



## Decreased Complete Oxidation Capacity of Fatty Acid in the Liver of Ketotic Cows<sup>a, \*</sup>

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**ABSTRACT** : Complete oxidation of fatty acid in the liver of ketotic cows was investigated. Serum non-esterified fatty acid (NEFA), beta-hydroxybutyric acid (BHBA) and glucose concentrations were measured using biochemical techniques. Carnitine palmitoyl transferase II (CPT II), 3-hydroxy acyl-CoA dehydrogenase (HAD) and oxaloacetic acid (OAA) concentrations in the liver were detected by ELISA. Serum glucose was lower in ketotic cows than controls ( $p < 0.05$ ). Serum BHBA and NEFA concentrations were higher in ketotic cows than controls ( $p < 0.05$ ). OAA, CPT II, and HAD contents in the liver of ketotic cows were lower than in controls ( $p < 0.05$ ). There were negative correlations between serum NEFA concentration and OAA, CPT II and HAD, but no correlation between serum BHBA concentration and capacity for complete oxidation of fatty acid. Overall, the capacity for complete fatty acid oxidation in the liver of ketotic cows might have been decreased. High serum NEFA concentrations may be unfavorable factors for the pathway of complete oxidation of fatty acid in the liver. (**Key Words** : Dairy Cow, Ketosis, Liver, Fatty Acid Oxidation)

### INTRODUCTION

Ketosis is a major metabolic disorder in cattle and commonly affects many dairy farms. Ketosis is associated with excessive negative energy balance (NEB), accompanied by relatively high levels of ketone bodies and a concurrent low concentration of blood glucose (Grummer, 1995; Melendez et al., 2006). A plasma BHBA concentration of greater than 1,200  $\mu\text{mol/L}$  is a common standard used for diagnosis of subclinical ketosis (Duffield, 2000; Geishhauser et al., 2000).

The main physiological characteristic of peripartum

cows is negative energy balance (Xu et al., 2008). Negative energy balance initiates fat mobilization and a subsequent increase in serum NEFA concentrations. Elevated blood NEFA is closely associated with negative energy balance (Xu et al., 2008). NEFAs are metabolised via four pathways in liver: fatty acid complete oxidation pathway producing  $\text{H}_2\text{O}$ ,  $\text{CO}_2$  and large amounts of energy; fatty acid incomplete oxidation pathway producing ketones and less energy; and NEFAs esterified in the liver and stored as fat or exported as a constituent of very low-density lipoproteins (VLDL) (Grummer, 2008).

Some researchers have reported the details of the pathway of NEFA esterification and exportation. This pathway is enhanced by adding dietary ruminally-protected choline, which can prevent excessive storage of triacylglycerol in the liver, but the ketosis was not changed in those conditions (Kristensen and Raun, 2007; Grummer, 2008). When ketosis occurs, synthesis and secretion of VLDL in the liver decreases (Oikawa, 1997; Yamamoto, 2001). In our view, those pathways may not be the key to prevention of ketosis. Fatty acid complete oxidation in the liver can improve the metabolic process and provide more ATP and diminish ketone concentrations in blood or triacylglycerol accumulation in the liver. However, little research is available for fatty acid complete oxidation in the

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liver of ketotic cows, especially *in vivo* experiments, and the findings are contradictory.

