Attenuation of Mammary Duct Development by Menhaden Oil in BALB/c Mice (43184)

S. ABRAHAM,* L. J. FAULKIN,† AND D. J. MITCHELL†

Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center of Northern California, Oakland, California 94609 and Department of Anatomy,† School of Veterinary Medicine, University of California, Davis, California 95616*

Abstract. **The predominant polyunsaturated fatty acids of the *n*-6 family found in corn oil (CO) are crucial for normal mammary duct formation when fed to animals. However, as shown here, not all polyunsaturated fatty acids are equally effective in stimulating mammary gland development. The *n*-3 fatty acids in a 10% menhaden oil (MO) diet fed to mice effectively reduced both the diameter and the length of the growing mammary ducts. Previously, we demonstrated a similar reduction in duct growth by feeding a 10% fat diet high in those saturated fats found in hydrogenated cotton seed oil. The inhibited rate of duct maturation caused by hydrogenated cotton seed oil was reversed when the mice were allowed to mature on a diet containing *n*-6 fatty acids prior to feeding the saturated fat diet. The addition of 1% CO to a 9% hydrogenated cotton seed oil diet fed to immature mice was also sufficient to restore duct growth. Mice fed menhaden oil diets, on the other hand, continued to show impaired ductal growth well into adulthood. Examination of the ovaries from MO-fed mice as compared with CO-fed mice revealed significantly fewer *corpora lutea*. When exogenous progesterone was given to MO-fed mice, ductal growth was partially restored, but not to the extent seen in mice fed corn oil diets. Investigation of the fatty acid contents of livers of these mice revealed reduced amounts of arachidonate (20:4) in MO-fed mice when compared with CO-fed animals. The addition of 1% CO to the 9% MO diets did not alter the arachidonate content, indicating a block in the conversion of linoleate (18:2) to 20:4 by the *n*-3 fatty acids. Hence, dietary *n*-6 fatty acids are essential for normal mammary ductal development when fed prior to maturation. Although saturated rats are ineffective, *n*-3 fatty acids can partially substitute for the required *n*-6 fatty acids in both ductal and ovarian development.** [P.S.E.B.M. 1991, Vol 196]

Earlier studies showed that mammary ducts grew more rapidly in young mice fed diets containing polyunsaturated fatty acids of the *n*-6 family (linoleate) than in those fed diets with monounsaturated or saturated fatty acids (1, 2). Diets containing corn oil (CO) as the only fat accelerated the growth of normal mammary epithelium in intact pubescent mice above that found in mice fed diets high in the saturated fatty acids of hydrogenated cotton seed oil (HCTO) (3). Similar observations were made with transplanted pieces of mammary ducts growing in cleared fat pads of weanling mice. Since this enhanced rate of mammary growth with polyunsaturated fat diets could be retarded when indomethacin (a cyclooxygenase inhibitor) was added to the diet, we suggested that prostaglandin production was involved in this process (1, 2).

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Examination of the ovaries from mice fed HCTO diets revealed that normal development of mature follicles and their subsequent transformation to *corpora lutea* were inhibited, which should have resulted in reduced progesterone production. On the other hand, the ovaries in those mice fed diets containing the *n*-6 polyunsaturated fatty acid (CO) had a normal appearance (3). These data led us to conclude that the observed effect of dietary fat on the developing mammary gland was mediated by the ovary (3). This conclusion was substantiated when we examined the influence of dietary fat on the growth of normal ducts transplanted into cleared fat pads of female mice which were mature when the diets were fed. Such duct transplants grew at similar rates in both the CO and the HCTO diet groups (3).

In this report we present data from a series of experiments which compare the influence on mammary gland growth of another polyunsaturated fat, menhaden oil (MO) extracted from menhaden fish, which is rich in the fatty acids of the *n*-3 family. These

new results demonstrate that not all polyunsaturated fatty acids are equally effective in stimulating duct growth. Indeed, the *n*-3 (those in MO) and *n*-6 (those in CO) families act quite differently with respect to mammary gland development. A preliminary report containing some of the data has already appeared (4). Subsequently, Welsch and O'Connor (5), in an extensive investigation of the influence of dietary fat on the development of mammary glands in female BALB/c mice, demonstrated that fish oil (MO) significantly reduced such growth in both immature and mammotrophic hormone-treated mature mice. In those studies, menhaden oil only suppressed gland development in mice whose mammae were in a state of intense proliferation and not in those possessing relatively quiescent mammary glands.

