Article

Effects of Maternal Clofibrate Supplementation During Gestation and Lactation on Intestinal Fatty Acid Oxidation of Suckling Piglets

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Effects of Maternal Clofibrate Supplementation during Gestation and Lactation on Intestinal Fatty Acid Oxidation of Suckling Piglets

Brandon Pike, Jinan Zhao, Julie A Hicks, Feng Wang, Paige Meisner, Lin Yang, Hsiao-Ching Liu, Jack Odle and Xi Lin *

Laboratory of Developmental Nutrition, Department of Animal Sciences, North Carolina State University, Raleigh, NC 27695, USA

*   Correspondence: xilin@ncsu.edu; Tel.: +01-919-515-4014

Abstract

To accelerate maturation of intestinal function and promote growth and development, the effect of maternal clofibrate on intestinal fatty acid metabolism was investigated in postnatal piglets. 27 pregnant sows were fed either 0, 0.25, or 0.5% clofibrate during late gestation and early lactation. [1-^{14}C]-Oleic acid metabolism was measured in vitro in intestinal mucosa of piglets with/without L-carnitine and/or malonate. Clofibrate increased oleic acid metabolism on d1, and the increase was higher from 0.5 than 0.25% of maternal clofibrate ($p < 0.005$). Flux to CO_2 increased with age while flux to acid soluble products (ASP) remained constant after d1. Flux to esterified products (ESP) increased on d7, but the increase was dampened by clofibrate ($p < 0.0001$). Carnitine increased flux to CO_2 and malonate decreased it ($p < 0.0001$), but neither affected ASP or ESP. Intestinal NEFA and TG levels decreased linearly, and CPT activity increased quadratically with age. Clofibrate increased ACOX abundance but decreased the ratio of CPT1A and CPT1B on d1. Postnatal age increased FABP2 but decreased PPARα and RXRα. In conclusion, maternal supplementation of clofibrate promotes intestinal energy generation from fat oxidation in postnatal piglets, but the promotion is influenced by age, in which ACOX, FABP2 and CPT1 might play modulatory roles.

Keywords: clofibrate; fatty acid; intestinal mucosa; maternal; suckling piglets

1. Introduction

The permeability of placenta to fatty acids (FAs) is highly restricted, consequentially; the primary energy substrate for developing fetus is glucose. Ketone bodies may be used as fuel and lipogenic substrates by the fetus when the mother is under fasting conditions using FA for ketogenesis [1]. However, milk lipids become the principal substrate for oxidative metabolism after birth. More than 55% of the dietary energy for neonates is milk fat [2] depending on species, suggesting that highly efficient oxidation of FA from milk fat is very important for the newborns' development and growth. Unlike human infants, newborn piglets and rats [3] have very limited fat stores. Energy is derived primarily from absorbed FA from the milk lipids, implying that intestine plays a critical role for neonates to acquire energy efficiently after birth.

Lipids and other nutrients from milk are taken primarily from the small intestinal lumen into the lymph system/bloodstream via the intestinal epithelial cells. This process undergoes significant development throughout life with key changes happening during fetal development and neonatal growth. The changes during development affect the efficiency of lipid uptake depending on species and energy needs at different stages. Fetal and neonatal pigs, like immature infants, are enriched in fetal-type cells in the developing intestine [4]. The fetal-type cells need to mature into adult enterocytes with fully functional absorptive cells at birth and during the suckling period. This transformation is crucial for their survival, development and growth after birth, as they have limited

energy storage and developmental delays. To have adult absorptive and secretory functions, therefore, the energy requirement of intestine is very high [5] including the needs for synthetic or digestive enzymes and the fast renewal of enterocytes.

The role of peroxisome proliferator-activated receptor alpha (PPARα) in FA oxidation in the liver has been well established, as the liver is the primary location of FA metabolic and ketogenic pathways to generate energy for use by other tissues. Compared to liver, the intestine utilizes lipids for energy itself [6]. This characteristic has driven us to evaluate the regulatory function of PPARα in the intestine. So far, most studies examining the effects of PPARα activation on intestinal lipid metabolism were performed in rodents [7–10]. Specifically, the expression of genes associated with PPARα activation by clofibrate, its pharmaceutical agonist, was determined in the developing small intestine in rat [11]. The results revealed that the PPARα target genes such as acyl-CoA oxidase 1 (ACOX 1) and FA-binding proteins (FABPs) might be coordinately regulated during postnatal development. Moreover, increased FA oxidation was observed also in enterocytes treated with the PPARα agonist bezafibrate [8]. As non-peroxisome-proliferating species [12], however, the kinetic responses of intestinal FA oxidative metabolism of pigs to PPARα activation have not been explored during neonatal-suckling period. Optimizing the metabolism of FA in the small intestine is beneficial for the survival and development of newborn pigs, because high neonatal piglet mortality has remained a major economic and animal welfare problem in the swine industry worldwide for decades.

The high mortality observed in neonatal swine is associated with the low energy intake and utilization after birth [13]. Increasing milk lipid utilization can improve growth and development, subsequently reducing the high mortality. Results from our previous studies showed that supplementation of clofibrate to neonatal piglets promoted hepatic and extrahepatic PPARα-dependent gene expression and increased FA oxidation [14,15]. Specifically, we found that maternal clofibrate could be transferred to the fetus across the placenta membrane and increase FA oxidation at birth. However, the stimulated FA oxidation is diminished with time [16], suggesting that sustained PPARα activation is required to maintain the induced high FA oxidation rate after birth. In addition, lactation transfer of clofibrate was observed in suckling rats with increased hepatic and renal cytochrome P450 4A mRNA levels [17,18]. With this knowledge, we hypothesized that supplementation of clofibrate to sows during late gestation and early lactation may stimulate the utilization of milk FA in the rapidly developing intestine of their offspring. To test our hypothesis, the capacity of energy generation from fatty acids was evaluated over time in intestine of suckling piglets from sows treated with or without clofibrate during late gestation and early lactation.

2. Results

2.1. Fatty Acid Oxidation

2.1.1. Effects of Maternal Supplementation of Clofibrate on Fatty Acid Metabolism in the Intestinal Mucosa of Piglets During the Neonatal-Suckling Period

- Significant interactions between maternal supplementation of clofibrate and postnatal age were observed for the ^{14}C accumulation in CO_2 ($p < 0.005$), ASP ($p < 0.05$), and ESP ($p < 0.0001$) as well as the total oxidation ($p < 0.05$) and total metabolism ($p < 0.0001$) from oleic acid (Table 1).
- The $^{14}CO_2$ accumulation was stimulated linearly with the dose of maternal clofibrate on d1 ($p < 0.0005$), but the stimulation was diminished after d7 depending on the dose. The accumulation rate measured on d1 was 1.1 and 1.6-fold greater in piglets from sows with 0.25 and 0.5% clofibrate than controls. The difference was not detectable on d7, while the accumulation was 60% higher on d19 from 0.25% and 44% and 72% higher on d 14 and 19 from 0.5% clofibrate treated sows than from the controls ($p < 0.05$).
- The ^{14}C accumulation in ASP on d1 was 1.3 and 1.7-fold higher in pigs from sows with 0.25 and 0.5% clofibrate than from the controls ($p < 0.05$), but the maternal supplementation had no effects

on the ^{14}C accumulation in ASP in pigs after d1. The ^{14}C accumulation in ASP was higher from d1 than all other ages (p < 0.0001), and no differences were observed between all other ages.

