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Differential incorporation of dietary conjugated linolenic and linoleic acids into milk lipids and liver phospholipids in lactating and suckling rats

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Abstract

Interest in health benefits of conjugated fatty acids is growing. The present study compared the incorporation pattern of dietary conjugated linolenic acids (CLnA) into milk with that of conjugated linoleic acids (CLA). Lactating Sprague-Dawley rats (Day 1) were divided into five groups fed the control diet (n=4) or one of four experimental diets supplemented with 1–2% CLA or CLnA mixture (n=8 each). Supplementation of 1% and 2% CLA led to enrichment of 4.17% and 8.57% CLA, respectively, while supplementation of 1% and 2% CLnA resulted in enrichment of only 0.98% and 1.71% CLnA in the milk lipids, demonstrating the transfer of CLnA from maternal diet to milk was discriminated. When the lactating rats were given a diet containing a CLnA mixture of *9t,11t,13t*-, *9c,11t,13t*- and *9c,11t,13c*-CLnA isomers, two CLA isomers, namely, *9t,11t* (0.59–0.90%) and *9c,11t* (1.21–1.96%), were found in the milk, suggesting that three CLnA isomers were Δ-13 saturated. Dietary CLnA at 1–2% had no effect on liver phospholipid (PL) fatty acid composition of both maternal and suckling rats, whereas dietary CLA increased docosahexaenoic acid (*4c,7c,10c,13c,16c,19c*-22:6) and palmitic acid (16:0) proportionally in the PL of maternal rats, but it suppressed 16:0 in the PL of suckling rats. It is concluded that maternal rats incorporate CLnA isomers into milk differently from that of CLA isomers. Most interesting is that maternal rats can metabolically convert CLnA to CLA.

Keywords: Conjugated linoleic acids; Conjugated linolenic acids; Milk; Δ-13 Saturation

1. Introduction

There are two types of structural-related conjugated octadecaenoic acids available in human diets; namely, conjugated linoleic acids (CLA) and conjugated linolenic acids (CLnA). CLA refers to a group of positional and geometrical isomers of conjugated octadecadienoic acids that have two conjugated double bonds. Silver-ion HPLC (Ag-HPLC) analysis has demonstrated that there are at least 12 CLA isomers [1]. In contrast, CLnA is a group of octadecatrienoic acid isomers that have three conjugated double bonds. Our previous study has characterized eight CLnA isomers in tung and pomegranate seed oil [2]. CLA and CLnA are found in various types of foods with CLA being present mainly in dairy foods and ruminant-derived meats [3], while CLnA being rich only in several kinds of seeds from bitter melon, pomegranate, tung and catalpa [4]. In addition, both CLA and CLnA can be formed during the processing of vegetable oils as a result of isomerization or dehydration of secondary oxidation products of linoleic acid and α-linolenic acid [5,6].

CLA has been extensively studied for its beneficial biological activities in improvement of immune function and prevention of chronic diseases including atherosclerosis, cancer, obesity and hypertension [7]. In contrast, information concerning the biological activity of CLnA is scarce except that some reports have demonstrated that CLnA is a potent suppressor of growth of certain human tumor cells [8–10]. In this regard, both CLA and CLnA have demonstrated to suppress mammary tumorigenesis via various mechanisms [11–13].

It is known that the fatty acid composition of milk reflects that of diets consumed by lactating women [14]. Both

Abbreviations: CLA, conjugated linoleic acids; CLnA, conjugated linolenic acids; GLC, gas-liquid chromatograph.

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ruminant and human milk contain conjugated fatty acids [15]. In view of their biological activity, we have interest to examine the enrichment of CLA and CLnA in milk by dietary supplementation. In this regard, dietary CLA is capable of incorporating into milk of cows [16], rats [17] and humans [18]. Our previous study showed that CLA isomers were able to incorporate in liver phospholipids (PL) of suckling rats [19]. Interestingly, metabolic conversion of some CLnA isomers to CLA isomers has been reported in rats [20,21]. However, no studies to date have investigated the transfer of CLnA from maternal diet to milk, and conversion of CLnA to CLA in lactating rats. The objectives of the present study were therefore (i) to compare the relative incorporation pattern of dietary CLA into rat milk with that of CLnA; (ii) to investigate whether dietary CLnA was able to be converted to CLA in milk when rats were fed only a CLnA diet; and (iii) to ascertain whether supplements of CLA and CLnA in maternal diet would affect fatty acid composition of PL in suckling rats.

2. Materials and methods

2.1. Materials

A mixture of CLA (Tonalin, 90%) was purchased from Larodan Fine Chemicals (Malmö, Sweden). This CLA mixture contained almost equal amounts of *9c,11t* (45.3%) and *10t,12c* (44.6%) isomers. Other fatty acids in the mixture were *9c*-18:1 (9.2%), 18:0 (0.2%) and *9c,12c*-18:2 (0.7%). CLnA (>91% purity) isomers were purified from solidified tung oil, bitter gourds and pomegranate seeds oil as we previously described [22]. After saponification and acidification, CLnA isomers were purified by repeat crystallization from 90% ethanol at 0°C. CLnA isomer prepared from pomegranate seed oil was punicic acid (*9c,11t,13c*-18:3, 91.4%). CLnA isomers from bitter gourds seeds oil contained two isomers, namely, α-eleostearic acid (*9c,11t,13t*-18:3, 61.7%) and β-eleostearic acid (*9t,11t,13t*-18:3, 29.1%). CLnA isomer prepared from solidified tung oil was β-eleostearic acid (*9t,11t,13t*-18:3, 94.3%). Crude CLnA isomer from pomegranate seeds oil (183 g), CLnA from bitter gourds seeds oil (315g) and CLnA from solidified tung oil (30g) were melted and then mixed to produce a final CLnA mixture consisting of mainly three isomers: *9t,11t,13t* (23.3%), *9c,11t,13t* (36.8%) and *9c,11t,13c* (31.7%). The other fatty acids in the CLnA mixture were 16:0 (4.2%), 18:0 (2.1%) and *9c*-18:1 (1.6%) and *9c,12c*-18:2 (0.9%).