Fatty acid complete oxidation consists of four key steps as mentioned above. CPT II is a important enzyme involved in fatty acid activation, and is positively correlated with transfer velocity of acyl-CoA into mitochondria (Hoppel, 1982). HAD is also an important enzyme for the velocity of fatty acid  $\beta$ -oxidation (Murondoti, 2004). OAA is a key metabolite, which determines the velocity of acetyl CoA into TCA, and finishes complete oxidation of fatty acid. Activity of carnitine palmitoyl transferase I (CPT I), with similar function to CPT II, is positively correlated with liver total lipid, liver triglyceride, liver triglyceride to glycogen ratio, and serum NEFA. However, primary ketosis did not greatly affect CPT I activity or sensitivity, which indicates that alterations of CPT I may not be a major factor in the etiology of primary ketosis (Dann and Drackley, 2005). Instead, the reduction of complete oxidation of fatty acid, which will increase partial oxidation of fatty acid, following parturition may contribute to postpartum ketosis, because of the decreased activity of acetyl-CoA carboxylase, fatty acid synthase and 3-hydroxy-acyl-CoA dehydrogenase (Murondoti et al., 2004). Therefore, it seems that fatty acid metabolism (complete oxidation or partial oxidation) in the liver and the development of ketosis are closely related (Grummer et al., 1993). The objective was to determine the metabolic process of fatty acid complete oxidation, during conditions of high NEFA concentration, in the liver of ketotic cows.

## MATERIALS AND METHODS

### Animal selection and sample collection

All animals used in this study were treated according to the International Guiding Principles for Biomedical Research Involving Animals. From a commercial dairy farm (Qingxin) located in Heilongjiang Province, China, we selected 20 lactating cows of the same breed, age, milk production, and body condition score at calving. Cows were fed the same diets during the dry period and early lactation. All samples were collected before feeding and milking. At

30 days after calving, blood samples were taken from the jugular vein before the liver biopsies were obtained. Jugular vein blood was centrifuged immediately after collection at 4,000 g for 10 min, and plasma was collected and frozen at  $-20^{\circ}\text{C}$ . All blood samples were handled similarly and decoagulant was not used. Cow liver tissue samples were taken from the 11th or 12th right intercostal space by liver transfixion pin. All liver tissue biopsies were quickly washed in a cold rinse buffer containing 0.2 mM protease inhibitor cocktail (protease inhibitor, Roche), oxacillin (25  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich), gentamycin (50  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich), penicillin (100 U/ml; Sigma-Aldrich), streptomycin (100  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich), amphotericin B (0.25  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich), and nistatin (50 U/ml; Sigma-Aldrich) to remove cell debris and blood, and were then frozen by immersion in liquid nitrogen (Chuang and Zhe, 2008). According to blood parameters and clinical signs, ten cows were diagnosed as ketotic ( $\beta$ -hydroxybutyric acid  $>1.2$  mmol/L, glucose  $<2.5$  mmol/L, and NEFA  $>500$   $\mu\text{mol}/\text{L}$ ), and ten cows were considered normal ( $\beta$ -hydroxybutyric acid  $<0.60$  mmol/L, glucose  $>3.75$  mmol/L, and NEFA  $<400$   $\mu\text{mol}/\text{L}$ ). The standard for ketosis in the cow is a plasma  $\beta$ -hydroxybutyric acid  $>1.2$  mmol/L (Ingvarsen 2006; Melendez et al., 2006), while plasma glucose and NEFA are the reference factors. All ketotic cows had the following clinical parameters confirmed: loss of appetite, decrease in milk production, rapid deterioration of body condition, hypoglycemia and hyperketonemia. All control cows had normal body temperature, pulse rate and breathing. Plasma results of the ten ketotic and ten normal cows are shown in Table 1.

### Serum concentrations

The concentration of plasma glucose (glucose oxidase method, Yulan Biotechnology Research Institute),  $\beta$ -hydroxybutyric acid (enzymatic-rate method, Randox Clinical Diagnostic Company), and NEFA (enzymatic method, Yulan Biotechnology Research Institute) were analyzed according to operating manuals (Xu and Wang, 2008).

