Animal, Page 1 of 8 © The Animal Consortium 2018
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Molecular mechanisms of lipid metabolism disorder in livers of ewes with pregnancy toxemia

Y. F. Xue, C. Z. Guo, F. Hu, D. M. Sun, J. H. Liu and S. Y. Mao†

Jiangsu Key Laboratory of Gastrointestinal Nutrition and Animal Health, Laboratory of Gastrointestinal Microbiology, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, Jiangsu Province, China

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Pathogenesis of pregnancy toxemia (PT) is believed to be associated with the disruption of lipid metabolism. The present study aimed to explore the underlying mechanisms of lipid metabolism disorder in the livers of ewes with PT. In total, 10 pregnant ewes were fed normally (control group) whereas another 10 were subjected to 70% level feed restriction for 15 days to establish a pathological model of PT. Results showed that, as compared with the controls, the levels of blood β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFAs) and cholesterol were greater ($P < 0.05$) and blood glucose level was lower ($P < 0.05$) in PT ewes. The contents of NEFAs, BHBA, cholesterol and triglyceride were higher ($P < 0.05$) and glycerol content was lower ($P < 0.05$) in hepatic tissues of PT ewes than those of the controls. For ewes with PT, excessive fat vacuoles were observed in liver sections stained with hematoxylin–eosin; furthermore, inner structures of hepatocytes including nuclei, mitochondria and endoplasmic reticulum were damaged seriously according to the results of transmission electron microscope. Real-time PCR data showed that compared with the controls, the expression of hepatic genes involved in fatty acid oxidation (FAO) and triglyceride synthesis (TGS) was enhanced ($P < 0.05$) whereas that related to acetyl-CoA metabolism (ACM) was repressed ($P < 0.05$) in PT ewes. Generally, our results showed that negative energy balance altered the expression of genes involved in FAO, ACM and TGS, further caused lipid metabolism disorder in livers, resulting in PT of ewes. Our findings may provide the molecular basis for novel therapeutic strategies against this systemic metabolic disease in sheep.

Keywords: pregnancy toxemia, fatty acid, β-hydroxybutyrate, cholesterol, triglyceride

Implications

Sheep during late gestation often suffer from negative energy balance, which might trigger body fat mobilization and modulate metabolic pathways, leading to lipid metabolism disorder and pregnancy toxemia (PT). The present study established the pathological model of PT by feed restriction and evaluated the alteration of lipid metabolism-associated pathways in livers of PT ewes. This is the first report to illustrate the molecular mechanisms of lipid metabolism disorder in livers of ewes with PT, which may provide a theoretical basis for novel therapeutic methods of PT.

Introduction

Morbidity of PT approximately ranges from 5% to 20%, and mortality can reach 80% in untreated animals (Rook and Herdt, 2000), resulting in a significant economic loss (Lima *et al.*, 2012). Pregnancy toxemia is the most common metabolic disease for ewes during late gestation, when insufficient energy intake due to the extruded abdomen is unable to meet the increased energy demands of fetuses. Negative energy balance can trigger body fat mobilization and prompt ketone body production and hepatic lipidosis. Thus, PT is characterized by elevated blood β-hydroxybutyrate (BHBA) (Firat and Ozpinar, 2002; Ismail *et al.*, 2008) and non-esterified fatty acids (NEFAs) (Lacetera *et al.*, 2001; Calpereyra *et al.*, 2009 and 2015) as well as accumulated hepatic triglyceride (Calpereyra *et al.*, 2009; Wang *et al.*, 2014). However, to the best of our knowledge, information on the molecular mechanisms underlying lipid metabolism disturbance and the contribution of this to the pathogenesis of PT remains unclear.

We hypothesize that negative energy balance may alter the expression of key enzyme genes involved in fatty acid oxidation (FAO), acetyl-CoA metabolism (ACM) and triglyceride synthesis (TGS), then cause the disorder of lipid metabolism in the liver, resulting in PT of ewes. Therefore, the objective of the present study was to explore pathways including FAO, ACM and TGS in livers of PT ewes, and to

† E-mail: maoshengyong@njau.edu.cn

clarify the underlying molecular mechanisms of lipid metabolism disorder.

Material and methods

Animals, diets and treatment

In all, 20 purebred Hu sheep (BW 60.6 ± 4.9 kg) carrying multiple fetuses with similar body condition scores (3.0 to 3.5 on a 1 to 5 scale) and similar parity (2 to 3 parity) and gestating for 108 days were enrolled in this study. All ewes were fed *ad libitum* in individual pens for a 7-day adaption period. After that, these ewes were assigned to two groups (10 ewes in each group) randomly at 115 days of gestation (considered as day 0 of the experimental period). Ewes in the control (CON) group were fed normally (1.56 kg total mixed ration, dry matter basis; according to the feed intake calculated in adaption period), whereas ewes in the treated (TR) group were subjected to a 70% level feed restriction (0.47 kg total mixed ration, dry matter basis) for 15 days, with free access to water. Ingredients and nutritional compositions of the total mixed ration are presented in Table 1.

Samples collection

Blood samples were collected from the jugular vein every 5 days before morning feeding at days 0, 5, 10 and 15. After 15 days of treatment, all ewes were sacrificed, and livers were separated and weighed to calculate the liver index (liver weight/BW). Then, hepatic samples of ewes in the CON group and TR group were all collected from the left lobes within 30 min. A part of each liver sample was stored in liquid nitrogen immediately to analyze the metabolites and the expression of genes, whereas another part was promptly fixed in either 4% paraformaldehyde or 2.5% glutaraldehyde to make hematoxylin–eosin staining sections or electron transmission microscopic sections, respectively.

