

Differentially expressed serum proteins associated with calcium regulation and hypocalcemia in dairy cows

Shi Shu¹, Yunlong Bai¹, Gang Wang¹, Xinhuan Xiao¹, Ziling Fan¹, Jiang Zhang¹, Chang Zhao¹, Yang Zhao¹, Cheng Xia^{1,*}, and Hongyou Zhang¹

* **Corresponding Author:** Cheng Xia
Tel: +86-4596819207, **Fax:** +86-4596819207,
E-mail: xcwlyxf2014@163.com

¹ College of Animal Science and Veterinary Medicine,
Heilongjiang Bayi Agricultural University, Daqing
163319, China

Submitted Aug 18, 2016; Revised Sept 22, 2016;
Accepted Nov 2, 2016

Objective: Hypocalcemia is an important metabolic disease of dairy cows during the transition period, although the effect of hypocalcemia on biological function in dairy cows remains unknown.

Methods: In this study, proteomic, mass spectrum, bioinformatics and western blotting were employed to identify differentially expressed proteins related to serum Ca concentration. Serum samples from dairy cows were collected at three time points: 3rd days before calving (day -3), the day of calving (day 0), and 3rd days after calving (day +3). According to the Ca concentration on day 0, a total of 27 dairy cows were assigned to one of three groups (clinical, subclinical, and healthy). Samples collected on day -3 were used for discovery of differentially expressed proteins, which were separated and identified via proteomic analysis and mass spectrometry. Bioinformatics analysis was performed to determine the function of the identified proteins (gene ontology and pathway analysis). The differentially expressed proteins were verified by western blot analysis.

Results: There were 57 differential spots separated and eight different proteins were identified. Vitamin D-binding protein precursor (group-specific component, GC), alpha-2-macroglobulin (A2M) protein, and apolipoprotein A-IV were related to hypocalcemia by bioinformatics analysis. Due to its specific expression (up-regulated in clinical hypocalcemia and down-regulated in subclinical hypocalcemia), A2M was selected for validation. The results were consistent with those of proteomic analysis.

Conclusion: A2M was as an early detection index for distinguishing clinical and subclinical hypocalcemia. The possible pathogenesis of clinical hypocalcemia caused by GC and apolipoprotein A-IV was speculated. The down-regulated expression of GC was a probable cause of the decrease in calcium concentration.

Keywords: Dairy Cows; Hypocalcemia; Proteomic; Bioinformatics

INTRODUCTION

Hypocalcemia is an important nutritional metabolic disease of dairy cows that occurs from 0 to 3 days after calving and is characterized by a reduction in serum calcium (Ca) concentration that does not return to normal for several days. Previous studies have reported that the incidence of hypocalcemia in dairy cows is 3.45% in North America, 6.17% in Europe, and 3.5% in Australia [1].

Hypocalcemia can be divided into two stages: clinical hypocalcemia (CH) and subclinical hypocalcemia (SH). According to the Merck Veterinary Manual (<http://www.merckmanuals.com/vet/>), the normal serum Ca concentration in the cow is 2.25 to 2.5 mmol/L, while the Ca concentrations in CH and SH are defined as 0.5 to 1.75 and <2.0 mmol/L, respectively. Hypocalcemia reduces the ability of immune cells to respond to stimuli [2], thus contributing to the

onset of infection, such as mastitis. Furthermore, hypocalcemia reduces smooth muscle contraction, leading to reduced rumen and abomasal motility, resulting in abomasal displacement and reduced feed intake. The effects of hypocalcemia on muscle contraction prevent efficient teat closure, which also contributes to the onset of mastitis [3]. It is well known that Ca concentration is the “gold standard” for the diagnosis of hypocalcemia at 0 to 3 days after calving. Upon diagnosis, cows will rapidly succumb to hypocalcemia, especially CH. In fact, many proteins are known to affect Ca regulation in dairy cows. Shu and Wang reported 12 differentially expressed proteins related to CH and SH by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry [4,5].

