# Angiopoietin-like protein 4 regulates breast muscle lipid metabolism in broilers

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**ABSTRACT** The objective of this study was to determine the effects of angiopoietin-like protein 4 (**ANGPTL4**) on breast muscle lipid metabolism in broilers. In experiment 1, 36 thirty-five-day-old male Arbor Acres broilers were randomly allocated into 6 treatment groups with 6 birds in a completely randomized design. The broilers were subjected to intravenous injection of His-SUMO-ANGPTL4 at the dose of 0 (injection of normal saline [**NS**]), 20, 100, 500, 2,500, or 12,500 ng/kg BW, respectively. The results showed that broilers at 30 min after His-SUMO-ANGPTL4 at the level of 12,500 ng/kg BW intravenous injection had higher ($P < 0.05$) concentrations of triglyceride and non-esterified fatty acid in the serum, higher ($P < 0.05$) adipose triglyceride lipase and carnitine palmitoyltransferase 1 mRNA expression in the breast muscle, but lower ($P < 0.05$) lipoprotein lipase (***LPL***) mRNA expression in the breast muscle. In experiment 2, 18 thirty-five-day-old male Arbor Acres broilers were randomly allocated into 3 treatment groups with 6 birds in a completely randomized design. The broilers were subjected to intravenous injection of NS, His-SUMO, or His-SUMO-ANGPTL4 (12,500 ng/kg BW) in order to rule out the effect of His-SUMO tag. It's confirmed that ANGPTL4 could increase ($P < 0.05$) concentrations of triglyceride and non-esterified fatty acid in the serum, enhance ($P < 0.05$) adipose triglyceride lipase mRNA expression in the breast muscle, and decrease ($P < 0.05$) *LPL* mRNA expression in the breast muscle. In experiment 3 and 4, co-culture experiments of chicken primary myoblasts and NS, His-SUMO, or His-SUMO-ANGPTL4 (250 pg/mL, physiological dose) were set up to monitor the cytotoxicity of ANGPTL4 and the changes of lipid metabolism-related genes expression. It was found that cell viability was not affected but *LPL* mRNA expression in chicken primary myoblasts was highly reduced ($P < 0.05$) by ANGPTL4. In conclusion, ANGPTL4 could promote lipodieresis and inhibit LPL in the breast muscle of broilers.

**Key words:** angiopoietin-like protein 4, broiler, breast muscle, lipid metabolism

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## INTRODUCTION

The improvements in carcass characteristics and meat quality are beneficial to our consumers. In recent years, the better body composition with higher intramuscular fat in the breast muscle and lower abdominal fat has gained increasing interest in poultry industry (Cui et al., 2018; Xing et al., 2020). Previous studies demonstrated that intestinal microbiota could influence intramuscular fat in the breast muscle of broilers (Yang et al., 2010; Zhao et al., 2018), however, the molecular mechanism is not clear.

Angiopoietin-like protein 4 (**ANGPTL4**), also known as peroxisome proliferator-activated receptor $\gamma$ angiopoietin-related protein, has been well characterized as a secretory protein (Yoshida et al., 2002; Xu et al., 2005; Altun et al., 2018). Previous studies demonstrated that ANGPTL4 has various physiological effects including fat metabolism, food intake regulation, plasma glucose level and tolerance regulation in rodent and human (Grootaert et al., 2012; Liu et al., 2017). However, information is lacking on the exact role of ANGPTL4 in broilers. Mandard et al. (2006) and Grootaert et al. (2011) reported that the intestinal microbiota could directly (by cell contact) or indirectly (by metabolite or secretion factors) modulate ANGPTL4 secretion, which not only regulates lipoprotein lipase (***LPL***) but also is a potent regulator of fatty acid oxidation. Ge et al. (2004) showed that adenoviral overexpression of ANGPTL4 potently increases plasma TG levels of mice by a mechanism independent of food intake or hepatic very low-density



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lipoprotein **(VLDL)** secretion. Besides, our previous study showed that intestinal microbiota could increase the breast muscle intramuscular fat content and at the same time reduce the serum ANGPTL4 concentration of broilers (Zhao et al., 2018). Therefore, we infer that ANGPTL4 may play an important role in increasing intramuscular fat in the breast muscle of broilers. In birds, it is important to study the process of lipid uptake and lipolysis of skeletal muscle in order to obtain increasing breast muscle intramuscular fat content of broilers. Therefore, the objectives of this study were to determine the effects of ANGPTL4 on these 2 aspects of lipid metabolism in the breast muscle of broilers.

## MATERIALS AND METHODS

### Ethics Statement

The animal care and use protocol was approved by the Animal Care and Use Committee of the Linyi University (Linyi, Shandong, China).

### Description of Recombinant Chicken ANGPTL4

The amino acid sequence (serial number F1NUQ4) and signal peptide part (1−18 amino acids) of chicken ANGPTL4 were found by UniProt. After removing the signal peptide and tagging the N-terminal with His-SUMO, the codon preference of *Escherichia coli* was compared. According to the codon annexation, the gene sequence encoding His-SUMO-ANGPTL4 was modified and replaced by the codon preferred by *Escherichia coli*. A new gene sequence encoding His-SUMO-ANGPTL4 was obtained and sent to Invitrogen Life Technologies (Shanghai, China) for full gene synthesis. The synthetic gene of His-SUMO-ANGPTL4 was cloned into Nde I and Xho I sites of pET21a(+) vector to construct recombinant plasmid pET21a-His-SUMO-ANGPTL4. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) and the bacteria were induced with 1 mmol/L isopropy-$\beta$-D-thiogalactoside (**IPTG**) at 37°C for 3 h. After lysis by ultrasonication, inclusion body solubilization and refolding (refolding buffer: 20 mmol/L PB, 240 mmol/L NaCl, 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 0.4 mol/L Sucrose, 0.5 mol/L Arg, 0.05%Triton X-100,1 mmol/L GSH, 0.1 mmol/L GSSG, pH 6.5), the refold protein was purified by Chelating SFF (Ni) column and the expected size of fusion protein His-SUMO-ANGPTL4 was obtained.

