diet did not significantly alter uterus and placenta AA. Diets containing 2.0 en% LnA significantly increased the percentage of DHA in uterus by 71%, but in the placenta there was no significant difference. As expected, DHA diets significantly increased both uterus and placenta DHA by 160–180% for the 0.7 en% DHA diet, and by 250–350% for the 2.0 en% DHA diet in comparison to the 0.7 en% LnA and 2.0 en% LnA diets, respectively.

Table 3
Uterus and placenta percentage of n-3 and n-6 fatty acids (mean±S.E.M.)

Diet en%	0.7 en% LnA	2.0 en% LnA	0.7 en% DHA	2.0 en% DHA
Uterus				
LA	12.2±2.5^{a,b}	9.3±0.9^{a,b}	7.6±0.5^{b}	11.3±1.4^{a}
LnA	0.6±0.3	1.1±0.4	1.1±0.5	0.7±0.4
EPA	1.1±0.4	1.2±0.6	1.2±0.6	0.9±0.3
DHA	2.4±0.4^{a}	4.1±0.3^{b}	6.3±0.3^{c}	8.5±0.3^{d}
AA	14.1±2.3^{a,b}	16.0±0.7^{a}	15.6±1.2^{a}	12.9±1.1^{b}
Placenta				
LA	12.8±0.2^{a}	14.3±0.5^{b}	12.8±0.3^{a,b}	13.6±0.6^{a,b}
LnA	1.4±0.3	1.3±0.2	1.2±0.3	1.5±0.2
EPA	0.5±0.3	0.5±0.2	0.5±0.4	0.7±0.2
DHA	2.4±0.6^{a}	3.7±1.5^{a,b}	6.7±0.1^{b}	10.7±0.4^{c}
AA	17.5±0.9^{a}	16.0±0.5^{a,b}	14.1±0.4^{b}	10.9±0.7^{c}

$n=8$–10 per group. Data were analyzed by one-way ANOVA. Comparisons between treatment groups were determined using the LSD method when ANOVA results were statistically significant ($P<.05$). Values with different letter superscripts are significantly different ($P<.05$).

3.2. Uterus and placenta PG production

Preliminary experiments established that PGE_2 and $PGF_{2\alpha}$ production rates by both uterus and placenta homogenates were linear from 2 to 15 min ($r^2 \geq .95$, $P<.001$).

In the uterus, increasing dietary LnA from 0.7 to 2.0 en% did not significantly decrease either PGE_2 or $PGF_{2\alpha}$ production rates (Table 4). Substituting DHA for LnA at 0.7 en% significantly reduced both uterus PGE_2 and $PGF_{2\alpha}$ production rates by approximately 50% in comparison to 0.7 en% LnA. When DHA was provided at 2.0 en% the suppression of both uterus PGE_2 and $PGF_{2\alpha}$ production rates was approximately 60% in comparison to 0.7 en% LnA. However, there were no significant differences in either PGE_2 or $PGF_{2\alpha}$ production rates by uterus between the 0.7 en% and 2.0 en% DHA diets.

As expected, placenta PGE_2 production rate was similar to that of uterus while uterus $PGF_{2\alpha}$ production was greater than that of placenta [27]. Placenta PGE_2 production was significantly reduced by approximately 40% with 2.0 en% DHA, but not with 0.7 en% DHA in comparison to 0.7 en% LnA diet. Placenta $PGF_{2\alpha}$ production was not significantly altered by diet.

3.3. Matrix metalloproteinase production

Significant effects of DHA-containing diets were found only for production of active forms of MMP-2 and MMP-9 in the placenta. Both placental active MMP-2 and active MMP-9 production were significantly reduced by approximately 25–40% by both 0.7 en% DHA and 2.0 en% DHA diets in comparison to 0.7 en% LnA diet (Table 4). There were no significant differences in placental active MMP-2 and MMP-9 production between the 0.7 en% and 2.0 en% DHA diets. Furthermore, placenta active MMP-2 and MMP-9 production with 2.0 en% LnA diet was not significantly different from either the 0.7 en% LnA diet or both of the DHA diets (Table 4). There were no significant diet effects (P value for ANOVA $F>.05$) for placenta pro-MMP-2 and pro-MMP-9, and for both the pro and active forms of MMP-2 and MMP-9 in uterus (Table 4).