Proteomics is a relatively new technology in bovine research and is widely used in other areas of research, such as human cancers [6], plant science [7], immunology, and parasitology. Two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry offers a comprehensive proteomic approach [8], while fluorescence two-dimensional differential gel electrophoresis (2D-DIGE) technology adds a quantitative component to conventional 2-DE analyses, allowing for direct comparison of changes in protein abundance across multiple samples simultaneously, with statistical confidence and without interference due to gel-to-gel variation [9,10]. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) is often used to identify proteins discovered by 2D-DIGE.

Serum samples from healthy dairy cows and those with CH and SH were analyzed by 2D-DIGE/MALDI-TOF-MS combined with bioinformatics to identify differentially expressed proteins that affect serum Ca regulation and to elucidate the cause of the reduction in serum Ca concentration after calving and determine why the Ca concentration is not restored to normal for several days after calving.

MATERIALS AND METHODS

Experimental animals

All experimental animals were obtained from an intensive dairy farm in accordance with the requirements of the Veterinary Medical Ethical Committee of the Local Agricultural Department of Mishan, Heilongjiang province, China. All experimental animals were maintained in accordance with the International Guiding Principles for Biomedical Research Involving Animals. All cows were fed a total mixed ration (TMR) during the pre-partum period, which consisted of 9 kg of concentrated feed, 17 kg of silage maize, 4 kg of hay, and 400 g of fat. The nutritional analysis of the TMR was 55.60% dry matter, 16% crude protein, 5.60% fat, 39.10% neutral detergent fiber, 20.30% acid detergent fiber, and 1.75 mcal/net energy for lactation, 180 g of Ca, 116 g of phosphorus.

Grouping

Blood samples were collected from randomly selected Holstein cows at three time points: 3rd day before calving (day -3), day of calving (day 0), and 3rd day after calving (day +3). None of the cows showed any clinical symptoms on day -3 and diseases other than hypocalcemia on day 0 were excluded. The cows were assigned to one of three groups based on serum Ca concentration on day 0: the CH group (n = 9), obvious clinical symptoms of hypocalcemia and Ca concentration of <1.80 mmol/L; the SH group (n = 9), no clinical symptoms of hypocalcemia and Ca concentrations of 1.80 to 2.00 mmol/L; and a control (C) group (n = 9), no clinical symptoms of hypocalcemia and Ca concentrations of 2.00 to 3.00 mmol/L.

Blood parameter analysis

Blood samples (10 mL) from all tested cows were collected from the caudal vein at three time points in accordance with the International Guiding Principles for Biomedical Research Involving Animals. The samples were centrifuged immediately at 4,000 g for 10 min. Then, the serum fraction was frozen in liquid nitrogen and stored at -80°C until subsequent analyses. The serum Ca concentration was detected using a commercial kit (651564-01; Roche Diagnostics, Mannheim, Germany) with an automatic biochemical analyzer (Modular DPP; Roche Diagnostics, Germany). The serum Ca concentrations at the three time points are shown Figure 1 and group values are shown in Supplementary Table S1.

To reduce individual differences, every sample for 2D-DIGE was obtained from serum samples of three animals. The test design is depicted in Table 1 and the study strategy is shown in Supplementary Table S2.

Protein processing

Serum samples were thawed at room temperature and pooled in accordance with the study design. Protein concentrations were measured using the Bradford protein assay. Lysis buffer and 3.5

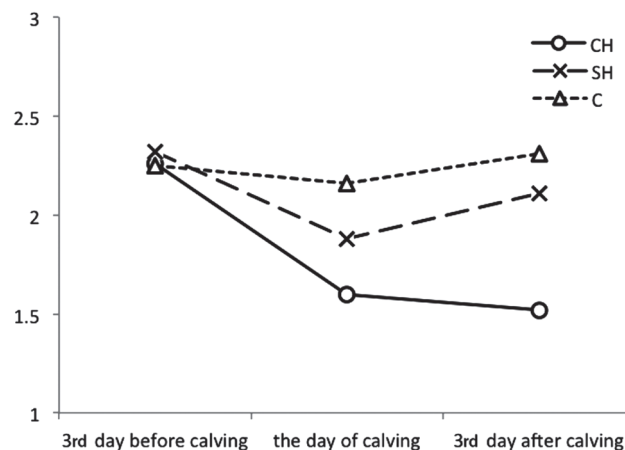


Figure 1. Ca concentration at three time points. The mean Ca concentration in each group at three time points.