- The ^{14}C accumulation in ESP in piglets from control sows increased from d1 to 7 but decreased greatly after d7 (p < 0.005). The accumulation was on average 61% and 81% lower from d 14 and 19 than d1 and d7. No difference was detected between d 14 and 19. Maternal clofibrate had no impact on the accumulation in ESP on d 1, 14 and 19, but decreased the accumulation in ESP on d 7 (p < 0.0001). The decrease was greater from maternal clofibrate level 0.5% than 0.25%.Third bullet.

- The ^{14}C accumulation in total oxidation (CO_2 + ASP) was 37% and 79% higher in piglets on d1 from sows with 0.25 % and 0.5 % clofibrate than from the controls (p < 0.05), but supplementation had no effects on the ^{14}C accumulation after d1. The ^{14}C accumulation in CO_2 + ASP was higher from d1 than that measured from other ages (p < 0.0001), but no differences were observed between all other ages.

- The ^{14}C accumulation in the total metabolism (CO_2+ASP+ESP) in piglets from control sows increased from d1 to 7 (p < 0.005) but had no difference on d 14 and 19. The accumulation was 3.1 and 4.6-fold higher on average from d1 and 7 than d14 and 19 (p < 0.0001). Maternal supplementation of 0.5% clofibrate increased the total metabolism on day 1 (p < 0.005) but decreased the accumulation in ESP on d 7. The decrease was greater from maternal clofibrate level 0.5% than 0.25%.

Table 1. Effects of maternal clofibrate and postnatal age on oleic acid metabolism in small-intestinal mucosa of suckling pigs.

Clofibrate	Con				Clof 0.25				Clof 0.50					p-Value		
Age	D1	D7	D14	D19	D1	D7	D14	D19	D1	D7	D14	D19	SEM	Clof	Age	Clof*Age
Oxidation					µmol/g protein. h											
CO_2	0.31a	0.73bcd	0.80bcd	0.60abc	0.65bc	0.93de	0.82bcde	0.96de	0.82cde	0.54ab	1.15e	1.03de	0.11	0.0018+	0.0020	0.0045*
ASP	8.9b	1.07a	1.01a	1.35a	11.96c	1.14a	1.04a	1.86a	15.34d	0.75a	1.29a	1.57a	1.13	0.0990	0.0001+^	0.0310
CO_2+ASP	9.22b	1.79a	1.81a	1.94a	12.61c	2.07a	1.86a	2.82a	16.16c	1.29a	2.43a	2.60a	1.15	0.0588	0.0001+^	0.0246
Metabolism					µmol/g protein. h											
ESP	7.56c	22.83e	3.56ab	3.39ab	7.14c	16.17d	2.78a	3.53ab	7.41c	6.12bc	1.79a	2.37a	1.22	0.0001+	0.0001+^	0.0001*
CO_2+ASP+ESP	16.81b	24.57d	5.36a	5.44a	19.87bc	18.24bc	4.63a	6.34a	23.57cd	7.42a	4.25a	4.96a	1.81	0.0408	0.0001+	0.0001

Data are the least square means and pooled standard error means (SEM). ^{abcde}Means within a row lacking a common letter differ. Con, Control sows, Clof 0.25, sows received 0.25% of dietary clofibrate and Clof 0.5, sows received 0.5% dietary clofibrate. ASP, acid soluble products and ESP, esterified products. * Indicating the difference between clofibrate and control from the analysis of contrast. +linear response. ^quadratic response.

2.1.1. Effects of Maternal Supplementation of Clofibrate on Fatty Acid Metabolism in the In-Testinal Mucosa of Piglets During the Neonatal-Suckling Period

- The % CO_2 in total FA oxidation increased, and % of ASP in Total FA oxidation decreased linearly with maternal clofibrate dose (p < 0.05) and postnatal age (p < 0.0001). No interactions (p > 0.1) were detected between maternal clofibrate and postnatal age (Table 2).

- The % of CO_2, ASP and ESP in total FA metabolites had significant (p <0.005) interactions (Table 2) between the maternal clofibrate and age. The % of CO_2 in total metabolites increased with age from d1 to d14 (p < 0.0001), but the increase was greater in pigs from sows fed 0.25% clofibrate on d7. The % CO_2 in pigs on d14 and 19 from sows fed 0.5% clofibrate were higher than that from control sows (p < 0.05). The % of CO_2 in pigs from sows fed   0.25% clofibrate was lower at d 19 compared to d14 (p< 0.005), but the % measured in pigs from sows fed 0 or 0.5% clofibrate showed   no difference. The % of ASP in total metabolites measured in piglets from control sows was decreased from d1 to d7 and then increased from d7 to d19 (p < 0.0001). Maternal clofibrate increased the % of ASP, but the increase varied with age and clofibrate dose. The % was higher in piglets on d1 and 14 from sows fed 0.25% clofibrate than control sows, and on d7 and d14 from sows fed 0.5% clofibrate than the controls (p < 0.05). The % of ESP in total metabolites in pigs from control sows increased from d1 to d7 but decreased after d7 (p < 0.005).   As opposed to the % of ASP, maternal supplementation of clofibrate decreased the % of ESP and the decrease varied with the age and dose of maternal clofibrate supplementation. The decrease in % of ESP

from 0.5% of clofibrate was similar as 0.25% of clofibrate on d1 and d7 but was greater than 0.25% of clofibrate on d14 and 19 ($p < 0.05$).

- There was no interaction between maternal clofibrate and age on the ratio of CO_2 and ASP (C/A). Maternal clofibrate also had no impact on the ratio, but the ratio increased with age. On average the ratio increased by 2.75-fold after d7 compared to d1 ($p < 0.0001$). The ratio of oxidized and metabolized products (O/M) in piglets from the control sows decreased by 34% from d1 to d7 but increased by 106% from d7 to d14 and by 37% from d14 to d19 ($p < 0.001$). Maternal clofibrate had no impact on the ratio at d1 and d7 but increased greatly after d7. The ratio in piglets from clofibrate-fed sows was on average 13% and 39% higher than control sows ($p < 0.005$).