### Birds and Treatments

Male Arbor Acres (AA) broiler chicks were obtained from a commercial hatchery (Xiling Family Farm, Tai'an, Shandong, China) at 1 d of age and housed in an environmentally controlled room. The temperature was maintained at 35°C during the first 3 d, between 28° and 30°C during the next 2 weeks, and at 25°C until the end of the experiment. Overhead light was provided continuously for the entire period of the experiment. The experimental diets were in pellet form and were formulated to meet or slightly exceed the nutrient requirements recommended by the National Research Council (1994). The diet compositions are shown in Table 1. All birds were fed *ad libitum* and had free access to water throughout the entire experiment.

**Table 1.** Ingredients and nutrient composition of experimental diets (% as fed unless noted).

| Item | Starter (1 to 21 d) | Grower (22 to 42 d) |
|---|---|---|
| Ingredients | | |
| Corn | 54.60 | 60.40 |
| Soybean meal (44.2% CP) | 35.20 | 30.20 |
| Soy oil | 2.65 | 3.52 |
| Corn gluten meal | 3.18 | 2.00 |
| Calcium hydrogen phosphate | 2.00 | 1.65 |
| Limestone | 1.25 | 1.25 |
| Sodium chloride | 0.35 | 0.35 |
| DL-Met | 0.17 | 0.10 |
| L-Lys HCl | 0.08 | 0.08 |
| Vitamin premix [1] | 0.03 | 0.03 |
| Mineral premix [2] | 0.20 | 0.20 |
| Choline chloride (50%) | 0.26 | 0.20 |
| Ethoxyquin (33%) | 0.03 | 0.03 |
| Chemical composition, analyzed | | |
| ME, calculated (kcal/kg) | 2,950 | 3,050 |
| CP | 21.50 | 19.00 |
| Calcium | 1.00 | 0.91 |
| Available phosphorus | 0.46 | 0.40 |
| Lys | 1.15 | 1.01 |
| Met | 0.50 | 0.40 |
| TSAA | 0.84 | 0.71 |
| Thr | 0.82 | 0.73 |
| Trp | 0.25 | 0.23 |

[1]Supplied per kilogram of diet: vitamin A, 12,500 IU; cholecalciferol, 2,500 IU; vitamin E, 30 IU; vitamin $K_3$, 2.65 mg; thiamin, 2 mg; riboflavin, 6 mg; pantothenic acid, 12 mg; cobalamin, 0.025 mg; niacin, 50 mg; biotin, 0.0325 mg; and folic acid, 1.25 mg.

[2]Supplied per kilogram of diet: Mn, 100 mg; Fe, 80 mg; Zn, 75 mg; Cu, 8 mg; I, 0.35 mg; and Se, 0.15 mg.

***Experiment 1*** At 35 d of age, 36 broilers with similar body weight (**BW**) (2.17 ± 0.03 kg) after being fasted for 12 h with access to water were randomly allocated into 6 groups of 6 birds and subjected to one of the 6 following treatments: intravenous injection (wing vein) of recombinant chicken ANGPTL4 at the dose of 0 (injection of normal saline [**NS**]), 20, 100, 500, 2,500, or 12,500 ng/kg BW (denoted as **control, ANGPTL4 20, ANGPTL4 100, ANGPTL4 500, ANGPTL4 2500**, and **ANGPTL4 12500**, respectively).

***Experiment 2*** At 35 d of age, 18 broilers with similar BW (2.15 ± 0.05 kg) after being fasted for 12 h with access to water were randomly allocated into 3 groups of 6 birds and subjected to one of the three following treatments: intravenous injection (wing vein) of NS, His-SUMO, or His-SUMO-ANGPTL4 (recombinant chicken ANGPTL4 at a dose of 12,500 ng/kg BW).

## Sample Collection

Before (−1 d, after being fasted for 12 h with access to water) and at 30 min after intravenous injection, 2.5 mL blood samples were taken from the wing vein of all birds using sterilized needles (0.7 × 25 mm) and non-heparinized tubes (5 mL). The blood samples were incubated at 37°C for 2 h and were then centrifuged at 1,500 × g for 10 min at 4°C. The resultant serum (supernatant) was stored in 0.5-mL Eppendorf tubes at −20°C. After bleeding, the same birds at 30 min after intravenous injection were then slaughtered by exsanguination under deep sodium pentobarbitone anaesthesia (30 mg/kg BW, i.v.). Some of the breast muscle samples (in the middle of the breast muscle, 1.0 × 1.0 × 1.5 cm) of each slaughtered AA broilers were washed with ice-cold NS, immediately frozen in liquid nitrogen, and stored at −40°C for analysis of enzyme activity, and some of the breast muscle samples (in the middle of the breast muscle, 0.3 × 0.3 × 0.5 cm) of each slaughtered AA broilers were immediately stored in RNAfixer (RP1302, BioTeke Co. Ltd, Beijing, China), preserved at 4°C overnight and transferred to −20°C for subsequent extraction of total RNA.