Table 1. The design of fluorescence-labeled samples¹⁾

Gel-ID	Label ²⁾		
	Cy2	Cy3	Cy5
Gel 1	Interior label	CH1	SH1
Gel 2	Interior label	SH2	C1
Gel 3	Interior label	C2	CH2
Gel 4	Interior label	CH3	C3
Gel 5	Interior label	SH3	CH2

¹⁾ The regulation of labeled is that sample of every group was not labeled only one kind fluorescence.

²⁾ "Interior label" is pooled all samples for reducing the individual difference. "CH1, CH2, CH3" are three samples in clinical hypocalcemia group. "SH1, SH2, SH3" are three samples in subclinical hypocalcemia group. "C1, C2, C3" are three samples in control group.

mg/mL of bovine serum albumin (BSA) at a ratio of 1:3 with ddH₂O at a ratio of 1:9 with the sample were added to a test tube. A 5- μ L aliquot of the sample, which was diluted according to the above method, was homogenized in BSA (standard protein buffer) and 875 μ L of protein assay buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein concentrations were quantified spectrophotometrically at 595 nm with BSA as a standard.

The abundance of albumin and immunoglobulins are varied in serum, and can account for up to 95% of proteins in serum. To increase resolution of the gels, highly abundant proteins were extracted and purified using a protein purification kit (GE Healthcare, Buckinghamshire, UK), while impurities were removed using a 3-kD ultrafiltration tube (UFC800396; Merck Millipore, Billerica, MA, USA), and then centrifuged at 10,000 g for 30 min. All protein concentrations were quantified twice according to the above method.

Fluorescent labelling of proteins

NaOH (50 mmol/L) was added to the samples to adjust the pH to 8.5 and lysis buffer was added to adjust the concentration to 5 mg/mL. The interior label was a mixed liquor of all nine samples and 400 pmol Cy2 fluorescent label was added to every 50 μ g of protein in the interior label. The samples were randomly labeled with Cy3 and Cy5. The CH1 sample was added twice to the different gels because of the odd number of total samples. This design was chosen to reduce the occurrence of technical errors and increase replication.

Cy3- and Cy5-labeled samples (50 μ L) were mixed with the interior label and then two-fold buffer (7 M urea, 2 M thiourea, 4% CHAPS (3-[[3-Cholamidopropyl] dimethylammonio] propanesulfonic acid), 1% Bio-Lyte, pH 3 to 10, 20 mg/mL dithiothreitol (DTT) and hydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Bio-Lyte, 10 mg/mL DTT) were added for a final volume of 410 μ L.

Two-dimensional gel electrophoresis

Immobiline DryStrips (length, 18 cm; GE Healthcare, UK), containing immobilized ampholytes forming a linear pH gradi-

ent of 4 to 7, were used for isoelectric focusing (IEF) in the first dimension. The strips were rehydrated in 100 μ L of a buffer containing 7 mmol/L urea, 2 mmol/L thiourea, 4% CHAPS, 30 mmol/L Tris buffer, and deionized water. Immobilized pH gradient (IPG) rehydration buffer contained 4.5 mg of 1% DDT, 4.5 μ L of 40% ampholyte, and trace amounts of bromophenol blue. The samples were applied to the strips following a cup loading protocol and IEF was performed at room temperature using the IPGPhor IEF system (Amersham Biosciences, Amersham, UK) at 50 V per strip for 13 h, according to the following program: fast gradient at 200 V for 30 min (to remove impurities), linear gradient at 1,000 V for 6 h, focusing at 10,000 V for 60,000 vh, and fast gradient at 500 V to the end of the run.

After IEF, the IPG strips were incubated in equilibration buffer I (6 mol/L urea, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.375 mol/L Tris-HCl, 0.1 g DDT) while shaking for 15 min, and then in equilibration buffer II (buffer I in which DTT was replaced by 0.4 g iodoacetamide) for a further 15 min. Thereafter, the strips were individually applied to the surface of a vertical 12% polyacrylamide gel and sealed with 0.5% agarose dissolved in electrophoresis buffer (3 g Tris-alkali, 14.4 g of glycerol, 0.1% SDS) with traces of bromophenol blue. SDS-polyacrylamide gel electrophoresis (PAGE) Broad Range or All Blue Precision Plus Protein (Bio-Rad Laboratories, Inc., USA) molecular weight standards were loaded at each end of the strip. Second-dimension electrophoresis was performed using an EttanDALT II gel at a constant gain of 30 V for 60 min, then 80 V for 16 h.