Table 1 *Ingredients and nutrient composition of the experimental diets for pregnant sheep (*Ovis aries*) in this study*

Items	Diets
Ingredient composition (% DM)	
Rye silage	42.3
Oat hay	34.6
Maize	12.0
Soybean	5.8
Barley	4.2
Premix[1]	1.1
Nutrient composition	
Metabolic energy (MJ/kg DM)	11.64
CP (% DM)	14.71
Crude fat (% DM)	2.95
NDF (% DM)	48.32
ADF (% DM)	29.09
Crude ash (% DM)	7.35
Calcium (% DM)	0.50
Phosphorus (% DM)	0.37

DM = dry matter basis.
[1]The premix (per kg) contained: vitamin A 64.8 mg, vitamin D_2 1.35 mg, vitamin E 1080 mg, nicotinic acid 353 mg, Mn 537 mg, Cu 540 mg, Zn 2422 mg, Co 7.2 mg, I 32 mg, Se 18 mg, P 66 g, Ca 130 g, $NaHCO_3$ 89 g and NaCl 140 ~ 180 g.

Biochemical analysis

The BHBA level in the blood was determined using the Free Style Optium Blood Glucose and Ketone Monitoring System (Abbott Diabetes Care Ltd, Oxon, UK). Glucose, NEFA, cholesterol, triglyceride and glycerol levels in plasma as well as NEFA, BHBA, cholesterol, triglyceride, glycerol and protein contents in livers were all determined by commercially available kits (Jian-Cheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Microscopic study

Hepatic tissues were prepared for light microscopy histological analysis using methodology previously described by Odongo *et al*. (2006). In brief, 4% paraformaldehyde (Sigma, St. Louis, MO, USA)-fixed, paraffin-embedded specimens were sectioned at a 6-μm thickness, stained with hematoxylin–eosin and mounted for analysis. In addition, hepatic tissues were prepared for transmission electron microscope using methods reported by Graham and Simmons (2005). Briefly, hepatic tissues were fixed in 2.5% glutaraldehyde immediately for 24 h, post-fixed for 1 h in 1% osmium, and then, dehydrated in ethanol solutions with different concentration gradients. Subsequently, the dehydrated specimens were transferred into a mixture of Spurr resin and acetone (1:1) for 30 min, followed by immersion in 100% resin for 10 h. Specimens containing resin were shifted to molds and polymerized at 40°C or 60°C for 48 h. Semithin sections (0.25 to 0.5 μm) were cut with a glass knife and stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin sections (70 to 90 nm) were cut with a diamond knife, stained with methanolic uranyl acetate and lead citrate and finally observed and photographed using transmission electron microscope (Hitachi H-7650; Hitachi Technologies, Tokyo, Japan). The microscopist was blinded to treating methods between two groups during the microscopy histological analysis.

Ribonucleic acid extraction, purification and complementary DNA synthesis

Total RNA was extracted from frozen hepatic tissue samples (~100 mg for each sample) in the CON and TR groups using the TRIzol method (Takara Bio, Otsu, Japan), as described by Chomczynski and Sacchi (1987). Ribonucleic acid yield was detected using the Nanodrop spectrophotometer ND-1000 UV-Vis (Thermo Fisher Scientific, Madison, WI, USA). The quality of RNA extracted was also verified by ensuring all RNA samples had an absorption ratio (A260/A280) between 1.8 and 2.0, and the integrity of RNA was checked using 1% agarose gel electrophoresis. All RNA solution samples were diluted to the same concentration (500 ng/μl). Total RNA (1 μg) was reverse transcribed to complementary DNA using a PrimeScript reverse transcription kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Primer 5 software (Whitehead Institute, Cambridge, MA, USA) and the BLAST computer program (National Center for Biotechnology Information, Bethesda, MD, USA) were utilized to design the primers used in the present study. All primers were synthesized by a commercial supplier (Invitrogen Life Technology, Shanghai, China). Details of the primer sets used for real-time PCR in the current study are listed in Supplementary Table S1. After reverse transcription, complementary DNA was quantified by using the QuantStudio 5 real-time PCR Instrument (Thermo Fisher Scientific, Waltham, MA, USA). The PCR mixture contained 2.0 μl of complementary DNA template, 0.4 μl of each primer (10 μM), 10 μl of SYBRR Premix Ex TaqTM (2*), 0.4 μl of ROX Reference Dye (50*) and PCR-grade sterile water to a final volume of 20 μl. The program for PCR amplification was as follows: 95°C for 30 s for pre-denaturation, and 40 cycles of 95°C for 5 s, 60°C for 34 s, followed by a melting curve program (60°C to 99°C with a ramping rate of 0.1°C/s and fluorescence measurement). A melting curve was generated for each sample to ensure the purity of PCR-amplified products. The relative messenger RNA (mRNA) expression level of each studied gene was normalized to β-actin (as a housekeeping gene), and the data were analyzed according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analyses were carried out by using the SPSS software package (SPSS version 19.0; SPSS Inc., Chicago, IL, USA). Data were presented as means with their standard errors. Statistical analyses of blood indicators were performed using the GLM for repeated-measures analysis procedure with Bonferroni's *post hoc* testing. Univariate GLM covariance analysis, with Bonferroni adjustment, was used to test differences between groups, with the liver index, hepatic biochemical indicators and gene expression levels as the dependent factors, treatment variable as the fixed factor and BW change value as covariate. The Spearman's correlation coefficients (r) of biochemical indicators between blood (day 15) and liver of the 20 sheep were calculated with bivariate correlation analysis. P-values < 0.05 (two-sided) were considered statistically significant.

Results

Animal behavior description

Ewes in the CON group remained alert and active throughout the dietary treatment period. However, ewes in the TR group showed clinical signs of toxemia including anorexia, grinding teeth, languishment, hindlimb ataxia and locomotion disturbances.

