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Research Article
Lipid Formation and mRNA Expression of Key Adipogenic Genes in the Liver of Athens Canadian Random Breed and COBB Chicken Breeds During the Embryogenic Period

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Abstract

Objective: This study was designed in order to compare the lipid formation and the expression of key lipid metabolism genes in the livers between 2 types of chicken breeds during embryonic development. **Methodology:** A total of 36 fertilized eggs from Athens Canadian Random Breed (ACRB: Unselected chicken), breeder hens and commercial breeder hens (COBB: Selected for rapid growth) were incubated at 37.5°C and 60% relative humidity. Liver samples were collected at embryonic day (E)14 and 18. The mRNA expression of key adipogenic genes, such as adipocyte protein 2 (AP2) and peroxisome proliferator-activated receptor gamma (PPAR-ɣ), was detected using quantitative real time polymerase chain reaction (qRT-PCR). Lipid droplet accumulation was also analyzed and compared using a histological method between COBB and ACRB on E14 and E18. **Results:** In this study, the histological analyses of COBB and ACRB livers showed no significant difference in the amount of lipid droplets accumulated. The mRNA expression of AP2 and PPAR-ɣ also was not significantly different between COBB and ACRB on E14 and E18. **Conclusion:** The results indicate that lipid formation in the liver during embryonic development is not affected by any genetic selection, suggesting that lipid metabolism changes between two chicken breeds could be initiated after hatch.

Key words: Broiler, embryo development, liver, adipogenesis, genetic selection

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Athens Canadian Random Breed (ACRB) chickens are non-selected, meat-type broilers[1]. Another strain of chickens, COBB, are selected meat-type broilers for rapid growth and are one of the most widely consumed broiler types in the world[1,2]. Because of the difference in growth traits, these 2 types of chickens were good models to understand adipocyte formation and lipid metabolism in chicken[1,2]. Because broiler chickens are one of the major sources of protein, thus chickens have been selected for rapid growth over several generations to greatly increase meat yield[2]. In the near future, poultry consumption is projected to overtake pork, which is currently the leading source of meat and will continue to increase rapidly worldwide[2,3]. The COBB broiler chickens are the most widely consumed bird and contribute seventy percent of the world's poultry consumption[3].

However, this selection (COBB) has resulted in growth acceleration with significantly more excess fat deposits than unselected chickens (Athens Canadian Random Breed, ACRB), which has been shown with studies proving that both abdominal adipose tissue and adipocytes of COBB chickens are much larger and heavier than ACRB chickens[1].

More feed is required to increase the muscle mass of chickens with excess fat and customers are also unwilling to purchase meat with poor quality and undesirable appearances[4]. Although fat is a necessity for growth, development and provides energy for the body, excess fat formation causes adverse effects in chickens that could lead to obesity, fatty liver disease and hepatocyte steatosis, fast growing chickens must be controlled in order to prevent disadvantageous consequences for the poultry industry[5]. Although the liver is a key organ for fatty acid synthesis and lipid metabolism in chickens, there have been no studies that have researched the difference in the amount of key hepatic adipogenic gene expression between COBB and ACRB chickens during the embryogenic period, which would provide valuable information in understanding the cause of excess lipid production in COBB livers starting from the embryogenic period. Our hypothesis is that since there is a greater amount of fat tissue in the selected chicken for fast growth, there is also a greater amount of lipid formation and adipogenic gene expression in the liver during the embryonic period, causing the liver to synthesize excess lipids for body fat tissue formation. Moreover, since the dietary factors do not much influence during the embryonic period, effects of genetic selection could be evaluated in the study. The objectives of this study were (1) To compare lipid production in 18 days embryo livers of ACRB and COBB and (2) To investigate if the expression of key adipogenic genes (PPAR-ɣ and AP2) was greater in COBB chickens starting from the embryogenic period. The present study of detecting and comparing mRNA expression patterns of these genes in the liver of 18 days COBB and ACRB chickens during the embryogenic period is crucial in order to determine the cause of excess adipose tissue in COBB chickens, which decreases optimal meat quality, meat yield and feed efficiency, creating concerns for the poultry industry because of the loss in profit[2]. This study would help to influence the future studies to discover effective ways to reduce excess adipose tissue in chickens so that meat quality, yield and profit for the poultry industry are not compromised.