Table 2. Effects of maternal clofibrate and postnatal age on product distribution from oleic acid metabolism in small-intestinal mucosa from suckling pigs.

	Con				Clof 0.25				Clof 0.50				p-Value			
	D1	D7	D14	D19	D1	D7	D14	D19	D1	D7	D14	D19	SEM	Clof	Age	Clof*
Oxidation																
CO_2%	16.51	33.10	36.15	28.24	11.52	36.29	40.34	32.12	16.69	31.37	42.38	38.09	2.89	0.2410	0.0001+^	0.1514
ASP%	83.49	66.90	63.85	71.76	88.48	63.71	59.66	67.88	83.31	68.63	57.62	61.91	2.89	0.2410	0.0001+^	0.1514
C/A	0.27	0.99	1.04	0.58	0.14	0.91	0.96	0.54	0.27	0.70	0.98	0.73	0.13	0.6779	0.0001^	0.0769
Metabolism																
CO_2%	1.32[a]	6.51[b]	14.01[cde]	12.26[bcd]	2.87[a]	11.86[cd]	20.36[ef]	14.58[d]	3.63[a]	8.55[bc]	22.49[f]	20.43[e]	1.54	0.0001+	0.0001+^	0.0016*
ASP%[c]	28.48[d]	11.28[a]	18.58[c]	36.16[f]	34.56[ef]	12.34[ab]	28.47[d]	29.64[de]	33.53[def]	16.53[bc]	35.88[f]	31.51[def]	1.87	0.0001+	0.0001+^	0.0001*
ESP%[c]	70.19[ef]	80.33[g]	65.08[de]	51.58[bc]	62.57[d]	75.14[f]	50.15[bc]	55.78[c]	62.85[d]	73.90[f]	38.28[a]	48.05[b]	2.29	0.0001+	0.0001+^	0.0001*
O/M	0.30[b]	0.20[a]	0.35[b]	0.48[cd]	0.37[b]	0.25[a]	0.50[d]	0.44[c]	0.37[b]	0.26[ab]	0.62[e]	0.52[d]	0.02	0.0001+	0.0001+^	0.0001*

Data are least square means and pooled standard error means (SEM). [abcde]Means within a row lacking a common letter differ. Con, Control sows, Clof 0.25, sows received 0.25% of dietary clofibrate and Clof 0.5, sows received 0.5% dietary clofibrate. ASP, acid soluble products and ESP, esterified products. C/A, CO_2/ASP and O/M, oxidation/metabolism. *Indicating the difference between clofibrate and control from the analysis of contrast. +linear response. ^quadratic response.

2.1.3. Effects of Carnitine and Malonate on Fatty Acid Metabolism in the Intestinal Mucosa of Piglets During the Neonatal-Suckling Period

- No interaction (Figure 1A) was detected between maternal clofibrate ($p = 0.9$) and the tissue treatment with carnitine or malonate for CO_2 production. However, the supplementation of carnitine increased ^{14}C accumulation in CO_2 by 18% compared to control ($p < 0.05$). Supplementation of malonate decreased ^{14}C accumulation in CO_2 by 61% compared to control ($p < 0.0001$). No improvement was observed after adding carnitine to the treatment with malonate. Supplementation of carnitine or/and malonate in the mucosa incubation had no impact on the accumulation in ASP ($p > 0.1$). No interaction was detected between maternal clofibrate ($p > 0.1$) and the tissue treatment with carnitine or malonate. The tissues from all pigs treated with carnitine and malonate had no impact on the ESP production ($p > 0.1$).

- Total oxidized products (CO_2 + ASP) and the total metabolites (CO_2+ASP+ESP) were not affected by supplementation of carnitine or/and malonate ($p > 0.1$). No interaction was detected between maternal clofibrate (age) and the tissue treatment with carnitine or malonate (Figure 1B). Addition of carnitine increased the ratio of O/M regardless of malonate treatment ($p < 0.0001$), but no interaction was detected between maternal clofibrate (age) and the tissue treatment with carnitine or malonate.
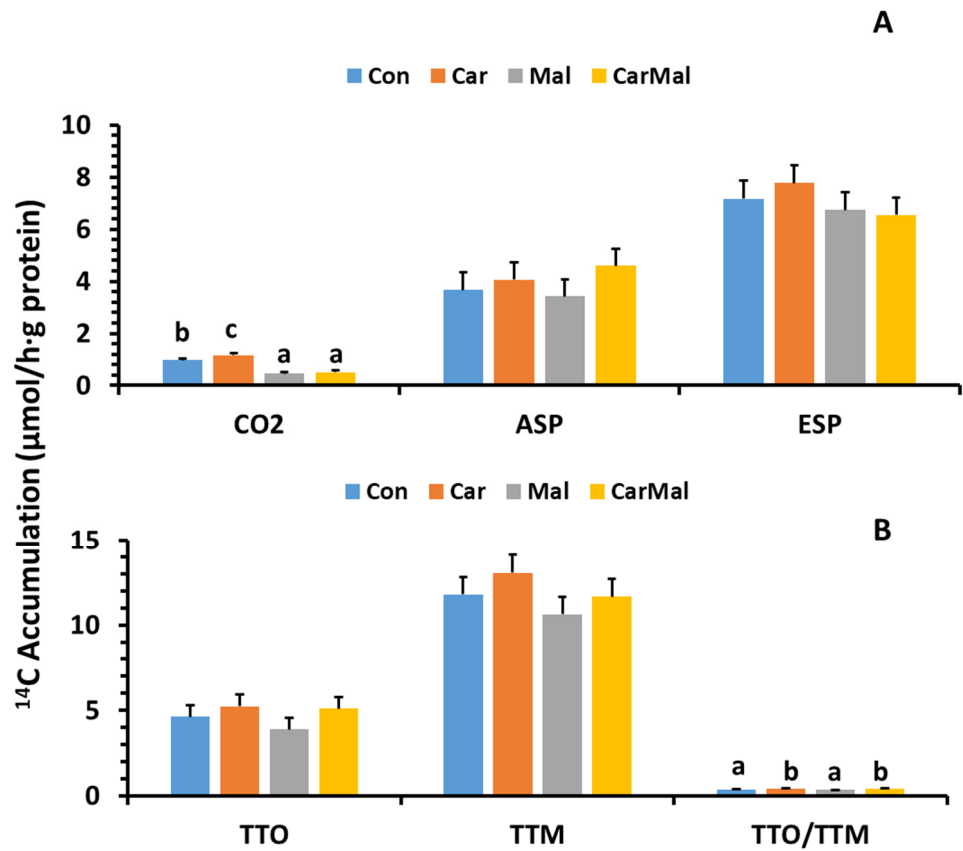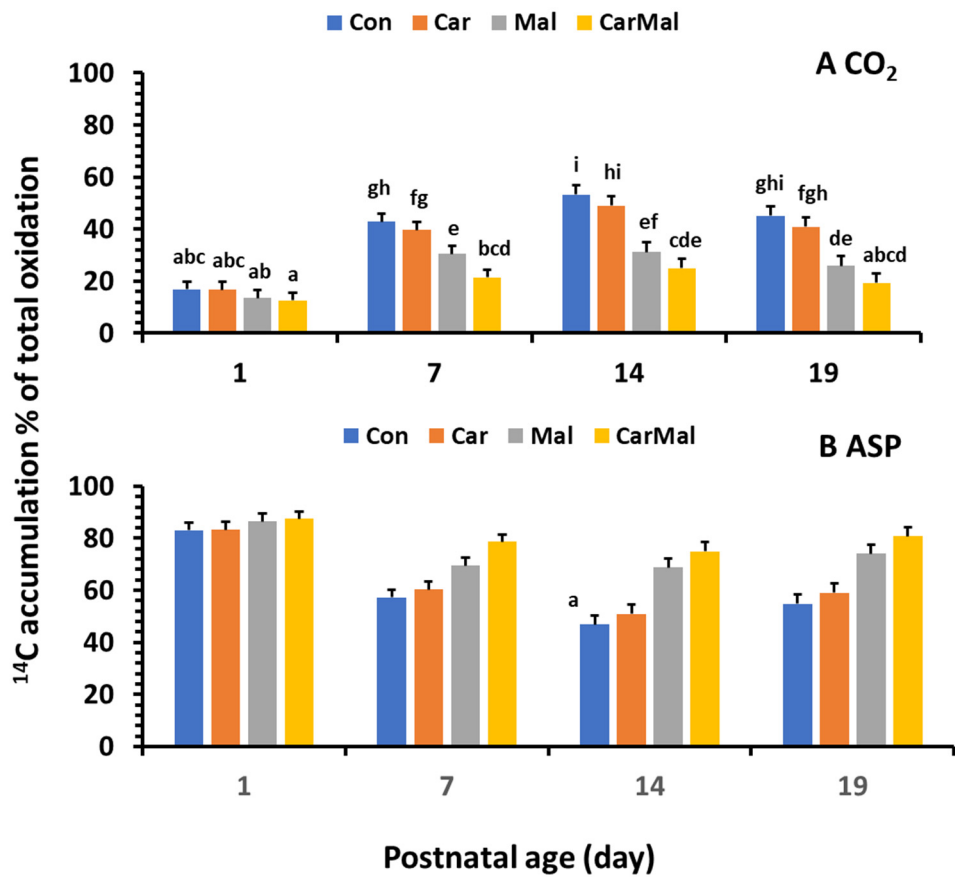
Figure 1