Biochemical indicators in blood and liver

As shown in Figure 1, with the prolongation of feed restriction for ewes in the TR group, blood BHBA level increased whereas blood glucose level decreased, and blood NEFAs level increased at the outset and subsequently decreased. Main effects of treatments for the levels of blood BHBA ($P < 0.001$), glucose ($P < 0.001$), NEFAs ($P < 0.001$) and cholesterol ($P < 0.001$) were significant, whereas those for the levels of blood glycerol ($P = 0.177$) and triglyceride ($P = 0.225$) were

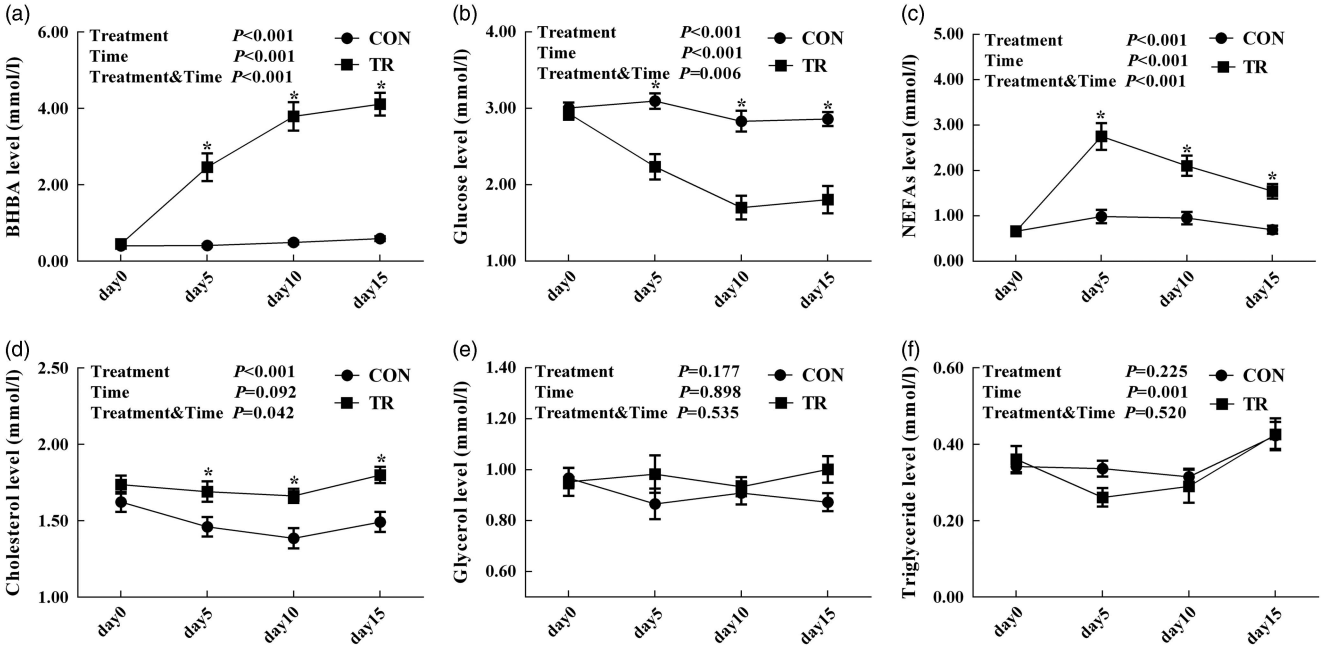

Figure 1 Blood biochemical indicators of pregnant sheep in the control (CON, fed in normal level) and treated (TR, restricted to 30% level of feed intake) group. (a) β-hydroxybutyrate (BHBA) level. (b) Glucose level. (c) Non-esterified fatty acids (NEFAs) level. (d) Cholesterol level. (e) Glycerol level. (f) Triglyceride level. Values are means ($n = 10$), with their standard errors represented by vertical bars. *Mean values with asterisks were significantly different between two groups ($P < 0.05$, GLM for repeated-measures analysis procedure with Bonferroni's *post hoc* testing).

nonsignificant. Blood BHBA levels ($P < 0.001$, <0.001, <0.001), NEFAs levels ($P = 0.002$, $= 0.002$, <0.001) and cholesterol levels ($P = 0.042$, $= 0.003$, $= 0.003$) at days 0, 10 and 15 of ewes in the TR group were significantly higher than those of the CON group, respectively. Blood glucose levels ($P = 0.005$, $= 0.006$, $= 0.002$) at days 0, 10 and 15 of ewes in the TR group were significantly lower than those of the CON group, respectively.

As shown in Figure 2, the contents of NEFAs ($P < 0.001$), BHBA ($P = 0.001$), cholesterol ($P = 0.033$) and triglyceride ($P < 0.001$) in livers of ewes upon feed restriction were higher compared with the CON group. Whereas, the content of hepatic glycerol in feed-restricted ewes was lower ($P = 0.035$) than that of the CON group.

Histological changes of livers in pregnancy toxemia ewes

Visual inspection showed that livers in the CON group were deep red, soft and elastic whereas livers in the TR group were swollen, deep yellow and fragile (Figure 3a). Moreover, there were local hemorrhages and regional degeneration on the surface of livers in the TR group (Figure 3b and c). Hepatic tissues stained with hematoxylin–eosin exhibited red coloration, which made apparent to see a large amount of fat vacuoles being distributed in the TR group (Figure 3e), yet only a few lipid droplets had been observed in the CON group (Figure 3d). Results of transmission electron microscope further showed the morphology of nuclei (Figure 3f), endoplasmic reticulum (Figure 3h) and mitochondria (Figure 3h) was normal in hepatocytes of the CON group. However, in hepatocytes of the TR group, nuclei were extruded by fat vacuoles and lost their basic shapes (Figure 3g). Dilatation and vesiculation occurred in the endoplasmic reticulum of hepatocytes in the TR group, and many polyribosomes originally attached to the endoplasmic reticulum had desquamated (Figure 3i). The double-layer membrane structure and inner cristae structure of mitochondria had partly disappeared, making the appearance of swell and vacuolation (Figure 3i). In addition, the liver index of the TR group (1.49%) was significantly higher ($P = 0.004$) than that of the CON group (1.15%).

