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Original Article

Effect of Conjugated Linoleic Acid in Ovo Feeding to Broiler Embryos on Breast Muscle Transcriptome Changes

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ABSTRACT

To better understand the effects of conjugated linoleic acid (CLA) administered at the early stages of embryonic development in poultry, it is necessary to analyze the molecular background. The molecular interpretation of poultry muscle after *in-ovo* administration of CLA remains to be reported. The purpose of the present study was to analyze the molecular background of muscle development based on gene expression parameters. On embryonic day (E) 11, the eggs were randomly divided into two groups (20 eggs per replicate). The CLA group was injected with 4.5 mg CLA in 100 mL of ethanol. The control group was injected with 100 mL of ethanol. At hatch, breast muscle samples were collected from four individuals per group for RNA-Seq. The result showed that 278 genes were differentially expressed ($p<0.01$, fold change >2) between the control group and the CLA group. 112 genes were upregulated in the CLA group, while 166 genes were upregulated in the control group. Function annotation showed that muscle activity (Ventricular cardiac muscle tissue morphogenesis, Adrenergic signaling in cardiomyocytes, Cardiac muscle contraction) and metabolism (Aromatic amino acid family metabolic process, Tyrosine metabolism, Retinol metabolism, Nitrogen metabolism, Cysteine, and methionine metabolism) were significantly enriched, including 11 genes(such as *TNNI1*, *TNNC1,* and *MYL3*) that were considerably upregulated in CLA group likely related to CLA processing by influencing the muscle activity processes and metabolic processes. It was concluded that *in ovo*, CLA supplementation could change the gene expression pattern of the breast muscle, and might affect muscle development.

INTRODUCTION

Conjugated linoleic acid (CLA) is a chemical compound consisting of a group of geometrical and positional isomers of linoleic acid, containing a conjugated double bond system. CLA has received much attention because of its attractive biological benefits. It has since been shown also to have antiatherogenic properties, normalize impaired glucose tolerance, stimulate immune function, and act as a potent cancer-preventive agent (den Hartigh *et al.*, 2019; Basak *et al.*, 2020; Dachev *et al.*, 2021; Fleck *et al.*, 2021). In animal science, CLA, as a growth factor, has been shown to reduce body fat, increase lean body mass, and feed efficiency in pigs, chicks, and mice (Wang *et al.*, 2019; Ao *et al.*, 2020; Gao *et al.*, 2022; Panisson *et al.*, 2022).

In ovo technology first became available for vaccination delivery in broiler hatcheries. In recent years, researchers have used *in ovo* technology to explore *in ovo* feeding on poultry hatching, development, and lipid metabolism. *In ovo* VE supplementation improved the chicks' oxidative state, which led to improved incubation results, chick quality,

and performance results (Araújo e*t al.,* 2019). L-carnitine added to commercial vaccine diluent at levels between 0.5 and 8.0 mg/100 µL for the commercial injection of broiler hatching eggs may decrease liver glucose and increase pipping muscle moisture concentrations of chicks on day 0 and 3 post-hatch, respectively (Keralapurath e*t al.,* 2010). Glycerol inoculation at E18 enhanced liver glycogen deposition (p=0.001) and improved broilers' performance at 7 d (Dal Pont e*t al.,* 2019). Xu *et al.* (2022) 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. *In ovo* stimulation with *lactobacillus plantarum* with raffinose family oligosaccharides triggered the most potent and favorable changes in the pectoral muscle metabolic gene expression in broiler chickens (Dunislawska e*t al.,* 2020). According to recent research conducted by Lu *et al.* (2022), the inclusion of IOF of Arg has shown to enhance the antioxidant capacity of the breast muscle of slow-growing chickens during the starter period. This improvement, in turn, may have a favorable impact on the overall health status of these birds post-hatch.

Most studies have focused on the phenotypic characteristics of chickens after in-ovo administration of nutrients, but the underlying molecular mechanisms remain to be reported. Therefore, to better understand the effects of CLA administered at the early stages of embryonic development in poultry, this study aims to explain the molecular background of chicken muscle change after CLA injection on day 12 of egg incubation, based on the RNA-seq of the breast muscle.

MATERIAL AND METHODS

Animals and treatment

In the present study, 40 fertility specific pathogen-free White Leghorn breeder (30 weeks of age) eggs with similar weights were purchased from Shandong Haotai Experimental Animal Breeding Co., Ltd. (Jinan, Shandong, China). The eggs were incubated in a forced draught incubator. The incubation approaches followed by Xu *et al.* (2022). On the embryonic day (E) 11, the eggs were randomly divided into two groups (20 eggs per group). The CLA group was injected with 4.5 mg t10, c12-CLA (Sigma-Aldrich, St Louis, MO) in 100 mL of ethanol. The control group was injected with 100 mL of ethanol. At embryonic age 18 d, the eggs from different groups were transferred into extra breathable mesh bags and returned to the incubator. The *in-ovo* feeding procedure was performed after removing dead sperm eggs.

