Analysis of Liver Transcriptome in Broilers with Ascites and Regulation by L-Carnitine

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Two experiments were conducted using male Ross-308 broilers. Exp. 1 was to determine the differences in liver transcriptomic profile of healthy broilers at 39 days (D39) vs. 21 days of age (D21), and of ascites vs. healthy broilers at D39 by GeneChips, under a low-temperature environment. Differentially expressed genes related to liver antioxidative capacity and energy metabolism were screened. Exp. 2 was to analyze the regulatory roles of L-carnitine on genes and biochemical parameters involved in liver oxidoreduction and energy metabolism. The results showed that the expression of 790 genes in D39 vs D21 healthy broilers (386 up- and 404 down-regulated) and expression of 178 genes in ascites vs. healthy broilers on D39 (128 up- and 50 down-regulated) was changed. The analysis of pathway and GO indicated that genes related to amino acid metabolism, fatty acid metabolism, Krebs cycle, carbohydrate metabolism, vitamin metabolism, cell apoptosis, immune response, oxidoreduction, angiogenesis, nitric oxide formation and erythrocyte differentiation were involved in the development of ascites. Liver MDA level of ascites broilers was significantly increased, but the activity of T-SOD was significantly decreased than healthy broilers. The activity of liver HK, MDH, PK and Na+−K+−ATPase was significantly lower in ascites broilers. The expression of liver HKDC1 and PCK1 was significantly down-regulated, and that of HNF-4α and AKR1B10 expression was significantly up-regulated. L-carnitine supplementation significantly increased the activity of T-SOD and PK, and significantly up-regulated HKDC1 and HNF-4α expression. It can be concluded that genes involved in many biological processes are differentially expressed due to ascites. Liver oxidation damage and energy generation obstruction were found in broilers with ascites. L-carnitine can alleviate the liver oxidative damage and promote the generation and utilization of energy. The present results demonstrated that L-carnitine could serve as a potential regulatory agent to reduce ascites susceptibility and mortality.

Key words: broiler ascites, L-carnitine, nutritional regulation, transcriptome

Introduction

Ascites syndrome (AS) is a metabolic disorder, characterized by hypoxaemia, increased work-load of the cardiopulmonary system, central venous congestion, hypertrophy of the right ventricle and a flaccid heart, an excessive accumulation of fluid in body coelomic cavities, and finally death (Riddell, 1991; Julian, 1993; Olkowski et al., 1999; Luger et al., 2003).

It has been shown that histologic lesions (Mohammadpour, 2007), significant reduction of hemoglobin content respective to red blood cell count (Shlosberg et al., 1998; Luger et al., 2003), decreased production of vessel dilating active substances (Hamal et al., 2009), abnormal metabolism of corticosteroids and triiodothyronine (Luger et al., 2003) and down-regulation of lung inflammatory chemokine genes (Hamal et al., 2010) are involved in the occurrence of ascites. However, despite intensive investigations of ascites syndrome, its pathogenesis remains unclear (Crespo and Shivaprasad, 2003). Nutrigenomics can be used to explain the pathogenesis of metabolic disorders resulting from the interaction between genes and nutrition, and can provide theoretical basis for the effective prevention and control of ascites. However, to date, research on gene expression of ascites has focused mainly on individual or several genes and lack the information of gene transcriptomic profile.

Reports on the relationship between nutrition and ascites indicate that dietary supplementation with nutrients such as vitamin E (Bottje et al., 1997; Villar-Patino et al., 2002), L-arginine (Lorenzoni and Ruiz-Feria, 2006; Tan et al., 2007) or L-carnitine (Buyse et al., 2001; Geng et al., 2004) can
reduce the incidence of ascites. The major metabolic role of L-carnitine is to transport long-chain fatty acids into mitochondria for \( \beta \)-oxidation and energy production. It is generally accepted that endogenous L-carnitine synthesis together with its dietary intake should be sufficient for normal transport function. However, in cases of increased metabolic rate (in fast growing broilers and cold temperature) when energy demands are elevated, the availability of L-carnitine may become a limiting factor for oxidative metabolism (Baghbanzadeh and Decuypere, 2008). Olkowski et al. (2007) reported that the L-carnitine level in the hearts of broilers with heart failure was lower than in normal broilers. Under these conditions, additional exogenous L-carnitine might prove beneficial. Previous studies in our laboratory have shown that diets supplemented with L-carnitine can improve liver antioxidative capacity and reduce the mortality due to ascites (Geng et al., 2004; Geng et al., 2007), but molecular mechanisms underlying these effects are yet to be investigated.