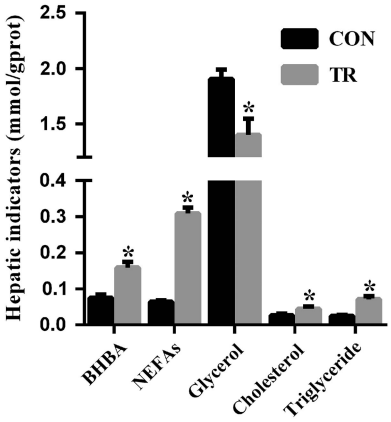

Figure 2 Hepatic biochemical indicators of pregnant sheep in the control (CON, fed in normal level) and treated (TR, restricted to 30% level of feed intake) group. Values are means ($n = 10$), with their standard errors represented by vertical bars. *Mean values with asterisks were significantly different between two groups ($P < 0.05$, univariate GLM covariance analysis with Bonferroni adjustment). BHBA = β-hydroxybutyrate; NEFAs = non-esterified fatty acids.

Fatty acid oxidation in liver

The expression levels of genes involved in FAO, including long-chain acyl-CoA synthase (*ACSL*) ($P < 0.001$), carnitine palmitoyltransferase 1 (*CPT*1) ($P = 0.001$), long-chain acyl-CoA dehydrogenase (*ACADL*) ($P < 0.001$), hydroxy acyl-CoA dehydrogenase ($P = 0.002$), enoyl-CoA hydratase ($P < 0.001$), 3-ketoacyl-CoA thiolase ($P = 0.001$) and acetyl-CoA acyltransferase 1 ($P = 0.002$), in the TR group were higher than those of the CON group, respectively. Whereas, carnitine palmitoyltransferase 2 ($P = 0.505$), acyl-CoA oxidation 1 ($P = 0.166$) and acyl-CoA oxidation 2 ($P = 0.074$) expression in the TR group were unchanged compared with the CON group (Figure 4).

Acetyl-CoA metabolism in liver

As the product of FAO, some acetyl-CoA will be incompletely oxidized to ketone bodies and cholesterol. The expression levels of genes involved in ketogenesis and cholesterol synthesis, including β-hydroxy-β-methylglutaryl CoA (HMG-CoA) synthase 2 (*HMGCS*2) ($P = 0.007$), HMG-CoA lyase ($P = 0.020$), HMG-CoA synthase 1 (*HMGCS*1) ($P < 0.001$) and HMG-CoA reductase (*HMGCR*) ($P = 0.006$), in the TR group, were lower than those of the CON group, respectively. Whereas, the expression level of acetyl-coA acyltransferase ($P = 0.156$) in the TR group remained unchanged compared with the CON group (Figure 5).

Triglyceride synthesis in liver

In addition to FAO, NEFAs can also be esterified to triglycerides, which are stored in hepatocytes as lipid droplets. The expression levels of genes involved in TGS, including glycerol kinase ($P < 0.001$), glycerol-3-phosphate dehydrogenase ($P < 0.001$), mitochondrial glycerol-3-phosphate acyltransferase ($P = 0.002$) and diacylglycerol acyltransferase ($P = 0.004$) in the TR group were greater than those of the CON group, respectively (Figure 6).

Correlations between blood and liver biochemical indicators

To further understand the material flow, correlations between blood (day 15) and liver biochemical indicators of the 20 sheep were evaluated. Results showed that blood NEFAs level was positively correlated with the contents of NEFAs ($r = 0.605$, $P = 0.005$), BHBA ($r = 0.646$, $P = 0.002$), cholesterol ($r = 0.573$, $P = 0.008$) and triglyceride ($r = 0.906$, $P < 0.001$) in liver. Blood glucose level was negatively correlated with the contents of NEFAs ($r = -0.571$, $P = 0.009$), BHBA ($r = -0.483$, $P = 0.031$) and triglyceride ($r = -0.525$, $P = 0.017$) in liver. Blood BHBA level was linked with the contents of NEFAs ($r = 0.776$, $P < 0.001$), BHBA ($r = 0.671$, $P = 0.001$), triglyceride ($r = 0.714$, $P < 0.001$) and glycerol ($r = -0.569$, $P = 0.009$) in liver. Blood cholesterol level was

