Effects of in ovo feeding of t10,c12-conjugated linoleic acid on hepatic lipid metabolism and subcutaneous adipose tissue deposition in newly hatched broiler chicks

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ABSTRACT The purpose of this study was to investigate whether in ovo feeding of t10,c12-conjugated linoleic acid (**CLA**) could regulate hepatic lipid metabolism and decrease lipid accumulation in newly hatched chicks. Three hundred and sixty fertily specific pathogen-free hatching eggs were selected and randomly divided into 6 groups. On embryonic day 11 of incubation (**E11**), 0, 1.5, 3.0, 4.5, 6.0, or 7.5 mg t10,c12-CLA were injected into the eggs. The results indicated that in ovo feeding of t10,c12-CLA significantly decreased the subcutaneous adipose tissue (**SAT**) mass and the relative SAT weight of newly hatched chicks in linear and quadratic manners ($P < 0.05$). In liver, the levels of triglycerides were reduced linearly and quadratically and total cholesterol were reduced quadratically as the dose of t10,c12-CLA increased ($P < 0.05$). Meanwhile, the hepatic carnitine palmitoyltransferase-1a (**CPT1a**) content and polyunsaturated fatty acid proportion were increased quadratically in t10,c12-CLA groups ($P < 0.05$), accompanied by the decrease of malondialdehyde level and the increase of glutathione peroxidase and total antioxidant capacity activities ($P < 0.05$). In addition, in ovo feeding of t10,c12-CLA decreased the mRNA expression levels of fatty acid synthase, acetyl-CoA carboxylase 1 in linear and quadratic manners ($P < 0.05$), and decreased the mRNA expression of adipose triacylglyceride lipase and stearoyl-CoA desaturase significantly in liver ($P < 0.05$), accompanied by upregulating the mRNA expression of CPT1a quadratically and AMP-activated protein kinase α linearly and quadratically ($P < 0.05$). In SAT, the mRNA expression of peroxisome proliferator-activated receptor γ ($PPAR\gamma$) and sterol regulatory element-binding protein-1c were decreased linearly and quadratically ($P < 0.05$), and the expression of $PPAR\alpha$ and $CPT1a$ genes were increased linearly and quadratically as the dose of t10,c12-CLA increased ($P < 0.05$). In conclusion, our findings demonstrate that in ovo feeding of t10,c12-CLA alleviates lipid accumulation in newly hatched chicks by suppressing fatty acid synthesis and stimulating lipolysis in the liver and inhibiting adipocyte differentiation in subcutaneous adipose tissue.

Key words: t10,c12-conjugated linoleic acid, in ovo feeding, liver, lipid metabolism, newly hatched chicks

INTRODUCTION

Metabolic disorder diseases, such as obesity and diabetes, are a growing concern worldwide, as the incidence rate is increasing due to the modern lifestyle of high-energy diets and reductions in physical activity (Dominguez-Salas et al., 2014; Stephenson et al., 2018; Zheng et al., 2018). Maternal obesity increases the risk of obesity in children, which further continues into adulthood, accompanied by an elevated risk of cancer, type 2 diabetes, cardiovascular disease, and high blood pressure (Yu et al., 2013; Gaillard, 2015). Numerous studies have shown that nutrition intervention during pregnancy and the infant period could benefit offspring in achieving standard weight gain and adequate metabolic control, which shows that the development of nutrient regulatory strategies in earlier stages of life is crucial (Fernandez-Twinn and Ozanne, 2010; Ross and Desai, 2013; Vickers, 2014). Such strategies provide an

opportunity to promote healthy fetal development, which could subsequently lead to healthy offspring that are less likely to develop metabolic disorder diseases during pregnancy and the infant period with nutrition intervention.

Conjugated linoleic acid (**CLA**) has been widely investigated as an inducer of delipidation that acts by modulating energy expenditure, apoptosis, fatty acid oxidation, lipolysis, stromal vascular cell differentiation, and lipogenesis (House et al., 2005). Among all CLA isomers, t10,c12-CLA was found to be responsible for fat-reducing effects (House et al., 2005). Previous studies showed that maternal supplementation with a 0.5% CLA mixture increased t10,c12-CLA incorporation in the serum, liver, and adipose tissue of offspring and decreased triglyceride (**TG**) accumulation and adipose tissue deposition (Reynolds et al., 2015; Lavandera et al., 2017; González et al., 2020; Fu et al., 2021). However, CLA treatment also decreased the synthesis of monounsaturated fatty acids (**MUFA**s) from saturated fatty acids (**SFA**s) by inhibiting Δ-9 desaturase activity in hen livers and increasing the ratio of SFAs:MUFAs in yolk sacs (Muma et al., 2006; Fu et al., 2021). Considering that chick embryonic development depends on the nutrients that are available in egg yolk (Nasir and Peebles, 2018; Givisiez et al., 2020), whether the decrease in fat deposition in chick offspring was a direct result of t10,c12-CLA incorporation in the yolk or liver of chicks or a change in fatty acid composition remains unclear. The effect of t10,c12-CLA incorporation in the yolk of fertilized eggs on the lipometabolism of chick offspring requires further study.

The nutritional state of the embryonic period is very important in regulating the development of tissues and organs in the growth stages of broilers, as it requires the absorption of specific nutrients (Nasir and Peebles, 2018). The in ovo feeding approach has been used for many years as a useful, convenient, and cost-effective method to investigate early nutrition during artificial incubation (Willemsen et al., 2008; Chen et al., 2009, 2010; Dai et al., 2020; Fatemi et al., 2020). Several studies on in ovo feeding of amino acids, minerals, carbohydrates, plant extracts, and vitamins have shown to improve chick hatching conditions and benefit the physiological and biochemical stages related to embryo development (Yair et al., 2015; Neves et al., 2017; Zhang et al., 2018; Araújo et al., 2019).