Sample Collection and RNA Extraction

Randomly selected individuals from the experimental group and the control group were sacrificed after hatching. Breast muscle samples were collected from four individuals per group into 2 mL Eppendorf tubes and snap frozen in liquid nitrogen and held at -80 °C until RNA extraction. Total RNA was extracted using a Trizol reagent (Thermo Fisher, Waltham, MA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analyzed by Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA). The sequencing library was prepared from high-quality RNA samples with RIN > 7.0.

Library Construction and Sequencing

mRNA was isolated from total RNA (5ug) using Dynabeads Oligo (dT) (Thermo Fisher, Waltham, CA, USA) with two rounds of purification. After the mRNA was purified, it was fragmented into smaller pieces by heating it with divalent cations (Magnesium RNA Fragmentation Module (NEB, USA) under 94 °C 5-7min). Then the cleaved RNA fragments were reverse-transcribed to create the cDNA by SuperScript™ II Reverse Transcriptase (Invitrogen, CA, USA), which was next used to synthesize U-labeled second-stranded DNAs with E. coli DNA polymerase I (NEB, USA), RNase H (NEB, USA) and dUTP Solution (Thermo Fisher, Waltham, CA, USA). Each strand's blunt ends received an A-base to prepare them for ligation to the indexed adapters. For ligation to A-tailed fragmented DNA, each adaptor had a T-base overhang. Dual-index adapters were ligated to fragments, and AMPureXP beads were used for size selection. After the heat-labile UDG enzyme (NEB, USA) treatment of the U-labeled second-stranded DNAs, the ligated products were amplified with PCR by the following conditions: initial denaturation at 95 °C for 3 min; 8 cycles of denaturation at 98 °C for 15 sec, annealing at 60 °C for 15 sec, and extension at 72 °C for 30 sec; and then final extension at 72 °C for 5 min. The average insert size for the final cDNA libraries was 300±50 bp. At last, we performed the 2×150bp paired-end sequencing (PE150) on an Illumina Novaseq™ 6000 (LC-Bio Technology CO., Ltd., Hangzhou, China) following the vendor's recommended protocol.

RNA-Seq Reads Mapping and DEG Analysis

We aligned reads to the genome of Gallus_gallus 6.0 using HISAT (https://daehwankimlab.github.io/hisat2/,

version:hisat2-2.0.4) package (Kim e*t al.,* 2015), which initially removed reads based on quality information accompanying each read and then mapped the reads to the reference genome. StringTie (http://ccb.jhu.edu/software/stringtie/, version:stringtie-1.3.4d) constructed each sample's mapped reads (Pertea e*t al.,* 2015). Gffcompare software (http://ccb.jhu.edu/software/stringtie/gffcompare.shtml, version:0.9.8) was used to rebuild a complete transcriptome from sample transcriptomes. StringTie and ballgown (http://www.bioconductor.org/packages/release/bioc/html/ballgown.html) were used to estimate the expression levels of all transcripts and calculate FPKM values for mRNAs after the final transcriptome was generated. DESeq2 analyzed gene expression in two groups. Differentially expressed genes (DEGs) were selected with log2 (fold change) > 1 or log2 (fold change) less than −1 with statistical significance (p<0.01). GO functions and KEGG pathways were enriched in differentially expressed genes.

qRT-PCR Confirmation

To confirm our differential expression results, we conducted quantitative reverse transcription PCR (qRT-PCR) for six selected genes (MYL10, MYHC1, G0S2, TNNC1, and USP24). The total RNA was used for first-strand cDNA synthesis using a commercial kit (TaKaRa, Dalian, China). cDNA was subsequently used for qRT-PCR analyses with an ABI 7500 Detection System (Applied Biosystems, Foster City, CA, USA) and primers designed using Primer Premier version 5.0 (PREMIER Biosoft, Palo Alto, CA, USA), as listed in Table 1. mRNA abundance of candidate genes was determined using the KAPA SYBR® FAST qPCR Master Mix (2×) Universal Cocktail (KAPA Biosystems, Boston, MA, USA). qRT-PCR was performed following the instructions of ABI 7500 with default parameters. A corrected Ct (ΔCt) was calculated by subtracting the β-actin Ct value from the target genes for each sample (Kaminski & Wong, 2018). To compare with the sequencing-based results, we converted the mean 2−ΔCt value for each group to fold change by dividing it by the mean value for the control as previously described (Yi e*t al.,* 2015).