## Determination of Serum Biochemical Parameters

The concentrations of triglyceride (**TG**) (A110-1-1), total cholesterol (**TC**) (A111-1-1) and non-esterified fatty acid (**NEFA**)(A042-2-1) in the serum were measured by colorimetric enzymatic methods using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

## Hormone-Sensitive Lipase Activity Assay

The hormone-sensitive lipase (**HSL**, EC 3.1.1.3) activity in the breast muscle was assayed using the same procedure as described by Fredrikson et al. (1981) and Huang et al. (2006). The enzyme activity unit description of LPL was pointed out by Zhao et al. (2013).

## Real-Time Quantitative PCR Analysis of Gene Expression

The real-time quantitative PCR analysis of gene expression in the breast muscle was performed using the same procedures as described by Zhao et al. (2016). For the production of cDNA, 400 ng of total RNA was reverse transcribed in a 10 $\mu$L reverse transcription system. cDNA was amplified in a 20 $\mu$L PCR reaction containing 0.2 $\mu$mol/L of each specific primer. The gene-specific primers for fatty acid transport protein 1 (***FATP1***), heart-fatty acid-binding protein (***H-FABP***), adipocyte fatty acid-binding protein (***A-FABP***), adipose triglyceride lipase (***ATGL***), carnitine palmitoyltransferase 1 (***CPT1***), carnitine palmitoyltransferase 2 (***CPT2***), long-chain acyl-CoA dehydrogenase (***LCAD***), ***LPL***, and glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***) are listed in Table 2.

## Cells and Treatments

Chicken primary myoblasts were isolated from the pectoralis muscle of SPF chicken embryos at 13 embryonic age. The culture of chicken primary myoblasts was performed using the same procedure as described by Shahjahan et al. (2016).

***Experiment 3*** Chicken primary myoblasts were seeded in 96-well culture plates ($10^4$ cells per well) and were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air until 80% confluence and myotubes forming. Twenty-four hours before stimulation, the confluent cell cultures were washed and cultured in fresh medium without FBS and antibiotic. Chicken primary

**Table 2.** Gene-specific primer of the lipid metabolism-related genes.

| Gene[1] | GenBank accession No. | Primer position | Primer sequences(5'→3') | Product size (bp) | Reference |
|---|---|---|---|---|---|
| *FATP1* | DQ352834 | Forward | TACCGCATCGCTGCCTTTG | 98 | This study |
| | | Reverse | ATGATGTTACCTGCGGAGCG | | |
| *H-FABP* | NM_001030889 | Forward | TGACCAAACCCACCACCATCA | 203 | Wang et al. (2012) |
| | | Reverse | TGTCTCCTTCCCATCCCACTTC | | |
| *A-FABP* | NM_204290 | Forward | AAGACTGCTACCTGGCCTGA | 104 | This study |
| | | Reverse | CCCACACCCAGCTCTTTCATA | | |
| *ATGL* | EU852334 | Forward | TCCTTCACCTTCAGCGTCCA | 113 | Cai et al. (2009) |
| | | Reverse | AGTGTTGTCCTCCATCTGGTC | | |
| *CPT1* | DQ314726 | Forward | GATGTTCGACCTCAACCGCT | 137 | This study |
| | | Reverse | ACCGTTTGGAGGAGATGTGG | | |
| *CPT2* | NM_001031287 | Forward | TGTTAGGGAACCGTCCAAGC | 109 | This study |
| | | Reverse | AAATCCCTGACCCATAGCAGC | | |
| *LCAD* | NM_001006511 | Forward | CGTGGTGATTGTGGTTACGGTTA | 203 | Wang et al. (2012) |
| | | Reverse | TGTTCTCTTTCCCAAGCAAGGC | | |
| *LPL* | NM_205282 | Forward | ACTTGAAGACCCGTGCTCAG | 97 | Huang et al. (2015) |
| | | Reverse | GGCTGGTCTACCTTGGTCAC | | |
| *GAPDH* | NM_204305 | Forward | AGAACATCATCCCAGCGTCC | 133 | Wen et al. (2014) |
| | | Reverse | CGGCAGGTCAGGTCAACAAC | | |

[1]Abbreviations: *FATP1*, fatty acid transport protein 1; *H-FABP*, heart-fatty acid-binding protein; *A-FABP*, adipocyte-fatty acid-binding protein; *ATGL*, adipose triglyceride lipase; *CPT1*, carnitine palmitoyltransferase 1; *CPT2*, carnitine palmitoyltransferase 2; *LCAD*, long-chain acyl-CoA dehydrogenase; *LPL*, lipoprotein lipase; *GAPDH*, glyceraldehydes-3-phosphate dehydrogenase

myoblasts were treated with 100 $\mu$L of DMEM/F-12 (HyClone, Waltham, MA) containing NS, His-SUMO, or His-SUMO-ANGPTL4 (250 pg/mL, physiological dose). After 24 h incubation, the cell viability was evaluated by cell counting kit-8 (**CCK-8**) (CK04, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

The experiment was performed three times on different days, and at least six wells per treatment. In each experiment, the cells were taken from 40 SPF chicken embryos and pooled.

