SHORT COMMUNICATION

Urine Proteomics Analysis of Dairy Cows with Fatty Liver

C Xu[1,§], YH Sun[1,§], W Yang[1], L wu[1], HY Zhang[1] and C Xia[1,2,3]*

[1]College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, 163319, P.R. China; [2]Synergetic Innovation Center of Food Safety and Nutrition, Northeast Agricultural University, Harbin, 150030, P.R. China; [3]Key Laboratory of Veterinary Medicine of Heilongjiang Bayi Agricultural University in Heilongjiang Provincial University, Daqing 163319, P.R. China
*Corresponding author: xcwlxyf2014@163.com

A B S T R A C T

The objective of this study was to screen and identify differential proteins in the urine samples from cows with fatty liver disease (FLD) in order to provide new biomarkers for FLD. In this trial, twenty urine specimen were collected and assigned into two groups. After labelling with an i-TRAQ reagent, differential proteins in the urine samples were separated using two dimensional chromatography, and then identified by tandem mass spectrometry. The results showed that 110 differential proteins were screened: 50 were upregulated and 60 were down-regulated in the urine of cows with FLD. Among of these proteins, vitronection (VTN), neutrophil gelatinase-associated lipocatin (LCN2), prothrombin (F2), and clusterin (CLU) were the key regulatory factors determined by gene function, signaling pathway, and gene networks analysis. So, it is believed that the 4 key regulatory factors may become important biomarkers of FLD, which will provide new clinicopathologic insights into FLD in dairy cows.

To Cite This Article: Xu C, Sun YH, Yang W, Wu L, Zhang HY and Xia C, 2016. Urine proteomics analysis of dairy cows with fatty liver. Pak Vet J, 36(1): 109-111.

INTRODUCTION

Fatty liver disease (FLD) in dairy cows, also known as fatty cow syndrome, is an energy metabolism disorder that usually occurs at calving or during the transition period (Yin *et al*., 2015; Yang *et al*., 2015). To date, two problems have not been solved in cow FLD. The first is lack of early and specific diagnosis; the second is the low cure and high mortality rates due to the lack of effective drugs. Currently, measurement of liver fat is the most common method for clinical diagnosis. However, this method has some defects, such as injury, infection, and higher cost. Some biochemical parameters in the blood are also important indicators to assist in the diagnosis of cow FLD, such as BHBA, ALT, and AST. However, these parameters have a low specificity (Xu *et al.*, 2008). So, we aimed to screen and identify biomarkers, and to investigate proteomics profiles for FLD using i-TRAQ technology, in order to lay the theoretical and methodological basis for early diagnosis and etiology of FLD.

§Contributed equally to this work.

MATERIALS AND METHODS

Animals: The study was administrated strictly according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. One hundred productive Holstein cows in 7th to 24th day postpartum were selected from one commercial dairy farm. These Holstein cows had the similar age (3-5 years old), parity (1-3), and feed according to the Chinese experimental test standard during the dry period and early lactation.

Hepatic TG content test and grouping: The hepatic TG content test is a particularly effective method to detect bovine FLD, called "gold standard". At first, the cows were conducted with local anesthesia in procain on the skin around their 11th or 12th right intercostal space. Then, liver tissue samples were taken from this space by a tailormade biopsy needle. Subsequently, the liver TG content was calculated. Finally, ten cows with FLD (average 35.53% in liver TG contents) and ten healthy cows (average 13.32% in liver TG contents) were selected respectively as group T and C without other diseases in this study.

Urine samples: Each 20 ml of cow urine in group T and C was collected with catheters before feeding and milking in the morning. All samples were centrifuged at 6000 rpm for 10 min, then the supernatant was taken, sealed, and frozen at -80ºC. In addition, some biochemical parameters in two groups were measured (Table 1).

ITRAQ labeled peptides and mass spectrometry: Group T and C were labeled for the use of the iTRAQ kit (SCIEX Company, Framingham, Massachusetts, USA). Samples were automatically loaded onto a peptide clip (Agilent Technologies, Wilmington, DE, USA), and were separated by chromatographic column (Agilent Technologies, Wilmington, DE, USA). The LTQ VELOS (Thermo Finnigan, San Jose, CA, USA) was used to perform mass spectrometry analysis for the hydrolysates, after these hydrolysates were desalted and separated by capillary HPLC.

Data analysis: Raw mass data analysis: The literature was searched for the Bovidae. REVERSED. fasta protein libraries, SEQUEST results filter parameters: False discovery rate (FDR)≤0.05. After collating the protein data, differential proteins were screened using two as the threshold. Gene Ontology (GO) analysis: Different functional concepts were organised into a directed acyclic graph (DAG) structure, or gene function classification system for each species, and a comprehensive summary of gene function using software R (http://www.r-project.org/).