Table 1 – The primers used in the qPCR.

Gene name	Primer	Size
TNNC1	F: GGGTGGACAAGTGCTGAAAT R: TGCTCAACCTGCAAGTGAAA	161
MYL10	F: ACAAGCAGACCGGTTCAGTC R: CTTTGAGCTGCGTTTCCTTC	154
G0S2	F: AGGATTTGGTGTTGGAGCAG R: CGCTCCCCCTTACCTGAG	170
MYH1C	F: ATCAGTCTTTGTGGCCCATC R: CAGGCTGCATAACGCTCTTT	231
USP24	F: CACGAAGTGGTGGAGACTGA R: GTGGATGCAATCAGGCACTT	170

RESULTS

Overall Assessment for Sequencing Data Mapping Statistics

We generated eight cDNA sequencing libraries. The libraries were sequenced, and eight sets of reads were obtained (Table 2). After filtering the low-quality raw reads, 48,225,038, 38,587,452, 51,007,780, 47,388,894, 42,437,314, 46,230,392, 42,926,074 and 51,258,668 clean reads were obtained from CLA_1, CLA_2, CLA_3, CLA_4, Control_1, Control_2, Control_3, and Control_4 samples, respectively. We mapped clean reads to the chicken reference genome Gallus_gallus 6.0 and found that 89.73–91.76% of the clean reads in the libraries were mapped to the chicken reference genome, and more than 60% of reads were uniquely mapped.

Table 2 – Summary of reads after quality control and mapping to the reference genome.

Sample	Clean reads	Mapped reads	Unique Mapped reads	Q30(%)
CLA_1	48225038	43970599(91.18%)	34161650(70.84%)	97.92
CLA_2	38587452	34625406(89.73%)	26749448(69.32%)	97.85
CLA_3	51007780	46210940(90.60%)	36087906(70.75%)	97.96
CLA_4	47388894	43013449(90.77%)	33292586(70.25%)	97.84
Control_1	42437314	38560939(90.87%)	30557674(72.01%)	97.91
Control_2	46230392	42174978(91.23%)	32940692(71.25%)	97.95
Control_3	42926074	39081440(91.04%)	30337643(70.67%)	97.98
Control_4	51258668	47034965(91.76%)	36438320(71.09%)	97.96

Differential Expression Analysis of Genes

A total of 23,971 transcripts were obtained (FPKM ≥ 0.01) from 16,692 genes (Table S1). Two hundred seventy-eight genes were differentially expressed (p<0.01, fold change > 2) between the control group and the CLA group (Table S2). 112 genes were upregulating in the CLA group, while 166 genes were upregulated in the control group.

G0S2, *MYL10*, *TNNC1*, *TNNI1*, *PLN*, *MYOZ2*, *CSRP3*, *MYL3*, *MKNK2*, and *ENSGALG00000045540* were significantly upregulating in the CLA group. *ENSGALG00000048899*, *IP6K3*, *UCP3*, *JUND*, *HIGD1A*, *CISH*, *SIK1*, *TFR2*, *MRI1*, and *MAT1A* were significantly upregulated in control group (Table 3).

Table 3 – Top 10 upregulated and downregulated genes in the CLA group.

Genes	CLA group (FPKM)	Control group (FPKM)	Fold change	Regulation
G0S2	571.42	218.8	2.61	Up
MYL10	273.33	9.42	29.02	Up
TNNC1	239.05	50.01	4.78	Up
TNNI1	230.21	25.75	8.94	Up
PLN	198.63	33.44	5.94	Up
MYOZ2	161.91	62.36	2.60	Up
CSRP3	105.56	26.11	4.04	Up
MYL3	103.88	3.27	31.80	Up
MKNK2	96.02	43.76	2.19	Up
ENSGALG00000045540	72.36	35.00	2.07	Up
ENSGALG00000048899	90.03	195.17	0.46	Down
IP6K3	56.84	139.06	0.416	Down
UCP3	55.30	119.51	0.46	Down
JUND	42.77	93.54	0.46	Down
HIGD1A	46.30	92.96	0.50	Down
CISH	32.28	67.36	0.48	Down
SIK1	30.45	63.46	0.48	Down
TFR2	18.77	54.47	0.34	Down
MRI1	22.14	52.72	0.42	Down
MAT1A	21.70	52.01	0.42	Down

GO annotation of differentially expressed genes

GO enrichment analysis results showed that DEGs significantly enriched 247 biological process categories, 37 cellular component categories, and 88 molecular function categories ($p<0.05$) (Table S3). The top 10 enriched GO terms ($p<0.001$) in the ontology classification "Biological Process" were selected and presented in Table 4. The results show that the GO terms for muscle activity processes (Ventricular cardiac muscle tissue morphogenesis, Regulation of the force of heart contraction, Cardiac muscle contraction) and metabolic processes (Aromatic amino acid family metabolic process, Tyrosine catabolic process, Nucleotide catabolic process) were significantly enriched, including 11 genes likely related to CLA processing by influencing the muscle activity processes and metabolic processes.