Figure 1. Effects of carnitine and malonate on intestinal oleic acid oxidation in suckling piglets. Oleic acid oxidation was measured with or without carnitine (1 mM) and/or malonate (5 mM) in intestinal mucosa of pigs from sows with or without clofibrate (Con, control; Car, carnitine; Mal, malonate; CarMal, carnitine+malonate). Columns represent oxidative products (μmol/g protein . h)and are least square means ± standard error means (CO_2; ASP, acid soluble product; ESP, esterified products; TTO, total oxidation product; TTM, total metabolic products; TTO/TTM, the ratio of TTO and TTM). [abc]Columns with differing letters denote significant differences ($p < 0.05$).

2.1.4. Effects of CARNITINE and malonate on Distribution (%) of CO_2, ASP and ESP in Total Fatty Acid Oxidation and Metabolism in the Intestinal Mucosa of Piglets During the Neonatal-Suckling Period

- There was no interaction between maternal clofibrate treatments and the treatments with carnitine and/or malonate ($p > 0.05$). However, significant interactions were detected between adding carnitine or/and malonate and postnatal age for % of CO_2 and ASP ($p < 0.0 1$) in total oxidation and the C/A ($p < 0.0001$) as well as the % of CO_2 ($p < 0.0001$) and ASP ($p < 0.01$) in the total metabolism. The interaction for ESP % in the total metabolism and the O/M also tended to be significant ($p = 0.052$).
- Addition of carnitine in the incubation medium had no influence on the % of CO_2 and ASP in the total oxidized products ($p > 0.1$), but addition of malonate reduced % of CO_2 (Figure 2A) and increased % of ASP (Figure 2B) in all ages ($p < 0.05$). The decrease was greater from the addition of carnitine+malonate than malonate when compared to the control. The ratio of CO_2/ASP in control group increased quadratically with age, but the increase was reduced by the addition of carnitine. Addition of malonate inhibited the ratio increase with age and kept the ratio with no difference from the d1.

Figure 2

Figure 2. Effects of carnitine and malonate on the distribution of intestinal oleic acid oxidation in piglets during the suckling period. Oleic acid oxidation was measured with or without carnitine (1 mM) or/and malonate (5 mM) in intestinal mucosa of pigs from sows with and without clofibrate (Con, control; Car, carnitine; Mal, malonate; CarMal, carnitine+malonate). Columns represent % of total oxidative products and are least square means ± standard error means for CO_2 (A) and ASP, acid soluble product (B). Columns lacking a common letter are different ($p < 0.05$).