***Experiment 4*** Chicken primary myoblasts were seeded in 6-well culture plates (Costar, Cambridge, MA) and were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air until 80% confluence and myotubes forming. Twenty-four hours before stimulation, the confluent cell cultures were washed and cultured in fresh medium without FBS and antibiotic. Chicken primary myoblasts were treated with 2 mL of DMEM/F-12 containing NS, His-SUMO, or His-SUMO-ANGPTL4 (250 pg/mL, physiological dose) and incubated in 5% $CO_2$ at 37°C for 24 h. The experiment was terminated by thoroughly washing the plates with ice-cold PBS and the cells were harvested for real-time PCR analysis as described above.

The experiment was performed three times on different days, and at least 6 wells per treatment. In each experiment, the cells were taken from 40 SPF chicken embryos and pooled.

### Data Calculations and Statistical Analyses

All data were subjected to analysis of variance using the general liner model procedure of Statistical Analysis System (**SAS**) 8.1 software (SAS Institute, Inc., Cary, NC). Orthogonal polynomial contrasts were used to determine linear and quadratic responses of broiler chickens to ANGPTL4 levels. The significance of differences among treatments was tested by Duncan's multiple-range test. A level of $P < 0.05$ was used as the criterion for statistical significance. Broiler was considered as the experimental unit.

## RESULTS

### Effect of Different Recombinant Chicken ANGPTL4 Levels on Serum Biochemical Parameters of Broilers

The concentrations of TG, TC and NEFA in the serum were similar among treatments before recombinant chicken ANGPTL4 injection (Table 3). However, as the level of recombinant chicken ANGPTL4 increased from 0 to 12,500 ng/kg BW, concentrations of TG and NEFA in the serum of broilers were linearly ($P < 0.05$) and quadratically ($P < 0.05$) increased after recombinant chicken ANGPTL4 injection. The serum of broilers in ANGPTL4 12500 contained the highest concentrations of TG and NEFA.

### Effect of Different Recombinant Chicken ANGPTL4 Levels on Lipid Metabolism-Related Gene Expression and Enzyme Activity in the Breast Muscle of Broilers

As the concentration of recombinant chicken ANGPTL4 increased from 0 to 12,500 ng/kg BW, *H-FABP, ATGL, CPT1* and *CPT2* mRNA expression in the breast muscle of broilers were linearly ($P < 0.05$) or quadratically ($P < 0.05$) increased or both, yet the *LPL* mRNA expression was linearly ($P = 0.012$) and quadratically ($P < 0.001$) decreased (Table 4). The *ATGL* mRNA expression in the breast muscle of ANGPTL4 20, ANGPTL4 100, ANGPTL4 500, ANGPTL4 2500 and ANGPTL4 12500 broilers was higher ($P < 0.05$) than that of control. Besides, among the groups injected with recombinant chicken ANGPTL4, breast muscle of ANGPTL4 2500 broilers appeared to contain the highest *ATGL* mRNA expression. Broilers of ANGPTL4 2500 and ANGPTL4 12500 had higher ($P < 0.05$) *CPT1* mRNA expression, but lower ($P < 0.05$) *LPL* mRNA expression in the breast muscle than those of control birds. However, no difference in *CPT1* and *LPL* mRNA expression in the breast muscle were observed between ANGPTL4 2500 and ANGPTL4 12500 birds. Hormone sensitive lipase activity in the breast muscle of birds was

**Table 3.** Effect of different recombinant chicken angiopoietin-like protein 4 levels on serum biochemical parameters of broilers.[1]

| | Recombinant chicken angiopoietin-like protein 4, ng/kg | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 20 | 100 | 500 | 2,500 | 12,500 | SEM | *P*-value | Linear | Quadratic |
| Before injection | | | | | | | | | | |
| Triglyceride, mmol/L | 0.32 | 0.30 | 0.31 | 0.31 | 0.29 | 0.31 | 0.033 | 0.988 | 0.934 | 0.816 |
| Total cholesterol, mmol/L | 3.47 | 3.36 | 3.52 | 3.15 | 3.28 | 3.44 | 0.152 | 0.547 | 0.731 | 0.590 |
| Non-esterified fatty acid, mmol/L | 0.89 | 0.86 | 0.72 | 0.76 | 0.85 | 0.88 | 0.046 | 0.076 | 0.188 | 0.417 |
| After injection | | | | | | | | | | |
| Triglyceride, mmol/L | 0.22[b] | 0.20[b] | 0.20[b] | 0.20[b] | 0.20[b] | 0.28[a] | 0.019 | 0.047 | 0.001 | 0.003 |
| Total cholesterol, mmol/L | 3.47 | 3.25 | 3.51 | 3.02 | 3.16 | 3.48 | 0.158 | 0.177 | 0.388 | 0.262 |
| Non-esterified fatty acid, mmol/L | 0.60[b] | 0.66[b] | 0.65[b] | 0.65[b] | 0.74[b] | 0.92[a] | 0.046 | < 0.001 | < 0.001 | < 0.001 |

[a,b]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 6 chickens.