MATERIALS AND METHODS

Animals: These experiments were performed according to the Institutional Animal Care and Use Committee of the University of Georgia. A total of 36 eggs from COBB (Cobb500: Selected broiler) and ACRB (non-selected broiler) breeder hens were incubated at 37.5°C and 60% relative humidity. Embryos were removed, sacrificed and liver samples were then collected for histology and gene expression at embryonic day E14 (n = 9/treatment) and E18 (n = 9/treatment).

Histology: Liver histological slides of COBB and ACRB at E14 and E18 were made to evaluate lipid accumulation in the liver. Liver tissues were fixed in 10% phosphate buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Samples were then embedded in paraffin wax and cut into sections 4.0 μm in thickness. Liver sections were stained with standard hematoxylin and eosin solution in order to visualize lipid droplets in the embryonic liver cells.

Quantification of mRNA using quantitative reverse transcription polymerase chain reaction (qRT-PCR): Total RNA was extracted from 80 mg of liver tissue using 1 mL of Qiazol lysis reagent (Qiazen, Valencia, CA, USA) following the manufacturer's protocol. RNA was then quantified by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). RNA purity was determined by ratios of absorption (260/280 nm = near 2.00). RNA was reverse transcribed with high capacity cDNA reverse transcription synthesis kits (Applied BioSystems, Life Technologies, CA, USA) according to the manufacturer's protocol. The qRT-PCR analysis was performed with the cDNA template, forward and reverse primers of each gene, nuclease free water and SYBR green as a detector (Bio rad, Herculis, CA, USA). Forward and reverse primers of GAPDH, AP2, PPAR-ɣ were designed and

Table 1: Primer sequences used for qRT-PCR gene expression

Name of Genes	Primer Sequence	Product length (bp)	Annealing temperature (°C)
GAPDH Fwd	GCT AAG GCT GTG GGG AAA GT	116	55
GAPDH Rev	TCA GCA GCA GCC TTC ACT AC		
AP2 Fwd	TGC TGG GCA TCT CAA TCA CA	106	57
AP2 Rev	GCA TTA GTC AGA ACG GGC CT		
PPARγ Fwd	TGA ATG TCG TGT GTG TGG GG	229	55
PPARγ Rev	GCA TTC GCC CAA ACC TGA TG		

examined for target identity with the National Center for Biotechnology Information (NCBI) (Table 1). Quantitative real time polymerase chain reaction (qRT-PCR) was performed using a StepOne Thermocycler (Applied Biosystem, Foster City, CA, USA) in duplicate reactions. Temperature cycles were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, annealing temperature for 20 sec and 72°C for 60 sec (Table 1). After 40 cycles, melting curves were assessed to confirm primer specificity. Data were generated with the Ct method by normalizing the expression of the target gene to a housekeeping gene, GAPDH. The values were reported as fold changes in mRNA expression of the target genes in the experimental groups compared with the control group.

Hepatic cell culture: Whole livers were collected from ACRB or COBB embryos at E18. The livers were minced into fine pieces with sterile scissors and scalpels and incubated with 10 mL of digestion buffer containing 0.25% collagenase (Sigma-Aldrich, MO, USA) in Dulbecco's modified eagle's medium (DMEM) (Mediatech Inc.,VA, USA) for 30 min at 37°C in a shaking water bath. After digestion, the contents were filtered through 40 µm nylon meshes and the filtrate was centrifuged at 1,200 rpm for 10 min. The supernatant was discarded and the hepatocyte pellet was resuspended with 10 mL DMEM containing 10% fetal bovine serum, L-glutamine, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycine (Mediatech Inc.,VA, USA). The cells were plated into 100 mm cell culture plates. The hepatocyte growth and morphology of ACRB and COBB were observed and pictured under a microscope.