Therefore, the present study was carried out to investigate the effect of in ovo feeding of t10,c12-CLA on the growth and lipid metabolism of newly hatched chicks. This study also focused on the changes in gene expression in the liver and subcutaneous adipose tissue (**SAT**), with particular emphasis on the genes involved in fatty acid synthesis and cholesterol metabolism. The liver is highly involved in oxidative metabolism. Previous studies demonstrated that CLA mixture supplementation increased the antioxidant capacity by increasing the oxidative stability of plasma and liver in laying hens, rats, and fish (Kim et al., 2005; Qi et al., 2011; Zuo et al., 2013). Therefore, we also evaluated the effect

of t10,c12-CLA treatment on hepatic antioxidant capacity in newly hatched chicks. The information obtained in this study can therefore be useful for understanding the role of t10,c12-CLA on embryo characteristics, lipid metabolism and antioxidant function in chicks.

MATERIAL AND METHODS

The experiment was approved by the Poultry Institute of Shandong Academy of Agricultural Sciences (Jinan, Shandong, China). All procedures in this study were performed in accordance with ethical guidelines for research in animal science.

Animals and Experimental Design

In the present study, 360 fertilely specific pathogen-free White Leghorn breeder eggs with similar weights (55.87 ± 3.47 g) were purchased from Shandong Haotai Experimental Animal Breeding Co., Ltd. (Jinan, Shandong, China). The eggs were incubated in a forced draught incubator (with a capacity of 880 eggs with 10 incubator trays and 88 eggs per tray) at 37.5 ± 0.3°C and 50 to 60% relative humidity, with automatic turning every 2 h. On embryonic day (**E**) 11, the eggs were randomly divided into 6 groups (6 replicates per group and 10 eggs per replicate) and injected with 0, 1.5, 3.0, 4.5, 6.0, or 7.5 mg t10,c12-CLA (Sigma-Aldrich, St Louis, MO) in 100 μL of ethanol. In order to ensure that all replicate of every treatment were arranged locally equalization as much as possible, 6 trays were used, and eggs were arranged on every tray, divided into 6 treatment groups with one replicate for every group. Six replicates of each treatment were therefore arranged on 6 different trays. At embryonic age 18 d, the eggs from different groups were transferred into different breathable mesh bags and returned to the incubator. The in ovo feeding procedure was performed after removing dead sperm eggs. The position of the yolk sac after candling was determined. Then, the air chamber was sterilized with 75% ethanol, and a hole was drilled with a diameter of approximately 1 mm. This was followed by a second sterilization. The reagent was slowly injected into the yolk sac of the embryonic egg with a 1 mL syringe. After the injection, the hole was sealed with melted paraffin, and the eggs were incubated until hatching. The in ovo feeding time of t10,c12-CLA was chosen according to previous studies (Liu et al., 2016; Zhu et al., 2020) to simulate the enrichment of maternal nutrition in the yolk sac of embryonic eggs.

Sample Collection

During incubation, dead eggs were removed and recorded. At hatching (D0), the hatchability of fertilized eggs (%) was calculated as follows: (the number of hatchlings/the number of fertilized eggs) × 100. Then, 2 chicks from each replicate were collected, weighed and sacrificed using pentobarbital anesthesia. Liver,

subcutaneous adipose tissue (**SAT**), and residual yolk sacs were collected, massed, and stored at −80°C for further analysis.

Measurement of Biochemical Indices in the Liver and SAT

The levels of triglycerides (**TG**s) and total cholesterol (**TC**) in the liver and SAT were measured using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The activities of glutathione peroxidase (**GSH-Px**) and total superoxide dismutase (**T-SOD**), the total antioxidant capacity (**T-AOC**) and the levels of malondialdehyde (**MDA**) in the liver were assayed using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocol. The levels of fatty acid synthase (**FAS**), acetyl-CoA carboxylase (**ACC**), and carnitine palmitoyltransferase-1a (**CPT1a**) in the liver were determined by ELISA kits (Wuhan ColorfulGene Biological Technology Co., LTD, Hubei, China). For standardization, the protein concentration was measured by the bicinchoninic acid method using a protein assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The experiments were strictly performed according to the manufacturer's instructions.

Histology of Lipids

To visualize the accumulation of cytoplasmic lipid droplets, frozen liver samples were embedded in optimal cutting temperature compound (Sakura Finetek USA Inc., Torrance, CA). Briefly, tissues were immediately frozen in liquid nitrogen, and sections (3 μm thick) were cut with a cryostat (Leica CM-1850, Wetzlar, Germany) at a temperature of −40°C. Then, the sections were fixed in 4% formaldehyde for 60 min and stained with filtered 0.5% red oil staining (Sigma-Aldrich, St Louis, MO) for 10 min before staining with hematoxylin (Sigma-Aldrich, St Louis, MO) for 1 min. Then, the sections were evaluated using a light microscope (Olympus CX-41, Tokyo, Japan).

Measurement of Fatty Acid Composition in the Liver and Yolk Sac

The fatty acid composition in the liver and yolk sac was determined using freeze-dried powder as previously described (Fu et al., 2021). Briefly, fatty acid methyl esters were prepared using chloroform/methanol and transesterified to methyl esters with BF3-methanol solution (13%). The fatty acid methyl esters were analyzed using direct transesterification by gas chromatography (Hewlett-Packard HP6890, CA), using the following conditions: 260°C injector temperature; 270°C detector temperature; He carrier gas; 1:50 split ratio; temperature program set for 100°C for 5 min, followed by an increase of 5°C/min to 240°C, and then maintained for 30 min. Peaks were identified by comparing the retention times with those of the corresponding standards from Sigma-Aldrich (St Louis, MO).

Real-Time PCR for mRNA Quantification

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription and quantification of mRNA were performed as previously described (Fu et al., 2017). Primer 5.0 software (Primer-E Ltd., Plymouth, UK) was used to design primers for exon-intron junctions. Primer sequences are listed in Table 1. Real-time PCR (Applied Biosystems 7500 Real-time PCR System; Applied Biosystems, Foster, CA) was performed according to the following protocol:

Table 1. Primers used in real-time quantitative PCR.