Materials and Methods

Animals and Diets. The mice used were BALB/c females from our breeding colony at Children's Hospital, Oakland, CA. They were held three or four to a cage and allowed access to food and water *ad libitum*. They were housed in a temperature-controlled room (22–23°C) on a 12-hr light-dark cycle.

The stock diet was Purina Laboratory Chow no. 5020 that contained 9% fat, of which 44% of the constituent fatty acids was linoleate (2). According to the manufacturer, this diet was composed of all natural ingredients, most of which were unspecified.

All test diets were based on a purified fat-free dry mixture (6) that contained, by weight, 60% glucose, 20% vitamin-free casein, 3.5% mineral mixture as described in the AIN recommendation of 1976, 1.0% vitamin mixture (AIN vitamin mixture 76A; Nutritional Biochemical, Cleveland, Ohio), 0.3% DL-methionine, 5% cellulose, 0.2% choline chloride, and 0.01% butylated hydroxytoluene. Enough diet to last several days was prepared by the addition of 10 g of either HCTO, CO, MO, or a specified combination of these oils to each 90 g of fat-free dry mixture. These 10% fat test diets were isocaloric and differed only in their fatty acid compositions. The test diets were stored in closed containers at 4°C and fresh diet was prepared twice a week. The fatty acid compositions and the commercial sources of the HCTO, CO, and MO used have been reported previously (7).

Tissue Preparations. The procedures used for clearing the inguinal mammary fat pads of ductal elements and the implantation of pieces of normal ductal tissue taken from virgin mice have been described earlier (8, 9). At the conclusion of the experimental period, mice were anesthetized with pentobarbital and the inguinal fat pads were removed. The fat pads were fixed in Tellyesniczky's fixative, defatted in acetone, stained with iron hematoxylin, and stored for examination in methyl salicylate (10). Morphologic observations were made using either a dissecting microscope, with which the percentage of the total fat pad filled with outgrowths was estimated to the nearest 10%, or a confocal microscope which was used to accurately quantitate duct size. This latter instrument (Bio-Rad Laboratories, Richmond, CA) employs a computer to generate planar images of whole-mount preparations of the inguinal mammary glands from which direct size measurements were made. Three of the largest ducts found at the nipple or proximal end of the gland were each measured at 10 different sites and compared with the diameter of the three largest ducts found at the growing, or distal, end of the gland. Six mammary glands from each diet group were measured, for a total of 180 determinations per site per diet group.

When required, the ovaries were removed, paraffin-embedded, and sectioned at 6 μm for microscopic examination. One medial section, which included the hilus of each ovary, was examined and the number of primary, secondary, and mature follicles, as well as corpora lutea and *corpora albicans* were counted. The representative section technique does not detect all of the listed structures in the ovary, but it does allow for a comparison of the major ones present in the ovaries taken from mice fed the different diets.

Biochemical Procedures. In some experiments, the liver was also removed after the animals were euthanized. A portion of the excised liver (0.5 g) was rinsed with 0.9% sodium chloride solution and the lipids were saponified by refluxing with 15% KOH in 50% aqueous methanol containing 0.01% hydroquinone. The nonsaponifiable lipids were then removed and the total free fatty acids were isolated according to previously published procedures (11). Portions of the free fatty acids were esterified with diazomethane and analyzed by gas chromatography.

The fatty methyl esters in hexane were automatically injected into a gas chromatograph (model 95A; Shimadzu Scientific Instruments, Columbia, MD) equipped with a 30-m × 0.025-mm fused silica column (model SP2330; Supelco, Bellefonte, PA) and a flame ionization detector. The carrier gas was helium and the column temperature program was 1 min at 100°C, 20°C/min to 170°C, 5 min at 170°C, 10°C/min to 240°C, and 8.5 min at 240°C. A variety of methyl ester standards, standard mixtures, and standards synthesized using published procedures were used to identify peaks based on their equivalent chain lengths calculated from the gas chromatography retention times. Quantitation of the methyl ester peaks was performed by a computer and was based on peak areas adjusted for the FID response (12). The value for each fatty acid is given as a percentage of the total fatty acids present in the liver.

Statistical Analysis. Comparisons between means were made with the use of the Mann-Whitney *U* rank

order test (13). Only those differences which had a probability of 0.05 or less were considered to be significant.

Results

In the initial series of experiments, groups of 3-week-old female BALB/c mice were weaned directly onto the special diets containing 10% fat (Table I). Each group of mice was allowed free access to the isocaloric diets which contained either (i) HCTO, CO, or MO as the sole lipid component or (ii) 1% corn oil with either 9% menhaden oil or 9% hydrogenated cotton seed oil. The animals were sacrificed after 3 or 6 weeks on their respective diets and the percentage of fat pads filled with ducts in both inguinal glands in each mouse was determined.