- A similar pattern of % of CO_2 (Figure 3A) as in oxidation was observed from control group. However, the % of ASP (Figure 3B) significantly decreased after d1, and the decrease was greater from d7 than d14 and 19. Addition of carnitine or/and malonate increased % of ESP (Figure 3C) and the increase was greater from carnitine+malonate than carnitine or malonate only. No difference was observed between d14 and 19.   The % of ESP increased on d7 and decreased after d7. The increase was reduced by the addition of carnitine and increased by the addition of malonate. No impacts were detected after d7. The ratio of oxidation and metabolism followed the same pattern as observed in % ASP.
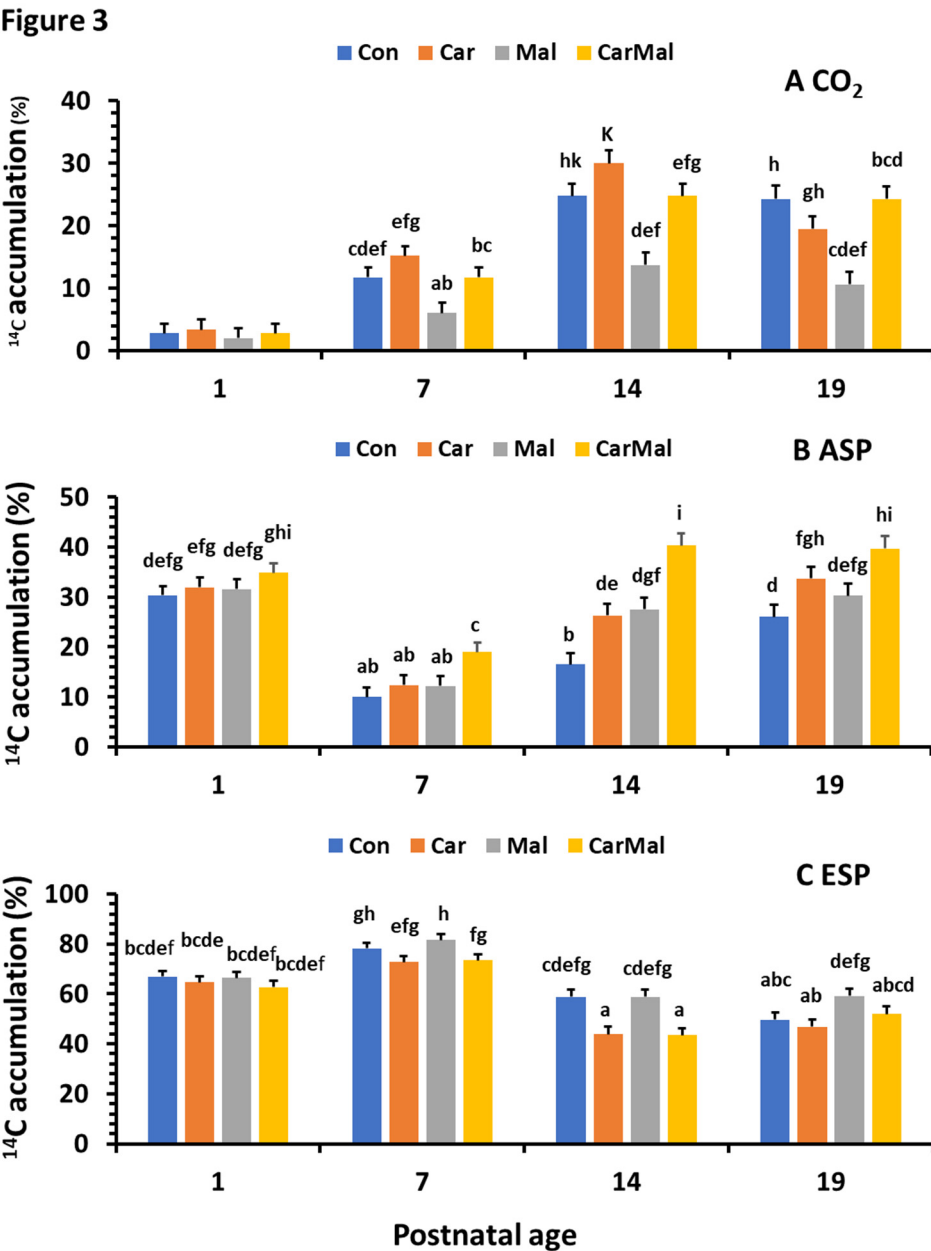
Figure 3. Effects of carnitine and malonate on the distribution of intestinal oleic acid metabolism in piglets during the suckling period. Oleic acid oxidation was measured with or without carnitine (1 mM) or/and malonate (5 mM) in intestinal mucosa of pigs from sows with and without clofibrate (Con, control; Car, carnitine; Mal, malonate; CarMal, carnitine+malonate). Columns represent % of total metabolic products and are least square means ± standard error means for CO_2 (A); ASP, acid soluble product (B); and ESP, esterified products (C). Columns lacking a common letter are different ($p < 0.05$).

2.2. NEFA and TG Concentrations

Maternal clofibrate supplementation had no impact on NEFA and TG concentration in the intestinal mucosa ($p > 0.1$). The average NEFA and TG level in the tissue were 52.94 ± 5.47 (µmol/g) and 2.39 ± 0.30 (µg/mg protein) respectively. However, both NEFA (Figure 4A) and TG (Figure 4B) varied greatly with postnatal age, in which a significant linear decrease with postnatal age was observed in the TG concentration and a quadratic response to the postnatal age was observed in NEFA concentration ($p < 0.005$). No interaction ($p > 0.05$) was detected between maternal clofibrate and postnatal age.
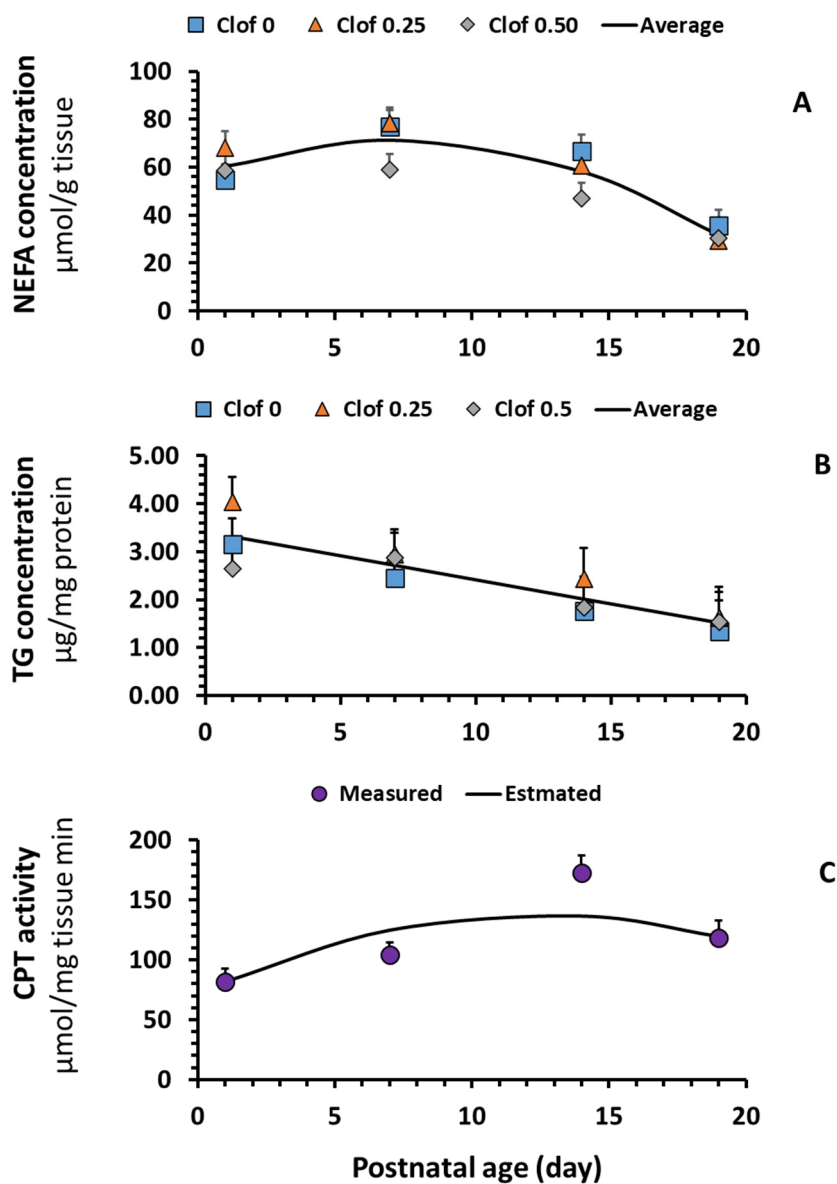
Figure 4. Effect of postnatal age on intestinal NEFA, TG and CPT activity in suckling piglets. NEFA (A) and TG (B) were determined using the commercial kits from MyBioSource Inc (MBS9719080 and MBS2556986; San Diego, CA, USA). The type and color of the markers represented data from maternal treatments (Clof 0, control; Clof 0.25 clofibrate 0.25; Clof 0.5 clofibrate 0.5) and the curve was from regression analysis with data from all treatments ($R^2 = 0.2$ and $p < 0.0001$) for NEFA and ($R^2 = 0.17$ and $p < 0.001$) for TG. CPT activity (C; $R^2 = 0.1$ and $p < 0.05$) was measured using radio-enzymatic assay [34].