Gene name	GenBank accession number	Primer sequences (5'→3')	Product size (bp)
β-actin	NM_205518.1	F: GAACCCCAAAGCCAACAG R: GGGCGTAGCCTTCATAGA	182
FAS	NM_205155.2	F: AATCTGCCGTCTGGAACTGAATGG R: CATCCTGTGACTGGTCGTGTTCTC	169
ACC1	NM_205505.1	F: TCCAGCAGAACCGCATTGACAC R: GTATGAGCAGGCAGGACTTGGC	187
CPT1a	NM_001012898.1	F: CGAGTCAGACACCACAGCAACAC R: CACCGTAACCATCATCAGCCACAG	99
ATGL	NM_001113291.1	F: AAGTCCTGCTGGTCCTCTCCTTG R: AGTGTTGTCCTCCATCTGGTCCTC	94
AMPKα	NM_001039603.1	F:GCCTCGCCATACTCTTGATGAGC R:TTCTTCCGTCGAACACGCAAGTAG	195
PPARα	NM_001001464.1	F: TGCTGTGGAGATCGTCCTGGTC R: CTGTGACAAGTTGCCGGAGGTC	166
SCD1	NM_204890.1	F: CACATGGCTTGGCTGCTGGTAC R: CTTGTAGTATCTCCGCTGGAACATCAC	117
SREBP-1c	NM_204126.2	F: TGGTGGTGGACGCCGAGAAG R: GTCGTTGATGGATGAGCGGTAGC	134
PPARγ	NM_001001460.1	F: AGGATTGCCAAAGTGCAATCAA R: TCCCCACACACGACATTC	133

Abbreviations: ACC, acetyl-CoA carboxylase; AMPKα2, AMP-activated protein kinase; ATGL, adipose triacylglyceride lipase; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; PPARα, peroxisome proliferator-activated receptor α; SCD1, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein-1c.

Table 2. Effect of in ovo feeding of 10,12-CLA on the embryo characteristics on the day of hatch.

	10,12-CLA, mg						P value		
Items	0	1.5	3.0	4.5	6.0	7.5	ANOVA	Linear	Quadratic
The number of fertile eggs	10.00 ± 0	9.83 ± 0.41	10.00 ± 0	9.83 ± 0.41	9.83 ± 0.41	10.00 ± 0	0.700	0.892	0.877
Hatchability, %	87.50 ± 2.02	77.78 ± 1.06	77.78 ± 1.06	88.19 ± 0.98	93.75 ± 8.84	86.11 ± 2.41	0.453	0.118	0.198
Body weight, g	40.48 ± 4.06	40.91 ± 5.92	40.46 ± 4.88	40.24 ± 3.59	38.87 ± 3.79	39.93 ± 2.33	0.929	0.263	0.404
Liver mass, g	0.87 ± 0.10	0.90 ± 0.11	0.85 ± 0.11	0.83 ± 0.07	0.83 ± 0.10	0.82 ± 0.06	0.569	0.093	0.246
Relative liver weight, %	2.09 ± 0.38	2.22 ± 0.33	2.12 ± 0.31	2.07 ± 0.20	2.21 ± 0.32	2.10 ± 0.16	0.844	0.771	0.950
SAT mass, g	0.91 ± 0.16a	0.60 ± 0.08c	0.76 ± 0.14b	0.60 ± 0.12c	0.55 ± 0.13c	0.61 ± 0.04c	<0.001	<0.001	<0.001
Relative SAT weight, %	1.89 ± 0.36a	1.62 ± 0.27ab	1.74 ± 0.39ab	1.54 ± 0.28b	1.50 ± 0.31b	1.57 ± 0.11ab	0.037	0.003	0.008
Yolk mass, g	6.40 ± 1.21	7.01 ± 0.93	5.93 ± 1.64	7.11 ± 0.96	6.16 ± 1.47	6.10 ± 1.05	0.315	0.598	0.640
Relative yolk weight, %	16.32 ± 2.68	17.06 ± 2.89	16.45 ± 4.03	17.65 ± 1.71	16.89 ± 3.23	15.52 ± 1.78	0.761	0.846	0.650

Values are presented as the mean ± SEM; n = 6 per group. Means within a row with no common superscripts differ significantly ($P < 0.05$).
Orthogonal polynomials were used to estimate the linear and quadratic effects of in ovo feeding of t10,c12-CLA.
Abbreviations: SAT, subcutaneous adipose tissue; Relative tissue weight, tissue mass/body weight.

95°C for 10 s; 40 cycles of 95°C for 5 s and 60°C for 40 s. A standard curve was plotted to calculate the efficiency of the PCR process. β-actin was used as in internal control to normalize and quantitate the mRNA levels of the target genes using the comparative CT method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). The specificity of the amplification products was verified by melting curve analysis. All samples were run in duplicate.

Statistical Analysis

All the data were analyzed by one-way ANOVA and polynomial regression analysis using the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC). Significant differences between the treatments were determined using Tukey's multiple range test. Significance was based on $P < 0.05$. Orthogonal contrasts were used to examine the linear and quadratic effects in response to in ovo feeding of t10, c12-CLA among different groups.

RESULTS

Embryo Characteristics on the Day of Hatch

The results showed that the inclusion of t10,c12-CLA had no significant influence on the hatchability of fertilized eggs (Table 2, $P > 0.05$). In addition, there were no significant differences in the body weight, liver weight or residual yolk weight of newly hatched chicks that received t10,c12-CLA feeding ($P > 0.05$). Compared to the control group, t10,c12-CLA treatment significantly decreased the subcutaneous adipose tissue (**SAT**) mass of chicks that received 1.5, 4.5, 6.0, and 7.5 mg of in ovo feeding of t10,

c12-CLA ($P < 0.05$). The relative SAT weight was significantly decreased for the chicks inoculated with 4.5 and 6.0 mg of t10,c12-CLA ($P < 0.05$), with no significant difference in the 1.5, 3.0, 7.5 mg t10,c12-CLA groups compared to the control group ($P > 0.05$). Moreover, the SAT mass and relative SAT weight were significantly decreased as the dose of t10,c12-CLA increased ($P < 0.05$), which got the minimum in 6.0 mg t10,c12-CLA group.

Hepatic Lipid Metabolic Parameters

The TG and TC levels in the liver were significantly decreased linearly and quadratically in response to the increase of in ovo feeding of t10,c12-CLA (Table 3, $P < 0.05$), and the level of FAS was also significantly increased quadratically in t10,c12-CLA groups ($P < 0.05$). Besides, 1.5 mg t10,c12-CLA significantly increased the level of ACC compared to the control and other t10, c12-CLA groups ($P < 0.05$). The level of CPT1a was significantly higher in all t10,c12-CLA-fed groups than in the control group, which also showed the quadratically increase as the dose of t10,c12-CLA increased ($P < 0.05$) and got a peak in 4.5 mg t10,c12-CLA group. The histological lipid analyses showed that in ovo feeding of t10, c12-CLA decreased the lipid droplets in hepatocytes in a dose-dependent manner (Figure 1).