It is clear from the data presented in Table I that those diets which either do not contain linoleate (HCTO), or have small amounts of linoleate (MO), do not allow normal development of the ductal elements. This inhibition of ductal growth was marginally apparent after 3 weeks on the diets (at 6 weeks of age), when Groups B and C were compared with Group A. The difference in duct growth was clearly significant following the 6-week period of dietary treatment when the mice were 9 weeks of age (Table I). Hence, the observations and conclusions made with the 10% HCTO versus 10% CO diets reported previously (1–3) were confirmed. These data also show that not all polyunsaturated fatty acid-containing diets result in similar effects on mammary ductal growth. Those fatty acids of the *n*-3 family do not adequately substitute for those of the *n*-6 family in this regard (compare CO with MO). The diet containing 1% CO and 9% HCTO had no significant inhibiting effect on ductal growth after 3 weeks (Table I, compare Group E with Group A). The addition of 1% CO to 9% MO produced no detectable change in growth at 3 weeks (compare Group D with Group A), but when fed for 6 weeks it restored normal growth (compare D with C). In addition, the data demonstrate that the stimulatory effect of corn oil after 3 weeks, but not after 6 weeks of growth, can be observed with as little as 5% CO in the diet. The values observed with Group B versus Group E show that this small amount of linoleate (0.6% of the diet) is sufficient to produce significant growth enhancement.

In the next series of experiments, all of the female mice had the glandular elements surgically removed from the no. 4 fat pads immediately after weaning at 3 weeks of age. For the next 7 weeks, Group F was allowed free access to either the 10% CO or the 10% MO diet, and Group G was given a standard laboratory chow. When the animals were 10 weeks old, all mice were given transplants of normal mammary tissue, using the established surgical procedure. From that point until the end of the experiment, when the mice were 16 weeks old, they were maintained on the special diets as shown in Table II. The results of these experiments again clearly demonstrate that 10% fat diets composed of menhaden oil, which does not have more than 1.5% linoleate, will not sustain a similar rate of ductal growth as those which possess about 60% of this essential polyunsaturated *n*-6 fatty acid (corn oil). In addition, the data obtained in this experiment (Table II) show that, whereas in previous experiments this influence of saturated fatty acids on ductal growth was observed only in immature mice, the inhibiting effect of the *n*-3 fatty acids in menhaden oil was still observed when ductal growth was measured in mice after reaching maturity. Hence, these new data suggest that *n*-3 fatty acids might actually inhibit the action of endogenous *n*-6 fatty acids. This suggestion is supported by the results from the experimental data recorded in Table I for the period during 3–6 weeks (compare Group D and E). From previous experience with this system, in which we suggested the possible involvement of pros-

Table I. Effect of Dietary Fat on the Growth of Mammary Ducts in Weanling BALB/c Mice Fed Test Diets for 3 or 6 Weeks

Group	Dietary fat	Age (weeks) on diet	Body weight (g ± SD)	No. of fat pads	% Fat pad filled[a]	P^b			
						vs A	vs B	vs C	vs D
A	10% CO	3–6	18.8 ± 1.4	10	35 ± 14				
	10% CO	3–9	17.8 ± 1.0	20	93 ± 5				
B	10% HCTO	3–6	16.2 ± 0.5	10	19 ± 12	<0.01			
	10% HCTO	3–9	18.9 ± 0.9	12	76 ± 22	<0.01			
C	10% MO	3–6	17.9 ± 0.8	16	29 ± 8	NS	<0.02		
	10% MO	3–9	18.4 ± 1.0	20	70 ± 14	<0.01	NS		
D	1% CO + 9% MO	3–6	18.2 ± 0.8	22	27 ± 13	NS	<0.05	NS	
	1% CO + 9% MO	3–9	18.3 ± 0.7	20	89 ± 12	NS	<0.05	<0.01	
E	1% CO + 9% HCTO	3–6	17.7 ± 0.5	22	51 ± 21	<0.05	<0.01	<0.01	<0.01
	1% CO + 9% HCTO	3–9	20.8 ± 0.8	20	79 ± 10	<0.01	NS	<0.05	<0.01

[a] Percentage of inguinal mammary fat pad filled with duct growth ± SD.
[b] Probability determined by the Mann-Whitney *U* rank order test (13). NS, not significant.