Cardiovascular and respiratory systems seem to interact to produce a cascade of events that culminate in ascites and multiple organs (heart, lungs and liver, etc) are involved in the development of ascites (Currie, 1999). However, liver plays a central role in whole body metabolism by regulating glucose and lipid homeostasis as well as protein synthesis, so any obstruction of liver energy metabolism may aggravate the occurrence of ascites. Coleman and Coleman (1991) reported that the peak incidence of ascites occurs in the fifth or sixth week of the growing period, the aetiology of the disease may be initiated much earlier. But the experiments done by our lab found the expression profiles of liver or heart in broilers before wk 2 were not significantly changed (unpublished data). So comprehensively integrating the hepatic expression profiles of broiler growing period and of healthy and ascites, finally obtaining the differentially expression genes related certain biological process are of importance. Therefore, the objectives of this study were two-fold. The first objective was determine the differences in the liver transcriptomic profile of healthy broilers at 39 days (D39) vs. 21 days of age (D21) and of ascites vs. healthy broilers at D39, by Affymetrix Chicken GeneChips. The second objective was to examine the regulatory roles of L-carnitine on gene expression and biochemical parameters associated with energy metabolism and antioxidative capacity of the liver.

Materials and Methods

Experimental Design

Two experiments were conducted using male broilers (Ross 308). In Exp.1, 196 day-old broilers were randomly assigned to fifteen replicate cages (2.4×0.6×0.6 m) of 14 birds each. The temperature in the house was 35°C during the first week and was lowered by 1°C every other day till 30°C was reached on day 10. From day 11 to the end of the experiment (42 days), all birds were exposed to a temperature cycle of 17°C during the day and 14°C at night in order to increase ascites susceptibility.

In Exp.2, a total of 392 day-old broilers were randomly assigned to two dietary treatments with fifteen replicates of fourteen birds each. The two treatment diets were supplemented with L-carnitine at levels of 0 or 100 mg/kg. The room temperature regime was similar to that of Exp. 1.

Animal Diets and Management

Diets (Table 1) were formulated to meet or exceed recommended requirements for all nutrients (NRC, 1994) and were pelleted. Treatment diets used in Exp.2 consisted of L-carnitine supplementation at levels of 0 or 100 mg/kg. The birds had free access to feed and water, with 23 h fluorescent illumination per day throughout the experimental period. Birds were vaccinated with newcastle disease-infectious bronchitis (ND-IB) vaccine at 7 and 21 days age, and infectious bursal disease (IBD) vaccine at 14 and 28 days age. The study protocol was approved and conducted in accordance with the Animal Ethics Committee guidelines of China Agricultural University.

Sampling and Measurement

Exp.1: On day 21, ten birds were randomly selected and killed by jugular bleeding after 8 h of feed deprivation. Liver tissue (200 g) was collected, put in RNase-free tubes, snap frozen in liquid N\(_2\) and stored at \(-80^\circ\text{C}\). Three samples from the ten were chosen for the analysis of liver transcriptomic profile.

On day 39 (the age at which a peak in the syndrome was
demonstrated previously; Peacock et al., 1988), 50 birds were selected and weighed after 8 h of feed deprivation. Whole blood samples were collected by venipuncture into EDTA-K3 anticoagulation tubes for the measurement of hematocrit (HCT) (Sysmex KX-21N Automatic blood analyzer, Kobe, Japan). The birds were then killed by jugular bleeding and, heart was removed and dissected. Weights of right ventricle (RV) and total ventricle (TV) were recorded to calculate the ascites heart index (AHI). Ascites heart index was calculated as (RV/TV) (Julian et al., 1989). Based on these results, liver tissue of four ascites and four healthy broilers was selected for the analysis of liver transcriptomic profile. The selection was based on the following three aspects: (1) HCT < 0.36, healthy; HCT ≥ 0.36, ascites; (2) AHI < 0.28, healthy; AHI ≥ 0.28, ascites; (3) Having effusion in abdominal cavity and pericardium, ascites, no effusion, healthy.

Exp. 2: on days 21 and 42, average daily gain (ADG), average feed intake (AFI), average body weight (ABW) and feed conversion ratio (feed intake: BW gain, FCR) were determined on pen basis, and the mortality due to ascites was calculated. On day 39, liver tissues of eight healthy and eight ascites broilers were selected for RT-PCR analysis according to the selection criteria mentioned above. Another set of liver tissues was collected for the determination of malondialdehyde (MDA, cat#: A001-1), Total superoxide dismutase (T-SOD, cat#: A001-1), glutathione peroxidase (GSH-Px, cat#: A005), hexokinase (HK, cat#: A077), succinyldehydrogenase (SDH, cat#: A022), malic dehydrogenase (MDH, cat#: A021-2), pyruvate kinase (PK, cat#: A076) and Na+ -K+ -ATPase (cat#: A016-2) activity. Malondialdehyde content was determined by the method of thiobarbituric acid (TBA). The enzyme activities were measured using commercially available colorimetric diagnostic kits (Nanjing jiancheng Bioengineering Institute, Nanjing, China; Tan et al., 2010). The protein content of liver tissue was measured by Coomassie Brilliant Blue G-250 reagent, with bovine serum albumin as the standard (cat#: A045-2).

Microarray Analysis
In exp. 1, total six microarray chips were used (three D39 and three D21 healthy broilers), and in exp. 2, total eight microarray chips were used (four ascites and four healthy broilers).