**Table 1.** Concentrations of serum glucose, BHBA and NEFA, and OAA, CPT II and HAD content in the liver of experimental cows (mean $\pm$ SEM)

	Glucose* (mmol/L)	BHBA (mmol/L)	NEFA ( $\mu\text{mol}/\text{L}$ )	OAA (mg/mgprot)	CPT II ( $\mu\text{g}/\text{mgprot}$ )	HAD (ng/mgprot)	Total protein in liver (mg)
Ketotic (ten cows)	2.86 $\pm$ 0.6 <sup>a</sup>	1.70 $\pm$ 0.30 <sup>a</sup>	1,154.29 $\pm$ 431.95 <sup>a</sup>	0.53 $\pm$ 0.18 <sup>a</sup>	4.10 $\pm$ 0.10 <sup>a</sup>	35.77 $\pm$ 8.21 <sup>a</sup>	2.88 $\pm$ 0.16 <sup>a</sup>
Healthy (ten cows)	4.74 $\pm$ 1.2 <sup>b</sup>	0.42 $\pm$ 0.15 <sup>b</sup>	208.99 $\pm$ 118.92 <sup>b</sup>	1.15 $\pm$ 0.73 <sup>b</sup>	8.30 $\pm$ 4.50 <sup>b</sup>	73.66 $\pm$ 36.92 <sup>b</sup>	3.02 $\pm$ 0.19 <sup>b</sup>

\* Ketotic cows were defined by a  $\beta$ -hydroxybutyric acid  $>1.2$  mmol/L; Normal cows were defined by  $\beta$ -hydroxybutyric acid  $<0.6$  mmol/L (Ingvarsen, 2006; Melendez et al., 2006); Ketotic cows had the following clinical parameters confirmed: loss of appetite, decrease in milk production, and rapid deterioration of body condition, hypoglycemia and hyperketonemia.

Different letters, <sup>a</sup> and <sup>b</sup>, indicate significant difference between the parameters of ketotic and healthy cows.

### Protein content of liver tissue

The purpose of determining protein content in the liver tissue was to calculate the weight of OAA, CPT II and HAD per unit of liver protein. The unit weights of OAA, CPT II and HAD are displayed as  $\mu\text{g}/\text{mg}$  protein and shown in Table 1. By using this method, we corrected for experimental error caused by liver weight.

Each liver sample (approximately 20 mg) was crushed in a mortar containing liquid nitrogen, and mixed with 400  $\mu\text{l}$  buffer solution (w/v of liver and solution buffer was 1:20) containing 50 mM Tris-HCl. Extracted liver tissue was centrifuged for 10 min at 2,500 g and the supernatant was collected. 10  $\mu\text{l}$  of sample supernatant was diluted 100 times and used to determine protein concentration. The protein concentration of liver tissue extracted from ketotic and normal cows was determined according to Bradford (Bradford, 1976) using BSA as a standard curve. The results are displayed in Table 1.

### OAA, CPT II and HAD content of liver tissue

OAA was measured using ADL goat anti-cow oxaloacetic acid kit, catalogue no. QRCT-3013321101101EIA\UTL, Lot no. RT110371 (Adlitteram Diagnostic Laboratories) according to the manufacturer's instructions. The minimum detectable concentration of OAA was 0.01 mg/ml. Briefly, standards and liver samples from ketotic and normal cows were incubated with biotinylated OAA in micro-titration wells, which had been coated with another OAA of defined and unique epitope specificity. After incubation and washing, the wells were treated with streptavidin labeled with horseradish peroxidase (HRP). The wells were again washed, a TMB substrate solution was added to the wells and color developed in proportion to the amount of bound OAA. The stop solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

CPT II was measured using ADL goat anti-cow carnitine palmitoyl transferase 2 kit, catalogue no. QRCT-3013321101223EIA\UTL, Lot no. RT110371 (Adlitteram Diagnostic Laboratories) according to the manufacturer's instructions. The minimum detectable concentration of CPT II was 0.01  $\mu\text{g}/\text{ml}$ . HAD was measured using ADL goat anti-cow hydroxylacyl-CoA dehydrogen kit, catalogue no. QRCT-3013321011011EIA\UTL, Lot no. RT110371 (Adlitteram Diagnostic Laboratories) according to the manufacturer's instructions. The minimum detectable concentration of HAD was 1.0 ng/ml. The determination mechanism, method and procedure of CPT II and HAD were the same as OAA. The OAA, CPT II and HAD content of liver tissue from ketotic and healthy cows are presented in Table 1.

