

Effects of feed restriction and dietary fat type on mRNA expression of liver fatty acid-binding protein (L-FABP) in broilers

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Abstract:

BACKGROUND: Liver fatty acid-binding protein (L-FABP) is the main cytosolic binding site for long chain fatty acids in hepatocytes. FABPs enhance the uptake of fatty acids into the cell by increasing their concentration due to decreasing concentration of unbound fatty acids inside the cell. **OBJECTIVES:** The aim of this study was to evaluate the effects of dietary unsaturated to saturated fatty acid ratio and feed restriction on L-FABP mRNA expression. **METHODS:** A total of 720, 10-day old male Ross 308 broiler chicks were fed diets with unsaturated to saturated fatty acid ratio (U/S) of 2, 3.5, 5, or 6.5 as ad libitum or skip-a-day feeding schedule (during 18–28 days of age). Relative expression of L-FABP mRNA in hepatocytes of broilers was determined using quantitative reverse transcription real-time polymerase chain reaction. **RESULTS:** Our results show that feed restriction induced the expression of L-FABP gene in the liver of broilers. Moreover, L-FABP gene expression was increased by dietary U/S ratio of 6.5. There was no interaction between dietary U/S and feed restriction on the L-FABP gene expression. **CONCLUSIONS:** Results indicate that birds have a mechanism for regulation of fatty acid transfer under different nutritional conditions.

Introduction

In broilers, because of the limited capacity of digestive tract, vegetable or animal fats or their mixtures form an important component of their high-energy diets. Fats with high unsaturated fatty acid content have more absorbability. In addition, there is a known synergism between saturated and unsaturated fatty acids (Freeman, 1984; Hulan et al., 1984). Moreover, age of birds affects the digestibility of fat, because at earlier ages, there is an inadequate production of digestive enzymes

from liver that metabolize fat (Yu and Robinson, 1992). In general, the metabolism of fat is highly regulated in which several genes are involved.

The intracellular fatty acid-binding proteins (FABPs) comprise a family of 14–15 kDa proteins that bind long chain fatty acids (Glatz and vander Vusse, 1996; Coe and Bernlohr, 1998). Members of this family have evolved over approximately one billion years by subsequent duplication and diversification of an ancestral intracellular lipid-binding protein gene thereby generating a large number of tissue-specific homologs (Storch and Corsico, 2008).

In general, tissues with an active fatty acid metabolism, such as liver, express high levels of FABPs that harmonize fatty acid absorption and utilization. Liver has a major role in the metabolism of fat, and considerable amounts of fatty acids are transferred into and out of this organ.

Evidence shows that L-FABP does not undergo major modulation during metabolic changes from fed to fasted to re-fed states, during which rapid and considerable transitions in hepatic fatty acid flux, fatty acid oxidation, and triglyceride biosynthesis take place (Bass, 1988). In addition, starvation activates comprehensive adaptative metabolic reactions including energy metabolic responses, a route involving tissue-specific changes in gene expression, and in which, liver plays an essential role.

FABPs may modulate lipid metabolism via involvement in the fatty acid uptake or export process. In this study, the proposed mechanisms are the regulation of substrate and/or product concentrations in the cytosolic compartment as a whole or more locally near particular enzymes, and/or specifically delivering or removing fatty acids to/from particular enzymes (Storch and Thumser, 2000). FABPs enhance the uptake of fatty acids into the cell by increasing their concentration, due to decreasing concentration of unbound fatty acid inside the cell (Weisiger, 1996).

It is suggested that L-FABP may function in the partitioning of fatty acids to different lipid metabolic pathways (Storch and Corsico, 2008). At present, the control of tissue-specific expression of FABP is only poorly understood. Often, the expression of FABPs in a given tissue reflects its lipid metabolizing capacity. In addition, increased fatty acid exposure leads to a marked increase in FABP expression (Haunerland and Spener, 2004).

Liver performs various functions; for example, it has an important role in fatty acid absorption (Prows et al., 1995; Newberry et al.,

2003), it directs fatty acids to particular metabolic pathways (Storch and Corsico, 2008), it is involved in lipoprotein biosynthesis (Spann et al., 2006), and it transports peroxisome proliferator-activated receptor (PPAR) ligands to the nucleus and modulates the target gene expression (Wolfrum et al., 2001). Furthermore, Gao et al., (2015) suggested that L-FABP may affect lipid metabolism by regulating PPAR α and liver bile acid-binding protein (L-BABP) in chicken liver.