RNA Preparation and cDNA Assay
Total hepatic RNA was extracted and purified using the RNeasy Mini Kit (Invitrogen Life Technologies, P/N 15596-018), and its quality was assessed using UV spectrophotometer (ND-1000, NanoDrop) and 1.5% formaldehyde degeneration agarose gel electrophoresis (CBC/UVP I-D001, Capital-Bio). mRNA was isolated using RNeasy MinElute Cleanup Kit (Qiagen, 74204) as per manufacturer’s recommendations, cDNA was prepared and purified by oligo-dT-primed reverse transcription using Poly-A RNA Control kit (Affymetrix, P/N 900433).

Synthesis of cRNA Labeled with Biotin and Fragmentation
Biotinylated dNTPs were incorporated into the generated cRNA during transcription using MessageAmp™ II-Biotin RNA Amplification Kit (Ambion, 17910). The RNA products were fragmented into strands of 200 bp or less using 5 X fragmented buffer.

Hybridization, Washing and Staining
After prehybridization in 300 μl 1× hybridization buffer at 45°C for 10 min, the microarrays were incubated for 16 h at 45°C with constant rotation (60 rpm) in hybridization oven 640 (Affymetrix), using Hybridization Control Kit (Affymetrix, P/N 900454). Following hybridization, the microarrays were washed on a fluids station (Fluidics Station 450, Affymetrix). Microarrays were stained with stain cocktail 1, followed by 10×4 wash cycles to finish the staining.

Microarray Scanning and Data Analysis
The microarrays were scanned using GeneChip® Scanner 3000 (Affymetrix). Raw data sets were normalized to total fluorescence, which represented the total amount of cRNA hybridized to a microarray, using Affymetrix® GeneChip® Operating Software Version1.4. Data sets were excluded if the absolute call (Abs call) was A (absent) or M (marginal) according to the detection p-value in all microarrays. Only the expression transcripts (the Abs Call was P, present) were used in further analysis.

Differentially expressed genes were identified from normalized data using the significance analysis of microarrays (SAM) algorithm. According to the SAM algorithm, genes were identified as differentially expressed on the basis of expression differences among the samples and the consistency of these differences. A gene is deemed ‘significant’ if fold change value (FC) surpasses a certain threshold. If FC ≥ 2.0, represents genes were up-regulated, and FC ≤ 0.5 represents genes were down-regulated. Clustering was achieved using average linkage clustering. Clustering image was displayed in TreeView. Gene functions and specific biological pathways were analyzed by molecular comments platform (MAS 3.0).

qRT-PCR
Differential expression of selected genes identified by microarray analyses was validated by qRT-PCR using the same RNA samples for microarray analysis. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. qRT-PCR was performed using Power SYBR Green® PCR Mix Master (ABI) in 7900 HT Fast RT-PCR system (ABI), result was expressed as 2ΔΔCt = (CtGAPDH-Ct_i) - (CtGAPDH-Ct_i), where CtgADPH and Ct_i are the Ct for the GAPDH and gene i in a sample. Fold change was expressed as the abundance ratio of ascites and healthy broilers. Finally, correlation coefficients between microarray and qRT-PCR were analyzed by Pearson correlation.

Statistical Analysis
The statistical analysis was performed with SPSS 16.0 software for Windows. Data (ABG, AFI, FCR and mortality) were analyzed by independent sample T-test was. The pen average was used as the experimental unit. Mortality was calculated on pen basis and analyzed after arcsin transformation. Data (MDA level, the activity of T-SOD, GSH-Px, HK, SDH, MDH, PK and Na+ -K+ -ATPase, the
expression of HKDC1, PCK1, HNF-4α and AKR1B10) from Exp. 2 were analyzed by two-way ANOVA for the following main effects: L-carnitine (0 or 100 mg/kg) and ascites (healthy or ascites), followed by Fisher’s protected least significant difference test considering the interaction between factors. Differences were considered statistically significant at \( P \leq 0.05 \).

**Results**

**Effects of L-carnitine on Growth Performance and Mortality in Broilers**

The effects of L-carnitine on ABW, FCR and mortality due to ascites are shown in Table 2. ADG of broilers on day 1–21 and day 21–42, and AFI on day 1–21 were not influenced by L-carnitine supplementation, but AFI on day 21–42 was significantly reduced by dietary L-carnitine supplementation. Both ABW and FCR were not influenced by L-carnitine supplementation in broilers during 0–3 wk and 3–6 wk, but mortality due to ascites (especially during wk 4 to wk 6) was significantly reduced.

**Screening of Differentially Expressed Genes of D39 vs. D21 Healthy Broilers, and Ascites vs. Healthy Broilers on D39**

In order to identify the differentially expressed genes, a two-class SAM analysis was performed on the log transformed data matrix. This led to the identification of a total 790 differentially expressed genes: 386 up-regulated and 404 down-regulated in D39 vs. D21 healthy broilers (Fig. 1), of the total 178 differentially expressed genes in ascites vs. healthy broilers on D39 (Fig. 2), 128 up-regulated and 50 down-regulated.