Hepatic Antioxidant Parameters in Liver

In the present study, 1.5-7.5 mg of t10,c12-CLA treatment had no significant influence on hepatic T-SOD activity (Table 4, $P > 0.05$). The 1.5-4.5 mg t10,c12-CLA treatment yielded higher GSH-Px in the livers of

Table 3. Effect of in ovo feeding of 10,12-CLA on the lipid metabolism parameters in the liver of newly hatched chicks.

	10,12-CLA, mg						P value		
Items	0	1.5	3.0	4.5	6.0	7.5	ANOVA	Linear	Quadratic
TG, mmol/g protein	1.40 ± 0.19a	0.89 ± 0.04b	1.07 ± 0.09b	0.88 ± 0.07b	0.95 ± 0.10b	0.91 ± 0.06b	<0.001	<0.001	<0.001
TC, mmol/g	2.74 ± 0.08a	2.25 ± 0.20b	2.27 ± 0.08b	2.40 ± 0.07b	2.38 ± 0.22b	2.43 ± 0.12b	0.008	0.112	0.001
FAS, pg/mg protein	30.41 ± 2.34b	46.78 ± 7.19a	42.70 ± 8.06a	42.73 ± 7.95a	40.43 ± 5.72a	40.94 ± 5.25a	0.004	0.212	0.017
ACC1, pg/mg protein	224.15 ± 19.50b	256.40 ± 24.49a	222.28 ± 23.26b	220.29 ± 26.09b	212.92 ± 10.16b	227.49 ± 12.31b	0.040	0.147	0.343
CPT1a, ng/mg protein	27.51 ± 2.80b	38.01 ± 6.06a	36.24 ± 4.14a	38.84 ± 5.07a	33.36 ± 5.93a	35.12 ± 4.70a	0.005	0.191	0.009

Abbreviations: TG, triglyceride; TC, total cholesterol; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; CPT, carnitine palmitoyl transferase. Values are presented as the mean ± SEM; n = 6 per group. Means within a row with no common superscripts differ significantly ($P < 0.05$).
Orthogonal polynomials were used to estimate the linear and quadratic effects of in ovo feeding of t10,c12-CLA.
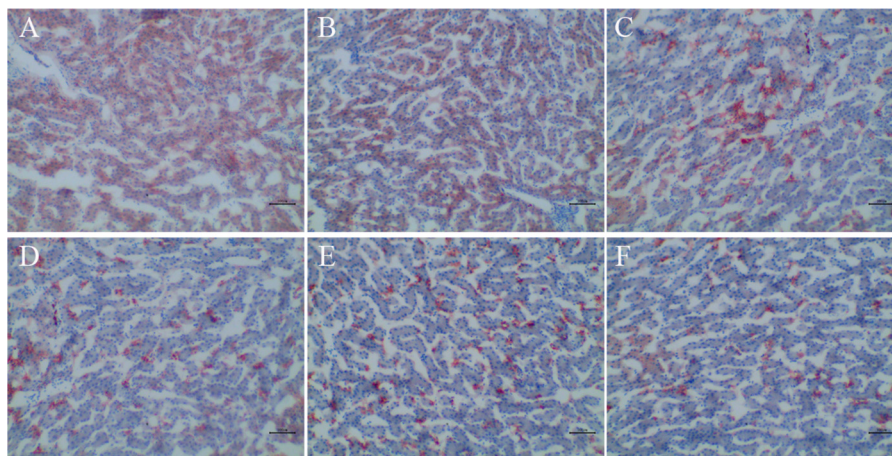
Figure 1. Effect of in ovo feeding of 10,12-CLA on lipid accumulation in the liver of newly hatched chicks. (A) Control group; (B) 1.5 mg t10, c12-CLA feeding group; (C) 3.0 mg t10,c12-CLA feeding group; (D) 4.5 mg t10,c12-CLA feeding group; (E) 6.0 mg t10,c12-CLA feeding group; (F) 7.5 mg t10,c12-CLA feeding group. Scale bar: 100 μm.

chicks compared to the control group ($P > 0.05$). And the hepatic T-AOC activities were increased quadratically as the dose of t10,c12-CLA increased ($P < 0.05$), with highest activity appeared in 4.5 and 6.0 mg t10,c12-CLA groups. The level of MDA in the liver of chicks was significantly decreased in all t10,c12-CLA-fed groups, especially in the 1.5 mg t10,c12-CLA group ($P < 0.05$).

Fatty acids composition in residual yolk sac and liver

The in ovo feeding of t10,c12-CLA had no significant influence on the proportion of MUFAs in the residual yolk sac or the proportions of SFAs and MUFAs in the liver of newly hatched chicks (Table 5, $P > 0.05$). In 7.5 mg t10,c12-CLA group, the SFA proportion was significantly higher in residual yolk than the control and 6.0 mg t10,c12-CLA groups ($P < 0.05$), which might contribute to the increased proportion of C12:0, C16:0, C18:0, C21:0, and C22:0 (Table S1, $P < 0.05$). In liver, the proportions of C18:2n6, C20:3n6, C20:4n6, C20:3n3, C22:2, and C22:6n3 were significantly increased by t10,c12-CLA treatment (Table S2, $P < 0.05$), which resulted in a higher proportion of PUFAs and n-3 to n-6 PUFA ratio in chicks that received in ovo feeding of t10,c12-CLA ($P < 0.05$). It also showed that the PUFA and

Table 4. Effect of in ovo feeding of 10,12-CLA on the antioxidant ability in the liver of newly hatched chicks