The basic liver-type fatty acid binding protein (Lb-FABP) is the only FABP that is not expressed in mammals; however, it is found in the liver of birds, fishes, reptiles, and amphibians (DiPietro et al., 1999). In chicken, Lb-FABP gene is expressed only in the hepatocytes, whereas L-FABP gene is expressed in both liver and intestinal tissues (Murai et al., 2009). Murai et al., (2009) identified little amounts of L-FABP and Lb-FABP mRNAs in the chicken liver during embryogenesis, but at the onset of hatching, a remarkable increase in mRNA expression was detected for both genes suggesting that the expression of L-FABP and Lb-FABP genes is coordinated from the developmental stages (Murai et al., 2009).

L-FABP gene expression alters a number of factors that greatly impact hepatic fatty acid metabolism, including feed restriction, high-fat diets, and PPARs (Bass, 1988; Veerkamp, 1995). Ockner and Manning (1974) were the first researchers who reported the effect of dietary fat levels on the production of FABPs in gastrointestinal tract. Huang et al., (2008) showed a dose-dependent increase in L-FABP gene expression of the chickens fed with soybean lecithin. Reverse Northern dot blot and semi-quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) showed that the chicken L-FABP gene was over-expressed in the hepatocytes of the common hybrids compared to their parental lines, which is consistent with the higher abdominal fat deposition and wider inter-muscular fat

width in the common hybrids (Yu et al., 2011). Zhang et al., (2013) suggested a genetic basis for the effects of strain on the chicken L-FABP gene expression; they suggest that a higher L-FABP mRNA expression enhanced the rate of lipogenesis and ultimately lipid deposition. They also demonstrated that L-FABP gene expression in fat chickens was higher than that in lean chickens.

Richards et al., (2003) showed that feed restriction reduced the expression of genes involved in lipogenesis, but enhanced the expression of genes like L-FABP that involve in fatty acid transport in hepatocytes of broiler breeder chickens. Murai et al., (2009) showed that feeding motivation was an important factor inducing Lb-FABP gene expression. In contrast, feeding stimulation only somewhat encouraged expression of the L-FABP gene, and was not at all times its main determinant.

We could not find any report on the coincided effects of feed restriction and dietary fat saturation on the L-FABP gene expression in broiler chickens; therefore, this study was designed to study the effects of skip-a-day feeding and dietary fat type on L-FABP mRNA expression in male broiler chickens.

Materials and Methods

A total of 720, 10-day old male chicks (Ross 308) were used in the study. The birds were randomly assigned according to their initial body weights to a completely randomized design with a 2×4 factorial arrangement with 3 replicates. Following were the experimental factors: (1) skip-a-day or free feeding at days 18–28 of age and (2) diets with different unsaturated to saturated fatty acid (U/S) ratios (2, 3.5, 5, and 6.5) formulated using different levels of sunflower oil and beef tallow. Fatty acid content of beef tallow and sunflower oil were determined by gas chromatography. The birds were fed a grower diet until 28 days of age followed by a finishing diet at 29 to 42 days of

age. All birds were handled in accordance with the Ross 308 broiler chickens Management Guide specifications. The basal diets were formulated using Ross 308 guideline. The fatty acid profile of the ingredients and the formulation and chemical composition of experimental diets are shown in Tables 1 and 2, respectively.

At the end of grower (28d) and finisher (42d) periods, four broilers per treatment selected at random were sacrificed by decapitation, and the livers were rapidly dissected out and flash-frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$. Total RNA of each tissue sample was extracted by TRIZOL Reagent kit (Invitrogen Inc., CA, USA) according to manufacturer's instruction. The integrity and purity of RNA were tested by measuring the optical density (ratios at 260 and 280 nm being greater than 1.9) and by electrophoresis using ethidium bromide staining. Total RNA was treated by RNase-free DNase I (Roche, Mannheim, Germany) and was stored at $-80\text{ }^{\circ}\text{C}$ until use. Real-time one-step RT-PCR was performed by iCycler instrument (BIO-RAD, Hercules, CA, U.S.A.) using a SYBR Green I RT-PCR quantitative kit (QBIogene, Inc.).

A master mix of 20 μL containing 1 μL diluted RNA, 0.1 μL AMV-RT, 4 μL SYBR Green I PCR Master Mix, 1 μL forward primer (18 pM/ μL), 1 μL reverse primer (18 pM/ μL), and 12.9 μL distilled water was prepared to perform real-time RT-PCR. The forward and reverse primers for L-FABP and β -actin cDNA were derived from a previous study (Richards et al., 2003). Primer sequences are shown in Table 3. The primers span exon/exon boundaries on the mRNA and did not amplify genomic DNA.