Table 4 – Top 10 biological process terms from GO analysis.

GO Term	Enriched differentially expressed genes
Ventricular cardiac muscle tissue morphogenesis	TNNI1, TNNC1, MYL2, MYL3
Regulation of postsynaptic neurotransmitter receptor activity	ENSGALG00000003074, CACNG5, CACNG4
Regulation of the force of heart contraction	CSRP3, MYL3, PLN
Aromatic amino acid family metabolic process	TAT, FAH, HPDL
Cardiac muscle contraction	TNNC1, CSRP3, MYL2, MYL3
Notochord formation	TBXT, EPHA2
Tyrosine catabolic process	TAT, FAH
Organic cation transport	SLC22A16, SLC22A3
Nucleotide catabolic process	UPP1, UPP2
Negative regulation of prostatic bud formation	BMP7, SULF1

Pathway enrichment analysis of differentially expressed genes

KEGG pathway enrichment analysis revealed that DEGs significantly enriched eight pathways (Fig. 1). Specifically, many genes were significantly enriched in signaling pathways closely related to muscle activity pathways (Cardiac muscle contraction, Adrenergic signaling in cardiomyocytes) and metabolic pathways (Tyrosine metabolism, Retinol metabolism, Nitrogen metabolism, Cysteine, and methionine metabolism, Glutathione metabolism, and MAPK signaling pathway). These results were consistent with the GO analysis results.

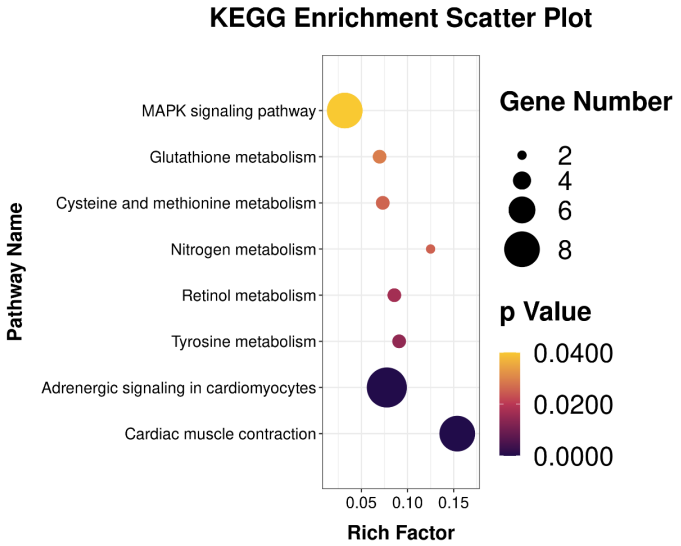

Figure 1 – KEGG pathway analysis of differentially expressed genes.

The advanced bubble chart shows the enrichment of differentially expressed genes in signaling pathways. The Y-axis label represents the pathway, and the X-axis label represents the rich factor (rich factor = amount of differentially expressed genes enriched in the pathway/amount of all genes in the background gene set). The size and color of the bubble represent the amount of

differentially expressed genes enriched in the pathway and enrichment significance, respectively.

Validation of DEGs by qRT-PCR

To validate the accuracy of mRNA-sequencing and our predictions, a subset of five crucial DEGs (*TNNC1*, *MYL10*, *G0S1*, *MYHC1*, and *USP24*) was selected for qRT-PCR validation. The comparative results of the fold changes predicted by RNA-Seq and qRT-PCR are displayed in Figure 2.

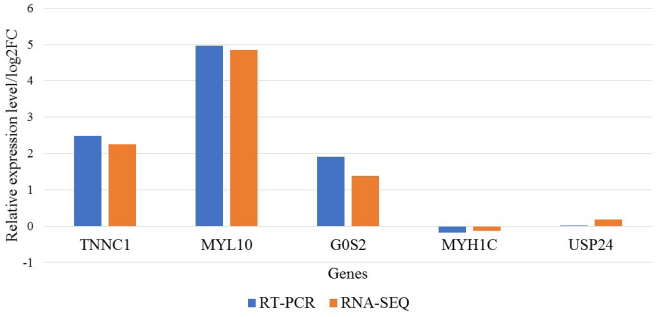

Figure 2 – Illustration of quantitative reverse transcription PCR confirmation results for five selected differentially expressed genes.