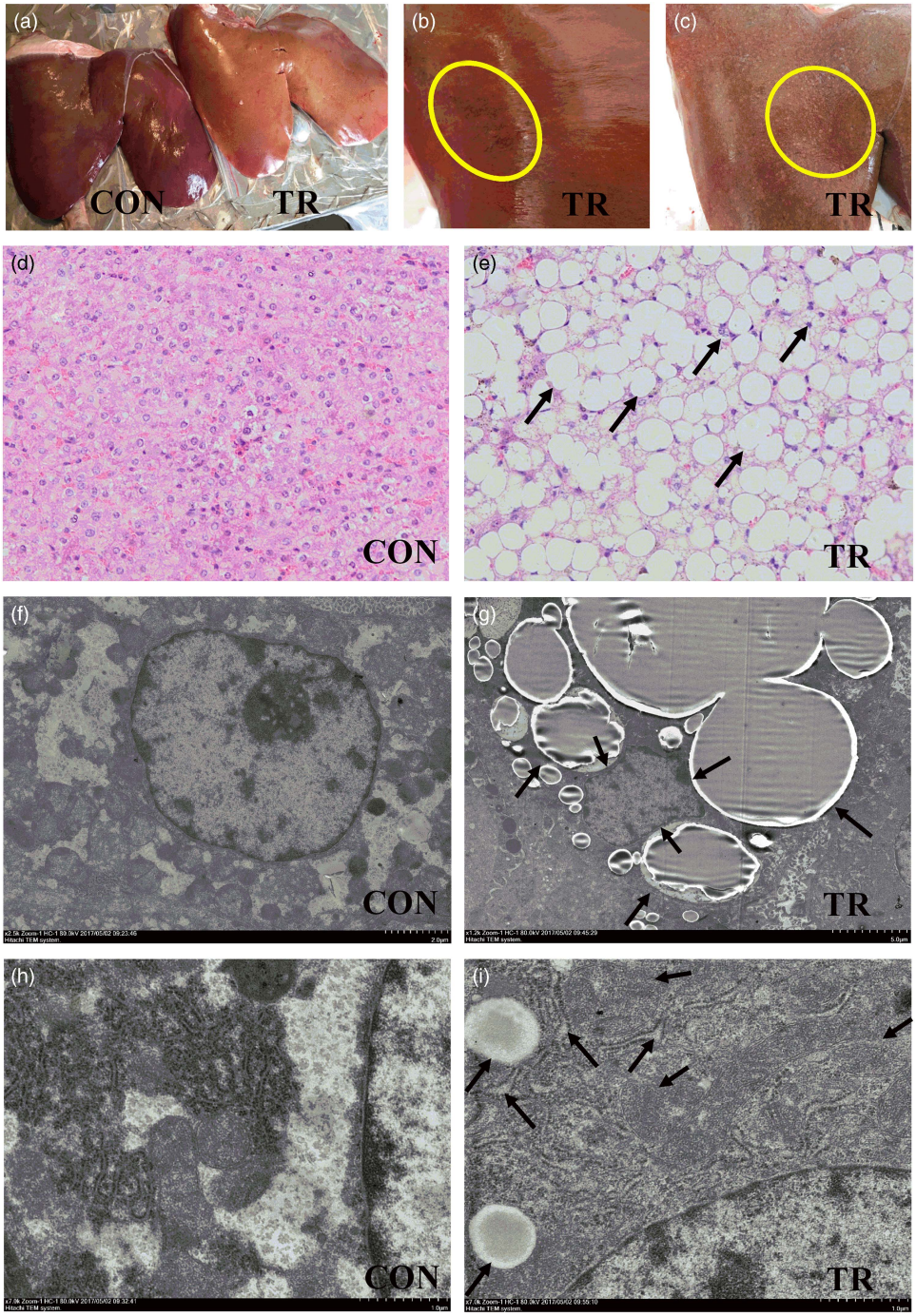

Figure 3 (Color online) Hepatic visual inspection, microstructure analysis and ultrastructure analysis of pregnant sheep in the control (CON, fed in normal level) and treated (TR, restricted to 30% level of feed intake) group. (a–c) Visual inspection of livers in the two groups, indicated by deep red color for livers in the CON group but the deep yellow color (a), local hemorrhage (b) as well as regional degeneration (c) for livers in the TR group. (d, e) Hematoxylin–eosin staining of liver sections from the two groups, indicated by normal microstructure for livers in the CON group (d) but many fat vacuoles for livers in the TR group (e). (f–i) Transmission electron microscopic images of livers in two groups indicated normal nuclei (f), rough endoplasmic reticulum and mitochondria (h) for livers in the CON group, but extruded and shape-modified nuclei (g), dilated and vesiculated rough endoplasmic reticulum, as well as swollen and disaggregated mitochondria for livers in the TR group (i). Magnification ×200, 200, 2500, 1200, 7000 and 7000 was used from (d) to (i), respectively.

positively correlated with the contents of NEFAs ($r = 0.498$, $P = 0.025$), BHBA ($r = 0.529$, $P = 0.016$) and triglyceride ($r = 0.658$, $P = 0.002$) in liver. Blood glycerol level was positively linked with the contents of BHBA ($r = 0.621$, $P = 0.004$) and triglyceride ($r = 0.465$, $P = 0.039$) in liver.

Discussion

Although PT is a rather commonly studied disease, available information about this disease mainly focuses on clinical features, biochemical indicators, diagnosis and conventional

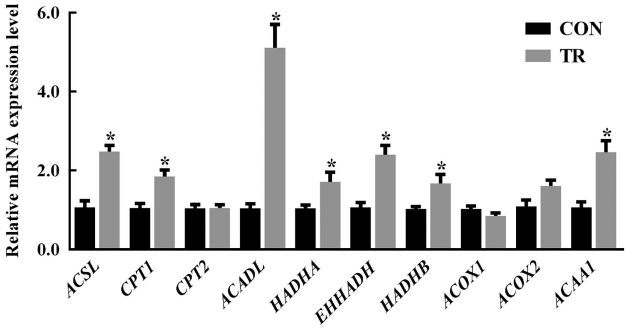

Figure 4 Relative messenger RNA (mRNA) expression levels of genes related to fatty acid oxidation (FAO) in livers of pregnant sheep in the control (CON, fed in normal level) and treated (TR, restricted to 30% level of feed intake) group. Values are means ($n = 10$), with their standard errors represented by vertical bars. *Mean values with asterisks were significantly different between two groups ($P < 0.05$, univariate GLM covariance analysis with Bonferroni adjustment). *ACSL* = long-chain acyl-CoA synthase; *CPT1* = carnitine palmitoyltransferase 1; *CPT2* = carnitine palmitoyltransferase 2; *ACADL* = long-chain acyl-CoA dehydrogenase; *HADHA* = hydroxy acyl-CoA dehydrogenase; *EHHADH* = enoyl-CoA hydratase; *HADHB* = 3-ketoacyl-CoA thiolase; *ACOX1* = acyl-CoA oxidation 1; *ACOX2* = acyl-CoA oxidation 2; *ACAA1* = acetyl-CoA acyltransferase 1.