**Table 4.** Effect of different recombinant chicken angiopoietin-like protein 4 levels on lipid metabolism-related gene expression in the breast muscle of broilers.[1]

| | Recombinant chicken angiopoietin-like protein 4, ng/kg | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 20 | 100 | 500 | 2,500 | 12,500 | SEM | *P*-value | Linear | Quadratic |
| Fatty acid transport protein 1 | 1.00 | 0.78 | 0.79 | 0.91 | 0.97 | 1.00 | 0.070 | 0.126 | 0.155 | 0.257 |
| Heart-fatty acid-binding protein | 1.00 | 1.14 | 1.03 | 0.94 | 0.83 | 1.04 | 0.071 | 0.120 | 0.935 | 0.034 |
| Adipocyte-fatty acid-binding protein | 1.00 | 0.84 | 1.01 | 1.13 | 0.94 | 0.78 | 0.103 | 0.288 | 0.118 | 0.290 |
| Adipose triglyceride lipase | 1.00[c] | 1.45[ab] | 1.39[ab] | 1.45[ab] | 1.58[a] | 1.31[b] | 0.079 | 0.001 | 0.985 | 0.030 |
| Carnitine palmitoyltransferase 1 | 1.00[b] | 0.97[b] | 0.92[b] | 1.03[ab] | 1.18[a] | 1.18[a] | 0.049 | 0.003 | 0.003 | <0.001 |
| Carnitine palmitoyltransferase 2 | 1.00 | 1.14 | 1.33 | 1.38 | 1.44 | 1.69 | 0.177 | 0.167 | 0.021 | 0.049 |
| Long-chain acyl-CoA dehydrogenase | 1.00 | 0.94 | 1.03 | 0.90 | 0.96 | 1.05 | 0.062 | 0.519 | 0.253 | 0.432 |
| Lipoprotein lipase | 1.00[a] | 0.97[a] | 1.00[a] | 1.04[a] | 0.74[b] | 0.82[b] | 0.048 | 0.002 | 0.012 | < 0.001 |

[a,b,c]Means within a row with different letters differ significantly ($P < 0.05$).
[1]Data are means for 6 chickens.

**Table 5.** Effect of different recombinant chicken angiopoietin-like protein 4 levels on hormone sensitive lipase activity in the breast muscle of broilers.[1]

| | Recombinant chicken angiopoietin-like protein 4, ng/kg | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | 0 | 20 | 100 | 500 | 2,500 | 12,500 | SEM | *P*-value | Linear | Quadratic |
| Hormone-sensitive lipase, U/mgprot | 1.46 | 1.78 | 1.70 | 1.91 | 1.93 | 2.01 | 0.155 | 0.212 | 0.106 | 0.129 |

[1]Data are means for 6 chickens.

not significantly affected by the injection of recombinant chicken ANGPTL4 (Table 5).

### *Effect of Angiopoietin-Like Protein 4 on Serum Biochemical Parameters of Broilers*

The concentrations of TG, TC and NEFA in the serum were similar among treatments before ANGPTL4 injection (Figure 1A, B, C). Broilers after His-SUMO-ANGPTL4 injection had higher ($P < 0.05$) concentrations of TG and NEFA in the serum than those of NS and His-SUMO injection broilers (Figure 1A, C). However, no difference in the concentrations of TG and NEFA in the serum was observed between NS and His-SUMO injection birds.

### *Effect of Angiopoietin-Like Protein 4 on Lipid Metabolism-related Gene Expression and Enzyme Activity in the Breast Muscle of Broilers*

Broilers of His-SUMO-ANGPTL4 injection had higher ($P < 0.05$) *ATGL* mRNA expression, but lower ($P < 0.05$) *LPL* mRNA expression in the breast muscle than those of NS and His-SUMO injection broilers (Figure 2). However, no difference in *ATGL* and *LPL* mRNA expression in the breast muscle were observed between NS and His-SUMO injection birds. His-SUMO-ANGPTL4 and His-SUMO injection broilers had higher ($P < 0.05$) *CPT1* mRNA expression in the breast muscle than that of NS injection birds. However, no difference in the *CPT1* mRNA expression in the breast muscle was observed between His-SUMO-ANGPTL4 and His-SUMO injection birds. Besides, no difference in HSL activity in the breast muscle was observed among NS, His-SUMO, and His-SUMO-ANGPTL4 injection broilers (Figure 3).

### *Effect of Angiopoietin-Like Protein 4 on Viability of Chicken Primary Myoblasts*

As shown in Figure 4, no difference was observed in the viability of chicken primary myoblasts after exposed to NS, His-SUMO, or His-SUMO-ANGPTL4 (250 pg/mL) for 24 h.

### *Effect of Angiopoietin-Like Protein 4 on Lipid Metabolism-Related Gene Expression in Chicken Primary Myoblasts*

Figure 5 showed that chicken primary myoblasts after exposed to His-SUMO or His-SUMO-ANGPTL4 for 24 h had higher ($P < 0.05$) *CPT1* mRNA expression than NS cells. However, no difference in *CPT1* mRNA expression was observed between His-SUMO and His-SUMO-ANGPTL4 supplemented cells. Besides, *LPL* mRNA expression of His-SUMO-ANGPTL4 chicken primary myoblasts was lower ($P < 0.05$) than that of chicken primary myoblasts in NS and His-SUMO. However, no difference was observed in *LPL* mRNA expression between NS and His-SUMO chicken primary myoblasts.