Oil red O staining: In order to measure lipid accumulation in hepatocytes, the cells were stained with Oil Red O. Oil Red O stained neutral lipids in the cells in red.

Statistical analysis: Research data were analyzed by using the general linear model procedure of the Statistical Analysis System (SAS) Institute version 9.4. Duncan test was used for evaluating the mean separation. The level of significance in the gene expression study was $p<0.05$.

RESULTS AND DISCUSSION

In the present study, the mRNA expression of two key adipogenic genes, AP2 and PPAR-ɣ, was compared between the 18 day embryos of COBB and ACRB chickens. These genes are crucial for the regulation of adipogenesis in chickens[6].

Peroxisome proliferator-activated receptor gamma (PPAR-ɣ) is a master regulatory gene of lipid metabolism[7]. This central gene regulator in adipose tissue enhances the expression of other key adipogenic genes, such as AP2[8]. The PPAR-ɣ gene is highly expressed in fat chicken lines and enhances fat deposition in the abdomen[6]. It has been shown that there was a higher PPAR-ɣ expression in the abdomen of 6 week old chickens when compared to younger chickens, which suggests that this gene plays a key role in expanding abdominal fat tissue in chickens. The PPAR-ɣ also helps to release fatty acids from triglycerides and transport fatty acids out of the cell[8]. PPAR-ɣ is significantly induced in chickens with fatty liver disease[9]. According to immunohistochemical studies, there is an increase in PPAR-ɣ mRNA expression in the nuclei of hepatocytes of chickens with fatty liver disease, suggesting that when this gene is significantly induced, it is an indicator of excess lipid accumulation[9,10].

Another gene involved in adipogenesis is the adipocyte fatty acid binding protein (AP2), an important regulator of lipid metabolism[6,7]. Its main role is acting as a carrier protein to facilitate the transport of fatty acids to the endoplasmic reticulum and regulating storage of lipid droplets in the cytoplasm, which is crucial for adipose tissue development[10]. Increased mRNA expression of AP2, which is controlled by PPAR-ɣ, is associated with obesity because of AP2's role of resisting insulin[11]. This protein is often expressed in areas of high lipid metabolism, which is in the liver for chickens[7,11]. Greater amounts of AP2 correlate with greater lipid metabolism and in livers where fat liver buildup is significant, the expression of AP2 mRNA increases, an indicator of fatty liver disease[6,11].

In the present study, when analysing the gene expressions of the two key adipogenic genes in 18 day embryo livers, both AP2 and PPAR-ɣ showed no significant fold difference in lipogenic gene expression between