Gel scanning and analysis

Following electrophoresis, signals from the three fluorophores in each gel were sequentially scanned using a Typhoon 9410 fluorescence imager (GE Healthcare, UK). The corresponding excitation and emission wavelengths for Cy3 and Cy 5 were 532 and 580 nm, and 633 and 670 nm, respectively. Cy2 images were obtained at wavelengths of 488 and 520 nm. The DeCyder (V6.5) was used for analysis of the DIGE gel to determine differences in protein content. The *t*-test was used to identify significant differences in protein expression. A probability (*p*) value of ≤ 0.05 and an increase in expression of >1.5-fold were considered statistically significant.

Mass spectrometry analyses and database search for protein identification

According to the results of the second protein quantification, the preparation gel, which was used for MS, was used for all samples according to the above-mentioned method. The analysis gel, which was used to determine differences in protein expression, was used to compare the preparations to ensure the places of proteins exhibiting differences in expression profiles. Then, spots containing proteins were carefully excised using a scalpel. After freeze-drying, the spots were mixed with trypsin solution and then subjected to enzymolysis at 37°C in a water bath for

16 h. Before MS analysis, peptides were extracted by the addition of 5% trifluoroacetic acid (TFA) solution. Then, 0.5 μ L of each peptide sample was placed on target of the mass spectrograph. After addition of 5 mg/mL of matrix solution (α -cyan-4-hydroxycinnamic acid, 0.1% TFA, 50% acetonitrile), the peptide samples were analyzed by MALDI-TOF MS using an ABI-4800 mass spectrometer (Applied Biosystems, Foster City, CA, USA) under the following parameters: scanning mode, reflection-type; range, 700 to 4,000 Da; laser energy of full scan, 5,000; second scan, 5,500. All proteins were identified by a search of the National Center for Biotechnology Information (NCBI) database.

Bioinformatics analysis

Gene ontology (GO) analysis is an important analytical method in bioinformatics software for high-throughput data. Cytoscape (<http://www.cytoscape.org/>) is an open source software platform for analysis of high-throughput data. The many plugins with Cytoscape are used for GO analysis and prediction of protein structure, and so on. In this study, the Bin/GO tool, a plugin of Cytoscape (version 2.8.3), was used for GO analyses.

All identified proteins were used for pathway analysis with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

Western blot analysis

The MS results showed that expression of the alpha-2-macroglobulin (A2M) protein was up-regulated in the CH group and down-regulated in the SH group, as compared with the C group. To verify the results of 2D-DIGE/MALDI-TOF-MS, differentially expressed proteins were chosen for western blot (WB) analysis using rabbit anti-A2M antibody (bs-9505R; Bioss, Inc., Woburn, MA, USA).

Crude serum proteins from three groups (CH group, n = 3; SH group, n = 2; C group, n = 3) at day -3 before calving were used for WB analysis. Equal amounts of proteins were separated by 5% to 20% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk powder for 2 h at 37°C, target proteins were incubated with primary antibodies for 2 h while shaking overnight at 4°C. Then, the membranes were incubated with secondary antibodies overnight at 4°C. Afterward, the membranes were dried to form a gel for scanning. The A2M protein content of the three groups was analyzed to verify relative expression levels.

RESULTS

Change in Ca concentrations from before calving to after

The Ca concentrations of the samples are shown in Figure 1. All data are provided in Supplementary Table S1. The Ca concentrations of all three groups were decreased on day 0, and there were no significant difference on day -3. Although the Ca concentrations of three groups were decreased on day 0, that of the

C group was still in the normal range. The Ca concentration in the C group was restored to a normal range on day +3, but not the other two groups, especially the CH group.