DISCUSSION

In the case of commercial poultry, provision of nutrients at the time of pre-hatching is a most effective and beneficial tool and affect the performance of growth till the last age. *In ovo*, inoculation is the source of nutrients for the developing embryo, which increases the viability of embryo by 90% and decreases the stunted growth after hatching (Saeed e*t al.,* 2019). CLA is a mixture of positional and geometric isomers of linoleic acid, containing a conjugated double bond system. Several studies have demonstrated that CLA increases lean body mass and enhances physical performance (Choi, 2009). The present study aimed to explain the molecular background of chicken muscle after CLA administration *in ovo* based on gene expression changes.

In this study, 278 genes were differentially ex-pressed between the control group and the CLA group. Function annotation showed that these DEGs were mainly involved in muscle amino acid metabolism and energy metabolism, such as Tyrosine metabolism, Cysteine and methionine metabolism, Glutathione metabolism, Nucleotide catabolic process, and MAPK signaling pathway. Protein and energy metabolism are tightly coupled, and the energy is needed for protein turnover during skeletal muscle development (Liu e*t al.,* 2020). MAPK signaling pathway is essential for the expression of muscle-specific genes. For example, the MAPK signaling pathway was shown to be needed for the early presentation of Myf5 and the term of several muscle structural genes (Keren e*t al.,* 2006). Moreover, DEGs were also related to muscle activity processes, including ventricular cardiac muscle tissue morphogenesis and cardiac muscle contraction. *TNNI1*, *TNNC1*, and *MYL3* that were significantly upregulated in the CLA group and were enriched in these biological processes and pathways. Troponin I and troponin C are the constituent protein of the troponin complex. TnI-skeletal-slow-twitch (*TNNI1*) and troponin-C type 1 (*TNNC1*) genes are switched on during skeletal muscle myogenesis (Shu e*t al.,* 2019). *MYL3* is a member of the MYL (myosin light chain) family. *MYL3* can bind calcium ions, promote muscle development, and participate in the contraction of striated muscles (Zhang e*t al.,* 2016). These genes are highly expressed during the early periods of muscle development in chickens (Ouyang e*t al.,* 2017).

In conclusion, our result showed that CLA administration *in ovo* changed the muscle gene expression that is involved in muscle activity processes, amino acid metabolism and energy metabolism. It provides an idea for the study of the effect of embryonic egg injection on muscle development, and provides theoretical guidance for the application of CLA administration *in ovo*.

ABBREVIATIONS

CISH, cytokine inducible SH2 containing protein; CSRP3, cysteine and glycine rich protein 3; G0S2, G0/G1 switch 2; HIGD1A, HIG1 hypoxia inducible domain family member 1A; IP6K3, inositol hexakisphosphate kinase 3; JUND, JunD proto-onco, AP-1 transcription factor subunit; MAT1A, methionine adenosyltransferase 1A; MYH1C, myosin heavy chain 1C; MKNK2, MAP kinase interacting serine/threonine kinase 2; MRI1, methylthioribose-1-phosphate isomerase 1; MYL3, myosin, light chain 3; MYL10, myosin light chain 10; MYOZ2, myozenin 2; PLN, phospholamban; SIK1, salt inducible kinase 1; TFR2, transferrin receptor 2; TNNC1, troponin-C type 1; TNNI1, TnI-skeletal-slow-twitch; UCP3, uncoupling protein 3; USP24, ubiquitin specific peptidase 24.

SUPPLEMENTARY MATERIALS

Table S1. The expression of all identified genes.

Table S2. Information of differentially expressed genes (DEGs).

Table S3. Results of the functional enrichment analysis of GO terms for DEGs.

ETHICS STATEMENT

All animal experiments were conducted under the Guidelines for Experimental Animals, established by the Ministry of Science and Technology (Beijing, China), and the study complies with the ARRIVE guidelines (https://arriveguidelines.org). Animal experiments were approved by the Science Research Department of the Shandong Animal Husbandry General Station (Ji'nan, China).

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

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AUTHORS' CONTRIBUTIONS

JY performed experiments and data analysis and draft writing. ZH contributed to animal experiments. KZ designed experiments, supervised and coordinated the study. All authors reviewed the manuscript.

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