### Statistical analysis

The data were analyzed using SPSS software. Results were expressed as the mean $\pm$ SD. Comparisons between ketotic and normal cows for serum and liver parameters were made using ANOVA. *p* values of  $\leq 0.05$  were considered significant.

## RESULTS

### BHBA, HEFA and glucose concentrations of ketotic and normal cows

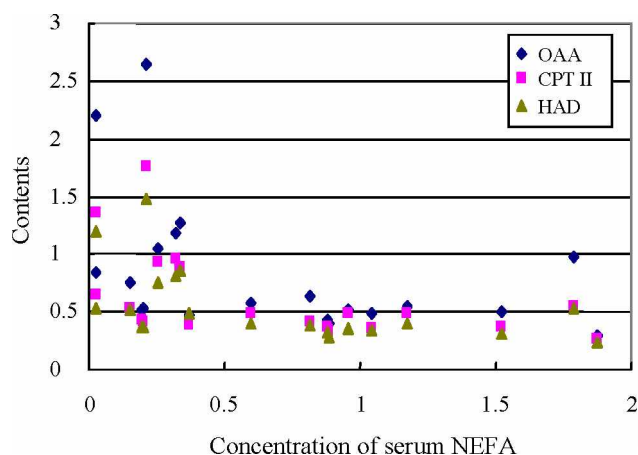
The concentration was expressed as mean $\pm$ standard deviation. Serum BHBA concentration of the ketotic group was  $1.70\pm 0.30$  mmol/L, serum glucose concentration was  $2.86\pm 0.60$  mmol/L and NEFA concentration was  $1,154.29\pm 431.95$   $\mu\text{mol}/\text{L}$ . Serum BHBA concentration of the control group was  $0.42\pm 0.15$  mmol/L, glucose concentration was  $4.74\pm 1.20$  mmol/L and NEFA concentration was  $208.99\pm 118.92$   $\mu\text{mol}/\text{L}$  (Table 1). The differences in BHBA, NEFA and glucose concentrations between ketotic and normal cows were significant ( $p < 0.05$ ). Compared with control cows, ketotic cows were hypoglycemic, hyperketonemic and had higher NEFA in the blood.

### OAA, CPT II and HAD content in the liver of ketotic and normal cows

Liver OAA content of the ketotic group was  $0.53\pm 0.18$  mg/mg protein, CPT II was  $4.10\pm 0.10$   $\mu\text{g}/\text{mg}$  protein and HAD was  $35.77\pm 8.21$  ng/mg protein. Liver OAA content of the control group was  $1.15\pm 0.73$  mg/mg protein, CPT II was  $8.30\pm 4.50$   $\mu\text{g}/\text{mg}$  protein and HAD was  $73.66\pm 36.92$  ng/mg protein (Table 1). The differences in liver OAA, CPT II and HAD content between ketotic and control cows were significant ( $p < 0.05$ ). The OAA, CPT II and HAD contents in the liver of ketotic cows were lower than normal cows, which indicated lower complete oxidation of fatty acid capability in liver of ketotic cows.