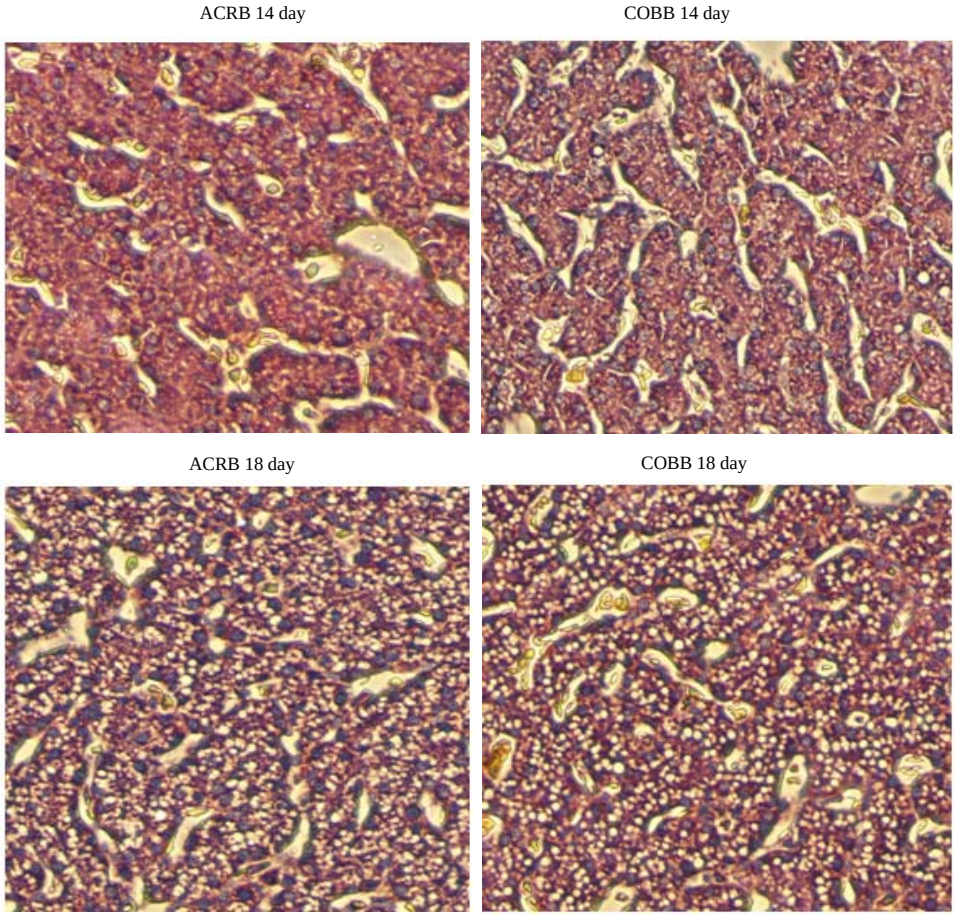

Fig. 1: Liver histology pictures of Athens Canadian Random Breed (ABRB) and COBB embryos at embryonic day E14 and E18

ACRB and COBB chickens (Fig. 2). Although there seems to be a significantly greater fold difference in the expression of AP2 in 18 day COBB embryo liver when compared to ACRB embryo liver, because of the high standard deviations and data variabilities in both chickens, it is unlikely that there actually is a difference in gene expression. Based on the histology of both 14 and 18 day liver embryo of ACRB and COBB chickens, there is not a significant difference in the amount of fat accumulation between them.

When comparing COBB chickens to ACRB chickens, the quantitative RT-PCR data in the current study showed no difference in the the adipogenic gene expression of AP2 and PPAR-ɣ during the embryogenic period, suggesting that the difference in lipid production between the two breeds occurs after hatching.

A study by Cartwright *et al.*[1], showed that both body weight and abdominal fat pads of selected commercial broiler chickens were greater than unselected broilers at 28 and 54 days. The fat pads of the selected broilers continued to increase until day 54, while those of the unselected broilers were not significantly affected by age. Based on this information, there is a possibility that after hatching, COBB chicken livers produce more fatty acids and transport more fatty acids to other tissues, such as muscle and adipose tissue, developing more fat formations.

In chickens, the liver is the major organ involved with lipid synthesis[4,12,13]. While other mammals can generate fat in different organs in the body, such as adipose tissue, chickens produce fatty acids mostly in the liver, which is why over 90% of fat in adipose tissue has been generated by fatty acids synthesized in the liver, instead of in the adipose tissue itself[12,13]. Fatty acids, which are produced in the liver or are absorbed into the body through food consumption, provide long-term energy for the chickens but if in excess, are stored as fat[12].