## DISCUSSION

Angiopoietin-like protein 4 has been proposed as a circulating mediator between gut microbiota and lipid metabolism in rodent and human (Grootaert et al., 2012). Our previous study showed that intestinal

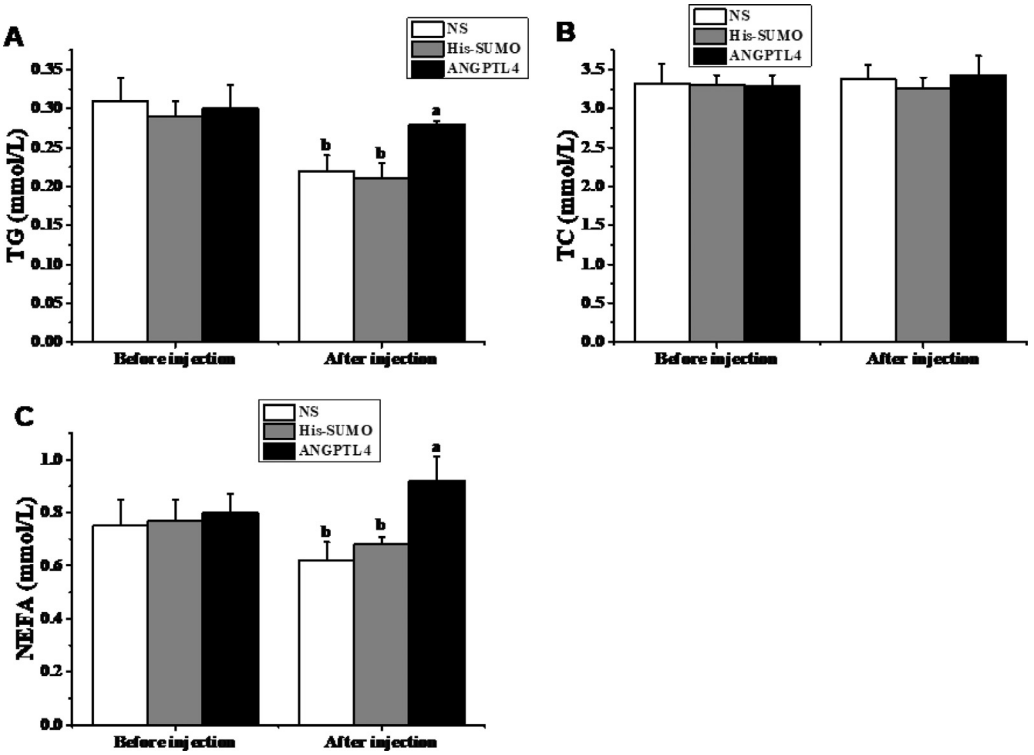


**Figure 1.** Effect of angiopoietin-like protein 4 (12,500 ng/kg BW, i.v.) on serum biochemical parameters of broilers. Before and at 30 min after intravenous injection, serum was obtained and triglyceride (**TG**) (A), total cholesterol (**TC**) (B), and non-esterified fatty acid (**NEFA**)(C) levels were measured for normal saline (**NS**), His-SUMO, or His-SUMO-ANGPTL4 (**ANGPTL4**). Value of each treatment is the mean of 6 chickens, and the vertical bar represents standard error. Means with different letters differ significantly ($P < 0.05$).

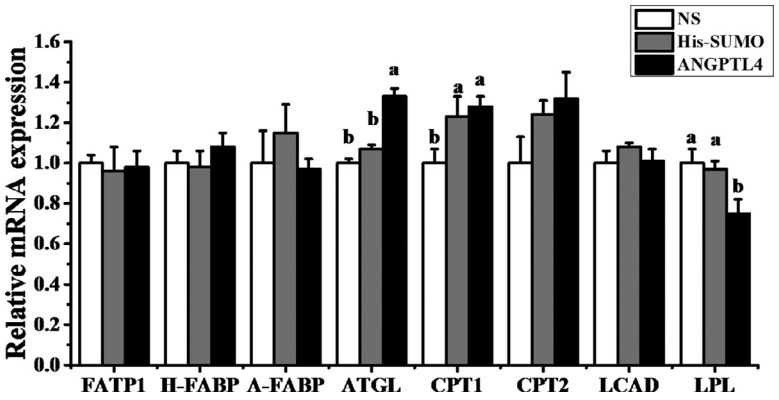


**Figure 2.** Effect of angiopoietin-like protein 4 (12,500 ng/kg BW, i.v.) on lipid metabolism-related gene expression in the breast muscle of broilers. At 30 min after intravenous injection, fatty acid transport protein 1 (***FATP1***), heart-fatty acid-binding protein (***H-FABP***), adipocyte-fatty acid-binding protein (***A-FABP***), adipose triglyceride lipase (***ATGL***), carnitine palmitoyltransferase 1 (***CPT1***), carnitine palmitoyltransferase 2 (***CPT2***), long-chain acyl-CoA dehydrogenase (***LCAD***), and lipoprotein lipase (***LPL***) mRNA expression in the breast muscle were measured for normal saline (**NS**), His-SUMO, or His-SUMO-ANGPTL4 (**ANGPTL4**). Value of each treatment is the mean of 6 chickens, and the vertical bar represents standard error. Means with different letters differ significantly ($P < 0.05$).

microbiota could increase the breast muscle intramuscular fat content and at the same time reduce the serum ANGPTL4 concentration of broilers (Zhao et al., 2018). Based on the phenomenon above, we postulated that ANGPTL4 could exert an intramuscular fat-promoting function in the breast muscle of broilers. In chickens, the quantity of intramuscular fat in the breast muscle is the net outcome of lipid uptake and lipolysis, so it is important to study the process of these two aspects of lipid metabolism in order to explain whether ANGPTL4 play an important role in increasing intramuscular fat in the breast muscle of broilers. Therefore, our research mainly focuses on the effects of ANGPTL4 on the lipid uptake and lipolysis in the breast muscle of broilers by animal experiments and chicken primary myoblasts culture.