	10,12-CLA, mg						P value		
Items	0	1.5	3.0	4.5	6.0	7.5	ANOVA	Linear	Quadratic
T-SOD, U/mg protein	281.44 ± 49.89	266.68 ± 49.37	266.15 ± 22.33	281.15 ± 40.70	317.24 ± 40.89	271.20 ± 46.92	0.388	0.180	0.392
MDA, nmol/mg protein	0.46 ± 0.02a	0.28 ± 0.02d	0.36 ± 0.04c	0.41 ± 0.02b	0.36 ± 0.05c	0.37 ± 0.04c	<0.001	0.346	0.208
GSH-Px, U/mg protein	13.34 ± 1.58c	15.53 ± 1.31ab	16.13 ± 1.31a	15.24 ± 0.84ab	14.01 ± 2.32bc	14.71 ± 1.75abc	0.032	0.783	0.082
T-AOC, U/mg protein	0.74 ± 0.11c	1.02 ± 0.15b	0.94 ± 0.22b	1.35 ± 0.16a	1.31 ± 0.13a	0.90 ± 0.18bc	<0.001	0.050	<0.001

Values are presented as the mean ± SEM; n = 6 per group. Means within a row with no common superscripts differ significantly ($P < 0.05$).
Orthogonal polynomials were used to estimate the linear and quadratic effects of in ovo feeding of t10,c12-CLA.
Abbreviations: T-SOD, total superoxide dismutase; MDA, malondialdehyde; GSH-Px, glutathione perosidase; T-AOC, total antioxidant capacity.

Table 5. Effect of in ovo feeding of 10,12-CLA on the fatty acid profile in the residual yolk sac and liver tissue (in % of total fatty acid methyl esters) of newly hatched chicks.

		10,12-CLA, mg						P value		
Items		0	1.5	3.0	4.5	6.0	7.5	ANOVA	Linear	Quadratic
Yolk sac	SFA	34.39 ± 0.63bc	34.98 ± 1.46abc	35.24 ± 0.65ab	34.94 ± 0.43abc	33.55 ± 0.74c	36.49 ± 0.87a	0.026	0.307	0.430
	MUFA	48.97 ± 3.35	47.33 ± 3.84	47.40 ± 0.63	46.98 ± 3.29	48.40 ± 1.35	46.78 ± 2.82	0.915	0.513	0.750
	PUFA	18.25 ± 0.17ab	19.08 ± 0.28ab	17.32 ± 0.02b	19.81 ± 1.43a	19.05 ± 1.44ab	18.06 ± 0.64ab	0.104	0.767	0.903
Liver	SFA	21.54 ± 0.98	20.96 ± 1.13	21.23 ± 1.74	20.32 ± 1.50	21.06 ± 1.31	20.23 ± 0.77	0.779	0.219	0.479
	MUFA	58.38 ± 3.53	55.01 ± 2.41	55.92 ± 1.71	55.41 ± 3.12	54.21 ± 1.83	56.30 ± 0.35	0.424	0.265	0.180
	PUFA	19.07 ± 1.25b	24.03 ± 1.31a	22.85 ± 2.91a	24.27 ± 1.71a	24.73 ± 1.37a	23.47 ± 0.99a	0.017	0.064	0.023
	n-3/n-6	0.08 ± 0.01c	0.14 ± 0.02ab	0.13 ± 0.02b	0.16 ± 0.02a	0.15 ± 0.01ab	0.13 ± 0.01b	0.001	0.072	0.001

Values are presented as the mean ± SEM; n = 6 per group. Means within a row with no common superscripts differ significantly ($P < 0.05$).
Orthogonal polynomials were used to estimate the linear and quadratic effects of in ovo feeding of t10,c12-CLA.
Abbreviations: SFA, saturated fatty acids (C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0); MUFA, monounsaturated fatty acids (C14:1 + C16:1 + C18:1n9 + C20:1 + C22:1 + C24:1); PUFA, polyunsaturated fatty acids (C18:2n6 + C18:3n3 + C20:3n6 + C20:4n6 + C20:3n3 + C22:6n3).
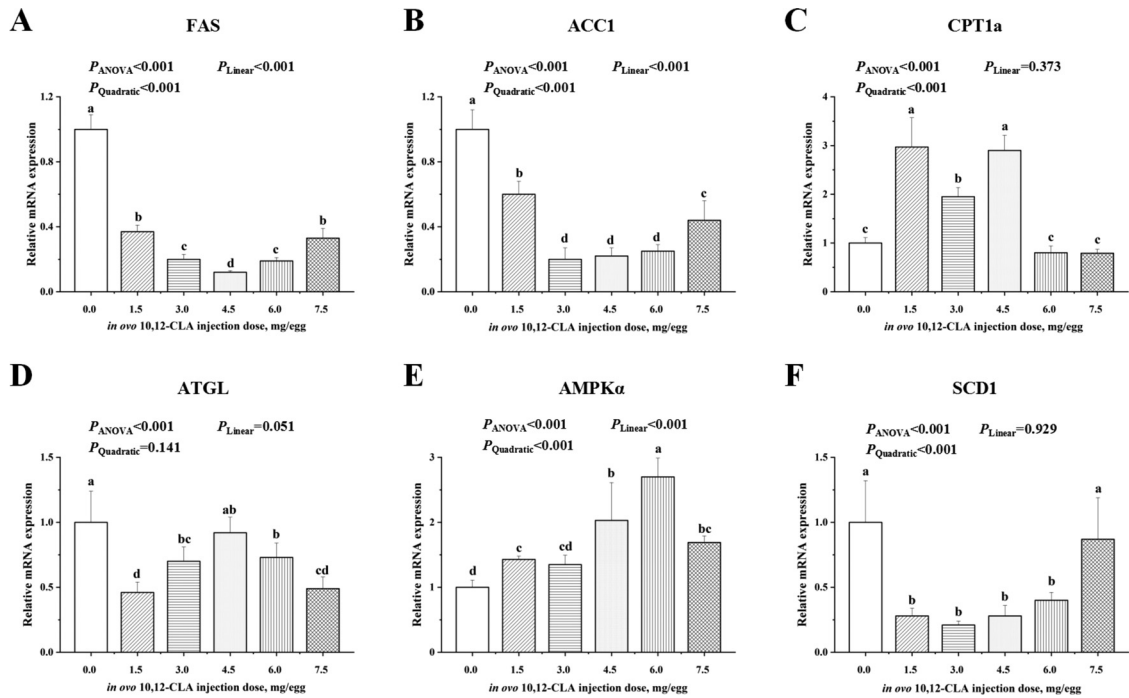
Figure 2. Effect of in ovo feeding of 10,12-CLA on the mRNA expression of *FAS* (A), *ACC1* (B), *CPT1a* (C), *ATGL* (D), *AMPKα* (E), and *SCD1* (F) in the livers of newly hatched chicks. Values are presented as the mean ± SEM; n = 6 per group. Orthogonal polynomials were used to estimate the linear and quadratic effects of in ovo feeding of t10,c12-CLA. Mean values with different letters are significantly different ($P < 0.05$). Abbreviations: ACC1, acetyl-CoA carboxylase 1; AMPKα, AMP-activated protein kinase; ATGL, adipose triacylglyceride lipase; CPT1a, carnitine palmitoyl transferase 1a; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase.

n-3 to n-6 PUFA ratio were increased quadratically as the dose of t10,c12-CLA increased ($P < 0.05$).