3.4. Collagenase activity

The collagenase activity assay measures the combined activity of MMP-1, MMP-8 and MMP-13 (collectively the Type 1 collagenases) by ELISA analysis of the cleavage products of biotinylated collagen substrate. Placental collagenase activity was significantly depressed 50–60% by 2.0 en% LnA and by both the 0.7 en% and the 2.0 en%

Table 4
Uterus and placenta PG and MMP production (mean±S.E.M.)

	Diet fatty acid and en%			
	LnA		DHA	
	0.7	2.0	0.7	2.0
Placenta PGE_2 (pg mg pro^{-1} min^{-1})	104.7±13.6[a]	104.5±14.6[a]	76.9±12.8[a,b]	62.6±5.4[b]
Uterus PGE_2 (pg mg pro^{-1} min^{-1})	172.4±37.8[a]	123.6±20.2[a,b]	91.0±11.1[b]	70.3±12.2[b]
Placenta $PGF_{2\alpha}$ (pg mg pro^{-1} min^{-1})	66.6±8.9	63.0±7.8	65.4±12.1	52.8±7.0
Uterus $PGF_{2\alpha}$ (pg mg pro^{-1} min^{-1})	379.8±81.3[a]	274.0±32.5[a,b]	201.0±26.5[b]	158.4±48.6[b]
Uterus collagenase activity (ng μg pro^{-1})	345.9±41.0	254.6±30.5	292.5±36.5	386.0±42.6
Placenta collagenase activity (ng μg pro^{-1})	48.4±8.9[a]	23.7±3.9[b]	19.2±7.2[b]	19.4 ± 3.8[b]
Placenta pro-MMP-2 (ng mg pro^{-1})	9.7±0.7	9.7±1.3	8.8±1.0	8.0±0.5
Placenta active MMP-2 (ng mg pro^{-1})	5.3±0.7[a]	4.2±0.7[a,b]	3.0±0.3[b]	3.7±0.3[b]
Placenta pro-MMP-9 (ng mg pro^{-1})	5.6±0.6	6.0±0.5	5.9±0.6	5.2±0.7
Placenta active MMP-9 (ng mg pro^{-1})	2.6±0.3[a]	2.5±0.3[a,b]	2.0±0.2[b]	1.9±0.1[b]
Uterus pro-MMP-2 (ng mg pro^{-1})	8.6±1.5	10.5±1.4	8.2±1.0	6.9±0.9
Uterus active MMP-2 (ng mg pro^{-1})	5.5±0.7	6.6±0.8	6.0±0.7	4.8±0.4
Uterus pro-MMP-9 (ng mg pro^{-1})	2.2±0.4	1.8±0.3	1.7±0.2	1.2±0.1
Uterus active MMP-9 (ng mg pro^{-1})	2.2±0.4	2.5±0.5	2.8±0.3	1.8±0.2
Placenta TIMP-2 (ng mg pro^{-1})	0.8±0.1	1.0±0.2	0.7±0.1	0.9±0.2
Uterus TIMP-2 (ng mg pro^{-1})	1.2±0.2	1.1±0.2	0.9±0.2	1.1±0.1
Placenta uPA (ng mg pro^{-1})	2.9±0.2	2.8±0.2	2.9±0.2	2.6±0.2
Uterus uPA (ng mg pro^{-1})	3.2±0.2	3.3±0.3	2.8±0.2	3.1±0.2

n = 8–10 per group. Data were analyzed by 1-way ANOVA. Comparisons between treatment groups were determined using the LSD method when ANOVA results were statistically significant (P <.05). Values with different letter superscripts are significantly different (P <.05).

DHA diets in comparison to the 0.7 en% LnA diet (Table 4). There were no significant differences in placental collagenase activity between the 2.0 en% LnA and either of the DHA diets. Uterus collagenase activity was not influenced by diet (P value for ANOVA F >.05).