**The pathway Analysis of Differentially Expressed Genes**

Gene functions and biological pathways were analyzed using KEGG database by MAS 3.0. The results showed that differentially expressed genes of D39 vs. D21 healthy broilers were mainly involved in amino acid metabolism, fatty acid metabolism, Krebs cycle, gluconeogenesis, vitamin metabolism, \( \text{Ca}^{2+} \) and mTOR signal transduction, cell apoptosis (Fig. 3). Differentially expressed genes of ascites vs. healthy broilers on D39 were mainly involved in immune response, oxidoreduction, lipid metabolism, amino acid metabolism, gluconeogenesis, insulin regulation, angiogenesis, and nitric oxide formation (Fig. 4).

**GO Term Analysis of Differentially Expressed Genes**

Biological processes of differentially expressed genes were analyzed by MAS 3.0. The results of GO term showed that differentially expressed genes of D39 vs. D21 ascites broilers were mainly involved in immune response, oxidoreduction, lipid metabolism, carbohydrate metabolism, fatty acid \( \beta \)-oxidation, Krebs cycle, signal transduction, cell apoptosis, oxidative stress response and erythrocyte differentiation (Fig. 5). Differentially expressed genes ofascites vs. healthy broilers on D39 were mainly involved in immune response, oxidoreduction, lipid metabolism, amino acid metabolism, gluconeogenesis, insulin regulation, angiogenesis, and nitric oxide formation (Fig. 6).

**Differentially Expressed Genes Related to Liver Oxidoreduction and Energy Metabolism**

Integrated results of pathway and GO term were showed in Table 3, it was indicated that genes associated with cell oxidoreduction were AKR1B10, CP, CYP, DECR1, DLD, GPX8, IDH3A, LAC, LDHA, NDUFS2, NNT, PCYOX1, Catalase, RETSAT, and SOD3. As shown in Table 4, genes associated with cell energy metabolism were COX7C, IDH3A, NDUFS3, OGDHL, PCK1, SDHD, UQCR1, HKDC1, and HNF-4α.

**Validation of Microarray Result**

The expression of HKDC1, PCK1, NDUFS3, HNF-4α, and AKR1B10 were analyzed by qRT-PCR using the same RNA samples for microarray analysis. The primer pair information was shown in Table 4. The results of the microarray and qRT-PCR were compared (Table 6). Pearson correlation between microarray and qRT-PCR indicated: \( R = 0.931, P = 0.022 \). qRT-PCR fold change values were corresponded closely with the microarray results.

**Effects of L-carnitine on Gene Expression Related to Energy Metabolism and Oxidoreduction**

HKDC1, PCK1, HNF-4α and, AKR1B10 expressions in liver tissues from ascites and healthy broilers on D39 were analyzed by qRT-PCR (Table 7). The expressions of liver HKDC1 and PCK1 of ascites were significantly down-regulated, and those of HNF-4α and AKR1B10 were significantly up-regulated. Dietary supplementation of L-carnitine significantly up-regulated the expression of HKDC1 and HNF-4α. Numerical, but not significant, changes in the ex-
pression of PCK1 and AKR1B10 were observed with L-carnitine supplementation.

**Effects of L-carnitine on Biochemical Parameters Related to Energy Metabolism and Oxidoreduction**

As shown in table 8, liver MDA levels in ascites broilers were significantly increased, and T-SOD activity was significantly decreased with L-carnitine supplementation. The activity of liver HK, MDH, PK and Na\(^+\)-K\(^+\)-ATPase in ascites broilers was significantly decreased. Dietary supplementation with L-carnitine significantly increased the activity of T-SOD and PK, and tended to increase the activity of SDH and Na\(^+\)-K\(^+\)-ATPase.

**Discussion**

It is estimated that 5% of broilers and 20% of roaster birds die of ascites (Balog, 2003). Given that an estimated 40 billion broilers are produced annually around the world, it is evident that the economic losses due to ascites are significant. Genetic, physiological, environmental, and management factors all seem to interact to produce a cascade of events that culminate in the ascites syndrome. Despite intensive investigations of the syndrome for many years, the exact cause of ascites is yet to be understood. The transcriptomic results from the current study indicated that genes involved in oxidoreduction, lipid metabolism, carbohydrate metabolism, immune response, signal transduction, cell apoptosis and erythrocyte differentiation were differentially expressed in the development of ascites (Fig. 3-6).

Khoshkhoo et al. (2006) found L-carnitine could significantly improve body weight gain only in 35-49 days old, but no effect in earlier ages. Hossininezhad et al. (2011) reported that feed conversion ratio was significantly decreased in total period by dietary L-carnitine supplementation. In contrast, Kidd et al. (2009) failed to observe any effects of L-carnitine supplementation on broiler performance. Zhang et al. (2010) reported that addition of ALC resulted in lower (linear effect, \(p<0.05\)) ADG and AFI. The current study observed the effect of L-carnitine supplementation on broiler performance was not significant (Table 2). Owen et al. (1996) reported differences in dosage level of L-carnitine, and physiological status of the animals may be responsible for the discrepancies between published studies. In addition, it is generally assumed that exogenous L-carnitine supplementation is only beneficial in the case of metabolic burdens such as cold or exercise. Geng et al. (2004) reported that dietary L-carnitine supplementation can increase broiler ascites-resistance and significantly reduce the mortality due to ascites. This study also found that the mortality due to ascites was significantly reduced by L-carnitine supplementation under a low temperature environment.