2.2. Diets

The control diet was prepared by mixing the powdered ingredients in the following concentrations (g/kg diet): casein, 235; canola oil, 100; maize starch, 338; sucrose, 243; mineral mix, 35; vitamin mix, 10; cellulose, 32; choline bitartrate, 4; DL-methionine, 3. Four experimental diets were prepared by adding 1% CLA (CLA1), 2% CLA (CLA2), 1% CLnA (CLnA1) and 2% CLnA (CLnA2) to the control diet (Table 1). The diets were stored frozen at −20°C. The reason for adding 1–2% CLA or CLnA in diet is that human diet in general contains no more than 2% of these fatty acids.

2.3. Animals

Lactating Sprague-Dawley rats (Day 1, n=34) were divided into five groups, namely, the control (n=4), CLA1 (n=8), CLA2 (n=8), CLnA1 (n=8) and CLnA2 (n=8). The maternal animals were switched immediately from the chow diet to one of the five diets. Maternal rats were housed individually in an animal room at 23°C with 12 h–12 h light–dark cycles. Litter sizes were averaged to 10–12 pups per dam. The fresh diets were given to the maternal rats daily, and uneaten food was discarded. Food intake and body weight were measured daily. The rats were allowed free access to food and water. The protocol was reviewed and approved by the Animal Experimental Ethical Committee, The Chinese University of Hong Kong. At Days 9, 12 and 15, four pups from each dam were decapitated at 9:00 to 10:00 a.m. The abdomens were opened and the milk in the stomach was collected and stored at −78°C until analyzed. The organs including liver, kidney, brain and heart of the pups were also retained and stored at −78°C before analysis. At the end of the experiment, maternal rats were also killed under a light carbon dioxide anesthesia and the liver was retained for lipid analysis.

2.4. Fatty acid analysis

Total lipids in milk and liver were extracted using chloroform–methanol (2:1, v/v) containing heptadecanoic acid as an internal standard. To determine the milk fatty acid composition, acid-catalyzed methylation was used in the

Table 1
Fatty acid composition of five diets (g/kg)[#]

Fatty acid	Control	CLA1	CLA2	CLnA1	CLnA2
16:0	5.98	5.69	5.27	6.59	7.60
16:1	0.24	0.21	0.22	0.22	0.22
18:0	2.77	2.65	2.57	3.41	3.69
9c-18:1	56.66	56.52	56.95	54.86	53.28
11c-18:1	3.63	3.69	4.07	3.39	3.36
9c,11c-18	19.87	19.74	19.51	20.52	21.01
9c,12c,15c-18:3	9.22	9.13	8.81	9.03	8.76
10t,12c-CLA	0.00	3.98	8.50	0.00	0.00
9c,11t-CLA	0.00	4.05	8.74	0.00	0.00
Total CLA	0.00	8.03	17.24	0.00	0.00
20:0	0.54	0.49	0.52	0.59	0.64
11c-20:1	1.16	1.09	1.12	1.14	1.12
13c-22:1	0.21	0.22	0.23	0.19	0.18
9t,11t,13t-CLnA	0.00	0.00	0.00	2.11	5.11
9c,11t,13t-CLnA	0.00	0.00	0.00	3.29	7.22
9c,11t,13c-CLnA	0.00	0.00	0.00	2.82	5.73
Total CLnA	0.00	0.00	0.00	8.22	18.05
Lipid/diet (g/kg)	100.27	107.47	116.52	108.16	117.90

[#] Four experimental diets were prepared by adding 1% CLA (CLA1), 2% CLA (CLA2), 1% CLnA (CLnA1) and 2% CLnA (CLnA2) to the control diet.

present study. In brief, the milk lipids (10–20 mg) were transesterified in 2 ml of 1.5 mol/L H_2SO_4/methanol solution under N_2. The methylation tube was placed in a heat block at 40°C for 1 h and then cooled at room temperature. Hexane (4 ml) and distilled water (1 ml) were then added and mixed thoroughly. After centrifugation, the top hexane layer containing fatty-acid methyl esters (FAME) was saved and subjected to gas chromatographic (GC) analysis.

Liver phospholipids were separated on a thin-layer chromatographic (TLC) plate. L-α-Phosphatidylcholine diheptadecanoyl was added as an internal standard to quantify PL [23]. Total lipids in the liver were dissolved in chloroform and then applied onto a TLC plate (20×20 cm; precoated with 250-mm silica gel 60 Å, Macherey-Naged, Duren, Germany) to separate different lipid classes. A solvent system of hexane-diethyl ether-acetic acid (80:20:1, vol/vol/vol) was used for development. The band containing PL was scratched off the plate, and the lipids extracted were converted to methyl esters as described above.

The FAME mixtures obtained from milk and liver were analyzed on a flexible silica capillary column (HP-INNOVAX, 30 m×0.32 mm I.D., Agilent Technologies, Santa Clara, CA, USA) in a Shimadzu GC-2010 gas–liquid chromatograph equipped with a flame-ionization detector and an automated injector (Shimadzu, Tokyo, Japan). Column temperature was programmed from 120°C to 220°C at a rate of 2°C/min and then held for 20 min. Injector and detector temperature was set at 250°C and 270°C, respectively. Helium was used as a carrier gas at a head pressure of 105 kPa.

2.5. GC-MS Analysis

It is known that FAME cannot be used to identify the location of double bonds due to the shift of double bonds along the carbon chain during GC-MS analysis. FAME (500 μg) was therefore converted into 4,4-dimethyloxazoline (DMOX) derivatives in 500 μl 2-amino-2-methylpropanol at 170°C for 2 h under N_2 protection. After heating, DMOX derivatives were extracted twice with 4 ml hexane after the addition of 4 ml saturated NaCl solution. GC-MS spectral analysis of DMOX derivatives was carried out on a Hewlett-Packard MSD 5970 B quadrupole with an ion source of 70 eV, fitted with a Hewlett-Packard 6890 II model chromatograph. The DMOX derivatives were eluted on a flexible silica capillary column (HP-INNOVAX, 30 m×0.32 mm I.D., Agilent Technologies). To obtain a better resolution for CLA and CLnA isomers, the column was operated for 1 min at 50°C, and the temperature was then increased to 120°C at a rate of 15°C/min, to 220°C at a rate of 2°C /min and then held for 15 min. Helium was used as a carrier gas at a head pressure of 105 kPa.