3.5. TIMP-2 and uPA activity

Despite differences in active MMP-2 and active MMP-9 production in placentae, there were no significant dietary effects on either placenta or uterus TIMP-2 and uPA activity.

4. Discussion

The formulation of the diet that included n-3 and n-6 PUFA was designed to address the significance of dietary n-3 form (LnA vs. DHA) and amount (0.7 and 2.0 en%) in altering rat reproductive tissue indices of gestational duration. The rat is considered to be a more effective converter of LnA to DHA and of LA to AA than humans [28–31], most probably due to higher Δ-6 and Δ-5 desaturase expression in the rat [28]. Consequently, we chose to compare the effects of dietary LnA and DHA in a model unlikely to give a false-positive result using the approach of human equivalent dietary amount (allometric scaling). We based this approach on the recent data of Jones and Whelan [32] where they found that the percentage of total diet calories (en%) provided by n-3 fatty acids was the best method to extrapolate intakes of n-3 fatty acids from rodents to humans. This en% allometric scaling approach proved to be much superior to the metabolic-size approach of Kleiber [33] where the dose of n-3 fatty acids is adjusted based on metabolic size expressed as body weight raised to the 0.75 power.

The conversion of LnA to DHA is achieved by desaturation and elongation steps, with the rate-controlling step in this process being the Δ-6 desaturase that converts LnA to stearidonic acid, and which also converts n-3 DPA to DHA [34]. Since typical human diets provide 6–7% of calories as LA, and only 0.7% of calories as n-3 PUFA with 90% of this provided by LnA [15], the conversion of LnA to DHA has been suggested to be impaired by LA competition [15–17], thus inhibiting DHA production. More recently, it has been shown that high intakes of PUFA inhibit the expression of both the Δ-6 and Δ-5 desaturases involved in DHA production from LnA [18,19].

Several studies in humans have shown that LnA conversion to DHA is small. Brenna [16] has estimated that whole body conversion of LnA to DHA in humans is less than 1.0%. Pawlosky et al. [17] have estimated that less than 0.2% of plasma LnA is converted to EPA and only 23% of this EPA is converted to DHA. A study by Li et al. [35] have examined both diet n-3/n-6 ratios and amount of dietary LnA on tissue LC n-3 fatty acids in vegetarian men. Varying the intake of LnA from a low intake of 0.4 en% to either a moderate intake of 2.0 en% or a high intake of 6.3 en%, regardless of the dietary n-3/n-6 ratio that varied between 1:1.1 and 1:40, had no effect on platelet and plasma phospholipid DHA concentration [35]. Increasing dietary LnA to the moderate or high levels did significantly increase plasma and platelet phospholipids EPA, but not DHA. Short-term supplementation of vegan diets with 2.0 g day^{-1} LnA, resulting in a reduction of the n-6/n-3 ratio from 13.7 to 6.8,

produced no changes in DHA concentrations in red blood cell, platelet, plasma phospholipids, plasma cholesterol esters and plasma triglycerides [36]. These human data suggest that increasing dietary LnA is ineffective in increasing tissue DHA, the functional end product of LnA metabolism.

In this study, we constructed diets with approximately constant LA (range, 5.4–6.3 en%) approximating the current US diet intake of LA [15], with two levels (0.7 and 2.0 en%) and two forms (LnA and DHA) of dietary n-3 fatty acids. We chose 0.7 en% since this is the current US diet intake of n-3 fatty acids, with 90% of this intake provided by LnA. In addition, we chose an enhanced but achievable 2.0 en% intake (equivalent to 4.4 g n-3 fatty acids per day for humans consuming a 2000-calorie diet) in order to examine the influence of increasing dietary n-3 fatty acid amount. The rat diets contained 14 en% from lipid, which is lower than the typical 28–36 en% of human vegan, lacto-ovo-vegetarian, and omnivore diets [37].

We have shown that substituting 0.7 en% DHA for either 0.7 en% LnA or 2.0 en% LnA is more effective than 2.0 en% LnA in increasing uterus and placenta DHA concentrations (Table 3). Similarly, 2.0 en% DHA is even more effective (Table 3). Furthermore, these rat studies were conducted with relatively constant dietary LA, which ranged from 5.4 to 6.3 en%, values close to the LA content of typical human diets [15]. Although 2.0 en% LnA increased DHA in uterus, but not in placenta, it was not as effective as DHA. DHA decreased AA in uterus and placenta, particularly when provided at 2.0 en%.