Reverse transcription was carried out at $55\text{ }^{\circ}\text{C}$ for 15 min. The reverse transcriptase enzyme was heat inactivated at $95\text{ }^{\circ}\text{C}$ for 4 min. The incubation temperature and duration of each cycle of the PCR were 30 s at $95\text{ }^{\circ}\text{C}$ for denaturation, 10 s at $63\text{--}59\text{ }^{\circ}\text{C}$ for annealing as a touchdown program, and 10 s at $72\text{ }^{\circ}\text{C}$ for

extension. The amplification was carried out for 40 cycles.

Real-time quantification was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA at the end of each amplification cycle. At the end of PCR, dissociation was performed by slowly heating the samples from 55 to 95 °C and continuous recording of the decrease in SYBR Green fluorescence resulting from the dissociation of double-stranded DNA. The threshold cycle (Ct), defined as the cycle at which an increase in fluorescence above a defined baseline can be first detected, was determined for each sample. L-FABP mRNA levels were estimated on the basis of PCR efficiency and Ct deviation of an unknown sample versus a control according to $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The reference gene chosen was β -actin RNA. For the validation of the method, cDNA was synthesized from 10-fold serially diluted RNA samples and amplified by real-time PCR using target gene-specific primers. Each PCR run included a no-template control and replicates of control and unknown samples. Each run had four replicates performed.

The birds fed ad libitum with dietary U/S ratio of 3.5 was chosen as the calibrator treatment. Using $2^{-\Delta\Delta CT}$ method, the data were presented as the fold change in L-FABP gene expression normalized to the β -actin gene (endogenous control) and relative to the calibrator treatment. For the calibrator control sample, $\Delta\Delta CT$ equals zero and 20 equals one; so the fold change in gene expression relative to calibrator control equals one, by definition. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression relative to the calibrator control. Data are presented as means \pm standard deviation (SD). The statistical significance was determined using the Duncan's multiple range method following analysis of variance (ANOVA).

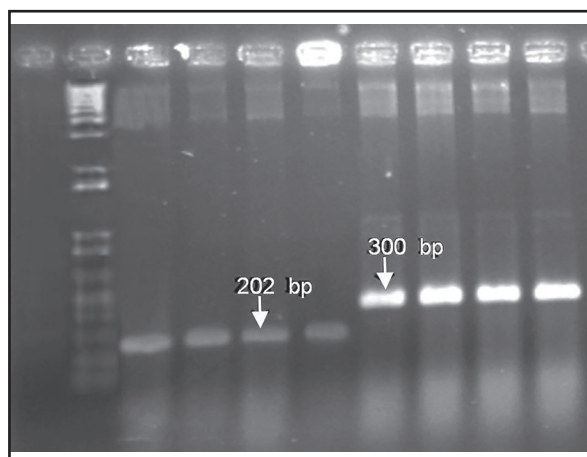


Figure 1. Agarose gel electrophoresis of L-FABP cDNA (202 bp) and β -actin cDNA (300 bp) amplification products.

Results

Melting curve analysis demonstrated that each of the primer pairs described (Table 3) amplified a single predominant product with a distinct melting temperature (T_m). In a pre-test, the real-time amplified RT-PCR product was sequenced (MWGBiotech, Ebersberg, Germany), and 100% homology to the chicken L-FABP sequence could be confirmed. Agarose gel electrophoresis demonstrates that the L-FABP product is a single band of the predicted size, 202 bp (Figure 1).

Table 4 illustrates the effects of different dietary ratios of U/S and skip-a-day feeding on L-FABP mRNA relative expression. At 28 and 42 days of age, no interaction was found between dietary U/S ratio and feed restriction. At 28 days of age, feed restriction significantly increased L-FABP mRNA expression ($p < 0.01$), but after re-feeding (42 days of age), no significant difference was found ($p > 0.05$). Dietary U/S ratio of 6.5 caused an increase in L-FABP mRNA expression at both 28 and 42 days of age ($p < 0.05$), but the changes did not follow a dose dependent manner.

Discussion

In this study, a durable feed restriction caused a temporary and notable increase in L-FABP

Table 1. Fatty acid composition of ingredients used for experimental diets formulation (g/kg DM).