Network analysis: We analyzed gene mutual relationships genome-wide to integrate the relationship between the gene networks. The network did graphics displays via Medusa software. Protein-protein interaction data from the Mammalian Protein-Protein Interaction (MIPS) database.

RESULTS

Differential proteins: Fifty species differential proteins were down-regulated proteins, including 34 cattle proteins included in the database, 13 human proteins included in the database, and three kinds of other animal proteins. In addition, 60 species were up-regulated proteins, in which 34, 20, and six were serially included in cattle, human, and swine species databases. The remaining proteins were included in other species databases.

GO analysis: As the Fig. 1 shown, one hundred and ten kinds of proteins were classified, then using a directed acyclic graph to represent the biological processes involved in cell location and molecular function.

Gene network analysis: As the Fig. 2 and Fig. 3, CLU, VTN, LCN2, and F2. CLU had the greatest correlation, appeared most, and was the control center of the gene network diagram, playing a crucial role in the fatty liver disease process, primarily because of greatest gene index, with CLU having the most radical and direct effect.
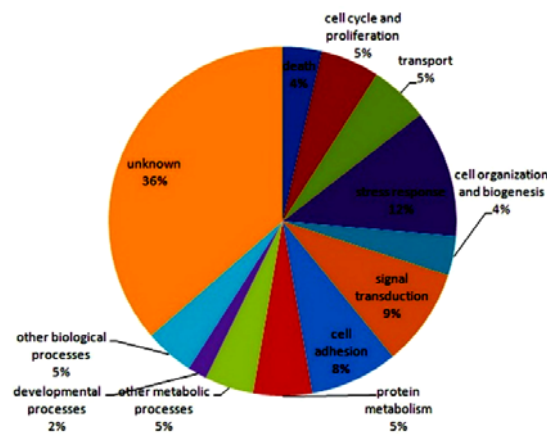

Fig. 1 GO classification of the proteome in the urine of cows with FLD. The 110 differential proteins were involved in various functions, including 5% cell cycle and proliferation, 5% transport, 36% unknown, and so on.
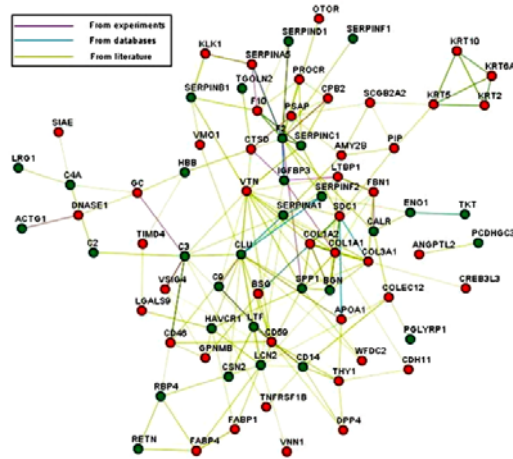

Fig. 2 Gene network analysis diagram. Note: Red circle represents the up-regulated proteins, green circle represents down-regulated proteins, line represents the link between each other, the genes have higher connection degree which is the gene network control centers, playing an important role.
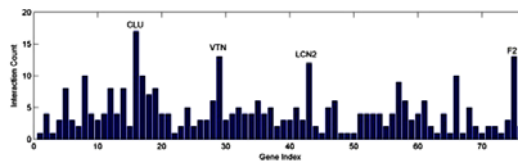

Fig. 3 Enrichment of relevant proteins in various biological processes. Notes: the abscissa represents the gene index and the ordinate axis represents the frequency of the protein.

DISCUSSION

Through analysis of related biological proteins, we found 4 critical factors that would influence the occurrence and development of FLD using different signaling pathways, following a specific adjustment procedure as described below.

Prothrombin (F2): F2 controls the synthesis of the prothrombin-thrombin precursor, shifting soluble fibrinogen into insoluble fibrin chains and catalyzing

Table I: Biochemical parameters in cows with fatty liver disease (n=10) and healthy cows (n=10)

Groups	Hepatic TG content (%)	BHBA (mmol/L)	TG (mmol/L)	GLC (mmol/L)	AST (U/L)
Affected Cows	35.53 ± 0.15^x	1.60 ± 0.62^x	0.28 ± 0.07^x	3.71 ± 1.11	73.30 ± 12.86
Control	13.32 ± 0.02^y	0.79 ± 0.11^y	0.16 ± 0.04^y	3.93 ± 1.35	76.32 ± 47.05
P value	0.023	0.018	0.035	0.161	0.833

Values (mean±SD) bearing different letters in a column differ significantly (P<0.05). TG=triglyceride; BHBA=β-hydroxybutyric acid; GLC=glucose; AST=aspartate aminotransferase.

many other coagulation reactions. According to results from the KEGG pathway database, F2 is mainly involved in actin cytoskeleton regulation; reduction of F2 can lead to F-actin decrease. Therefore, F2 is not only involved in regulation of blood coagulation, but also in regulating muscle contraction. Meanwhile, F2 is a potent inhibitor of calcium oxalate crystal growth, present in undiluted human urine and inorganic conditions.