2.6. Silver-ion high-performance liquid chromatography

FAME of CLA and CLnA isomers (10 μl of 20 mg/ml) were separated on a silver-ion impregnated column (250×4.6 mm i.d., 5 μm, Chrompack, Bridgewater, NJ, USA) in an HP-1100 HPLC equipped with a ternary pump delivery system. For CLA FAME, hexane containing 0.1% acetonitrile was chosen as a mobile phase at a flow rate of 1 ml/min and the absorbance was monitored at 233 nm. For

Table 2
Weights of stomach milk and organs of suckling rats[#]

	Control	CLA1	CLA2	CLnA1	CLnA2	P_{CLA}	P_{CLnA}
Maternal rat							
Body	282.5±20.6	271.3±18.9	284.0±19.5	283.8±24.5	275.6±22.9	0.93	0.43
Number of pups/dam	12.0±2.2	10.0±3.1	10.0±1.7	12.0±1.8	12.00±1.93	0.33	–
Day 9							
Body	17.4±3.1	22.6±3.6	23.9±±5.0	20.6±3.6	22.2±2.8	0.21	0.13
Brain	0.66±0.08	0.85±0.07	0.89±0.10	0.71±0.05	0.79±0.04	0.23	0.08
Milk	0.59±0.12	0.84±0.30	0.58±0.16	0.80±0.30	0.65±0.04	0.98	0.82
Heart	0.14±0.02	0.17±0.03	0.19±0.04	0.16±0.03	0.17±0.02	0.07	0.12
Liver	0.63±0.14	0.78±0.09	0.90±0.20	0.73±0.12	0.79±0.10	0.04	0.09
Kidney	0.23±0.15	0.30±0.05	0.36±0.07	0.30±0.06	0.31±0.06	0.03	0.26
Day 12							
Body	30.5±0.2	33.3±0.1	36.9±0.8	35.1±0.8	34.9±1.3	0.04	0.35
Brain	1.01±0.08	1.24±0.05	1.26±0.04	1.02±0.03	1.05±0.07	0.29	0.18
Milk	1.03±0.09	1.17±0.08	0.83±0.14	0.92±0.11	0.75±0.12	0.60	0.08
Heart	0.30±0.04	0.38±0.08	0.46±0.09	0.23±0.01	0.27±0.01	0.02	0.72
Liver	1.25±0.05	1.54±0.05	1.61±0.08	1.32±0.05	1.39±0.05	0.18	0.06
Kidney	0.48±0.03	0.66±0.08	0.73±0.06	0.49±0.01	0.47±0.05	0.16	0.67
Day 15							
Body	35.7±4.03	45.9±1.1	46.3±1.6	46.5±0.9	44.9±2.5	0.31	0.44
Brain	1.10±0.05	1.17±0.04	1.13±0.05	1.18±0.07	1.32±0.04	0.72	0.10
Milk	0.60±0.24	1.00±0.16	1.01±0.13	0.53±0.10	0.91±0.08	0.32	0.44
Heart	0.31±0.01	0.42±0.05	0.42±0.02	0.31±0.01	0.47±0.02	0.33	0.44
Liver	1.62±0.04	2.48±0.15	2.40±0.33	1.95±0.09	2.13±0.78	0.39	0.12
Kidney	0.60±0.08	0.86±0.02	0.93±0.08	0.66±0.01	0.83±0.05	0.20	0.17

[#] Four experimental diets were prepared by adding 1% CLA (CLA1), 2% CLA (CLA2), 1% CLnA (CLnA1) and 2% CLnA (CLnA2) into the control diet.

CLnA FAME, hexane containing 0.25% acetonitrile was chosen as a mobile phase at a flow rate of 0.5 ml/min and the absorbance was monitored at 268 nm. Each isomer was identified according to the retention time of authentic standards as we previously described [2].

2.7. Statistics

Data were expressed as means±S.D. The group means were statistically analyzed using one-way analysis of variance and *post hoc* LSD test on SigmaStat Advisory Statistical Software. Each treatment against the control was compared using Dunnett's method, while the significance for trend (P_{CLA}) across the control, CLA1 and CLA2 was assessed using Bonferroni's method. Similarly, the trend (P_{CLnA}) across the control, CLnA1 and CLnA2 was also assessed. Significance was defined as $P<.05$.

3. Results

3.1. Fatty acid composition of dietary fat

The control maternal rats were fed a diet containing canola oil, while the experimental groups were fed the same diet but supplemented with 1% and 2% CLA or CLnA mixture (Table 1). The fatty acid analysis found that two CLA diets had 8.03 and 17.24 g CLA/kg diet, whereas two CLnA diets had 8.22 and 18.05 g CLnA/kg diet.

3.2. Weights of stomach milk, body and organs of suckling rats

No trend in weight of stomach milk weight (the milk retained in the stomach of pups) among the five groups could be observed. No significant difference in body and organ weights was observed between CLA and its corresponding CLnA group. Statistically, significant increasing trends in body and organ weights of neonatal rats could not be observed with two doses of CLA (or CLnA) except for the liver and kidney at Day 9 and body weight at Day 12 (Table 2).