Ingredients	Crude fat (g/kg DM)	fatty acid type							
		16:0	16:1	18:0	18:1	18:2	18:3	Other Fatty Acids	Impurities
Corn	38.3	6.2	-	1	11.7	18.2	0.9	0.3	-
Soybean meal	10.2	2.4	0.1	0.5	1.6	4.7	0.7	0.2	-
Sunflower oil	1000	67	1	43	274	571	37	7	-
Beef tallow	921	250	42	227	370	25	3	4	79

Table 2. Composition and calculated nutrient contents of broiler grower (fed 10 to 28 days) and finisher (fed 29 to 42 days) diets with different unsaturated to saturated fatty acid ratios. (*) Provided per kilogram: 4,000,000 U vitamin A; 800,000 U cholecalciferol; 14,000 U vitamin E; 760 mg vitamin K3; 2,800 mg vitamin B2; 1,520 mg vitamin B6; 7.6 mg vitamin B12; 18,000 mg nicotinic acid; 560 mg folic acid; 4,400 mg pantothenic acid; 19,000 mg choline chloride; 45.3 mg biotin; 16,000 mg zinc; 25,600 mg manganese; 12,800 mg iron; 3,200 mg copper; 64 mg selenium; 320 mg iodine.

Ingredients (%)	US/S fatty acid ratio							
	2		3.5		5		6.5	
	grower	finisher	grower	finisher	grower	Finisher	grower	finisher
Corn	42.83	49.29	46.02	52.42	47.29	53.66	47.97	54.33
Soybean meal	42.38	36.35	41.75	35.72	41.5	35.48	41.38	35.34
Sunflower meal	1.49	1.32	4.36	4.14	5.5	5.26	6.12	5.86
Beef Tallow	9.07	8.85	3.64	3.52	1.48	1.4	0.33	0.27
CaCO ₃	0.99	1	1	1.01	1	1.01	1	1.01
Dicalcium phosphate	1.84	1.88	1.83	1.87	1.83	1.87	1.83	1.87
Common	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Vitamin and Mineral premix *	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL- Methionine	0.34	0.31	0.34	0.3	0.34	0.3	0.33	0.3
HCl-Lysine	0.18	0.13	0.18	0.14	0.19	0.15	0.19	0.15
Chemical composition (%)								
ME (Kcal/kg)	3150	3200	3150	3200	3150	3200	3150	3200
Crud Protein	21	19	21	19	21	19	21	19
Calcium	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Av. Phosphorus	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Sodium	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Lysine	1.2	1.05	1.2	1.05	1.2	1.05	1.2	1.05
Methionine	0.54	0.49	0.54	0.49	0.54	0.49	0.54	0.49
Met + Cys	0.85	0.78	0.85	0.78	0.85	0.78	0.85	0.78

gene expression. In the study of Murai et al., (2009), feed restriction and re-feeding did not result in unidirectional alterations in L-FABP gene expression; a 4-h feed restriction to some extent reduced the L-FABP mRNA level, while feed restriction for 20 h caused a 2.2-fold increase in L-FABP mRNA level compared with

that of the fed group. Moreover, re-feeding after feed restriction had an irrelevant effect on the L-FABP mRNA expression; therefore, Murai et al., (2009) suggested that feeding motivation may not be a major factor regulating L-FABP gene expression.

The effect of feed restriction on L-FABP

Table 3. Primer sequences and PCR product sizes of L-FABP and β -actin genes.

Gene	Primer sequences (5'-3') Forward and reverses	GenBank accession number	Product base pair
L-FABP	GAGCTCCAGTCCCATGAAAA TCAGCAGCTCCATCTCACAC	AF380999	202
β -Actin	TGCGTGACATCAAGGAGAAG TGCCAGGGTACATTGTGGTA	L08165	300

Table 4. Effects of feed restriction and dietary fat saturation degree on L-FABP relative mRNA expression¹. (**) $p < 0.01$; (*) $p < 0.05$. L-FABP: liver fatty acid-binding protein; U/S: dietary unsaturated to saturated fatty acid ratio; FR: feed restriction as skip-a-day feeding at 10–28 Days of age. a–d Values in the same column in each comparison group, with no common superscript differ significantly; $p < 0.05$. ¹Values are means of four determinations of L-FABP relative gene expression (4 birds per age/each feeding program/each U/S ratio).

Experiment variables		28 days of age	42 days of age
Dietary U/S	Feed Restriction		
2	-	1 ± 0.2 ^d	1 ± 0.28 ^c
2	+	3.89 ± 0.08 ^{ab}	1.42 ± 0.23 ^b
3.5	-	1.75 ± 0.15 ^d	1.62 ± 0.4 ^b
3.5	+	3.78 ± 0.24 ^{ab}	2.32 ± 0.34 ^a
5	-	3.07 ± 0.18 ^{bc}	1.85 ± 0.23 ^{ab}
5	+	3.61 ± 0.32 ^{ab}	1.54 ± 0.37 ^b
6.5	-	3.61 ± 0.32 ^{ab}	1.58 ± 0.18 ^b
6.5	+	4.65 ± 0.2 ^a	2.48 ± 0.1 ^a
Probability > F			
Analysis of Variance	U/S	FR	U/S × FR
28 days of age	*	**	NS
42 days of age	*	*	NS

gene expression enhancement agrees with the findings of Richards et al., (2003) in broiler breeder hens. In their report, feed restriction resulted in a decreasing expression of genes related to lipogenesis but an increase in expression of genes like L-FABP involved in lipids transportation.