### The relationship between serum glucose, BHBA, NEFA and OAA, CPT II, HAD content in the liver of ketotic and control cows

There was no obvious correlation between concentrations of serum glucose or BHBA and OAA, CPT II, and HAD content in the liver of experimental cows. There was negative correlation between serum NEFA concentration and OAA ( $r = -0.58$ ), CPT II ( $r = -0.61$ ), and HAD ( $r = -0.65$ ) content (Figure 1). The maximally suppressed concentration of NEFA was at approximately 0.5 mM. With rising serum NEFA concentration, the OAA, CPT II and HAD contents decreased. High concentrations



**Figure 1.** Correlation between serum NEFA concentration and OAA, CPT II and HAD content in the liver of experimental cows. There was a negative correlation between serum NEFA concentration and OAA ( $r = -0.58$ ), CPT II ( $r = -0.61$ ), HAD ( $r = -0.65$ ) in liver of experimental cows. With a rising serum NEFA ( $\mu\text{mol/L}$ ) concentration, the OAA (mg/mgprot), CPT II ( $\mu\text{g/mgprot}$ ) and HAD (ng/mgprot) content in the liver decreased. High serum NEFA caused down-regulation of fatty acid oxidation capacity in the liver of ketotic cows.

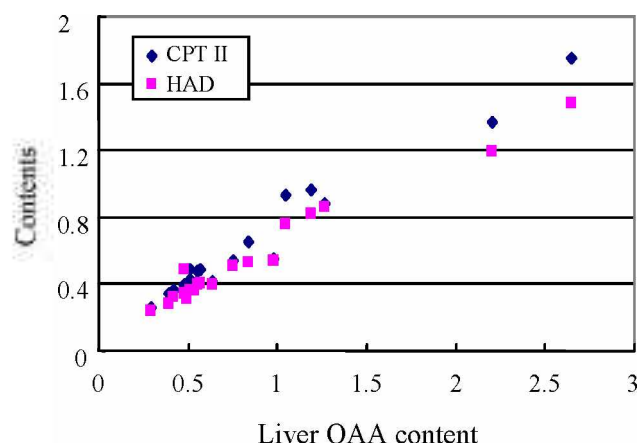
of NEFA may alter the metabolic process of fatty acid complete oxidation in the liver of ketotic cows. Indeed this needs more experimentation.

#### The relationship between OAA, CPT II and HAD in the liver of experimental cows

There were positive correlations between OAA and CPT II ( $r = 0.98$ ), HAD ( $r = 0.98$ ) in the liver. With rising liver OAA content, the HAD and CPT II content in the liver also increased (Figure 2).

### DISCUSSION

Ketosis is a major disease associated with negative energy balance. Increased energy demand because of lactation and/or fetal development and decreased energy intake because of depressed appetite before the disease results in ketosis will result in quick blood glucose drawdown and body fat mobilization (Xu et al., 2008). Therefore, blood NEFA concentration is subsequently increased. The liver is the most active metabolic organ for cows (Melendez et al., 2006). Therefore, metabolism of blood NEFA, in the liver, may be very important for NEB diseases such as ketosis. However, how metabolism of high concentrations of NEFA occurs in liver is not clear and little reported. Esterification and exportation of NEFA as VLDL in the liver can be enhanced through additional dietary ruminally-protected choline, but the storage of fat in liver was not relieved and ketosis was not prevented (Kristensen and Raun, 2007; Grummer, 2008). When ketosis occurred,



**Figure 2.** The correlation among OAA, CPT II and HAD in the liver of experimental cows. There were positive correlations between OAA and CPT II ( $r = 0.98$ ), and HAD ( $r = 0.98$ ) in the liver of experimental cows. With a rise in liver OAA content, HAD and CPT II in the liver also increased. The role of OAA, CPT II and HAD in fatty acid oxidation might be integrated.