3.3. Milk fatty acid composition

Fatty acid composition of milk samples collected on Days 9, 12 and 15 was very similar. To simplify the presentation, only data on Day 12 are shown hereafter. Firstly, supplementation of 1% and 2% CLA mixture in the maternal diet led to enrichment of CLA by 4.17% and 8.57% in the milk lipids, respectively (Table 3). Incorporation of dietary CLA into milk was dose dependent (trend: $P_{CLA}=.01$). Similarly,

Table 3
Milk fatty acid composition in the control, CLA- and CLnA-supplemented rats at Day 12 of lactation[#]

FA %	Control	CLA1	CLA2	CLnA1	CLnA2	P_{CLA}	P_{CLnA}
16:0	20.10±2.14^{a}	18.13±1.41^{b}	16.12±0.75^{b}	20.44±1.37^{a}	21.12±1.37^{a}	0.01	0.12
18:0	3.44±0.20^{b,c}	3.43±0.34^{c}	3.450.25^{b,c}	3.96±0.34^{b}	4.46±0.37^{a}	0.67	0.01
20:0	0.14±0.02	0.13±0.01	0.13±0.01	0.14±0.01	0.13±0.02	0.33	0.34
Total saturates	23.68±2.01^{a}	21.69±1.28^{b}	19.700.70^{b}	24.54±1.62^{a}	25.71±1.28^{a}	0.02	0.05
9c-18:1	39.53±2.78^{a}	37.18±1.23^{a,b}	34.91±0.83^{b,c}	35.33±2.11^{b}	32.15±1.39^{c}	0.01	0.05
11c-20:1	0.80±0.11^{a}	0.61±0.05^{b}	0.570.04^{b,c}	0.57±0.03^{b,c}	0.49±0.06^{c}	0.23	0.17
Total n-9	40.33±2.43^{a}	37.79±1.21^{a,b}	35.480.78^{b,c}	35.90±2.03^{b}	32.64±1.32^{c}	0.02	0.05
11c-18:1	3.36±0.27^{a}	2.92±0.10^{a,b}	2.74±0.07^{b}	2.81±0.33^{b}	2.62±0.24^{b}	0.15	0.17
9c,12c-18:2	9.81±0.71	10.14±0.47	10.25±0.34	9.94±0.55	9.71±0.72	0.18	0.72
11c,14c-20:2	0.30±0.03^{a}	0.21±0.04^{b}	0.20±0.02^{b}	0.23±0.02^{b}	0.20±0.03^{b}	0.28	0.14
8c,11c,14c-20:3	0.21±0.02^{a}	0.11±0.02^{c}	0.08±0.01^{c}	0.16±0.03^{b}	0.14±0.02^{b}	0.19	0.15
5c,8c,11c,14c-20:4	0.62±0.12^{a}	0.50±0.05^{a,b}	0.51±0.05^{a,b}	0.55±0.07^{a,b}	0.47±0.04^{b}	0.38	0.02
7c,10c,13c,16c-22:4	0.05±0.10	0.06±0.00	0.07±0.01	0.09±0.01	0.30±0.37	0.03	0.24
4c,7c,10c,13c,16c-22:5	0.17±0.04^{a}	0.10±0.02^{b}	0.11±0.02^{b}	0.14±0.03^{a}	0.11±0.01^{b}	0.42	0.04
Total n-6	11.16±1.03	11.12±0.61	11.22±0.48	11.11±0.93	10.93±1.03	0.59	0.20
9c,12c,15c-18:3	3.79±0.08	4.04±0.14	4.08±0.11	4.00±0.22	3.86±0.28	0.25	0.79
5c,8c,11c,14c,17c-20:5	0.21±0.02^{a}	0.14±0.03^{b}	0.12±0.03^{b}	0.21±0.02^{a}	0.19±0.01^{a}	0.20	0.33
7c,10c,13c,16c,19c-22:5	0.28±0.04^{a}	0.18±0.03^{b}	0.17±0.02^{b}	0.34±0.05^{a}	0.26±0.09^{a}	0.28	0.85
4c,7c,10c,13c,16c,19c-22:6	0.27±0.09^{a}	0.23±0.04	0.26±0.02	0.24±0.04	0.21±0.06	0.85	0.03
Total n-3	4.55±0.21	4.59±0.19	4.63±0.17	4.79±0.28	4.52±0.38	0.75	0.94
9t,11t-CLA	ND	0.02±0.00	0.05±0.00	0.59±0.01	0.90±0.02	0.07	0.12
10t,12c-CLA	ND	1.94±0.09	4.06±0.28	ND	ND	0.02	–
9c,11t-CLA	ND	2.21±0.05^{b}	4.46±0.29^{a}	1.21±0.14^{d}	1.96±0.02^{c}	0.01	0.05
Total CLA	ND	4.17±0.09^{b}	8.57±0.23^{a}	1.80±0.31^{d}	2.86±0.61^{c}	0.01	0.04
9t,11t,13t-CLnA	ND	ND	ND	0.03±0.00^{b}	0.06±0.00^{a}	–	0.05
9c,11t,13t-CLnA	ND	ND	ND	0.19±0.01^{b}	0.40±0.01^{a}	–	0.02
9c,11t,13c-CLnA	ND	ND	ND	0.76±0.09^{b}	1.25±0.26^{a}	–	0.05
Total CLnA	ND	ND	ND	0.98±0.17^{b}	1.71±0.36^{a}	–	0.05
Others	16.92±1.27	17.68±1.72	17.57±2.02	18.01±2.1	18.80±1.38	0.42	0.06
Lipid/milk (mg/g)	193.79±10.85^{a}	176.58±9.89^{b}	163.17±14.9^{b}	159.45±13.91^{b,c}	148.64±14.63^{c}	0.05	0.05

ND, not detected. ^{a,b,c,d}Mean values in the same row with different superscript letter differ significantly ($P<.05$).

[#] Four experimental diets were prepared by adding 1% CLA (CLA1), 2% CLA (CLA2), 1% CLnA (CLnA1) and 2% CLnA (CLnA2) into the control diet.

incorporation of dietary CLnA into milk was also dose dependent (trend: P_{CLA}=.05). However, supplementation of 1% and 2% CLnA in maternal diets resulted in enrichment of only 0.98% and 1.71% CLnA in the milk lipids (Table 3), demonstrating that incorporation of CLA into milk was greater than that of CLnA (P<.05). Secondly, supplementation of 2% CLnA mixture into the maternal diet suppressed the level of arachidonic acid (*5c,8c,11c,14c*-20:4, P<.05) compared with the control value. Thirdly, total milk fat was significantly reduced in both CLA- and CLnA-supplemented groups compared with the control (trend: P_{CLA}=.05; P_{CLnA}=.05, Table 3). CLnA had greater suppression on milk fat than CLA (P<.05). Fourthly, incorporation of CLA and CLnA isomers into milk was selective. In diet, the ratio of two CLA isomers, *10c,12t* and *9c,11t*, was almost 1:1, while in milk, the ratio of *10c,12t* to *9c,11t* was 9:10. Among the three CLnA isomers, the ratio of *9t,11t,13t*/*9c,11t,13t*/*9c,11t,13c* was 8:12:10; in contrast, the ratio was 1:6:25 in milk.