Gene Expression in Liver

T10,c12-CLA treatment significantly reduced the mRNA expression of *FAS* and *ACC1* in livers of chicks in linear and quadratic manner (Figure 2A, B, $P < 0.05$). The hepatic mRNA expression of *CPT1a* was significantly and quadratically upregulated in the 1.5 to 4.5 mg t10,c12-CLA treatments compared to the control group (Figure 2C, $P < 0.05$). The expression of *ATGL* and *SCD1* was reduced in liver of chicks that received in ovo t10,c12-CLA feeding compared to the control group (Figure 2D, F, $P < 0.05$). The expression of *AMPKα* was upregulated linearly and quadratically as the dose of t10,c12-CLA increased (Figure 2E, $P < 0.05$), which reached peak in the 6.0 mg t10,c12-CLA feeding group.

Gene Expression in SAT

The results showed that, compared to the control group, the mRNA expression of peroxisome proliferator-activated receptor α (**PPARα**) and *CPT1a* was significantly increased linearly and quadratically in the SAT of chicks as the dose of t10,c12-CLA increased (Figure 3A, B, $P < 0.05$). Sterol regulatory element-binding protein-1c (**SREBP-1c**) and *PPARγ* are the key adipogenic transcription factors in SAT, and the mRNA expression of *SREBP-1c* and *PPARγ* was down-regulated in the 3.0-7.5 mg t10,c12-CLA treatment

groups (Figure 3C, D, $P < 0.05$), which also showed linear and quadratic manner as the dose of t10,c12-CLA increased ($P < 0.05$).

DISCUSSION

The nutrition conditions of early life, such as maternal nutrition and nutrition intervention during the infant period, have a long-term influence on the development and health of offspring. The development of nutrient regulatory strategies in early stages of life is crucial. Previous studies have shown that maternal CLA mixtures decrease lipid deposition in embryos and posthatch chick offspring by altering hepatic lipid metabolism (Fu et al., 2020a, 2021). As one of the 2 major isomers of CLA, t10, c12-CLA has been demonstrated to increase energy expenditure and inhibit lipogenesis in adipocytes in vitro and in vivo. However, it is unknown whether maternal consumption of t10,c12-CLA has a direct effect on lipid metabolism in chick offspring. The in ovo feeding approach has been used for many years as a useful, convenient, and cost-effective in vivo method to investigate the early nutrition of poultry. In the present study, we found that in ovo feeding of t10,c12-CLA had no significant influence on the hatchability, body weight and liver mass of newly hatched chicks, but SAT deposition was reduced. Furthermore, fatty acid synthesis and lipid droplet deposition in the liver was decreased, and the ability of adipocytes to differentiate was decreased in SAT. These changes were due to the regulation of the expression of lipid metabolism-related genes and enzyme
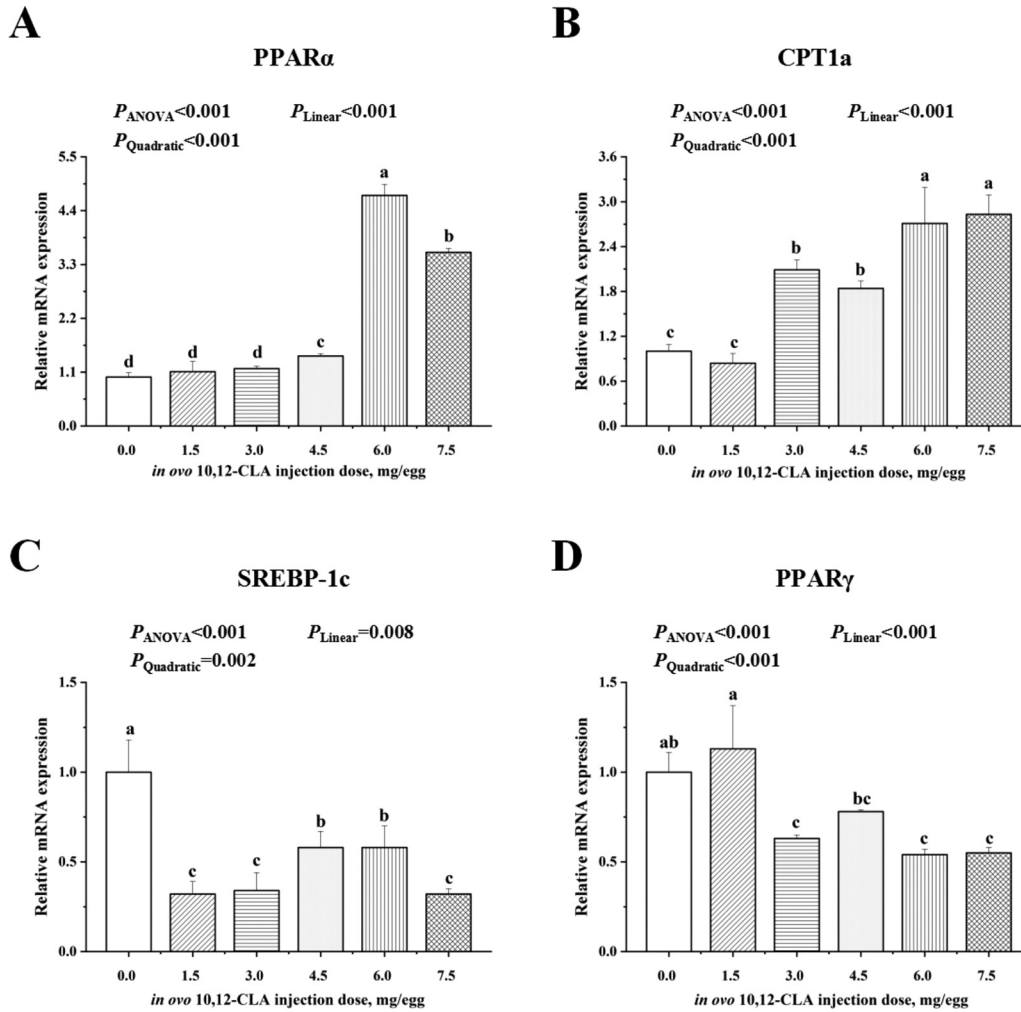
A