It is reported that the elevated production of reactive oxygen species (ROS) in broilers prone to ascites may po-
tentiate the development of ascites or aggravate ascites as it occurs (Enkvetchakul et al., 1993; Bottje and Wideman, 1995; Arab et al., 2006). The concentration of free radicals and MDA were increased in the serum and other tissues, but the activity of SOD and GSH-Px was significantly reduced (Geng et al., 2004). Pulmonary hypertension induced a large number of free radicals and MDA production in many tissues, causing lipid peroxidation in mitochondrial membrane, leading to over-consumption of antioxidant enzymes and inadequate synthesis. The current study indicated that gene expression of parameters relating to oxidation-reduction, such as AKR1B10, CYP2, CP, GPX8, Catalase, and SOD3, was significantly changed (Table 3), which further suggest that lipid peroxidation damage was significantly aggravated in the liver tissue of ascites broilers. AKR1B10, also called Aldose Reductase-Like 1 (ARL-1), is isolated from human hepatocarcinoma (Cao et al., 1998). AKR1B10 is a redox enzyme depending on NADPH. The prime biological function of AKR1B10 is involved in cell proliferation and the sensitivity to acrylaldehyde, butenoic aldehyde and malonaldehyde, protecting cells from toxicity damage caused by aldehyde (Martin and Maser, 2009). Zu et al. (2009) reported that AKR1B10 was mainly expressed in the small intestine and colon, and low in liver, thymus, prostate and testis. However, AKR1B10 expression was significantly up-regulated in hepatocellular and lung carcinoma, which suggest that AKR1B10 can be used as a potential medical marker for cancer remedy (Fukumoto et al., 2005; Pemiing, 2005). The present results indicate that AKR1B10 expression in ascites broilers was up-regulated to resist cell oxidative damage. Using the scavenging agents of free radical such as vitamin E and L-carnitine can enhance the defense function of antioxidative system, which can reduce the occurrence of ascites syndrome (Villar-Patino et al., 2002; Geng et al., 2004). L-carnitine can significantly increase the activity of T-SOD, and tended to down-regulate the expression of AKR1B10, indicating that cell damage suffered from toxic aldehydes was markedly decreased. Therefore, the protective role of AKR1B10 from endogenous carbonyl damage and the regulation role of L-carnitine may provide some theoretical mechanism for the etiology of metabolic diseases.

Liver has complex biological functions. Many important metabolic activities, such as carbohydrate metabolism, amino acid metabolism, blood glucose regulation, albumin generation, detoxification process, take place in the liver. These
activities can be suppressed when liver energy metabolism is blocked. The results of the current study indicated that expression of genes relating to energy metabolism, such as HKDC1, COX7C, DLD, NDUFS3, PCK1, SDHD, HNF-4α, and UQCRC1 was significantly changed (Table 4), which affects the energy generation.

HK gene mainly codes hexokinase (HK) to catalyze glucose into glucose-6-phosphate. This process can activate glucose to participate in the synthesis and catabolism. PCK1 mainly codes Phosphoenolpyruvate Carboxykinase (PEPCK), which plays an important role in gluconeogenesis. Diaz-Cruz et al. (1996) observed greater concentrations of glucose for ATP generation to meet the tissue demand of the liver of broilers affected with altitude-induced ascites and attributed it to gluconeogenesis. Daneshyar et al. (2009) reported that the concentration of blood sugar in fasted chickens at wks 4 and 6 was increased under a low ambient temperature, which suggested that gluconeogenesis level was increased in ascites chickens. HNF-4α is a nucleus hormone receptor transcription factor. Recent studies showed that HNF-4α participates in the regulation of expression of specific genes associated with carbohydrate and lipid metabolisms. HNF-4α is of importance in the regulation of energy metabolism by regulating PCK expression in hepatocytes. Target genes of PGC-1α/HNF-4α include two rate-limiting enzyme genes in gluconeogenesis, namely PEPCK and glucose-6-phosphatase. Yoon et al. (2001) reported that the stimulation of PGC-1α to liver gluconeogenesis depends on its direct contact with HNF-4α, which can promote the transcription initiation of PCK and directly regulating the efficiency of gluconeogenesis.