Table II. Effect of Dietary Fat on Growth of Mammary Ducts Transplanted into Cleared Fat Pads of 10-Week-Old Adult Female BALB/c Mice

Experimental group	Dietary fat	Age (weeks) on diet	Body weight (g ± SD)	No. of fat pads	% Fat pad filled[a]	P^b
F	10% CO	3–16	18.2 ± 0.8	12	86 ± 24	
	10% MO	3–16	23.6 ± 1.2	12	64 ± 31	<0.025
G	10% CO	10–16	20.6 ± 1.9	16	98 ± 3	
	10% MO	10–16	25.6 ± 1.3	17	72 ± 34	<0.005

[a] Percentage of inguinal mammary fat pad filled with duct growth ± SD.
[b] Probability determined by the Mann-Whitney *U* rank order test (13).

Table III. Effect of Dietary Fat on Growth of Mammary Ducts Transplanted into Cleared Fat Pads of 10-Week-Old Adult Female BALB/c Mice with and without Progesterone Treatment

Experimental group	Dietary fat	Age (weeks) on diet	Progesterone treated	Body weight (g ± SD)	No. of fat pads	% Fat pad filled[a, b]
H	10% CO	8–14	No	21.5 ± 0.5	9	98 ± 4a
	10% CO	8–14	Yes	19.8 ± 1.5	10	97 ± 7a
	10% HCTO	8–14	No	19.0 ± 1.0	9	94 ± 10a
	10% HCTO	8–14	Yes	18.8 ± 1.5	10	100 ± 0a
	10% MO	8–14	No	24.9 ± 1.5	7	74 ± 19b
	10% MO	8–14	Yes	28.1 ± 1.5	10	91 ± 10a

[a] Percentage of inguinal mammary fat pad filled with duct growth ± SD.
[b] Probability determined by the Mann-Whitney *U* rank order test (13). Values with different letters are significant at the level of $P < 0.01$.

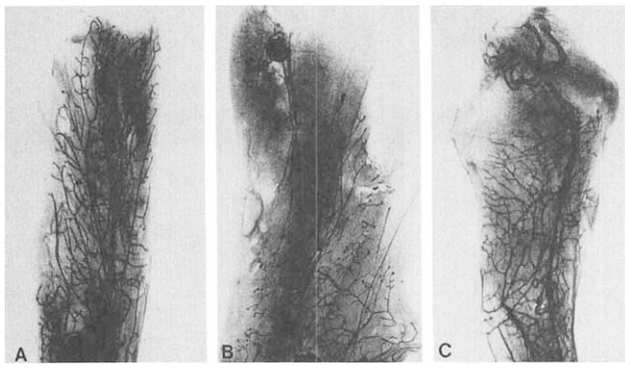

Figure 1. Whole mounts of mammary ducts transplanted into the inguinal mammary fat pads of BALB/c mice fed specific diets for 6 weeks. Treatments were: (A) Ten percent CO diet. (B) Ten percent MO diet. (C) Ten percent MO diet and mice treated with 1 mg of progesterone/day. Note the extensive branching in the hormone-treated group; however, duct diameters in these glands were smaller than those in the CO group (original magnification ×5).

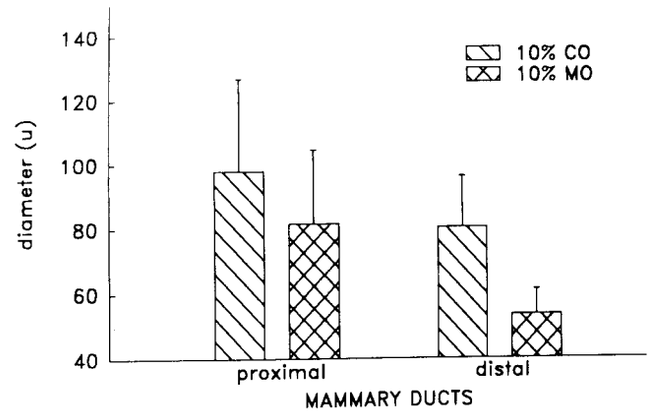

Figure 2. Comparison of mammary duct diameters from BALB/c mice fed diets supplemented with 10% CO or 10% MO. Ducts nearest the nipple end of the gland were termed proximal and those at the opposite end were termed distal. Values are given as averages of 10 sample diameters from each of the three largest ducts at both the proximal and distal ends of the fat pad. Six glands from each diet group were used.

taglandins (1–3), it appears that this inhibitory influence is located at the level of metabolism whereby linoleate is converted to arachidonate and subsequently, through the cascade of reactions, catalyzed by cyclooxygenase.