synthesis and secretion of VLDL in the liver was also decreased (Oikawa, 1997; Yamamoto, 2001). Increased supplementation of carnitine can promote transport of activated fatty acid into mitochondria and accelerate  $\beta$ -oxidation of activated fatty acid (Carlson et al., 2007). However, as a result of greater hepatic fatty acid partial oxidation, plasma  $\beta$ -hydroxybutyric acid was higher for carnitine supplementation groups (Carlson et al., 2006; Carlson et al., 2007). However, the concentration of non-esterified fatty acid in serum was not affected by carnitine (Carlson et al., 2007) and how the TCA cycle (complete oxidation pathway) is affected is unknown. So, the enhanced pathways of VLDL synthesis and secretion and NEFA esterification in liver do not relieve NEB. In our previous research using comparative proteomic techniques, we observed that the expression of acetyl-Coenzyme A acetyltransferase 2 and 3-hydroxyacyl-CoA dehydrogenase type-2, those enzymes involved in fatty acid degradation, were decreased in the liver tissues of ketotic cows, which suggested that the ability to utilize fatty acids to produce  $\text{CO}_2$ ,  $\text{H}_2\text{O}$  and ATP was decreased (Xu and Wang, 2008). As a consequence, fatty acids may be accumulated in hepatic cells (Jonas et al., 1978), and the fatty acid complete oxidation process decreased (Murondoti, 2004). Therefore, liver fatty acid complete oxidation may be a key factor involved in decreasing BHBA and providing more ATP to prevent ketosis, but details of this metabolic pathway, especially during high NEFA concentration, are unknown. Here, two key enzymes and one key metabolite of the fatty acid oxidation pathway were determined. OAA, CPT II and HAD can reflect the metabolic conditions in respective steps of fatty acid complete oxidation in the liver.

Serum BHBA and NEFA concentrations in ketotic cows

were higher than normal cows ( $p < 0.05$ ), but the serum glucose concentration was lower ( $p < 0.05$ ). This result was similar to previous findings (Xu et al., 2008). The OAA, CPT II and HAD contents in the liver of ketotic cows were lower than in normal cows ( $p < 0.05$ ), and there was a significant negative correlation between serum NEFA concentration and OAA, CPT II and HAD contents. This indicates that the pathways of acyl-CoA entry into mitochondria,  $\beta$ -oxidation of activated fatty acid and the TCA cycle were weakened in ketotic cows. As a consequence, the metabolic capability of fatty acid complete oxidation in the liver of ketotic cows might be weakened. There was significant correlation between concentrations of serum NEFA and liver OAA, CPT II and HAD content. So, the weakened fatty acid complete oxidation pathway may be related to high NEFA, but more experimentation is needed to confirm. Weakened metabolism of fatty acid complete oxidation may be the key factor of ketosis development. There was no correlation between serum BHBA or glucose and liver OAA, CPT II and HAD content. This indicates that the effect of high serum BHBA and low glucose on fatty acid oxidation was not obvious.

Murondoti reported that the activities of acetyl-CoA carboxylase, 3-hydroxy-acyl-CoA dehydrogenase and fatty acid synthase in the liver were decreased in experimental cows following parturition (Murondoti et al., 2004). Hepatic citrate synthase activity was decreased in experimental cows after parturition. Therefore, *de novo* fatty acid synthesis in the liver after parturition may be reduced. The reduction in fatty acid oxidation following parturition may contribute to postpartum ketosis (Murondoti et al., 2004). Our findings were in accordance with Murondoti et al. (2004). Using gene microarray and quantitative PCR methods, Loor et al. (2007) reported that the genes of fatty acid uptake or transport in the liver of ketotic cows were decreased. They also reported that the ability to produce ATP may have been decreased in ketotic cows. This implies that fatty acid complete oxidation capacity may be weakened. These results were similar to our findings.

In the present study, there were significant positive correlations between OAA, CPT II and HAD content in the liver of experimental cows. This result indicates that fatty acid oxidation, fatty acids entering mitochondria, and the TCA cycle were interrelated.

### CONCLUSIONS

Fatty acid complete oxidation capability was decreased in the liver of ketotic cows. Decreased complete oxidation of fatty acid in liver will provide little ATP, which cannot reduce NEB and may be a factor causing ketosis. There was

a significant negative correlation between the concentration of serum NEFA and capacity of fatty acid complete oxidation. A high concentration of serum NEFA may play a negative effect on the metabolic pathways of fatty acid complete oxidation.

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