Numerous studies have implicated eicosanoids in the regulation of gestational length, parturition and the initiation of labor [3,5,6]. PGE_2, $PGF_{2\alpha}$, LTC_4 and LTB_4 are elevated in the maternal circulation prior to the onset of spontaneous labor, and exogenous administration of either PGE_2 or $PGF_{2\alpha}$ induces cervical ripening, uterine contractions and emptying during both full-term and preterm labor [3,5,6]. Both PGE and PGF analogs are used in pharmacologic preparations to promote cervical dilation and to induce labor contractions.

PG synthesis increases during parturition at term, as evidenced by increased PGE_2 and $PGF_{2\alpha}$ and their metabolites in the amniotic fluid and in the peripheral circulation during labor [6]. Sadovsky et al. [6] demonstrated that selective inhibitors of COX-2 are effective in diminishing PGE_2 levels in amniotic tissue and thus may be useful in preventing preterm deliveries. The enhanced amniotic COX-2 expression was associated with 2- to 5-fold increases in PGE_2 production in all laboring women compared with nonlaboring women [6].

Since n-3 LC PUFAs are known to influence PG production, either by competition with AA for incorporation into phospholipid pools [38] or by suppressing COX-2 expression [39–41], we examined uterus and placenta PG production rates (Table 4). Both 0.7 and 2.0 en% DHA significantly depressed uterus PGE_2, uterus $PGF_{2\alpha}$ and placenta PGE_2 production rates in comparison to 0.7 en% LnA diet. Increasing dietary LnA from 0.7 to 2.0 en% did not significantly alter PG production in comparison to the 0.7 en% LnA group. Despite a trend in placenta $PGF_{2\alpha}$ production (Table 4), the data were not significant. The decreases in uterus PG production broadly reflect the changes in uterus DHA and AA concentrations, and these data suggest that provision of dietary DHA, even at a modest 0.7 en % of calories, is more effective than increasing dietary LnA.

Studies have shown that PG stimulates MMP expression. PGE_2 stimulates the expression of MMP-2 in cultured rat mesangial cells [42] and cultured human pancreatic cancer cells [43] and increases release of MMP-2 and MMP-9 in fetal rat hepatocytes [44]. $PGF_{2\alpha}$ has been shown to increase pro-MMP-1 and pro-MMP-3 secretion by cultured human ciliary smooth muscle cells [45]. COX-2 induction and exogenous PGE_2 addition increase murine macrophage MMP-9 expression [46].

Recently, PG and MMP have been investigated as having interdependent roles in parturition [47–49]. PG has been shown to play a role in the up-regulation of MMP expression and activity during labor [13,14]. Thus, the possible reduction of the 2-series PGs by preformed LC n-3 fatty acids could ultimately reduce the activity of MMP that is associated with labor, specifically with preterm premature rupture of membranes (PPROM). Ulug et al. [47] assessed the effect of PG synthesis on the expression and activity of MMP-2 and MMP-9 and TIMP-1 in fetal membranes of women who delivered by cesarean section and prior to the establishment of regular uterine contractions or rupture of membranes. Ex vivo cultures of amnion, chorion, and deciduas were examined in the absence or presence of $PGF_{2\alpha}$ or the COX synthesis inhibitor, indomethacin. In the decidua, significantly more MMP-2, MMP-9 and TIMP-1 were produced in the presence of $PGF_{2\alpha}$. $PGF_{2\alpha}$ caused an 11.3-fold increase in the active form of MMP-2 in the decidua and decreased decidual TIMP-1 production by 70%. However, $PGF_{2\alpha}$ produced no significant changes in MMP-2, MMP-9 or TIMP-1 production in the amnion and chorion [47]. In our study, we found changes in placenta Type 1 collagenase activity (MMP-1, MMP-8 and MMP-13 activity) paralleled those for placental MMP-2 and MMP-9 expression, which supports the PG reduction interpretation. MMP-2 and MMP-9 have been examined for their involvement in the process of fetal membrane rupture because of their roles in the degradation of collagen types I, IV and V [8,9]. Uchide et al. [48] observed that the ratio of MMP-9 to TIMP correlated with tensile strength in fetal membranes. In general, MMP-2 has been shown to be present at relatively consistent values before, during and after labor in fetal membranes, while MMP-9 has been shown to increase with the onset of labor and then decline again after delivery [49].