3.4. Conversion of CLnA to CLA in milk

The most interesting observation was that CLA was found in the milk of CLnA-fed maternal rats. Supplementation of 1% and 2% CLnA mixture led to enrichment of 1.80% and 2.86% CLA in the milk lipids, suggesting that part of CLnA was metabolically converted to CLA (Table 3). Conversion of CLnA to CLA in CLnA-fed maternal rats was dose dependent (trend: P=.01). The major CLA isomers in the milk of CLnA-fed rats were *9t,11t* (0.59–0.90%) and *9c,11t* (1.21–1.96%). The conversion of CLnA to CLA had been confirmed using GC, silver-ion high-performance liquid chromatography (Ag+-HPLC) and GC-MS. To be more specific, no CLA and CLnA isomers were detected in the control milk (Fig. 1A). In the CLnA-fed groups, one additional peak, which was suspected to be a CLA isomer, was detected in addition to three CLnA isomers (Fig. 1B). Having compared with GC chromatogram of CLA standards (Fig. 1C), we deduced that this new peak was *9t,11t/9c,11t*-CLA isomers (Fig. 1C). Milk FAME was then converted to its DMOX derivative and analyzed using GC-MS, confirming that the double bonds were conjugated and located at Positions 9 and 11 as characterized by having a molecular ion with *m/z* ratio of 333, fragments of *m/z* 182 and 262 (allylic cleavage), differences in 12 mass units between fragments of *m/z* 196 and 208, and between fragments of *m/z* 222 and 234. To determine the geometric configuration, this sample was analyzed using Ag+-HPLC. By comparing it with CLA standards, this converted CLA contained actually two isomers, namely, *9t,11t* and *9c,11t*-CLA isomers (Fig. 1E). Evidence supported that CLnA was converted metabolically to CLA in maternal rats.

3.5. Fatty acid composition in the PL of suckling rats

Supplementation of 1% and 2% CLA in the maternal diet led to incorporation of 0.69–1.67% CLA (trend: P_{CLA}=.05), while the same amount of CLnA in diet only led to incorporation of 0.07–0.08 % CLnA into the PL of suckling rats (Table 4), suggesting that CLA had greater incorporation than CLnA. Similarly, supplementation of CLnA in maternal diet also resulted in accumulation of 0.38–0.55% CLA in the PL of suckling rats, indicating the metabolic conversion of CLnA to CLA. In general, supplementation of CLA and CLnA at 1–2% maternal diet had no effect on fatty acid composition of PL except for 16:0 and *9c*-18:1 which showed a decrease trend with increasing amounts of CLA ($P_{16:0}$ =.04 and $P_{18:1n\text{-}9}$=.04; Table 4).

3.6. Fatty acid composition in the PL of maternal rats

The results on maternal PL analysis clearly demonstrated that CLA had greater incorporation (0.15–0.29%) than CLnA into the PL of suckling rats (0.06%; Table 5). Incorporation of CLA into maternal PL was dose dependent (trend: P_{CLA}=.05), while incorporation of CLnA into maternal PL was minimal and no dose-dependent pattern was observed. Similar to that in milk and PL of suckling rats, metabolic conversion of CLnA to CLA occurred also in the maternal liver, as 0.11–0.16% of CLA was found in the PL of CLnA-fed maternal rats (Table 5). Supplementation of CLnA at 1–2% levels did not change the fatty acid composition of maternal PL, while that of CLA at a similar level resulted in a decreasing level of 16:0 (P=.03) and an increasing level of docosahexaenoic acid (*4c, 7c,10c,13c,16c,19c*-22:6, P=.05).

4. Discussion

Interest in health benefits of conjugated fatty acids is growing, as they have been shown to possess antioxidant, antitumor, immunomodulatory and serum lipid-lowering activity [24,25]. Enrichment of CLA in milk by dietary supplement has been achieved in cow, rats and humans [16–18]. The present study is the first report on transfer of CLnA isomers from the maternal diet to the milk in rats. It demonstrated clearly that transfer of both CLA and CLnA from maternal diet to milk was dose dependent (trend: P_{CLA}=.01, P_{CLnA}=.05; Table 3). It was found that supplementation of CLnA in the maternal dietary fat at 1% and 2% levels in diet led to incorporation of only 0.98–1.71% CLnA into milk lipids (Tables 1–3). In contrast, supplementation of CLA at similar levels led to greater incorporation of CLA in milk lipids (4.21–8.66%), suggesting that both CLA and CLnA were able to transfer from maternal diet to milk with the former being preferentially incorporated into milk. Regarding the incorporation of CLA into milk, the present study was in agreement with our previous investigation reporting that addition of CLA in maternal diet at 2% had 7.8–8.6% CLA accumulated in the milk lipids [19]. The present results showed that transfer of CLnA from maternal diets was quantitatively minor compared with that of CLA.