Results presented in Table II demonstrate that the fatty acids in menhaden oil do not sustain a rate of duct growth similar to that in mice fed corn oil. Regardless of the mechanism, the system is quite sensitive to the composition of the dietary fatty acids, since significant differences were not observed between the growth rate of ducts in mice placed on MO diets at 10 weeks of age and those placed on diets 7 weeks earlier.

In the next series of experiments, we sought to obtain evidence for the role played by hormones in these processes (Table III). At 3 weeks of age, these mice had both their no. 4 mammary fat pads cleared of glandular elements and were then allowed to mature until 8 weeks of age. At that time, when they had developed normal ovaries, all mice received a normal duct transplant and were then divided into three groups, each of which was either treated or not treated with

Table IV. Influence of Dietary Fat on Ovary[a] Histology in BALB/c Mice with or without Progesterone Treatment

Group	Dietary fat	Age (weeks) on diet	Progesterone treated	No. of follicles[b,c] as:			*Corpora lutea*[b,c]	*Corpora albicans*[b,c]
				Primary	Secondary	Mature		
A	10% MO	3–9	No	6.8 ± 4.3	7.7 ± 2.9	1.2 ± 1.1	0.4 ± 0.9	1.8 ± 0.6
B	10% MO	3–9	Yes	6.0 ± 3.2	9.6 ± 1.7	1.7 ± 1.9	0	1.8 ± 1.2
C	10% CO	3–9	No	8.5 ± 2.6	9.5 ± 2.3	1.3 ± 1.0	1.1 ± 0.7	0.6 ± 0.6
D	10% MO	8–14	No	5.5 ± 1.5	5.0 ± 1.5	3.3 ± 1.8	0.1 ± 0.3	1.3 ± 1.3
E	10% MO	8–14	Yes	4.6 ± 2.5	5.4 ± 2.9	0.8 ± 0.7	0.1 ± 0.3	2.0 ± 0.9
F	10% CO	8–14	No	6.8 ± 3.2	7.4 ± 2.1	3.2 ± 1.6	1.9 ± 1.8	1.2 ± 1.4

[a] A single ovary from each of 10 mice was examined for each diet group.
[b] The values are given as averages ± SD.
[c] Probability determined by the Mann-Whitney *U* rank order test (13): secondary follicles: A versus D, $P = 0.01$; B versus E, $P = 0.025$; *corpora lutea*: A versus C, $P = 0.025$; D versus F, $P = 0.01$; *corpora albicans*: A versus C, $P = 0.005$.

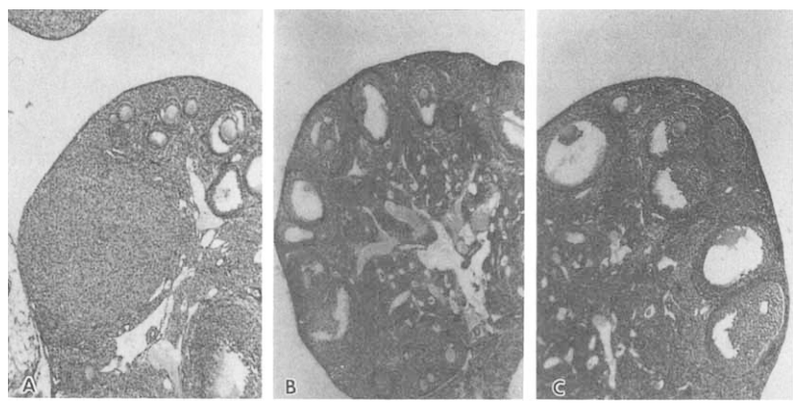

Figure 3. Histologic sections, cut at 6 μm, of ovaries taken from BALB/c mice fed either the 10% CO or the 10% MO diets. (A) CO diet showing normal development with a single, predominate *corpora lutea* and a progression of developing follicles. (B) Ovary from MO-fed mouse showing many large follicles but no *corpora lutea*. (C) Ovary from mouse treated with 1 mg of progesterone/day and fed MO diet showing few developing follicles and no *corpura lutea* (original magnification ×150).