2.3. CPT Enzyme Activity

Maternal supplementation of clofibrate had no significant impact on the activities of CPT1, CPT2 and Total (CPT1 + CPT2), except for the trends test using orthogonal polynomial coefficients that showed the CPT1 activity tended increase linearly with maternal clofibrate dose (p=0.067). Postnatal age had a greater impact on CPT2 activity and the total activity of CPT ($p < 0.001$), and the impact followed a quadratic pattern ($p < 0.01$).   The highest activity was observed in piglets at 14 days of age (Figure 4C).

2.4. Gene Expression (qPCR)

Maternal supplementation of clofibrate had no impact on the expression of genes examined in intestine mucosa of pigs (Table 3). However, postnatal age had an impact on the expression of CPT1A ($p < 0.05$), FABP ($p < 0.001$), PPARα ($p < 0.05$) and Retinoid X receptor alpha (RXRα; $p < 0.005$).   The expression FABP was on average 90% higher in pigs at d14 and 19 than d1 and 7, while the expression of CPT1A was 53% lower on d14 than on the average of d1, d7 and d19. PPARα and RXRα were on average 50% and 74% lower in pigs at d19 than d1 and d7. In addition, the interaction between maternal clofibrate and postnatal age was significant for ACOX expression ($p < 0.05$) and tended to be significant for the ratio of CPT1A and CPT1B ($p = 0.087$), in which maternal supplementation of clofibrate increased ACOX expression but decreased the ratio of CPT1A and CPT1B on d1.

Table 3. Effect of maternal clofibrate and postnatal age on intestinal mucosa gene expressions of piglets during neonatal suckling period.

Genes	Clofibrate (%)					Postnatal Age (day)						Interaction
	Clof 0.00	Clof 0.25	Clof 0.50	SEM	*p-Value*	D1	D7	D14	D19	SEM	*p-Value*	*p-Value*
	Fold change					Fold change						
INTESTINE												
ACOX1	2.75	2.60	2.34	0.24	0.423	2.68	2.46	2.63	2.50	0.28	0.915	0.037*
CPT 1A	1.01	1.04	1.19	0.19	0.737	1.48[b]	1.09[b]	0.58[a]	1.16[b]	0.22	0.046^	0.543
CPT 1B	1.14	0.98	1.18	0.21	0.772	1.39	1.35	0.65	1.02	0.25	0.161	0.177
FABP2	0.82	1.17	1.04	0.13	0.171[#]	0.56[a]	0.79[a]	1.25[b]	1.43[b]	0.15	0.001[+]	0.400
PPARα	1.23	0.89	1.10	0.15	0.266	1.28[b]	1.41[b]	0.96[ab]	0.66[a]	0.18	0.019[+]	0.106
RXRα	1.15	0.66	0.95	0.17	0.123[#]	1.22[bc]	1.34[c]	0.79[ab]	0.33[a]	0.20	0.003[+]	0.244
CPT1A/CPT1B												
Ratio	1.04	1.01	0.93	0.09	0.662	1.00[ab]	0.89[a]	0.84[a]	1.25[b]	0.09	0.034^	0.087*

Genes: *ACOX,* peroxisomal acyl-CoA oxidase; *CPT1A*, carnitine palmitoyltransferase 1A, *CPT1B*; carnitine palmitoyltransferase 1A; *FABP*, fatty acid binding protein; *PPARα*, peroxisome proliferator-activated receptor alpha; *RXRα*, retinoid X receptor alpha.
[#]Contrast control vs. clofibrate for *FABP* ($p = 0.078$); for *RXR* ($p = 0.095$); [+]linear response ($p < 0.05$) ^ quadratic response ($p < 0.05$).
*Significant interaction was observed for *ACOX*1 in which supplementation of clofibrate increased its expression on d1 but not after d1. The ratio of *CPT1A/CPT1B* decreased on d1.

3. Discussion

3.1. The Effect of Maternal Clofibrate on Intestinal Fatty Acid Metabolism in Suckling Piglets

Development and growth of the intestine is rapid after birth, and increasing FA oxidation could be important for the intestine to meet the energy requirement and promote the fetal-type enterocyte maturation because milk fat is the primary energy source at birth.   In this study, we evaluated the effects of PPARα activation on intestinal FA metabolism in piglets by feeding the sows clofibrate (a PPARα agonist) during late gestation and early lactation. The effects of clofibrate varied with dose and piglet postnatal age. In general, the intestinal oxidation of oleate to CO_2 increased linearly with the postnatal age. Maternal supplementation of clofibrate promoted the increase throughout the entire postnatal period. Consistent with the increase in CO_2 production, the TG and NEFA levels decreased with postnatal age, demonstrating the role and importance of TG and NEFA in intestinal energy supplies. Furthermore, stimulation of CO_2 production was impacted by clofibrate doses and by postnatal age despite clofibrate being undetectable in the milk. Similar results were not observed in liver metabolism with the same treatments after d1 [14]. Given the detection limit of measuring clofibrate, whether the variation was associated with a potential very trace amount of clofibrate in the milk was not known. In addition, the variations in CO_2 production were not in conjunction with an increase in CPT1 activity and gene expression of PPARα and RXRα in the present study although a higher CPT2 activity was observed on d14. CPT1A and CPT1B are present on the mitochondrial membrane in most tissues, while pig has an atypical molecular structure of CPT1, and the sensitivity of CPT1A and CPT1B to malonyl-CoA inhibition are different from other animals [19], in which CPT1A has a high sensitivity to malonyl-CoA inhibition and CPT1B has a low sensitivity to malonyl-CoA inhibition [20]. Therefore, we examined the CPT1A and CPT1B gene expression and were unable to detect any changes in intestine of piglets from sows with clofibrate supplementation in early