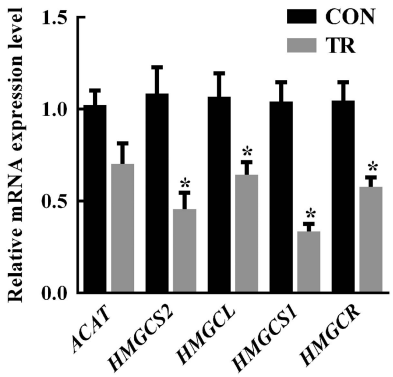

Figure 5 Relative messenger RNA (mRNA) expression levels of genes related to acetyl-CoA metabolism (ACM) in livers of pregnant sheep in the control (CON, fed in normal level) and treated (TR, restricted to 30% level of feed intake) group. Values are means ($n = 10$), with their standard errors represented by vertical bars. *Mean values with asterisks were significantly different between two groups ($P < 0.05$, univariate GLM covariance analysis with Bonferroni adjustment). *ACAT* = acetyl-CoA acyltransferase; *HMGCS2* = β-hydroxy-β-methylglutaryl CoA (HMG-CoA) synthase 2; *HMGCL* = HMG-CoA lyase; *HMGCS1* = HMG-CoA synthase 1; *HMGCR* = HMG-CoA reductase.

therapy (Marteniuk, 1988; Rook and Herdt, 2000; Calpereyra *et al.*, 2012). This is the first study to explore the molecular mechanisms of lipid metabolism disruption in livers of ewes with PT. Ewes subjected to feed restriction presented a series of symptoms, which were consistent with the clinical signs of PT in previous reports (Marteniuk, 1988; Rook and Herdt, 2000; Calpereyra *et al.*, 2012). The blood BHBA levels of feed-restricted ewes were all above the threshold (BHBA <0.8 mmol/l, normal; 0.8 to 1.6 mmol/l, subclinical PT; >1.6 mmol/l, clinical PT) (Firat and Ozpinar, 2002; Ismail *et al.*, 2008), further confirming the successful induction of the pathological model of clinical PT (West, 1996; Calpereyra *et al.*, 2009).

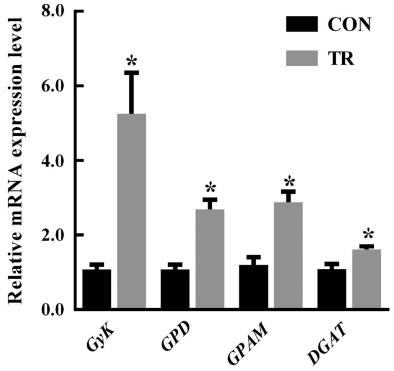

Figure 6 Relative messenger RNA (mRNA) expression levels of genes related to triglyceride synthesis (TGS) in livers of pregnant sheep in the control (CON, fed in normal level) and treated (TR, restricted to 30% level of feed intake) group. Values are means ($n = 10$), with their standard errors represented by vertical bars. *Mean values with asterisks were significantly different between two groups ($P < 0.05$, univariate GLM covariance analysis with Bonferroni adjustment). *GyK* = glycerol kinase; *GPD* = glycerol-3-phosphate dehydrogenase; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *DGAT* = diacylglycerol acyltransferase.

Previous studies reported that elevated body fat mobilization in feed-restricted animals induced liver degeneration and vacuolation (Calpereyra *et al.*, 2009). In the present study, the protoplasm of many hepatocytes appeared to be replaced by lipid, which was similar to Snook's description (Snook, 1939). Furthermore, the changed structures of hepatocytes, including nuclear shape alteration, mitochondrial vacuolation and endoplasmic reticulum derangement, were consistent with the findings reported by Wang *et al.* (2014). Some endoplasmic reticulum might be smooth reticulum, and their derangements were likely related to the disordered lipid metabolism in liver of PT ewes. Although some morphological changes might be the result from the fixation defects, however, when compared with the CON, severe alteration of nuclei, mitochondria and endoplasmic reticulum in the TR group implied that there was damage to hepatocytes' internal structures in PT ewes.

A significantly elevated liver index also certified the turgidity of livers in PT ewes. This could be due to the progressive accumulation of triglyceride that the livers of PT ewes became enlarged, deep yellow and degenerated (Wang *et al.*, 2014; Abba *et al.*, 2015). The increased level of triglyceride and decreased level of glycerol can be partly explained by the elevated expression of genes linked to TGS in livers of PT ewes. This finding corresponds to previous reports that increased plasma NEFAs results in up-regulated fatty acid esterification and triglyceride storage in livers of dairy cows (Grummer, 1993). Furthermore, previous studies have shown that only a very small part of triglyceride can be transported out of the liver by very low-density lipoprotein (Kleppe *et al.*, 1988). Officially because of this, no difference in plasma triglyceride level between two groups and no correlation between the level of triglyceride in plasma and liver was observed. Generally, the excessively accumulated triglyceride in livers of PT ewes caused fatty liver.