Table V. Effect of Dietary Fat on the Number of Successful Pregnancies in BALB/c Females Housed with Male Mice from 16 to 18 Weeks of Age

Dietary fat	Age (weeks) started on diet	Body weight at 18 weeks (g ± SD)	No. pregnant/ no. mice	% Pregnant	Pups[a]/ pregnant dam
10% CO	3	23.6 ± 0.9	16/16	100	6.6
	8	24.7 ± 0.8	13/13	100	7.2
10% HCTO	3	22.1 ± 1.1	2/14	14	5.0
	8	24.1 ± 1.1	7/16	44	5.2
10% MO	3	22.9 ± 0.4	9/15	60	4.7
	8	25.7 ± 0.6	12/17	71	3.4

[a] Probability determined by the Mann-Whitney *U* rank order test (13). The number of pups was significantly different ($P < 0.05$) at 3 weeks for CO versus MO, and at 8 weeks for CO versus HCTO and CO versus MO.

hormone, as given in Table III. The mice that received hormone were injected with 3 mg of progesterone, dissolved in triolein, every third day during the subsequent 6 weeks of ductal growth while consuming these same diets. The number of animals in each group varied from 7 to 10, as given in Table III. As previously demonstrated in mature mice, diets which are simply devoid of linoleate have no inhibiting effect on ductal growth. However, if, in addition to the lack of linoleate, the diet contained *n*-3 fatty acids, ductal growth was significantly retarded. Furthermore, these data demonstrate that exogenous progesterone can overcome the down-regulation caused by these marine oils (Table III). Exogenous progesterone stimulated ductal development in mice fed the MO diet, filling from 90 to 100% of the pad. However, these ducts showed much less robust growth with fewer branches and thinner ducts than were seen in the CO-fed mice (Fig. 1). It is interesting to note that the differences observed between diets were more pronounced when comparing the diameters of the distal ducts than those of the proximal ducts. Such data (Fig. 2) suggest that the *n*-3 fatty acids

Table VI. Fatty Acid Composition of Livers of BALB/c Mice Fed Diets Containing Various Fats

Fatty acid	% Total fatty acids[a] in liver of mice fed:					
	10% HCTO (5)[b]	10% CO (3)	10% MO (5)	1% CO + 9% HCTO (3)	1% CO + 9% MO (3)	1% CO (3)
14:0	0.56 ± 0.09	0.39 ± 0.04	0.78 ± 0.25	0.33 ± 0.06	0.59 ± 0.14	0.22 ± 0.02
16:0	20.70 ± 0.55	20.56 ± 0.13	26.19 ± 0.95	21.44 ± 0.25	22.90 ± 1.62	21.32 ± 0.65
16:1 n-7	4.52 ± 0.25	1.78 ± 0.23	3.97 ± 0.99	2.93 ± 1.32	3.75 ± 0.18	3.68 ± 0.50
18:0	7.00 ± 1.46	8.46 ± 1.23	10.14 ± 1.45	9.19 ± 2.28	10.46 ± 0.64	7.67 ± 0.51
18:1 n-9	46.30 ± 2.93	24.69 ± 2.19	20.00 ± 3.97	31.41 ± 0.56	17.73 ± 0.28	32.2 ± 0.46
18:2 n-6	2.62 ± 0.19	22.18 ± 0.81	4.19 ± 1.44	9.40 ± 1.19	6.23 ± 1.15	13.46 ± 1.55
18:3 n-6	1.62 ± 0.25	0.71 ± 0.05	0.30 ± 0.03	—	—	—
18:3 n-3	—[c]	—	0.28 ± 0.12	0.69 ± 0.23	0.43 ± 0.16	0.80 ± 0.23
20:0	—	0.27 ± 0.03	—	0.05 ± 0.01	0.34 ± 0.09	0.21 ± 0.06
20:2 n-9	0.52 ± 0.06	0.83 ± 0.01	0.31 ± 0.09	0.15 ± 0.02	—	0.29 ± 0.09
20:3 n-9	4.60 ± 0.73	—	—	0.31 ± 0.03	—	0.13 ± 0.04
20:3 n-6	0.60 ± 0.11	1.14 ± 0.02	0.56 ± 0.06	0.76 ± 0.08	0.64 ± 0.06	0.66 ± 0.26
20:4 n-6	5.60 ± 0.86	10.81 ± 1.16	4.12 ± 0.35	9.90 ± 0.23	4.94 ± 0.30	10.26 ± 2.21
20:5 n-3	0.60 ± 0.11	—	4.23 ± 1.52	—	7.20 ± 1.24	0.23 ± 0.08
22:4 n-6	0.29 ± 0.05	1.23 ± 0.28	1.20 ± 0.27	0.98 ± 0.16	0.71 ± 0.16	0.84 ± 0.23
22:5 n-6	1.44 ± 0.41	2.93 ± 0.54	0.19 ± 0.04	2.62 ± 0.10	0.14 ± 0.03	2.76 ± 0.43
22:5 n-3	0.06 ± 0.01	0.19 ± 0.12	2.75 ± 0.30	0.45 ± 0.15	3.10 ± 0.66	0.26 ± 0.05
22:6 n-3	2.02 ± 0.18	3.06 ± 0.35	17.60 ± 0.28	3.14 ± 0.35	20.55 ± 1.21	3.46 ± 0.85

[a] Percentage of total fatty acids ± SD.
[b] Number of mice fed each diet shown in parentheses.
[c] Denotes a value of less than 0.05.

have an effect on duct growth different from that of the *n*-6 fatty acids and may indeed act through a different mechanism.