Keller et al. (2011) investigated the effect of L-carnitine on the hepatic transcript profile in piglets, the results indicated that representative genes involved in cellular fatty acid uptake, fatty acid activation, fatty acid β-oxidation, glucose uptake, and glycolysis were significantly up-regulated by carnitine. In contrast, genes involved in gluconeogenesis were down-regulated by carnitine. The studies
have already shown that carnitine supplementation increases glucose disposal and glucose oxidation in animals as well as in healthy and diabetic patients due to activation of the pyruvate dehydrogenase complex (Calvani et al., 2000; Van Weyenberg et al., 2009). GCK (Hexokinase D) was up-regulated by 27-fold in the liver of piglets by L-carnitine supplementation, which indicates that L-carnitine has a dramatic effect on glucose metabolism. In contrast, genes involved in gluconeogenesis like PCK1 and FBP2 were significantly down-regulated (Keller et al., 2011). This indicates that the positive effect of L-carnitine on glucose utilization is explained not only by stimulation of glycolysis but also suppression of gluconeogenesis in the liver. The current results indicate that the expression of HKDC1 in ascites broilers was down-regulated (Table 7), thus reducing the activation and utilization of glucose. The expression of HNF-4α in ascites broilers was up-regulated, which was beneficial to promote the initiation of transcription of rate-limiting enzyme genes in gluconeogenesis. But the expression of PCK1 in ascites broilers was down-regulated, which is in contradiction with the increased blood sugar levels in ascites broilers. But dietary supplementation of L-carnitine can significantly up-regulate the expression of HKDC1 and HNF-4α, and numerically up-regulate the expression of PCK1. These findings suggest that L-carnitine can contribute to the activation and utilization of glucose and liver gluconeogenesis, which will be beneficial to the glucose supply and blood sugar stability. This shows that supplemental L-carnitine influences gene expression in the liver of broilers and indicates that at least some of the biological effects of L-carnitine are mediated by altering gene transcription.

HK, SDH, MDH and PK are the important enzymes in cell glycolysis and Krebs cycle. The activity of these enzymes is of importance to glucose utilization and ATP generation. Na⁺-K⁺-ATPase activity is an important indicator to measure mitochondria function for energy metabolism, and it has a close relationship with the decomposition of ATP. The current study indicated that the activity of liver HK, MDH, PK and Na⁺-K⁺-ATPase was significantly decreased (Table 8), which is consistent with the expression of genes associated with energy metabolism. It is speculated that the generation and utilization of ATP was blocked in broilers with ascites, which cannot meet the requirement for energy under a cold-temperature environment. In the current study, dietary supplementation with L-carnitine significantly...
Table 3. Differentially expressed genes associated with liver oxidation-reduction

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Gene</th>
<th>FC (D39/D21)</th>
<th>FC (Ascites/Healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gga.4869.1.S1_at</td>
<td>Aldo-keto reductase family 1, member B10</td>
<td>AKR1B10</td>
<td>2.17</td>
<td>2.03</td>
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<td>Gga.10928.1.S1_at</td>
<td>Cholesterol 25-hydroxylase</td>
<td>CH25H</td>
<td>2.60</td>
<td>1.23</td>
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<tr>
<td>Gga.12074.1.S1_at</td>
<td>Ceruloplasmin (ferroxidase)</td>
<td>CP</td>
<td>2.60</td>
<td>2.87</td>
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<tr>
<td>Gga.3621.2.S1_s_at</td>
<td>Crystallin, zeta (quinone reductase)</td>
<td>CRYZ</td>
<td>0.39</td>
<td>0.61</td>
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<tr>
<td>GgaAffx.21843.2.S1_s_at</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 18</td>
<td>CYP2C18</td>
<td>0.14</td>
<td>0.84</td>
</tr>
<tr>
<td>Gga.9634.1.S1_at</td>
<td>2,4-dienoyl CoA reductase 1, mitochondrial</td>
<td>DECR1</td>
<td>0.45</td>
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<tr>
<td>Gga.4909.2.S1_s_at</td>
<td>Dihydrolipoamide dehydrogenase</td>
<td>DLD</td>
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<td>0.95</td>
</tr>
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<td>GgaAffx.12041.1.S1_s_at</td>
<td>Follicular lymphoma variant translation 1</td>
<td>FVT1</td>
<td>0.48</td>
<td>0.53</td>
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<tr>
<td>Gga.11913.1.S1_at</td>
<td>Glutathione peroxidase 8 (putative)</td>
<td>GPX8</td>
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<td>Gga.1096.1.S1_at</td>
<td>Hydroxysteroid (17-beta) dehydrogenase 10</td>
<td>HSD17B10</td>
<td>0.47</td>
<td>0.65</td>
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<tr>
<td>Gga.13058.1.S1_s_at</td>
<td>Isozyme dehydrogenase 3 (NAD+) alpha</td>
<td>IDH3A</td>
<td>2.24</td>
<td>0.99</td>
</tr>
<tr>
<td>GgaAffx.22018.1.S1_at</td>
<td>L-amino-acid oxidase precursor</td>
<td>LAO</td>
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<td>3.54</td>
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<td>Gga.4398.1.S1_at</td>
<td>Lactate dehydrogenase A</td>
<td>LDHA</td>
<td>2.15</td>
<td>1.20</td>
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<tr>
<td>Gga.4778.1.S1_at</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa</td>
<td>NDUFV2</td>
<td>0.49</td>
<td>0.88</td>
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<td>GgaAffx.9445.1.S1_s_at</td>
<td>Nicotinamide nucleotide transhydrogenase</td>
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<td>GgaAffx.2637.2.S1_s_at</td>
<td>Catalase</td>
<td>RCMB04.1 ij22</td>
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<tr>
<td>Gga.3030.1.S1_at</td>
<td>Retinol saturase (all-trans-retinol 13,14-reductase)</td>
<td>RETSAT</td>
<td>0.47</td>
<td>0.81</td>
</tr>
<tr>
<td>Gga.1128.2.S1_a_at</td>
<td>Superoxide dismutase 3, extracellular</td>
<td>SOD3</td>
<td>6.71</td>
<td>2.89</td>
</tr>
</tbody>
</table>