Incorporation of three CLnA isomers into the milk was selective (Table 3). Results clearly showed that *9c,11t,13c*-CLnA isomer was preferentially incorporated into the milk

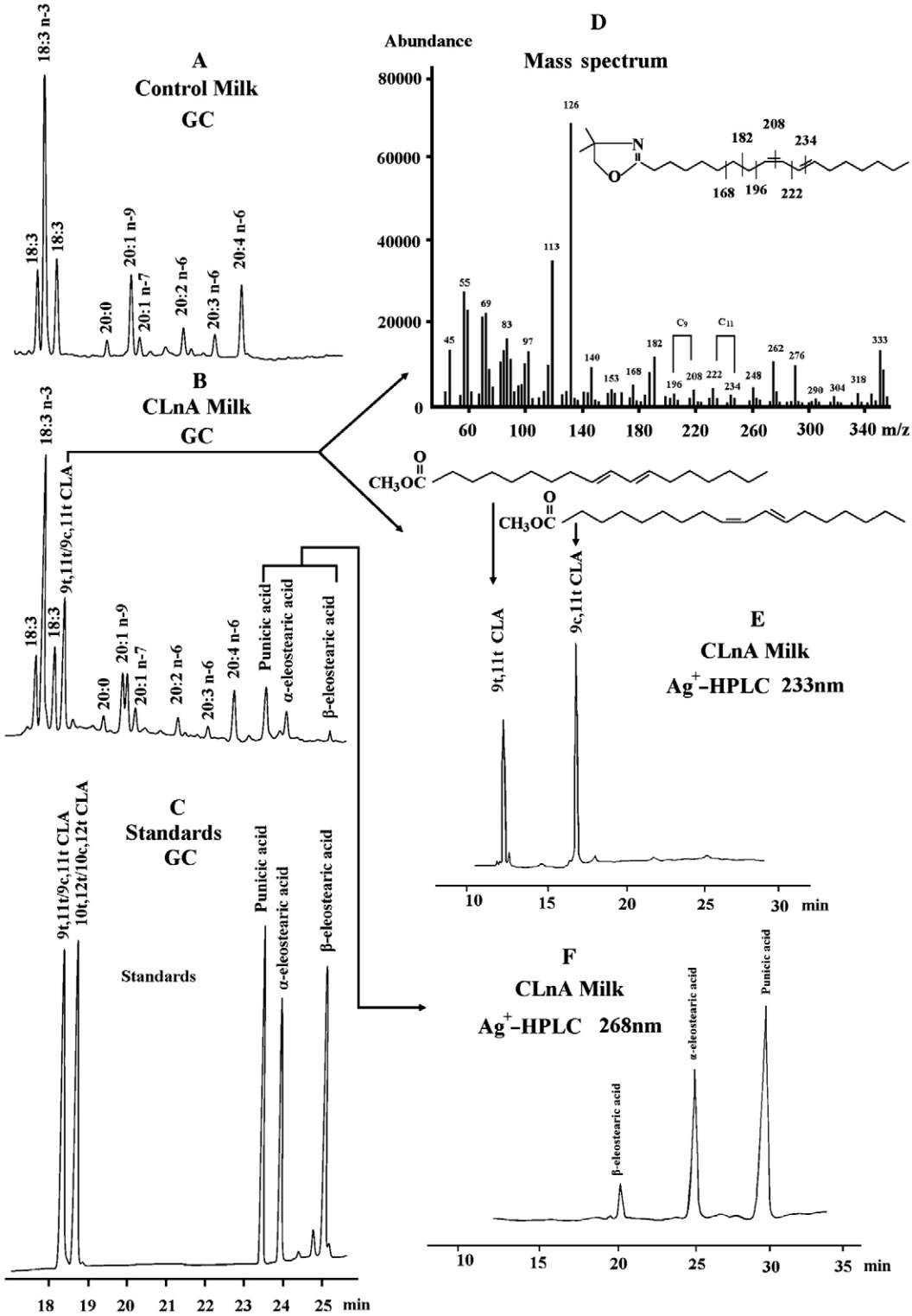

Fig. 1. Conversion of CLnA isomers to CLA in milk. (A) Partial GC FAME profile of the milk lipids in rats fed the control diet. (B) Partial GC FAME profile of the milk lipids in rats fed the CLnA diet. (C) Partial GC FAME profile of a standard mixture containing *9t,11t*/*9c,11t*, *10t,12t*/*10c,12t*, α-eleostearic acid (*c9,t11,t13*-CLnA), β-eleostearic acid (*t9,t11,t13*-CLnA) and punicic acid (*c9,t11,c13*-CLnA). (D) GC-MS spectrum of DMOX of *9t,11t*/*9c,11t*-CLA isomers. (E) Ag^{+}-HPLC chromatogram of the milk lipids in rats fed the CLnA diet at 233 nm. (F) Ag^{+}-HPLC chromatogram of the milk lipids in rats fed the CLnA diet at 268 nm.

Table 4
Fatty acid composition of PL in the control, CLA- and CLnA-supplemented suckling rats at Day 12[#]