To ascertain whether MO could influence ovarian development, glands from mice fed 10% MO, with and without hormone stimulation, were sectioned and analyzed. Although there were no apparent differences in primary, secondary, or mature follicles, regardless of age or diets fed (Table IV), we did observe a reduced number of *corpora lutea* in the MO-fed groups, either in the presence or absence of injected progesterone. Whether studied during the period from 3 to 9 weeks (Fig. 3) or from 8 to 14 weeks of age (Table IV), the mice that were fed the MO diet had fewer *corpora lutea* in their ovaries than did those that were fed the CO diet (Table IV). This reduction in active *corporea lutea* suggests a disruption in the normal ovarian cycle with a consequent diminution in available circulating progesterone. The administration of exogenous progesterone had no apparent effect on ovarian histology.

The influence of these dietary fats on ovarian function was judged by reproductive capability. Weanling mice were either fed the experimental diets from 3 weeks of age or allowed to eat the standard stock diet until they attained maturity at 8 weeks of age, and then were placed on the experimental diets. All females were placed with males for a 2-week period when they attained 16 weeks of age. The numbers of successful pregnancies, as well as the number of pups delivered by each female in the various diet and time groups, were recorded and appear in Table V. All of the dams in both the 3-week and the 8-week corn oil-fed groups showed 100% pregnancies with about seven pups per litter. However, a severe reduction (14 and 44%) in the percentage of pregnancies was noted in the case of the HCTO-fed groups, with a moderate reduction (60 and 71%) in the MO-fed groups. Clearly, those diets devoid of linoleate do not allow for normal fecundity. In addition, dietary *n*-3 fatty acids (MO), while allowing some measures of success, could not completely substitute for dietary *n*-6 fatty acids (CO), since the pregnancy rate with the MO diet was significantly below that observed with the CO diet.

The lipids in the virgin mammary glands are principally composed of triglycerides. This class of lipid does not contain many of the longer chain length fatty acids normally found in tissues. Hence, in order to shed light on the total fatty acids patterns as they are affected by the different lipid diets fed to these mice, we selected the liver as a representative tissue for such studies. As expected, analysis of the fatty acid composition of the livers of mice fed the various fat diets (Table VI) revealed that those mice fed the 10% HCTO were low in linoleate (18:2) and arachidonate (20:4) and had appreciable amounts of eicosatrienoate (20:3*n*-9), the fatty acid characteristic of animals that are not given essential fatty acids. The data also showed that those mice fed the 10% CO diet did not have any 20:3*n*-9, but contained high amounts of linoleate (22.2%) and arachidonate (10.8%), along with significant amounts of other longer *n*-6 fatty acids (1.2 and 2.9% of 22:4*n*-6, and 22:5*n*-6, respectively). Although those mice fed

the 10% MO diet had very significant amounts of these *n*-3 fatty acids, found in the dietary fish oil they consumed, they were also low in both linoleate and arachidonate (*n*-6 fatty acids).

When one compares the linoleate levels of the livers of mice fed 1% CO + 9% HCTO with those fed 1% CO + 9% MO (Table VI), it is clear that MO reduced the amount of 18:2*n*-6 in the liver. Furthermore, since the levels of 20:4 in the former group were 9.9% and in the latter were 4.9%, the *n*-3 fatty acids in the fish oil must have effectively reduced the conversion of 18:2 to 20:4. In Table VI we also include data from mice fed a 1% CO diet. Although these animals were not used to study mammary gland development, the data obtained from their livers could be used for comparisons of fatty acid patterns. It is interesting to note that the levels of 20:4 in the 1% CO + 9% HCTO group compared with the 1% CO group were almost the same, but significantly higher than those in the 1% CO + 9% MO group. such data substantiate the hypothesis that *n*-3 fatty acids in the MO diet must have an inhibitory effect on the conversion of 18:2 to 20:4.