Fold change (FC) ≥ 2.0 represents up-regulated, and FC ≤ 0.50 represents down-regulated.

Table 4. Differentially expressed genes associated with liver energy metabolism

<table>
<thead>
<tr>
<th>Input Symbol</th>
<th>Gene Title</th>
<th>Gene</th>
<th>FC (D39/D21)</th>
<th>FC (Ascites/Healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gga.9720.1.S1_at</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1</td>
<td>ATP5A1</td>
<td>0.47</td>
<td>0.93</td>
</tr>
<tr>
<td>Gga.11459.1.S1_at</td>
<td>Hexokinase domain containing 1</td>
<td>HKDC1</td>
<td>—</td>
<td>0.28</td>
</tr>
<tr>
<td>Gga.6171.2.S1_at</td>
<td>Cytochrome c oxidase subunit VIIc</td>
<td>COX7C</td>
<td>0.44</td>
<td>0.81</td>
</tr>
<tr>
<td>Gga.4909.2.S1_s_at</td>
<td>Dihydrolipoamide dehydrogenase</td>
<td>DLD</td>
<td>0.49</td>
<td>0.95</td>
</tr>
<tr>
<td>Gga.19277.1.S1_at</td>
<td>Hepatocyte nuclear factor 4, alpha</td>
<td>HNF-4α</td>
<td>1.43</td>
<td>2.26</td>
</tr>
<tr>
<td>GgaAffx.13058.1.S1_s_at</td>
<td>Isocitrate dehydrogenase 3 (NAD+) alpha</td>
<td>IDH3A</td>
<td>2.23</td>
<td>0.99</td>
</tr>
<tr>
<td>Gga.1290.1.S1_at</td>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa</td>
<td>NDUF3S</td>
<td>0.40</td>
<td>0.71</td>
</tr>
<tr>
<td>Gga.3232.1.S1_at</td>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa</td>
<td>NDUF4S</td>
<td>0.27</td>
<td>0.94</td>
</tr>
<tr>
<td>Gga.4778.1.S1_a_at</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa</td>
<td>NDUFV2</td>
<td>0.49</td>
<td>0.88</td>
</tr>
<tr>
<td>GgaAffx.15157.7.S1_at</td>
<td>Oxoglutarate dehydrogenase-like</td>
<td>OGDH</td>
<td>3.36</td>
<td>1.39</td>
</tr>
<tr>
<td>Gga.4447.1.S1_at</td>
<td>Phosphoenolpyruvate carboxykinase 1 (soluble)</td>
<td>PCK1</td>
<td>0.31</td>
<td>0.34</td>
</tr>
<tr>
<td>Gga.1161.1.S1_at</td>
<td>Succinate dehydrogenase complex, subunit D, integral membrane protein</td>
<td>SDHD</td>
<td>2.92</td>
<td>2.51</td>
</tr>
<tr>
<td>Gga.4338.3.S1_a_at</td>
<td>Succinyl-cytochrome c reductase core protein l</td>
<td>UQCRC1</td>
<td>0.42</td>
<td>1.01</td>
</tr>
</tbody>
</table>

FC ≥ 2.0 represents up-regulated, and FC ≤ 0.50 represents down-regulated.

Table 5. Primer pairs used to analyze gene expression by qRT-PCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward primer (5′→ 3′)</th>
<th>Reverse primer (5′→ 3′)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKDC1</td>
<td>GAAAGCTTGTGGGATGGAC</td>
<td>ACACATTGCTACCTGTTGTCGC</td>
<td>213</td>
</tr>
<tr>
<td>PKC1</td>
<td>GCTGACACTGTTCATGAATGCA</td>
<td>ACTGGTGAATTGCACCTGCA</td>
<td>106</td>
</tr>
<tr>
<td>NDUF3S</td>
<td>CTGGCTGCTTTGGAATGATGTT</td>
<td>GAGAAAGGACAGACGTGCAATT</td>
<td>128</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>ACATCTGATGATGGGATGTCG</td>
<td>ATCTGGAGGGATGTCGACAG</td>
<td>243</td>
</tr>
<tr>
<td>AKR1B10</td>
<td>ACAAGGATGATATTTCACACGTGG</td>
<td>TCAGTTATGAGTCGCACCTAA</td>
<td>196</td>
</tr>
</tbody>
</table>
increased the activity of PK, and numerically increased the activity of Na\(^+\)-K\(^+\)-ATPase by dietary L-carnitine supplementation.