Normally, when NEFAs overwhelm the capacity of fatty acid metabolism, the alternative way of re-esterification to triglyceride for NEFAs with free glycerol in liver proceeds substantially (Brickner *et al.*, 2009). In line with the enhanced FAO in ketotic (Loor *et al.*, 2007) and energy-restricted (Loor *et al.*, 2006) dairy cows, the increased expression levels of genes linked to fatty acid activation, acyl-CoA transportation and acyl-CoA oxidation in the present study imply that there is an enhancement of FAO in liver of PT ewes. It has been proven that the transcriptional levels of *ACSL*, *ACADL* and *CPT*1 are elevated significantly with increased NEFAs in cultured bovine hepatocytes (Li *et al.*, 2013b). Another research has also shown that CPT1, the rate-limiting enzyme of FAO, can be activated by long-chain fatty acids (Louet *et al.*, 2001). In the present study, NEFAs were accumulated both in livers and blood of PT ewes. Therefore, the intensified FAO in livers of PT ewes may be partly due to the rising level of NFEAs.

The major finding of the current study was that ACM was inhibited in livers of ewes with PT, which might further increase NEFAs accumulation and aggravate fatty liver. Acetyl-CoA, as the product of FAO, and its outlets include complete and incomplete oxidation. Nonetheless, the oxaloacetic acid pool is very limited, especially when ketosis occurs (Xu *et al.*, 2010), and represses acetyl-CoA to generate citric acid and enter citrate cycle for complete oxidation. The alternative pathway of incomplete oxidation to produce ketone bodies (mainly BHBA) and cholesterol may be activated. As a result, a significant increase of BHBA and cholesterol was detected in the blood and livers of PT ewes. It is well known that ketogenesis is controlled by substrate availability (Williamson and Whitelaw, 1978; McGarry and Foster, 1980) and ketogenesis-associated genes (Rodriguez *et al.*, 1994). It also has been proven that BHBA (more than 1.5 mmol/l) shows a dose-dependent inhibitory effect on *HMGCS*2 expression in bovine hepatocytes (Li *et al.*, 2013a). In the current study, substrates were abundant due to the up-regulated FAO, so the down-regulated ketogenesis pathway in livers of PT ewes might result from feedback regulation with the substantial accumulation of BHBA. In addition, down-regulated mRNA expression level of *HMGCS*2 (Li *et al.*, 2012) has also been reported in ketotic cows. For cholesterol synthesis, previous findings reveal that *HMGCS*1 expression is inhibited by fasting and by accumulated product (Royo *et al.*, 1991). Thus, the decreased expression of *HMGCS*1 in livers of PT ewes might result from the restricted feeding and accumulated cholesterol. Consistent with our results, down-regulated *HMGCR* has also been observed in ketotic cows (Loor *et al.*, 2007). In general, both ketogenesis and cholesterol synthesis have been down-regulated, which represses the metabolism of NEFAs.

In conclusion, negative energy balance could enhance FAO and TGS with the excessive deposition of NEFAs in the liver. However, ACM was repressed with the accumulation of BHBA and cholesterol in the liver. Fatty liver, which was always accompanied with PT, resulted from continuously accumulated triglyceride and caused severe liver damage.

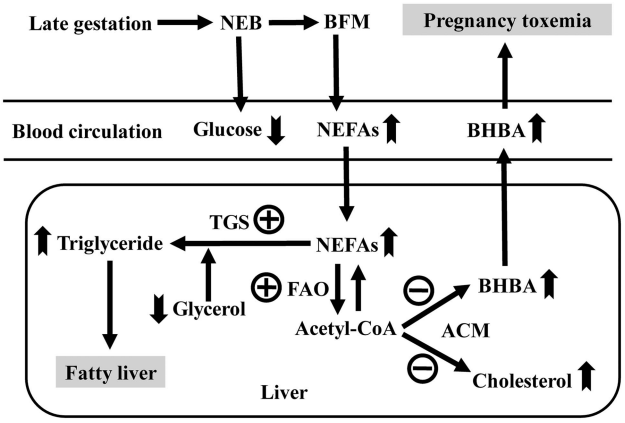

Figure 7 Integrated analysis of lipid metabolism disturbance in livers of ewes with nutrition-induced pregnancy toxemia. Up swallowtail arrows represent increased substances; down swallowtail arrows represent decreased substances; plus signs represent up-regulated pathways; minus signs represent down-regulated pathways. NEB = negative energy balance; BFM = body fat mobilization; NEFAs = non-esterified fatty acids; BHBA = β-hydroxybutyrate; TGS = triglyceride synthesis; FAO = fatty acid oxidation; ACM = acetyl-CoA metabolism.

When liver function was injured, decreased ability of energy conversion would trigger much more severe negative energy balance and body fat mobilization, resulting in more severe PT (Figure 7). Generally, as discussed above, several critical molecular mechanisms of lipid metabolism disorders were recognized in livers of PT ewes, and the findings of this research have important implications for developing the novel therapeutic strategies of PT.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731118002136

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The authors' contributions are as follows: S. Y. M. and Y. F. X. conceived and designed the study; Y. F. X., C. Z. G. and F. H. conducted the research; Y. F. X., D. M. S. and J. H. L. analyzed and interpreted the data; and Y. F. X. wrote the manuscript. All authors read and approved the final version of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest.

Ethics statement

The animal experimental design and procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University (SYXK(Su)2015-0656). This experiment was performed using Hu-sheep at a breeding farm in Shanghai, China.

Software and data repository resources

The authors declare that the data of this research are not deposited in any official repository.

References

Abba Y, Abdullah FFJ, Chung ELT, Sadiq MA, Mohammed K, Osman AY, Rahmat NRR, Razak IA, Lila MAM, Haron AW and Saharee AA 2015. Biochemical and pathological findings of pregnancy toxemia in Saanen doe: a case report. Journal of Advanced Veterinary & Animal Research 2, 236–239.

Brickner AE, Pires JAA, Gressley TF and Grummer RR 2009. Effects of abomasal lipid infusion on liver triglyceride accumulation and adipose lipolysis during fatty liver induction in dairy cows. Journal of Dairy Science 92, 4954–4961.