Although COBB breed chickens have more abdominal fat pads than ACRB breed chickens[1], based on the histology of the present study, there was little difference in the amount of lipid accumulation in ACRB and COBB chicken livers during the embryonic period (Fig. 1), indicating that there was a lack

of evidence of excess lipid accumulation in the liver of COBB chickens. Lipid accumulation at E18 was considerably higher than one at E14 regardless of the breeds, indicating that the embryonic period between E14-18 would be an important stage for lipid production in the livers of the chickens. Compared to chickens with typical fatty liver disease, which is characterized by the overaccumulation of lipids in the liver due to the increased expression of several adipogenic genes, our observations show that there was no lipid accumulation between COBB and ACRB because fatty liver disease usually expresses increased adipogenic genes such as PPAR-ɣ and AP2 mRNA[9,14,15]. However, in the current study, the expression of these key adipogenic genes, PPAR-ɣ and AP2, was also not induced significantly in this study. Therefore, there was no difference in lipid accumulation in the liver during the embryonic period. However, when isolated hepatocytes from the COBB or ACRB were grown in the cell culture, hepatocytes from the COBB accumulated more fat droplets than those from the ACRB (Fig. 3), indicating that lipid metabolism of the liver is under tight control during the embryonic period. After hatch or when hepatocytes are grown in culture system, the COBB birds or hepatocytes considerably increased lipid formation. This is a valuable evidence indicating that excess fat deposition in selected chickens is caused by factors after hatch.

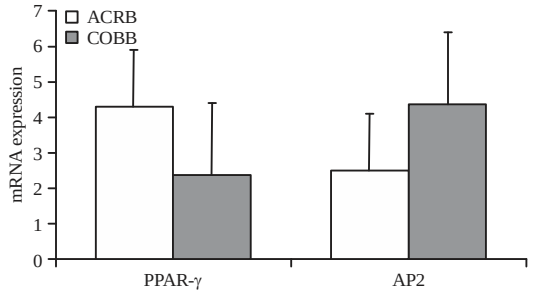

Fig. 2: The mRNA expression of two key adipogenic genes, PPAR-ɣ and AP2 in the livers of ACRB and COBB embryos at E18

One reason for why there is no evidence of lipid accumulation in the COBB chickens during the embryonic period is that there may be higher fatty acid and lipid export to other tissues from the COBB liver. Our findings provide some evidence that once the limit for the amount of lipid production is reached inside the liver in a normal physiological condition, the excess lipid is pumped out to other tissues through blood circulation. When the capacity for lipid production inside the liver is full, very low density lipoproteins (VLDL) may play the key role of transporting triglycerides into other tissues.

The VLDLs are crucial during the process of lipogenesis in other tissues, including adipose tissue, because they play a major role in transporting triglycerides to other tissues[4,15]. Consequently, the growth of fat tissue depends on the amount of triglycerides transported by VLDLs[4,15]. The VLDLs are synthesized in the liver, beginning at the endoplasmic reticulum and are then transported to the Golgi apparatus to be packaged and released from the cell[4].

Triglycerides are also synthesized in liver cells when three fatty acids bind to a glycerol molecule[16]. After fatty acids bind to a glycerol, the new triglyceride is recognized by VLDL lipoprotein receptors. Triglycerides, using VLDL transporters, are then released into the bloodstream[4]. Once the triglycerides reach the adipose tissue, the breakdown of triglycerides is catalyzed by lipoprotein lipase (LPL), which results in a fatty acid and diacylglycerol release, so that fatty acids can then be stored in adipose tissue and reform as triglycerides[15,17].

This pathway of fatty acid synthesis in the liver and excretion into adipose cells by VLDL transportation shows that