In the effective dose assay, the concentrations of TG and NEFA in the serum and *ATGL* and *CPT1* mRNA expression in the breast muscle were induced, but the *LPL* mRNA expression in the breast muscle was inhibited by His-SUMO-ANGPTL4 at the level of 12,500 ng/kg BW. There is considerable evidence that serum TG concentration is indicative of basal adjustment of fatty acid circulation between the adipose tissue and the liver (Mossab et al., 2002). Non-esterified fatty

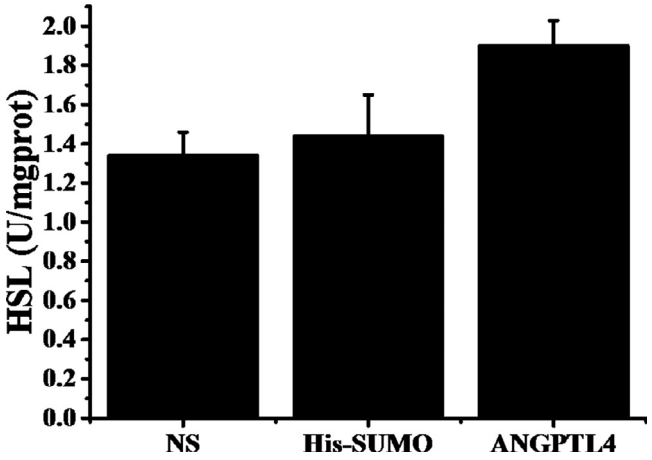


**Figure 3.** Effect of angiopoietin-like protein 4 (12,500 ng/kg BW, i. v.) on hormone sensitive lipase (**HSL**) activity in the breast muscle of broilers. At 30 min after intravenous injection, HSL activity in the breast muscle was measured for normal saline (**NS**), His-SUMO, or His-SUMO-ANGPTL4 (**ANGPTL4**). Value of each treatment is the mean of 6 chickens, and the vertical bar represents standard error.

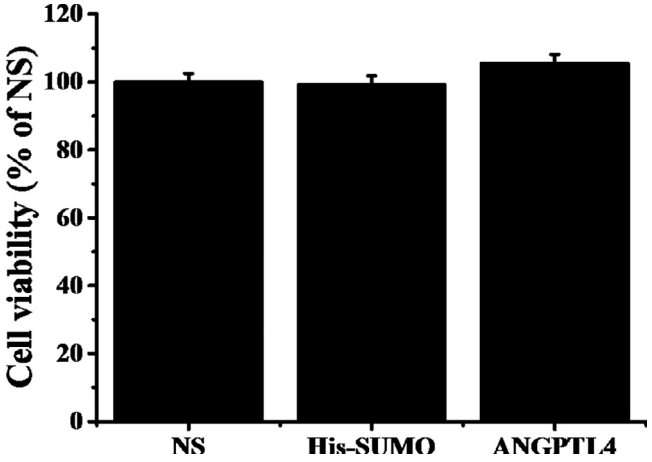


**Figure 4.** The viability of chicken primary myoblasts after exposed to normal saline (**NS**), His-SUMO, or His-SUMO-ANGPTL4 (**ANGPTL4**, 250 pg/mL) for 24 h. Value of each treatment is the mean of three independent experiments, and the vertical bar represents standard error.

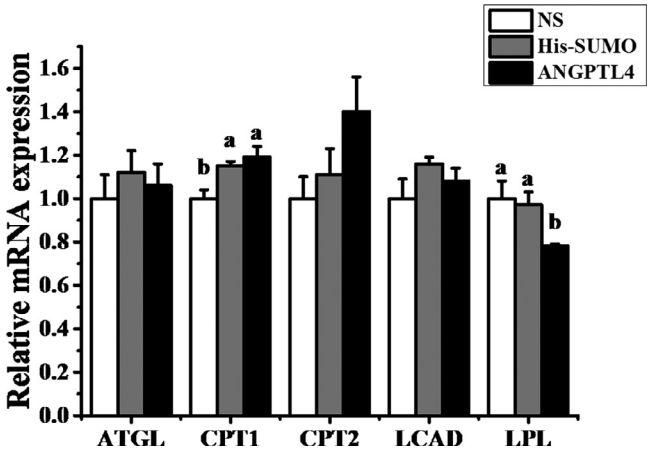


**Figure 5.** Adipose triglyceride lipase (***ATGL***), carnitine palmitoyltransferase 1 (***CPT1***), carnitine palmitoyltransferase 2 (***CPT2***), long-chain acyl-CoA dehydrogenase (***LCAD***), and lipoprotein lipase (***LPL***) mRNA expression in chicken primary myoblasts after exposure to normal saline (**NS**), His-SUMO, or His-SUMO-ANGPTL4 (**ANGPTL4**, 250 pg/mL) for 24 h. Value of each treatment is the mean of three independent experiments, and the vertical bar represents standard error. Means with different letters differ significantly ($P < 0.05$).