In our study, placenta active MMP-2 and active MMP-9 (the gelatinases) expression were significantly reduced by

23–43% by both the 0.7 and 2.0 en% DHA diets in comparison to the 0.7 en% LnA diet. However, increasing dietary LnA to 2.0 en% did not alter placenta active MMP-2 and MMP-9 expression. Despite these changes, we found no significant diet effects on pro (latent)-MMP-2 or pro-MMP-9 expression. Since reductions in placental MMP were paralleled by reductions in placenta PGE_2, these results, and the findings of other studies, suggest that reductions in PG may mediate the decreased MMP expression. McLaren et al. [50] showed that PGE_2 plays a role in the up-regulation of cultured human fetal membrane MMP-9 production and indomethacin suppressed MMP-9 production. In a study of PGE_2 infusion in nonpregnant virgin, preterm and term pregnant, and postpartum rats, PGE_2 induced a marked increase in plasma MMP-2 in both preterm and term pregnant rats [51]. Uterine MMP-2 levels rose approximately 10-fold in preterm pregnant rats and approximately 5-fold in the term uterus in response to PGE_2 infusion [51]. For MMP-9 production, PGE_2 infusion markedly increased uterine levels in preterm pregnant rats and more modestly increased MMP-9 in the nonpregnant uterus, term pregnant uterus and postpartum uterus [51]. Cervical MMP-9 showed a similar response to PGE_2 infusion as the uterus [51].

Despite significant changes in uterus PGE_2 and $PGF_{2\alpha}$ we found no significant differences in uterus pro and active MMP-2 and MMP-9 expression and collagenase activity. However, uterus MMP expression and collagenase activity trended lower as PG production decreased.

Since MMPs are expressed in their pro forms and then proteolytically activated to their active forms by uPA [8,9,11], and PGE_2 is known to increase uPA expression in macrophages [46], we measured placenta and uterus uPA activity. There was no effect of diet on uterus and placenta uPA activity despite significant reductions in placenta and uterus PGE_2 and uterus $PGF_{2\alpha}$ production.

MMP activity is controlled at the levels of transcription, proteolytic activation of the zymogen form and by inhibitors such as TIMP [8,9]. MMP can be activated by other MMPs [9] as well as by serine proteases such as uPA [9,11]. TIMP-1 is inducible by PGE_2 and binds with MMP-9 [52]. We were unable to measure TIMP-1 since the human ELISA assay does not respond with rat tissue extracts. The TIMP-2 ELISA assay functions with a variety of animal tissue homogenates, including the rat. TIMP-2 is constitutively expressed and binds with MMP-2 and it has been shown that PGE_2 is without effect on human uterus TIMP-2 production [51]. In our study, we found no significant diet effects on either uterus or placenta TIMP-2 production. The significant decreases observed in placenta active MMP-2 and MMP-9 production and placental collagenase activity were not associated with changes in either uPA or TIMP-2 production. Placental MMP decreases were paralleled by decreases in placental PGE_2 production, and PGE_2 is known to mediate MMP expression [10]. However, we cannot exclude the possibility that serine proteases other than uPA, or TIMP-1 expression, could account for these decreases in MMP.

In summary, this study suggests that a modest amount of DHA, included in diets at the expense of the current US diet intake of LnA, is effective in reducing Day 20 pregnant rat reproductive tissue PG and MMP production. These results suggest that DHA may be an effective nutrient in lengthening gestation and reducing premature delivery, and provides some insight into its potential mode of action. Furthermore, increasing LnA 3-fold from the current US dietary intake is not effective in reducing reproductive tissue indicators of shortened gestation. The form of dietary LC n-3 fatty acid (DHA vs. LnA) appears to be more important than the amount.

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