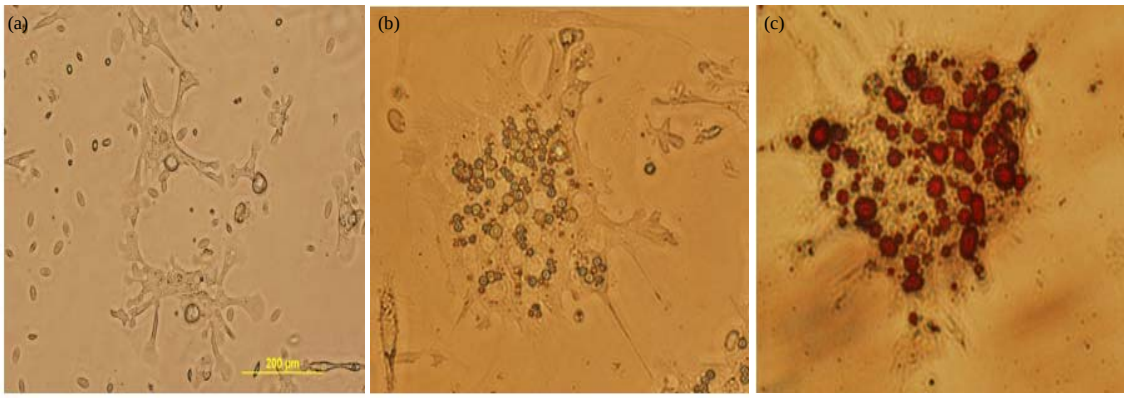

Fig. 3(a-c): (a) Hepatocyte pictures of ACRB, (b) COBB and (c) COBB with oil Red O staining for lipid accumulation in the cells

compared to ACRB chickens, COBB chickens have more excess fatty acids that must be excreted from the liver and stored in adipose tissue, which results in excess fat tissue formation because when more triglycerides are synthesized than broken down, there is a net accumulation of fat[17]. In liver cells of chickens with high VLDLs, lipogenic enzymes were very active and an increase in fatty acid synthesis was usually followed by an increase in lipid secretion[17,18]. Therefore, higher rates of triglyceride synthesis, due to enhanced synthesis of fatty acids in the liver, could be responsible for high amounts of abdominal adipose tissue in COBB chickens after hatch[6].

Large fat deposits in COBB chickens could also be caused by different external factors that occur post-hatch, including higher dietary fatty acid, energy intake, absorption, enhanced overall growth and nutrient absorption. Because of genetic selection for the highest possible muscle yield in broilers, voluntary feed intake and growth rates have increased significantly, which leads to increased body fat deposition in adipose tissue throughout the body[16,19]. High energy diets fed to commercial broiler chickens result in higher percentages of abdominal fat deposition, which suggests that by lowering the caloric intake of the chickens, fat accumulation can be significantly reduced[20,21]. However, it was also found that when protein levels in the feed were reduced, the percentage of body fat increased[22]. These results show that nutritional factors, such as dietary protein and caloric intake, influence lipid metabolism in commercial broiler chickens[23]. Although COBB chickens have benefited by genetic selection to increase their body weight, growth rate, muscle development and feed efficiency, this selection has resulted in chickens with higher body fat content compared to ACRB chickens[21,24,25]. As body and muscle mass, growth and feed efficiency have increased in the COBB breed, their feed intake and ability to absorb nutrients were also enhanced, resulting in higher fat depositions than the unselected breed, ACRB. In addition, fat tissue itself in the COBB may be more active in fatty acid uptake and fat accumulation compared to the ACRB after hatch.

CONCLUSION

In chickens, the development of adipose tissue is influenced by different factors, including genetic selection, nutritional intake and feed efficiency. In the current study, we evaluated liver fat synthesis between the COBB and ACRB during embryonic period because the liver is the key organ for fatty acid biosynthesis and body lipid metabolism in chickens. Key adipogenic genes, such as AP2 and PPAR-ɣ, are not significantly expressed during the embryogenic period of both selected and unselected chickens for fast growth, suggesting that the high amount of abdominal fat in COBB chickens is caused by factors after hatching. A more detailed understanding on mRNA expressions of various key adipogenic genes is essential in order to find solutions to improve the development of broiler chickens so that they can yield maximum muscle mass, feed efficiency and nutritional quality. This research provides an evidence that fat accumulation of selected chicken is not caused by any factors during embryonic period and may be caused by factors after hatch.

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