lactation. However, we noticed that the ratio of *CPT1A*/*CPT1B* decreased with postnatal age. The interaction between maternal clofibrate and piglet age for the ratio tended to be significant ($p = 0.087$) and the ratio was lower on d1 from clofibrate than control. The changes in *CPT1A* and *CPT1B* ratio might influence the sensitivity of CPT1 to malonyl-CoA inhibition. The relative decrease in highly malonyl-CoA sensitive CPT1A might be associated with the CO_2 increase. In addition, interaction between maternal clofibrate and postnatal age for ACOX1 expression was detected with a higher expression on d1. Also, FABP2 expression increased with age, indeed, suggesting that ACOX1 and FABP play a role in intestinal FA oxidation as observed in rats [11]. Furthermore, it is possible that genes associated with TCA that were not examined in this study contributed to the increase in CO_2 production. CO_2 production is indicative of increased ATP production through the TCA, and it has been reported that intermediates of the TCA were altered with clofibrate treatment [21].

Compared to CO_2, ASP production had completely different responses. The dose-dependent stimulation of oleate oxidation to ASP by maternal clofibrate was detected only on day1. Combing CO_2 and ASP together, the effect of maternal clofibrate on FA oxidation followed the same pattern as observed in ASP production. This result was consistent with that observed in liver of piglets receiving clofibrate from maternal supplementation, demonstrating clofibrate transferred from sows to fetal piglets via the placenta. After d1, ASP production decreased dramatically, and the reduced ASP production was not affected by maternal clofibrate supplementation or by further advancement in postnatal age. The lack of response to maternal clofibrate could be due to a low mammary transfer efficiency or dose of clofibrate supplementation, because the dose used in previous study with lactating rats was at least 7-fold [18] higher than the dose we fed to the sows. In addition, ketogenic capability was observed in the small intestine of rats [22] and can be induced by ketogenic diet [23], but the expression of ketogenic rate-limiting enzyme mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) was not detectable in the small intestine of neonatal pigs [24]. Butyrate was converted into ketone bodies observed in colonocytes with increasing concentrations of [1-^{14}C] butyrate [25], but gene expression almost was not detected in duodenum, jejunum and ileum until 8 weeks of age. Therefore, the reduced ASP production appeared to be associated with the inherent defect in ketogenic ability in intestine of neonatal piglets. In addition, the PPARα, and RXRα significantly decreased after d7. The reduced ASP production appeared to be associated with the lack of ketogenic ability and gene expression, while the high ASP production on d1 could be related to the high-level TG and NEFA as well as accumulation of the absorbed soluble carnitine esters after birth.

A previous study with hepatocytes isolated from suckling newborn and 15 d-old pigs showed that neonatal piglets have a huge rate of hepatic fatty acid esterification [26]. The rate of fatty acid esterification was associated with the rapid increase in fat- stores after birth [27]. Indeed, the hepatic esterification increased greatly with the postnatal age [14]. A similar increase in ESP production on d2 was also observed in a previous study with pig intestinal cells [28]. It is very interesting, however, that the intestinal esterification rate increased after birth and decreased greatly after 7 days, and maternal clofibrate had a great impact on the increase of the FA esterification on d7. The high ESP measured in pigs from control sows was reduced greatly compared to pigs from sows with clofibrate. The reduction was much greater from high maternal clofibrate than low maternal clofibrate, suggesting that maternal clofibrate could affect the ESP after birth and the degree of effect depended on the dosage of maternal clofibrate. However, no clofibrate effect was detected when the ESP decreased quickly with age after d7. These changes were consistent with the decrease in PPARα, RXRα expression and increase in FABP expression with age. The results from this study highlighted the high esterification capacity for FA in the newborn pig intestine, which may preserve this major colostrum FA for delivery to other tissues. Nutritional control of intestinal FA esterification, such as dietary fat intake, has been described in the literature [29]. Milk fat usually is high after farrowing and in the first several days after farrowing depending on species. Sow milk fat peaked around d3 and then decreased as lactation progressed [30]. We were interested in the potential influence of the decreased ESP on FA oxidation. The decrease in esterification on d7 and after d7 clearly illustrate that

it has limited impact on overall oxidation. Combining the oxidative products and ESP, the total metabolites were increased at d1 primarily due to an increase in oxidation induced by clofibrate, while the total metabolism was decreased at d7 primarily due to a decrease in esterification.

Although the stimulatory effect of clofibrate on fatty acid oxidation had a significant interaction with the postnatal age, the interaction did not affect oxidative product distributions between CO_2 and ASP. In general, the CO_2 increased with clofibrate dose and postnatal age while the ASP decreased with clofibrate and postnatal age. Both linear and quadratic responses were detected. However, the distributions of CO_2, ASP and ESP in total metabolism were impacted significantly by maternal clofibrate and postnatal age. In general, the % of oxidative products on average increased and the % of ESP decreased in piglets from sows receiving clofibrate during late gestation and early lactation. The effect of maternal clofibrate on the distribution of metabolic products appeared to be different from that in liver [14], implying the tissue specificity in which the absorption of dietary FA and converting them into TG for transport to other tissues is the primary pathway but not for producing ketone bodies and FA synthesis. The limited FA synthetic activity and rapid reduction in FA esterification are also the key factors in altering the metabolic allocations.

3.2. *The effect of Providing Carnitine and Inhibiting TCA Activity on Intestinal Fatty Acid Metabolism in Suckling Pigs*

Providing carnitine increased CO_2 production and the oxidative proportion of the total metabolism, suggesting that the stimulatory role of carnitine might be limited to the entry of FA into mitochondria. This was confirmed by inhibiting TCA cycle activity via the addition of malonate, an inhibitor of succinate dehydrogenase. As the product of β-oxidation, acetyl-CoA can be metabolized completely via TCA cycle or be converted to acetate, acetyl-carnitine, HMG-CoA for ketogenesis or malonyl-CoA for FA synthesis, which comprises most of the ASP. We expected that ASP would increase when carnitine was added into the system and both ASP and ESP would increase when TCA cycle was inhibited. However, no influences on ASP and ESP were detected by the addition of carnitine or/and malonate. Available evidence in the literature showed that lipogenesis in pigs is extremely low in tissues other than adipose [31]. Consistent with this observation, the activities of enzymes related to lipogenesis are also very low in intestine [32]. In addition to lipogenesis, the gene of the key enzyme HMGCS for ketogenic pathway is undetectable in the small intestine of neonatal pigs [24]. The lack of lipogenic and ketogenic activities appeared to be associated with the lower response of ASP to carnitine and malonate. Although the status of carnitine acetyltransferase and acetyl-hydrolase in pig intestine was not evaluated in this study, and are not available in literature, our results illustrated and supported that the energy generation pathway is the main flux within intestinal FA metabolism. Energy generated from FA oxidation is the primary fate in intestinal mucosal fatty acid metabolism such as the TG/FA absorption and FABP synthesis.