Discussion

This study emphasizes the fact that the growth control mechanisms involved in mammary gland development are affected differently by dietary polyunsaturated *n*-3 fatty acids than those of the *n*-6 family. For example, dietary menhaden oil, when compared with corn oil, significantly reduced the growth of mammary ducts as well as the size of terminal duct branches. Previously, we demonstrated (1–3) a similar reduction in the growth pattern of mammary glands in mice fed a saturated fat diet composed of HCTO. Although the systemic effect of the latter essential fatty acid-deficient diet eventually presented as a general scaly appearance of the mice with loss of body weight (2) (a finding which is very well known and classically described), the menhaden oil diet could have prevented these symptoms. In addition, when immature mice were fed the saturated fat diet (HCTO), their ovaries failed to develop properly (3). There is little doubt that underdevelopment of the ovaries resulted in a reduction in the levels of the primary stimulatory hormones necessary for normal mammary development. However, when mammary ducts were transplanted into the gland-free fat pads of mice with mature ovaries who were then fed the HCTO diet, no significant effect on duct growth was observed. Conversely, mature or immature mice fed diets supplemented with menhaden oil showed none of the weight loss or skin effects seen in mice fed the HCTO-*n*-6 fatty acid-deficient diet. Indeed, in some instances, these MO-fed mice actually accumulated more body weight, by as much as 20% at 16 weeks of age, than those of the corn oil-fed groups.

Although this fish oil was better than HCTO in promoting a gain in body weight, mammary duct development was still dramatically reduced by the fatty acids found in the MO diet. The results show reduction in elongation of ducts by as much as 25% can occur over a 6-week period. Hormone supplementation, in the form of exogenous progesterone, can reverse this effect and actually sitmulate ducts to grow to the same extent as those found in CO-fed mice. In this latter case, however, when the ducts were carefully analyzed, it was found that their average diameter was reduced by more than 50%, even in the hormone-supplemented group. Such results suggest that mammary glands develop much more slowly and with less arborization when menhaden oil diet is fed than when the 18:2*n*-6-rich corn oil diet is fed. Although the parameters for evaluating mammary duct growth used in this study were not the same as those used by Welch and O'Connor (5), we came to the same conclusion: *n*-3 fatty acids suppress mammary duct growth in immature as well as mature mice. The presence of shorter mammary ducts results in fewer mammary epithelial cells that are at risk of a genomic insult(s) that would lead to mammary cancer.

The ovaries in menhaden oil-fed mice did show an abnormal development when compared with those from corn-oil fed animals, which could account for some of the growth differences. We could document at least a 50% reduction in the number of *corpora lutea* found in the ovaries of the MO-fed mice compared with those seen in CO-fed groups. The ovaries from the MO-fed mice, however, were not "shut down" as dramatically as those in HCTO-fed animals, since the MO-fed mice were still capable of becoming pregnant and delivering healthy young. The decreased functional status of the ovaries in the MO-fed mice could be demonstrated by the fact that the number of first pregnancy pups was significantly less than from mice fed corn oil-supplemented diets. Furthermore, when mammary ducts were transplanted into the cleared fat pads of adult mice who were subsequently fed MO diets, the ductal growth was still significantly less when compared with that in CO or HCTO-fed animals. Clearly, the systemic effects of MO (*n*-3 fatty acids) are quite different than those of either the saturated or the *n*-6 dietary fatty acids.

In whole animals, as well as in cell culture experiments, an essential feature of mammary epithelial cell proliferation appears to be associated with a requirement for linoleate and its conversion to arachidonate (14) with subsequent transformation into prostaglandins and other oxygenated metabolites (15). The initial processing of linoleate occurs through its desaturation and elongation to arachidonate before its conversion to prostaglandins via the cyclooxygenase reaction (16). It is possible that *n*-3 fatty acids effectively block the conversion of linoleate to arachidonate, thus limiting

the amount of prostaglandins available to stimulate mammary gland proliferation. Indeed, the inhibition of *n*-6 fatty acid metabolism by *n*-3 fatty acids has been demonstrated in many biologic systems (17, 18). It would appear, however, that the quantity of arachidonate present, even in these mice, as judged by its presence in the livers of MO-fed mice, indicates that the quantities of C20:4 are apparently sufficient to satisfy minimum requirements.

Although there are many considerations which remain unresolved, our data indicate that the multiple effects elicited by dietary MO preclude a single site of action for mammary gland development. Furthermore, identification of the mechanistic interactions between *n*-3 and *n*-6 fatty acids which interfere with normal mammary growth controls could allow for manipulation of the cellular environment so as to reduce possible transformations which lead to hyperplasia in the mammary gland.

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