acid is mainly generated by the hydrolysis of TG in adipose tissue, and its concentration reflects the lipolytic activity in adipose tissue (Mersmann and MacNeil 1985). Adipose triglyceride lipase is the key enzyme catalyzing the initial step in TG hydrolysis (Zimmermann et al., 2004). Carnitine palmitoyltransferase 1, which mediate long-chain fatty acids cross mitochondrial membranes into mitochondria, is generally considered to be a rate-limiting enzyme of the fatty acid oxidation (Bartlett and Eaton, 2004; Motoki et al., 2012). Lipoprotein lipase catalyzes the hydrolysis of TG component of circulating chylomicron (CM) and VLDL, which is a rate-limiting step in the lipid transport into peripheral tissues (Cai et al., 2009; Dijk et al., 2018). Therefore, the higher concentrations of TG and NEFA in the serum, higher *ATGL* and *CPT1* mRNA expression in the breast muscle, but lower *LPL* mRNA expression in the breast muscle in group injected with His-SUMO-ANGPTL4 at the level of 12,500 ng/kg BW in this study indicated ANGPTL4 may play an important role in breast muscle lipid metabolism regulation. To further confirm this idea, we used the His-SUMO tag as a negative control in order to rule out the tag effect on breast muscle lipid metabolism regulation. The higher serum concentrations of TG and NEFA, higher breast muscle *ATGL* mRNA expression, but lower breast muscle *LPL* mRNA expression in group injected with His-SUMO-ANGPTL4 in this study indicated ANGPTL4 indeed play an important role in breast muscle lipid metabolism regulation, especially in fat degradation and LPL inhibition. As mentioned above, LPL is a key enzyme in the hydrolysis of TG from circulating CM and VLDL. Therefore, the increased serum TG concentration of broilers injected ANGPTL4 in this study is likely associated with the inhibition of clearance of circulating TG. Additionally, the higher serum NEFA concentration of broilers injected ANGPTL4 is consistent with the ANGPTL4 effect on fat degradation obtained in this study, suggesting that the increased serum NEFA concentration was partially attributed to the enhanced breast muscle *ATGL* mRNA expression produced by ANGPTL4. However, the ability of His-SUMO-ANGPTL4 and His-SUMO to stimulate breast muscle *CPT1* mRNA expression showed that the His-SUMO might be the modulators for breast muscle *CPT1* mRNA expression. Besides, the unchanged *FATP1, H-FABP* and *A-FABP* mRNA expression in the breast muscle in the groups injected with His-SUMO-ANGPTL4 and His-SUMO in this study indicated that ANGPTL4 may not alter the lipid uptake in the breast muscle, however, this needs to be verified by further research.

To further confirm the effect of ANGPTL4 on fat degradation and LPL inhibition in the breast muscle, chicken primary myoblasts culture was conducted. In the cell viability assay, the unchanged viability of

chicken primary myoblasts after exposure to His-SUMO-ANGPTL4 (250 pg/mL, physiological dose) or His-SUMO for 24 h in this study indicated that ANGPTL4 didn't negatively affect the cell viability. Therefore, the above concentration of ANGPTL4 was applied in the following experiment. In the stimulation assay, the *CPT1* mRNA expression in chicken primary myoblasts was highly induced by both His-SUMO and His-SUMO-ANGPTL4. The finding was consistent with the result of animal experiment above, which once again indicated that His-SUMO could contribute to the increased breast muscle *CPT1* mRNA expression. Besides, the lower *LPL* mRNA expression in the chicken primary myoblasts exposed to His-SUMO-ANGPTL4 in this study was also consistent with the result of animal experiment above, which indicated that the ANGPTL4 can directly inhibit breast muscle *LPL* mRNA expression. However, the *ATGL* mRNA expression in chicken primary myoblasts was not affected by His-SUMO-ANGPTL4, which was inconsistent with the result of animal experiment above. This implied that the breast muscle *ATGL* mRNA expression in animal experiments stimulated by ANGPTL4 might be in an indirect manner, such as ANGPTL4 need to be proteolytically cleaved to function and so on (Dijk and Kersten, 2014). However, this needs to be verified by further research.

Studies in mice and human have shown that ANGPTL4 is produced by a variety of tissues, and is secreted into the bloodstream in glycosylated, oligomerized, native and cleaved isoforms to modulate physiological events such as angiogenesis, cell differentiation and the crosstalk between liver, brain, adipose and muscle tissue in lipid and glucose metabolism (Grootaert et al., 2012). For example, the mechanistic action of the inhibition of ANGPTL4 on LPL, which is well known, is mediated by a relatively short amino acid sequence close to the N terminus (Lafferty et al., 2013; Dijk and Kersten, 2014). Information on the effects of ANGPTL4 on breast muscle lipid metabolism of broilers is lacking. Yoshida et al. (2002) observed that intravenous injection of ANGPTL4 did not affect the concentration of TC, but markedly increased concentrations of TG and NEFA in the plasma of mice at 30 min after injection. Ge et al. (2004); Köster et al. (2005); Lichtenstein et al. (2007) and Wang et al. (2016) reported that ANGPTL4 overexpression dramatically increased concentrations of TG and NEFA in the plasma of mice. Singh et al. (2018) observed that genetic loss of ANGPTL4 in brown adipose tissue enhanced plasma TG clearance of mice. Mandard et al. (2006) observed that *ATGL* mRNA expression was elevated by 50% in ANGPTL4 transgenic mice. Grootaert et al. (2012) reported that ANGPTL4 is a LPL inhibitor in mice. Greiner and Bäckhed (2011) reported that ANGPTL4 not only aggravates LPL inhibition and reduces LPL-mediated TG storage but also increases lipodieresis in skeletal muscle. It appeared that the serum biochemical parameters and breast muscle lipid metabolism-related gene expression response observed in this study was similar to those of mice in previous studies.

In conclusion, we demonstrated that ANGPTL4 is a hyperlipidemia-inducing factor in broilers and has the ability to promote lipodieresis and inhibit LPL in the breast muscle of broilers. This finding raises an interesting possibility that ANGPTL4 might play an important role in regulating intramuscular fat in the breast muscle of broilers.

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## DISCLOSURES

The authors declare that they have no conflict of interest.

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