Calpereyra L, Acostadibarrat J, Benech A, Silva SD, Martín A and González-Montaía JR 2012. Ewe pregnancy toxemia. Revista Mexicana De Ciencias Pecuarias 3, 247–264.

Calpereyra L, Benech A, Gonzálezmontaña J, Acostadibarrat J, Da SS and Martín A 2015. Changes in the metabolic profile of pregnant ewes to an acute feed restriction in late gestation. New Zealand Veterinary Journal 63, 141–146.

Calpereyra L, Borteiro C, Benech A, Rodas E, Abreu MN, Cruz JC and González-montaña JR 2009. Histological changes of the liver and metabolic correlates in ewes with pregnancy toxemia. Arquivo Brasileiro De Medicina Veterinária E Zootecnia 61, 306–312.

Chomczynski P and Sacchi N 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162, 156–159.

Firat A and Ozpinar A 2002. Metabolic profile of pre-pregnancy, pregnancy and early lactation in multiple lambing Sakiz ewes. 1. Changes in levels of plasma glucose, 3- hydroxybutyrate and cortisol levels. Annals of Nutrition & Metabolism 46, 57–61.

Graham C and Simmons NL 2005. Functional organization of the bovine rumen epithelium. American Journal of Physiology Regulatory Integrative & Comparative Physiology 288, R173–R181.

Grummer RR 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. Journal of Dairy Science 76, 3882–3896.

Ismail ZA, Almajali AM, Amireh F and Alrawashdeh OF 2008. Metabolic profiles in goat does in late pregnancy with and without subclinical pregnancy toxemia. Veterinary Clinical Pathology 37, 434–437.

Kleppe BB, Aiello RJ, Grummer RR and Armentano LE 1988. Triglyceride accumulation and very low density lipoprotein secretion by rat and goat hepatocytes in vitro. Journal of Dairy Science 71, 1813–1822.

Lacetera N, Bernabucci U, Ronchi B and Nardone A 2001. Effects of subclinical pregnancy toxemia on immune responses in sheep. American Journal of Veterinary Research 62, 1020–1024.

Li P, Li X, Fu S, Wu C, Wang X, Yu G, Long M, Wang Z and Liu G 2012. Alterations of fatty acid β-oxidation capability in the liver of ketotic cows. Journal of Dairy Science 95, 1759–1766.

Li P, Liu M, Long M, Guo Y, Wang Z, He J and Liu G 2013a. Effect of β-hydroxybutyrate on expression of 3-hydroxy-3-methylglutaryl-CoA synthase in bovine hepatocytes cultured in vitro. Feed Industry 21, 15.

Li P, Liu Y, Zhang Y, Long M, Guo Y, Wang Z, Li X, Zhang C, Li X and He J 2013b. Effect of non-esterified fatty acids on fatty acid metabolism-related genes in calf hepatocytes cultured in vitro. Cellular Physiology & Biochemistry 32, 1509–1516.

Lima MS, Pascoal RA and Stilwell GT 2012. Glycaemia as a sign of the viability of the foetuses in the last days of gestation in dairy goats with pregnancy toxaemia. Irish veterinary journal 65, 1–6.

Livak KJ and Schmittgen TD 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Loor J, Dann H, Guretzky N, Everts R, Oliveira R, Green C, Litherland N, Rodriguez-Zas S, Lewin H and Drackley J 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiological Genomics 27, 29–41.

Loor J, Everts R, Bionaz M, Dann H, Morin D, Oliveira R, Rodriguezzas S, Drackley JK and Lewin HA 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiological Genomics 32, 105–116.

Louet JF, Le MC, Pégorier JP, Decaux JF and Girard J 2001. Regulation of liver carnitine palmitoyltransferase I gene expression by hormones and fatty acids. Biochemical Society Transactions 29, 310–316.

Marteniuk JV and Herdt TH 1988. Pregnancy toxemia and ketosis of ewes and does. Veterinary Clinics of North America Food Animal Practice 4, 307–315.

McGarry J and Foster D 1980. Regulation of hepatic fatty acid oxidation and ketone body production. Annual review of biochemistry 49, 395–420.

Odongo N, AlZahal O, Lindinger M, Duffield T, Valdes E, Terrell S and McBride B 2006. Effects of mild heat stress and grain challenge on acid-base balance and rumen tissue histology in lambs. Journal of Animal Science 84, 447–455.

Rodriguez JC, Gil-Gómez G, Hegardt FG and Haro D 1994. Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. Journal of Biological Chemistry 269, 18767–18772.

Rook JS and Herdt TH 2000. Pregnancy toxemia of ewes, does, and beef cows. Veterinary Clinics of North America Food Animal Practice 16, 293–317.

Royo T, Ayté J, Albericio F, Giralt E, Haro D and Hegardt FG 1991. Diurnal rhythm of rat liver cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase. Biochemical Journal 280, 61–64.

Snook LC 1939. Fatty infiltration of the liver in pregnant ewes. Journal of Physiology 97, 238–249.

Wang M, Enzhong LI and Wang G 2014. Histopathological analysis of liver during pregnancy toxemia in small-tailed han sheep. Agricultral Sciences and Technology 3, 470–473.

West HJ 1996. Maternal undernutrition during late pregnancy in sheep. Its relationship to maternal condition, gestation length, hepatic physiology and glucose metabolism. British Journal of Nutrition 75, 593–605.

Williamson DH and Whitelaw E 1978. Physiological aspects of the regulation of ketogenesis. Biochemical Society Symposium 43, 137–161.

Xu C, Liu G, Li X, Xia C, Zhang H and Wang Z 2010. Decreased complete oxidation capacity of fatty acid in the liver of ketotic cows. Asian Australasian Journal of Animal Sciences 23, 312–317.