FA %	Control	CLA1	CLA2	CLnA1	CLnA2	P_{CLA}	P_{CLnA}
16:0	21.41±0.38	20.37±1.39	19.08±2.33	20.16±0.69	22.00±2.12	0.04	0.80
18:0	21.03±1.22	22.95±1.24	21.67±1.96	21.60±0.41	22.31±2.44	0.79	0.40
20:0	0.31±0.03[a,b]	0.28±0.00[b]	0.28±0.01[b]	0.38±0.09[a,b]	0.49±0.05[a]	0.33	0.08
Total	42.75±1.09	43.60±2.39	41.03±4.32	42.14±1.23	44.80±4.28	0.54	0.47
9c-18:1	8.66±0.43	8.22±0.39	7.67±1.63	7.84±0.64	8.12±1.57	0.04	0.55
11c-20:1	0.60±0.02	0.54±0.01	0.41±0.06	0.51±0.08	0.50±0.09	0.13	0.28
Total n-9	9.26±0.34	8.76±0.38	8.08±1.38	8.35±0.67	8.62±1.32	0.05	0.52
11c-18:1	2.63±0.02[a]	2.29±0.04[a,b]	2.28±0.04[a,b]	2.220.09[a,b]	2.17±0,23[b]	0.32	0.27
9c,12c-18:2	8.53±0.36	9.44±0.33	8.73±0.51	8.89±0.22	9.67±0.52	0.87	0.13
8c,11c,14c-20:3	0.76±0.06[a,b]	0.68±0.08[b]	0.59±0.00[b]	0.86±0.08[a]	0.93±0.06[a]	0.02	0.07
5c,8c,11c,14c-20:4	18.60±0.77	17.64±0.44	18.89±0.93	19.43±0.33	17.25±2.85	0.86	0.58
7c,10c,13c,16c-22:4	0.42±0.01	0.37±0.02	0.38±0.03	0.35±0.02	0.36±0.08	0.46	0.42
4c,7c,10c,13c,16c-22:5	0.24±0.01[a]	0.21±0.05[a]	0.14±0.01[b]	0.24±0.05[a]	0.23±0.15[a]	0.14	0.33
Total n-6	28.55±1.02	28.34±0.83	28.73±1.26	29.77±0.63	28.44±3.29	0.70	0.95
9c,12c,15c-18:3	0.35±0.00	0.38±0.01	0.27±0.02	0.27±0.03	0.31±0.06	0.50	0.68
5c,8c,11c,14c,17c-20:5	0.61±0.08	0.71±0.19	0.64±0.02	0.78±0.05	0.74±0.09	0.81	0.48
7c,10c,13c,16c,19c-22:5	2.99±0.07	2.47±0.09	1.51±0.12	2.32±0.33	2.32±0.72	0.11	0.33
4c,7c,10c,13c,16c,19c-22:6	11.92±0.65	11.75±1.09	14.61±1.28	12.71±0.55	10.96±2.67	0.37	0.63
Total n-3	15.87±0.67	15.31±1.24	17.03±1.35	16.08±0.88	14.33±3.41	0.91	0.92
9t,11t-CLA	ND	ND	ND	0.11±0.01	0.16±0.03	–	0.14
10t,12c-CLA	ND	0.42±0.02	0.98±0.06	ND	ND	0.05	–
9c,11t-CLA	ND	0.27±0.01	0.70±0.06	0.26±0.02	0.38±0.03	0.05	0.13
Total CLA	0.00±0.00[d]	0.69±0.03[b]	1.68±0.12[a]	0.38±0.04[c]	0.55±0.06[b]	0.05	0.14
9t,11t,13t-CLnA	ND	ND	ND	0.03±0.01	0.03±0.01	–	0.79
9c,11t,13t-CLnA	ND	ND	ND	0.03±0.01	0.03±0.01	–	0.18
9c,11t,13c-CLnA	ND	ND	ND	0.03±0.01	0.03±0.01	–	0.78
Total CLnA	ND	ND	ND	0.08±0.01[a]	0.07±0.01[a]	–	0.79
Others	0.94±0.02	1.01±0.04	1.17±0.10	0.98±0.03	1.02±0.05	0.09	0.25
PL/Liver (mg/g)	27.87±1.88	27.18±1.03	27.14±1.57	27.41±2.61	25.63±2.24	0.85	0.85

[a,b,c]Mean values in the same row with different superscript letter differ significantly ($P<.05$).
[#] Four experimental diets were prepared by adding 1% CLA (CLA1), 2% CLA (CLA2), 1% CLnA (CLnA1) and 2% CLnA (CLnA2) into the control diet.

followed by *9c,11t,13t* and *9t,11t,13t*, as reflected from the observation that the ratio of *9t,11t,13t*/*9c,11t,13t*/*9c,11t,13c* was 8:12:10 in the diet, but it became 1:6:25 in milk (Table 3). It was concluded that CLnA isomers were discriminately transferred from maternal diet to milk compared with other fatty acids. Similar to our previous study [19], incorporation of CLA isomers into milk was, however, very similar. The present study confirmed that incorporation of *9c,11t* and *10c,12t*-CLA into milk was proportional to that in the diet. In this regard, distribution of CLA isomers in adipose tissues of pig was similar to that in the diet, suggesting that the relative incorporation of all the CLA isomers was not selective [26]. However, it is known that Δ-9 desaturase converts *11t-18:1* to *9c,11t*-CLA endogenously and this may affect the incorporation pattern of exogenous CLA isomers [16,27].

The most interesting observation was that feeding maternal rats a CLnA diet could produce a milk containing CLA, indicating that CLnA was converted to CLA. In this regard, Tsuzuki et al. [20] have demonstrated that α-eleostearic acid (*9c,11t,13t*) was quickly converted to CLA in the liver, kidney and intestine of rats and speculated that there was an oxidoreductase which was able to carry out Δ13 saturation reaction and converted CLnA to CLA. In addition, the same authors found that *9c,11t,13t*-CLnA was poorly absorbed but it was efficiently converted to *9c,11t*-CLA [21]. It is possible that in intestine and liver, CLnA is converted to CLA, which is then transferred to the mammary gland. However, the possibility that *9c,11t,13t*-CLnA was directly converted to *9c,11t*-CLA in the mammary gland could not be eliminated. In this study, three natural CLnA isomers including *9c,11t,13t*-CLnA, *9t,11t,13t*-CLnA and *9c,11t,13c*-CLnA as a mixture were added into the maternal diets, showing that milk incorporated *9c,11t,13c* (one *trans* bond) mostly followed by *9c,11t,13t* (two *trans* double bonds) and *9t,11t,13t* (three *trans* double bonds) in a decreasing order. The results suggested that oxidoreductase of Δ13-saturation preferred substrates with a *trans*-configuration. Further studies are deemed necessary to investigate the underlying mechanism by which CLnA isomers with a *trans*-configuration are preferred by oxidoreductase. It should also be pointed out that conversion of CLnA to CLA is so far only reported in rats. To our best knowledge, no data are available to extrapolate that such conversion exists in humans and other mammals.