In conclusion, a total 790 differentially expressed genes (386 up- and 404 down-regulated) were screened from liver transcriptomic profile in D39 vs. D21 healthy broilers. A total 178 differentially expressed genes (128 up-regulated and 50 down-regulated) were screened in ascites vs. healthy broilers on D39. The analysis of pathway and GO term indicated that genes involved in amino acid metabolism, fatty

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**Table 6. Comparison of results from the microarray and qRT-PCR (n=4)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change (Ascites/Healthy)</th>
<th>Microarray</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKDC1</td>
<td>0.28</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>PCK1</td>
<td>0.34</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>NDUFS3</td>
<td>0.71</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>HNF4α</td>
<td>2.26</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>AKR1B10</td>
<td>2.03</td>
<td>5.75</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7. Effects of L-carnitine on gene expression related to hepatic energy metabolism and oxidation-reduction (n=6)**

<table>
<thead>
<tr>
<th>L-carnitine (mg/kg)</th>
<th>Ascites</th>
<th>HKDC1</th>
<th>PCK1</th>
<th>HNF4α</th>
<th>AKR1B10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.646</td>
<td>10.41</td>
<td>0.409</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.049</td>
<td>1.408</td>
<td>0.948</td>
<td>0.048</td>
<td></td>
</tr>
<tr>
<td>Poled SEM</td>
<td>0.267</td>
<td>1.357</td>
<td>0.165</td>
<td>0.024</td>
<td></td>
</tr>
</tbody>
</table>

Main effect

L-carnitine level

− 0.588 6.15 0.944 0.121

Ascites

− 1.173 7.00 1.467* 0.094

Source of Variation

L-carnitine 0.035 0.709 0.035 0.200

Ascites 0.049 0.000 0.021 0.000

L-carnitine × Ascites 0.113 0.085 0.051 0.117

“−” indicates healthy broilers, “+” indicates ascites broilers. * in the same column is significantly different (P<0.05) and ** P<0.01.

**Table 8. Effects of L-carnitine on liver biochemical parameters (n=8)**

<table>
<thead>
<tr>
<th>L-carnitine (mg/kg)</th>
<th>Ascites</th>
<th>MDA nmol/mgprot</th>
<th>T-SOD U/mgprot</th>
<th>GSH-Px U/mgprot</th>
<th>HK U/mg prot</th>
<th>SDH U/mg prot</th>
<th>MDH U/mg prot</th>
<th>PK U/g prot</th>
<th>Na(^+)-K(^+)-ATPase mmolPi/mgprot/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.87</td>
<td>74.26</td>
<td>16.81</td>
<td>53.78</td>
<td>3.28</td>
<td>1.61</td>
<td>36.52</td>
<td>8.017</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.08</td>
<td>71.82</td>
<td>16.69</td>
<td>53.78</td>
<td>3.99</td>
<td>1.21</td>
<td>28.63</td>
<td>5.452</td>
<td></td>
</tr>
<tr>
<td>Poled SEM</td>
<td>0.08</td>
<td>2.371</td>
<td>0.372</td>
<td>3.677</td>
<td>0.345</td>
<td>0.059</td>
<td>1.973</td>
<td>0.547</td>
<td></td>
</tr>
</tbody>
</table>

Main effect

L-carnitine level

− 0.99 72.90 16.74 45.87 3.66 1.41 33.37 6.735

Ascites

− 1.08 84.83** 16.43 57.25 5.08 1.56 38.45 8.393

Source of Variation

L-carnitine 0.421 0.004 0.534 0.204 0.065 0.164 0.019 0.081

Ascites 0.029 0.041 0.099 0.041 0.149 0.032 0.001 0.015

L-carnitine × Ascites 0.144 0.137 0.072 0.179 0.687 0.160 0.067 0.919

“−” indicates ascites-resistant broilers, “+” indicates ascites-susceptible. * in the same column is significantly different (P<0.05) and ** P<0.01.
acid metabolism, Krebs cycle, carbohydrate metabolism, vitamin metabolism, cell apoptosis, immune response, oxidation-reduction, angiogenesis, nitric oxide formation and erythrocyte differentiation are differentially expressed in the development of ascites. Liver oxidative damage in ascites broilers was aggravated, enzyme activity of energy metabolism was significantly decreased, and expression of genes associated with liver HKDC1 and PCK1 was significantly down-regulated. Dietary supplementation of L-carnitine significantly enhanced liver antioxidative capacity, and significantly up-regulated HKDC1 and HNF-4α expressions, which may promote the generation and utilization of ATP. The present results suggest that L-carnitine can serve as a potential regulatory agent to reduce ascites susceptibility and mortality.

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