4. Materials and Methods

4.1. *Animals and Treatments*

The animal study was described previously [14]. Briefly, a total of 27 gestating sows (in 3 blocks and 9 sows each block) were divided into three groups and fed a standard commercial corn/soybean-meal diet supplemented with three levels of clofibrate: 0% (Control), 0.25% (Clof 0.25), and 0.5% (Clof 0.5) of feed. The treated sows received either clofibrate dissolved in 15 mL of ethanol or 15 mL of ethanol vehicle. The sows were fed twice daily, but the ethanol with or without clofibrate was given to all the sows in the morning via mixing with and feeding in small amount of feed. The clofibrate supplementation started from one-week pre farrowing and ended with one-week post farrowing. The Institutional Animal Care and Use Committee (IACUC) at North Carolina State University, IACUC ID 16-142, approved animal care and all experimental procedures.

Piglets with average body weight from each litter at each time point were selected on days 1, 7, 14, and 19. The selected piglets were euthanized via American Veterinary Medical Association

approved exsanguination while under anesthesia. Intestinal mucosa was collected from the proximal and distal ends of the small intestine from the piglets at each time point in 30-centimeter segments. The segment was rinsed with 0.9% NaCl solution and then opened lengthwise. With the segment on a smooth flat surface, a glass microscope slide was used to scrape the mucosa free. The first 30 centimeters of the proximal end was used for fatty acid oxidation measurement and the second 30 centimeters were frozen in liquid nitrogen for later analysis.

4.2. Fatty Acid Metabolism Measurements

The collected mucosa was homogenized in a handheld glass homogenizer (7 mL, Dounce Tissue Grinder; Vineland, NJ 08360, USA) in a buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.1mM EDTA with a ratio of 1:4 (w:v). FA oxidation was measured in a reaction buffer incubated with the fresh mucosa homogenate using [1-^{14}C]-oleic acid as a substrate (1 mM; 0.25μCi/μmole). The measurements were performed in the presence or absence of L-carnitine (1 mM) with or without malonate (5 mM), an inhibitor of tricarboxylic acid cycle (TCA), in a reaction buffer reported by [33]. The CO_2, acid soluble products (ASP) and esterification products (ESP) generated from the measurements and the homogenate protein were determined following the procedures described previously [14].

4.3. Non-Esterified Fatty Acids (NEFA) and Triglycerides (TG) Assays

TG and NEFA were determined in the frozen mucosa using the commercial kits from MyBioSource Inc (MBS9719080 and MBS2556986; San Diego, CA, USA) by following the kit instructions.

4.4. Enzymatic Assay

Specific activity of carnitine palmitoyl transferase (CPT) was determined in homogenate from the frozen mucosa samples with and without addition of malonyl-CoA (10 mM). The determination was performed following the procedure as described previously [34].

4.5. RNA Isolation and RT-qPCR

Total RNA was isolated from the mucosa using Tri reagent with 50 mg of the frozen samples following the manufacturer's procedure. Quantification and quality control were performed using a Nanodrop spectrophotometer (ND-1000ThermoFisher, Wilmington, DE), and 0.8% agarose gel electrophoresis post DNase treatment. cDNA synthesis was performed using Super Script III Reverse Transcriptase as described previously [15]. Primers for RT-qPCR (Supplemental Table 1) were created using BLAST Primer Designer (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The RT-PCR reaction and the ct values were normalized to a housekeeping gene (RPL9) and a plate normalizer was included on each plate to account for run differences.

4.6. Chemicals

All chemicals for this study were sourced from Sigma-Aldrich, Inc (St. Louis, MO 63103), except for clofibrate that came from Cayman Chemical (Ann Arbor, MI 48108), Superscript from Thermo Fisher Scientific (Waltham, MA02451), and TurboDNase from Ambion. The ^{14}C radiolabeled oleic acid and acetyl-CoA were purchased from American Radiolabeled Chemicals (St Louis, MO 63146) and SYBR Green from BioRad (Hercules, CA94547).

4.7. Statistical Analysis

Data from enzymatic and RT-qPCR assays were analyzed according to a 3 (Control, Clof 0.25 and Clof 0.5) x 4 (d1, 7, 14 and 19) factorial randomized complete block design (blocked by sow (litter)). Data from in vitro fatty acid oxidation was analyzed according to split-plot design. The main plot was the 3 maternal dietary treatments on animals at 4 ages, and the sub-plot was the 4 treatments

with a 2 (± carnitine) x 2 (± malonate) factorial design on the tissues. Analyses were performed using the General Linear Models (GLM) procedure of SAS (SAS software 9.4; Cary, NC USA). Testing for trends (linear and quadratic) was performed also with the contrast statement using orthogonal polynomial coefficients. The least square means (Lsmeans) were calculated and the interactions between clofibrate x postnatal age, and between (clofibrate x postnatal age) x treatments were tested. The data from main effects were reported only if the interaction was not significant. Data are presented as Lsmeans ± standard error means unless specified otherwise. Difference was reported as significant when $p < 0.05$ and as trends when $0.05 < p < 0.1$.

5. Conclusions

Maternal supplementation of clofibrate at late gestation and early lactation improves energy generation via increasing β-oxidation activity, decreasing FA esterification and promoting TG and NEFA utilization. However, the improvement varies with the increase in postnatal age, in which the alterations of gene expression of ACOX and FABP as well as ratio of CPT1A/CPT1B may play regulatory roles. Addition of carnitine increases FA oxidation, but the increase is limited by the activity of TCA cycle. In addition, the dose of clofibrate and the efficiency in delivery of clofibrate via milk apparently need to be investigated in the future.

Abbreviations

The following abbreviations are used in this manuscript:

ACOX1	Acyl-CoA oxidase 1
ASP	Acid soluble products
ESP	Esterification products
FA	Fatty acid

FABPs	FA-binding proteins
HMGCS	3-hydroxy-3-methylglutaryl-CoA synthase
NEFA	Non-Esterified Fatty Acids
RXRα	Retinoid X receptor alpha
TCA	Tricarboxylic acid cycle
TG	Triglycerides

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