It seems that during lipolysis following feed restriction, body has to transfer more amounts of fat-hydrolyzed products, and this can lead to an increase in FABPs production. This agrees with the results of Wesinger (1996) that there was a positive correlation between FABPs gene expression and cellular fatty acid metabolism. It is assumed that the stimulation of L-FABP gene expression is activated by the raise in non-esterified fatty acid (NEFA) influx into the liver (Murai et al., 2009).

Duplus et al., (2000) found no significant difference in L-FABP gene expression by the

degree of saturation in dietary fat, whereas in our research, dietary U/S ratio of 6.5 interestingly raised L-FABP gene expression. Our finding agrees with the report of Yuan et al., (2012) who showed that compared with unsaturated soybean oil, the saturated poultry fat significantly decreased L-FABP mRNA expression.

In this study, no significant interaction between type of feeding and unsaturation degree of dietary fat indicates that these factors independently affect the L-FABP gene expression.

Higher L-FABP gene expression in the liver of the broilers following feed deprivation and high unsaturated fatty acids ratio might result in increased fatty acids transportation into the mitochondria or peroxisomes and enhance fatty acids oxidation and reduce fat depot. Therefore, the regulation of L-FABP gene expression might have an effect on the deposition of

fat in the chicken.

In conclusion, this study provides evidences to the physiological functions of L-FABP in the avian liver; however, there are many reports on the effects of other factors triggering L-FABP. For example, growth hormone increases L-FABP gene expression in the liver of rats (Berry et al., 1993; Carlsson et al., 1998). Our data propose that birds have a mechanism for the regulation of fatty acid transfer under different nutritional condition. However, further studies will be needed to explain the exact mechanism for the increase in L-FABP gene expression.

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اثر محدودیت غذایی و نوع چربی جیره بر بیان mRNA پروتئین متصل شونده به اسید چرب در کبد جوجه‌های گوشتی

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چکیده

زمینه مطالعه: پروتئین متصل شونده به اسید چرب مخصوص کبد (L-FABP) جایگاه اصلی اتصال سیتوزولی اسیدهای چرب زنجیر بلند در سلول‌های کبدی است. FABPs جذب اسیدهای چرب به داخل سلول را افزایش می‌دهند و این عمل را از طریق افزایش شیب غلظتی آنها به دلیل به حداقل رساندن میزان اسید چرب غیر متصل در سلول انجام می‌دهند. هدف: هدف از این تحقیق بررسی اثرات نسبت اسیدهای چرب غیر اشباع به اشباع جیره و محدودیت غذایی بر بیان mRNA پروتئین متصل شونده به اسید چرب مخصوص کبد بود. روش کار: تعداد ۷۲۰ قطعه جوجه گوشتی نر ۱۰ روزه از سویه رأس ۳۰۸ با جیره‌های بانسب‌های اسیدهای چرب غیر اشباع به اشباع (U/S) ۲، ۳/۵، ۵ یا ۶/۵ به صورت آزاد یا یک‌روز در میان تغذیه گردیدند (طی سن ۲۸-۱۸ روزگی). بیان نسبی mRNA مربوط به L-FABP در سلول‌های کبدی جوجه‌ها با استفاده از نسخه برداری معکوس طی روش real time PCR تعیین گردید. نتایج: نتایج به روشنی نشان دادند که محدودیت غذایی باعث القاء بیان ژن در کبد جوجه‌های گوشتی گردید. بیان ژن L-FABP توسط نسبت ۶/۵ اسیدهای چرب غیر اشباع به اشباع جیره افزایش یافت. اثر متقابلی بین U/S جیره و محدودیت غذایی بر بیان ژن L-FABP مشاهده نگردید. نتیجه‌گیری نهایی: این مشاهدات پیشنهاد می‌نمایند که پرندگان دارای سازوکاری برای تنظیم انتقال اسیدهای چرب تحت شرایط مختلف تغذیه‌ای هستند.

واژه‌های کلیدی: جوجه‌های گوشتی، نسبت اسیدهای چرب غیر اشباع و اشباع جیره، محدودیت غذایی، پروتئین متصل شونده به اسید چرب، ریل تایم پی سی آر

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