B

C

D

Figure 3. Effect of in ovo feeding of 10,12-CLA on the mRNA expression of the $PPAR\alpha$ (A), $CPT1a$ (B), $SREBP\text{-}1c$ (C), and $PPAR\gamma$ (D) genes in the SAT of newly hatched chicks. Values are presented as the mean $\pm$ SEM; n = 6 per group. Orthogonal polynomials were used to estimate the linear and quadratic effects of in ovo feeding of t10,c12-CLA. Mean values with different letters are significantly different ($P < 0.05$). Abbreviations: CPT, carnitine palmitoyltransferase; PPAR, peroxisome proliferator-activated receptor; SREBP-1c, sterol regulatory element-binding protein-1c.

activity, which were accompanied by an increase of the proportion of PUFAs in the liver. The changes in gene expression in the liver and SAT and lipid deposition suggest a regulatory role of in ovo feeding of t10,c12-CLA on lipid metabolism in chick offspring.

Previous studies have found that the addition of CLA mixtures to the diets of laying hens reduced the hatchability of fertile eggs (Muma et al., 2006; Aydin et al., 2009; Leone et al., 2009). In the present study, our results showed that in ovo feeding of t10,c12-CLA had no significant influence on the hatchability of eggs. Chick embryos rely on egg nutrients during incubation, and the interference of lipid transference from the yolk sac to the embryo significantly influences the development of the embryo. In the present study, the proportion of SFAs and PUFAs in posthatch residual yolk sacs was slightly higher by in ovo t10,c12-CLA feeding (less than 6.11% and 8.55%, respectively) but not MUFAs. However, maternal CLA feeding led to an approximately 30 to 60% increase in the SFA proportion and a 30 to 40% decrease in the MUFA proportion in the yolk sac of late-stage chick embryos (Leone et al., 2009, 2010; Fu et al., 2020a). This result suggests that the reduction in

hatchability of eggs might be related to the severe imbalance of fatty acid composition in the yolk sac and serious interference of lipid transference to the embryo but not CLA incorporation into the yolk sac. Furthermore, previous studies also confirmed that the addition of plant oil, such as soybean oil and corn oil, could recover the maternal CLA-induced loss of hatchability by balancing the fatty acid profile in yolk (Cherian et al., 2005; Muma et al., 2006; Fu et al., 2020b).

Although previous studies detected CLA isomer incorporation in the yolk sac and liver of embryos and hatching chicks (Fu et al., 2020a, 2021), while in the present study, we did not detect any incorporation of t10,c12-CLA in the yolk sac or liver of newly hatched chicks. This might be related to the feeding dose and the further catabolism of t10,c12-CLA. Compared to the effect of maternal CLA supplementation on the incorporation of CLA isomers in the yolk and liver, which had a stable influence on the fatty acid profile with long-term treatment, the influence of in ovo feeding of 1.5 to 7.5 mg t10, c12-CLA was short-term, acute and slight. Additionally, we found that the proportions of PUFAs, such as n-3 (C20:3n3, and C22:6n3) and n-6 (C18:2n6, C20:3n6,

and C20:4n6), in liver were significantly increased as the dose of t10,c12-CLA increased. It has been reported that upon absorption, CLA is converted into a conjugated C18:3 product by Δ-6 desaturase and is further elongated and desaturated into conjugated C20:3 and C20:4 (Sebedio et al., 2001; Berdeaux et al., 2002; Gruffat et al., 2003). These reports are consistent with our findings. In addition, increasing evidence has demonstrated that n-3 PUFAs alleviate alcoholic steatosis and alcohol-induced liver injury through multiple mechanisms, such as decreasing de novo lipogenesis and enhancing mitochondrial fatty acid β-oxidation in the liver, as well as decreasing lipid mobilization from adipose tissue (Sekiya et al., 2003; Song et al., 2008; Wang et al., 2019). Increasing the ratio of n-3 to n-6 PUFAs contributed to protection against obesity and insulin resistance (Riserus et al., 2009; An et al., 2012; Liu et al., 2013). In our study, the results demonstrated that in ovo feeding of t10,c12-CLA, especially the 4.5 mg t10,c12-CLA group, quadratically increased the ratio of n-3 to n-6 PUFAs in liver of chicks, which might also result in a decrease in lipid deposition.

Maternal CLA mixtures were reported to have a potential role in reducing body fat accumulation and modulating lipid metabolism in offspring during the gestation period and embryo incubation (Lavandera et al., 2017; Fu et al., 2021). Although there were equal amounts of the 2 main CLA isomers, c9t11-CLA and t10c12-CLA, in the CLA-oil mixture, their biological effects were reported to be different. T10c12-CLA was principally responsible for modulating fat accumulation by inducing lipolysis and fatty acid oxidation (Poirier et al., 2005; Yeganeh et al., 2017; Wang et al., 2019). The present study found that in ovo feeding of t10,c12-CLA reduced the deposition of lipid droplets in the liver and decreased the deposition of SAT in dose dependent manner linearly and quadratically in chicks, and the lowest SAT weight and relative weight was observed in 4.5 and 6.0 mg t10,c12-CLA group. These findings were stimultaneously accompanied by the decrease in hepatic TG and TC levels, which is consistent with previous studies. It has been reported that the liver is a key target for CLA (Aydin et al., 2001; Laliotis et al., 2010; Richards et al., 2010). In avians, the liver is the main site of de novo lipogenesis. In the liver, fatty acids are synthesized and packaged, and TG is transported to other tissues. Adipose tissue expansion is a result of the formation of adipocytes and the accumulation of TG-containing lipid droplets in these cells (Wang et al., 2017). The decrease in lipid deposition in the liver and SAT suggest that in ovo feeding of t10,c12-CLA can regulate lipid metabolism in the liver and SAT of newly hatched chicks. It should be noted that the delipidation effect of t10,c12-CLA on adipose tissue and the adipose tissue-dependent effect induced by t10,c12-CLA was fully investigated by previous studies. However, several studies also found that the antiobesity effect of t10, c12-CLA might promote fatty liver by enhancing fatty acid transport to the liver (Clement et al., 2002; Vyas et al., 2012; Cordoba-Chacon et al., 2019).