Vitronectin (VTN): According to the results of the KEGG pathway database, VTN was primarily present in the extracellular matrix-receptor response pathway (ECM), reducing the ECM, directly causing actin to decrease in the adhesion pathway. In other words, an increase in VTN can adjust downward against actin by the ECM process.

Actin exists in cells in two forms, including free monomer and filament (F-Actin) protein polymer. It was believed that ATP was the main factor of regulating actin assembly of kinetic instability behavior (Atkinson *et al.*, 2004). The extension of the actin assembly was regulated by ATP. When ATP-actin was binding to the end of the fiber, the conformation of actin would change and ATP would hydrolyze to ADP+Pi. ADP-actin had a lower affinity with the fiber ends easily fell off from the end, shortening the fiber. When cows were suffering from FLD, β-oxidation of fatty acid decreased and there was not enough acetyl coenzyme A accessing into the citric acid cycle and generating enough ATP (Rukkwamsuk *et al.*, 2005), so that concentration of ATP-actin reduced, finally, leading to F-Actin shortening.

When cows with FLD had F-Actin down-regulated, concentrations of VTN increased and that of F2 was decreased. Therefore we believed that VTN and F2 were closely related to with cows FLD and impacted disease development.

Neutrophil gelatinase-associated lipocalin (LCN2): LCN2 was also associated with liver regeneration, and can be used as a sensitive biomarker for liver function (Sultan *et al.*, 2013). In addition, FLD affects biological functions of proteins, especially high density lipoprotein (HDL). In cow FLD, HDL particles combined a large number of globin and plasma amyloid A, making apolipoprotein AI and apolipoprotein C-III concentration decreased. Apolipoprotein AI can activate lecithin-cholesterol acyl-transferase, making lecithin phosphate from cholesteryl and lysophosph atidylcholine esterifying to free cholesterol. In cow FLD, cholesterol esters, very low density lipoprotein (VLDL), low-density lipoprotein (LDL) declined, so that the transport of triglycerides decreased, resulting in FLD via a vicious cycle. In short, the damaged liver produced LCN2, affecting the function of the protein, and leading to a reduction of apolipoproteins; in turn, reduction of apoli-poproteins exacerbates FLD.

Clusterin (CLU): Clusterin was positively correlated to LDL, HDL, and cholesterol, and was negatively correlated with TG, which is key to metabolic syndrome (Hoofnagle *et al.*, 2010). In other words, when Clusterin was down-regulated, TG increased and LDL decreased. The reduction of LDL led to TG accumulation in the liver, coupled with elevated TG, commonly exacerbating the occurrence of FLD. So, down-regulated clusters were consistent with FLD, and can be considered as a biomarker of FLD.

Conclusions: F2 and VTN regulated energy metabolism through the signal pathway and LCN2 and CLU controlled fat accumulation in the liver through regulating VLDL-carried TG, closely contributing to the pathogenesis of FLD.

Author's contribution: CX and YHS executed the experiment and analyzed the sera and tissue samples. WY analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

REFERENCES

Atkinson SJ, Hosford M and Molitoris B, 2004. Mechanism of actin polymerization in cellular atp depletion. J Biol Chem, 279: 5194-5199.

Hoofnagle AN, Wu M, Gosmanova A, Becker J, Wijsman E *et al.*, 2010. Low clusterin levels in high-density lipoprotein associate with insulin resistance, obesity, and dyslipoproteinemia. Arterioscl Throm Vas, 30: 2528-2534.

Rukkwamsuk T, Rungruang S, Choothesa A and Wensing T, 2005. Effect of propylene glycol on fatty liver development and hepatic fructose 1, 6 bisphosphatase activity in periparturient dairy cows. Livest Prod Sci, 95: 95-102.

Sultan S, Cameron S, Ahmad S, Malik I, Schultze F *et al.*, 2013. Serum lipocalin2 is a potential biomarker of liver irradiation damage. Liver Int, 33: 459-468.

Xu C, Wang Z, Liu G, Li X, Xie G *et al.*, 2008. Metabolic characteristic of the liver of dairy cows during ketosis based on comparative proteomics. Asian-Aust J Anim, 21: 1003-1010.

Yang W, Wu DJ, Zhang RH, Deng QH, Ding HY *et al.*, 2015. Effects of propylthiouracil-induced hypothyroidism on nonalcoholic fatty liver disease in rats fed a high-fat and high-cholesterol diet. Pak Vet J, 35: 1-6.

Yin L, Qin X, Deng Q, Zhang Y, Lei L *et al.*, 2015. The isolation of pre-adipocytes from dairy cow adipose tissue and the development of pre-adipocytes into mature adipocytes. Pak Vet J, 35: 283-288.