Incorporation of CLA and CLnA was also found in the liver of both suckling and maternal rats. Firstly, incorporation of both CLA and CLnA in the PL was much lesser than that into milk lipids. To be specific, supplementation of 1–2% CLA in the maternal diet led to 4.17–8.57% CLA into

Table 5
Fatty acid composition of PL in the control, CLA- and CLnA-supplemented maternal rats[#]

FA %	Control	CLA1	CLA2	CLnA1	CLnA2	P_{CLA}	P_{CLnA}
16:0	10.61±0.66	11.31±0.70	11.91±0.26	10.03±1.72	11.15±1.86	0.03	0.68
18:0	34.12±0.15	35.14±0.99	34.01±0.35	34.52±1.31	33.95±2.31	0.94	0.81
20:0	0.05±0.01	0.03±0.02	0.03±0.00	0.04±0.02	0.05±0.01	0.33	1.00
Total	44.78±0.78	46.48±1.67	45.95±0.57	44.59±2.90	45.15±4.09	0.53	0.55
9c-18:1	6.14±0.57	4.80±0.54	4.59±0.60	5.97±0.34	5.73±0.45	0.25	0.06
11c-20:1	0.17±0.08	0.12±0.01	0.10±0.10	0.33±0.12	0.24±0.17	0.15	0.71
Total n-9	6.31±0.56	4.92±0.50	4.69±0.57	6.30±0.44	5.97±0.58	0.25	0.32
11c-18:1	2.17±0.47	1.36±0.19	1.52±0.22	1.53±0.14	1.52±0.37	0.45	0.33
9c,12c-18:2	5.39±0.52	5.79±0.40	5.55±0.30	6.47±0.61	6.95±0.96	0.74	0.14
8c,11c,14c-20:3	1.11±0.13	0.70±0.13	0.58±0.08	1.10±0.21	0.83±0.58	0.20	0.31
5c,8c,11c,14c-20:4	26.35±1.41	24.59±0.70	24.08±0.90	25.99±1.35	26.17±0.44	0.20	0.67
7c,10c,13c,16c-22:4	0.21±0.03	0.22±0.01	0.24±0.04	0.20±0.03	0.21±0.03	0.12	1.00
4c,7c,10c,13c,16c-22:5	0.26±0.09	0.30±0.04	0.30±0.08	0.26±0.07	0.30±0.11	0.33	0.33
Total n-6	33.32±2.56	31.60±1.26	30.75±1.24	34.02±2.18	34.46±2.09	0.13	0.08
9c,12c,15c-18:3	0.15±0.03	0.13±0.01	0.13±0.03	0.18±0.05	0.15±0.03	0.33	1.00
5c,8c,11c,14c,17c-20:5	1.46±9±0.24^{a,b}	0.88±0.14^{b}	0.61±0.08^{b}	2.02±0.50^{a}	1.590.62^{a,b}	0.13	0.86
7c,10c,13c,16c,19c-22:5	0.80±0.12^{a,b}	1.40±0.12^{a}	1.34±0.15^{a,b}	0.99±0.19^{a,b}	0.94±0.29^{b}	0.39	0.50
4c,7c,10c,13c,16c,19c-22:6	10.60±1.37^{b,c}	12.63±1.34^{a,b}	14.41±0.74^{a}	9.72±0.55^{c}	9.57±0.48^{c}	0.02	0.25
Total n-3	13.01±1.56^{b}	15.04±1.17^{a}	16.49±0.89^{a}	12.91±1.03^{b}	12.25±1.27^{b}	0.05	0.26
9t,11t-CLA	ND	ND	ND	0.08±0.01	0.11±0.02	–	0.16
10t,12c-CLA	ND	0.09±0.01	0.18±0.03	ND	ND	0.05	–
9c,11t-CLA	ND	0.06±0.01	0.11±0.01	0.03±0.01	0.05±0.01	0.03	0.07
Total CLA	ND	0.15±0.01^{b}	0.29±0.06^{a}	0.11±0.03^{b}	0.16±0.04^{b}	0.01	0.08
9t,11t,13t-CLnA	ND	ND	ND	0.02±0.01	0.02±0.01	–	0.33
9c,11t,13t-CLnA	ND	ND	ND	0.02±0.01	0.02±0.01	–	0.33
9c,11t,13c-CLnA	ND	ND	ND	0.02±0.01	0.02±0.01	–	0.33
Total CLnA	ND	ND	ND	0.05±0.03^{a}	0.06±0.02^{a}	–	0.33
Others	0.41±0.05	0.45±0.02	0.31±0.05	0.47±0.07	0.43±0.12	0.51	0.79
PL/Liver (mg/g)	26.21±1.09	25.70±1.06	29.73±3.02	28.47±1.52	27.54±1.63	0.41	0.60

[a,b,c]Mean values in the same row with different superscript letter differ significantly ($P<.05$).
[#] Four experimental diets were prepared by adding 1% CLA (CLA1), 2% CLA (CLA2), 1% CLnA (CLnA1) and 2% CLnA (CLnA2) to the control diet.

milk lipids, 0.69–1.68% CLA in the PL of suckling rats and 0.15–0.29% CLA in the PL of maternal rats. The incorporation pattern was in agreement with that of our previous study in rats fed a 2% CLA diet [19]. Similarly, feeding 1–2% CLnA diets had 0.98–1.71%, 0.07–0.08% and 0.05–0.06% CLnA incorporated into the milk lipids and PL of suckling and maternal rats, respectively. We are unaware of any studies investigating the incorporation pattern of CLnA into milk and other tissues, and therefore we have no data to compare. Secondly, incorporation of CLnA into milk and PL was discriminated compared with that of CLA. This is probably attributable to the conversion of CLnA to CLA by oxidoreductase [20], and, therefore, lesser CLnA was available for incorporation into the milk and liver. Thirdly, supplementation of CLnA at 1–2% had no effect on the PL fatty acid composition of both maternal and suckling rats. In contrast, dietary CLA at similar levels increased docosahexaenoic acid and 16:0 proportionally in the PL of maternal rats, while it decreased 16:0 and increased docosahexaenoic acid in the PL of suckling rats. The present study confirmed our previous observation in rats fed a 2% CLA diet [19]. It is therefore concluded that addition of CLA and CLnA into maternal diet at the current levels would not cause adverse change in liver fatty acid composition of both maternal and neonatal rats.

In summary, we found that incorporation of CLnA isomers into milk was partially discriminated compared with CLA isomers due to metabolic conversion of CLnA to CLA. Rats must possess a Δ-13 saturation system, which is capable of converting CLnA isomers to CLA isomers *in vivo*. This system is more effective in saturation of a CLnA isomer at the Δ-13 position with *trans*-configuration being preferred.

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