Interestingly, these findings were closely associated with the animal models used. In lean mice, t10,c12-CLA treatment induced steatosis in lipodystrophic models. These mice also demonstrated dyslipidemia due to white adipose tissue lipolysis, which led to hepatic insulin resistance and glucose intolerance (Wang et al., 2013; Qiang et al., 2016; Sakaguchi et al., 2017).

It is known that t10,c12-CLA reduces fat deposition by different mechanisms in different animal models; such mechanisms include decreased fatty acid uptake, increased fatty acid oxidation, and increased energy expenditure (Pariza et al., 2001). The liver plays a crucial role in regulating lipid metabolism. The CPT1a is the main enzyme involved in hepatic fatty acid oxidation. In the present study, we found that in ovo feeding of t10,c12-CLA qudratically increased the content of CPT1a in the liver of chicks, with a peak observed in 4.5 mg t10,c12-CLA group. Additionally, as the dose of t10,c12-CLA increased, the reduced mRNA expression of *FAS, ACC1*, and *SCD-1* and the increased expression of *CPT1a* in the liver, linearly and/or quadratically, also indicated an imbalance induced by in ovo feeding of t10, c12-CLA between lipogenesis and fatty acid oxidation. These findings are in agreement with previous findings (Choi et al., 2001; Maslak et al., 2015; Lavandera et al., 2017). Furthermore, the mRNA expression of *AMPKα* was increased linearly and quadratically as the dose of t10,c12-CLA increased, with the peak observed in 4.5 to 6.0 mg groups. Such an increase might further inhibit ACC activity and malonyl CoA production, resulting in the suppression of fat synthesis and the stimulation of fatty acid oxidation (Yang et al., 2009; Li et al., 2011; Fu et al., 2020a). Based on the above mentioned effects observed in the liver of chicks, in ovo feeding of t10,c12-CLA might program lipid metabolism and prevent lipid accumulation.

In addition to the liver, another major organ affected by CLA treatment is the SAT. Previous studies have shown that CLA, especially t10c12-CLA, increases energy expenditure, initiates apoptosis and modulates the differentiation of preadipocytes, resulting in a reduction of adipose tissue mass (House et al., 2005). As previously mentioned, in ovo feeding of t10,c12-CLA decreased the SAT mass and relative SAT weight in newly hatched chicks in the present study, especially in 4.5 and 6.0 mg t10,c12-CLA groups. Furthermore, we also found that t10,c12-CLA treatment increased the expression of *PPARα*, which is a transcription factor involved in lipid metabolism, and *CPT1a* in SAT and inhibited the expression of *PPARγ* and *SREBP-1c* in SAT in linear and quadratic manner.

Previous studies have demonstrated that t10,c12-CLA is a PPARγ antagonist, and the modulatory role of t10,c12-CLA in preadipocyte differentiation may be driven by downregulating *PPARγ* expression (Taylor and Zahradka, 2004). In differentiating adipocytes from humans and mice, t10,c12-CLA reduced PPARγ-dependent gene transcription and TG accumulation (Evans et al., 2000, 2001; Brown et al., 2003). SREBP-1c is a key lipogenic transcription factor in

cellular lipid metabolism and homeostasis. SREBP-1c has been reported to regulate the activity of lipogenic genes and has been demonstrated to be closely related to the regulation of lipid metabolism (Chen et al., 2004; Owen et al., 2012). In bovine mammary epithelial cells, t10,c12-CLA reduced lipogenesis and the mRNA expression of lipogenic genes by inhibiting the proteolytic cleavage of SREBP-1c (Peterson et al., 2003). PPARα has been shown to regulate the expression of enzymes associated with fatty acid oxidation (Kersten et al., 1999; Peters et al., 2001). Taking the present study into account, we propose that the delipidation effect of t10, c12-CLA in newly hatched chicks was attributed not only to the decrease in TG accumulation in the liver but also to the inhibition of adipocyte differentiation and increase in fatty acid oxidation in SAT.

The liver is highly involved in oxidative metabolism. Oxidative stress might also induce an inflammatory state due to the excessive lipolysis that accentuates the production of reactive oxygen species (**ROS**). Therefore, we determined the hepatic antioxidative ability and found that 1.5 to 4.5 mg t10,c12-CLA treatment yielded higher GSH-Px and T-AOC activities and decreased the level of MDA in the liver of chicks. These findings are consistent with previous findings (Qi et al., 2018; Liu et al., 2021). MDA is a major oxidation product of peroxidized PUFAs and an important indicator of lipid peroxidation. GSH-Px and T-AOC play important roles in antioxidative processes. It has been reported that CLA was the only PUFA that protected human fibroblasts against peroxidative damage without MDA or ROS overproduction (Qi et al., 2018). Consistent with previous studies, the t10,c12-CLA-induced hepatic antioxidative status might be related to the inhibition of the production of ROS and the transcription of proinflammatory cytokines (Dipasquale et al., 2018).

In conclusion, we demonstrated that in ovo feeding of t10,c12-CLA decreased lipid synthesis and accumulation in newly hatched chicks by decreasing lipogenesis in the liver and inhibiting adipogenesis in SAT. Furthermore, there was no significant influence on hatching body weight, liver mass or residual yolk sac. The change in lipid metabolism might be attributed to the regulation of lipometabolism by t10,c12-CLA in the liver and SAT and the change in fatty acid composition in the yolk sac and liver. Obviously, several questions remain unclear in the present study, which provides directions for future investigations. In addition, in vitro cell models may be used to better understand the mechanisms by which t10, c12-CLA mediates lipid metabolism in adipocytes and hepatocytes.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101797.

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