Analysis of differential protein expression

A representative fluorescent image is shown in Figure 2. All images are provided in Supplementary Table S3. Fifty-seven protein spots were separated. Each of these protein spots was significantly different between at least two groups (1.5-fold difference in abundance; $p < 0.05$). Twenty-seven spots were selected for MALDI-TOF-MS, each spot on the gel was required not to be connected to any other spot, and the blot was clear and complete.

Mass spectrometry identification of differentially expressed proteins

Thirteen peptide mass fingerprints (PMFs) of 27 spots were obtained after MALDI-TOF-MS identification. The NCBI database was searched to identify the 13 PMFs to confirm the identification of the proteins. Of the 13 PMFs, eight different proteins were identified.

The results of MS analysis are shown in Table 2. The eight differentially expressed proteins included third party data (TPA): vitamin D-binding protein precursor (GC), complement C4 precursor (C4), paraoxonase 1 (PON1), A2M, endopin 1 (EN1), TPA: apolipoprotein A-IV precursor (APOA4), alpha-1-antitrypsin, and haptoglobin (HP).

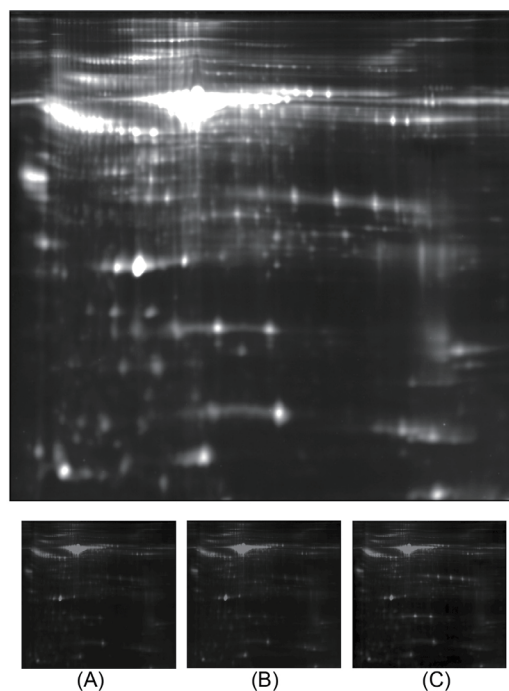


Figure 2. Two-dimensional differential gel electrophoresis (2D-DIGE) fluorescence image of one pair of CH and C samples with interior labels. (A) pooled interior label with Cy2. (B) MF labeled with Cy3. (C) C labeled with Cy5. The three pictures below: Individual channels of Cy2, Cy3, and Cy5.

Table 2. Identification of 16 differentially expressed proteins expressed among the three groups by mass spectrometry

No.	Spots No.	NCBI_ID	Name of proteins	Score	Molecular mass	Abbreviation	CH/C ^(1,2)	SH/C	CH/SH
1	1062	gi 296486435	TPA: vitamin D-binding protein precursor (<i>Bos taurus</i>)	253	53321	GC	↓	-	↓
2	630	gi 262050656	complement C4 precursor (<i>Bos taurus</i>)	117	192676	C4	-	↓	-
3	1082	gi 86826758	Paraoxonase 1 (<i>Bos taurus</i>)	75	39816	PON1	↓	-	↓
4	483	gi 157743038	A2M protein (<i>Bos taurus</i>)	543	167470	A2M	↑	↓	-
5	616	gi 157743038	A2M protein (<i>Bos taurus</i>)	216	167470				
6	181	gi 280771107	endopin 1 (<i>Bos taurus</i>)	273	46175	EN1	-	↓	↑
7	805	gi 280771107	endopin 1 (<i>Bos taurus</i>)	277	46175				
8	1083	gi 296480272	TPA: apolipoprotein A-IV precursor (<i>Bos Taurus</i>)	169	42963	APOA4	↓	-	↓
9	1131	gi 296480272	TPA: apolipoprotein A-IV precursor (<i>Bos taurus</i>)	92	42963				
10	1081	gi 42	alpha-1-antitrypsin (<i>Bos taurus</i>)	73	46075	AAT	↓	-	↓
11	1087	gi 42	alpha-1-antitrypsin (<i>Bos taurus</i>)	68	46075				
12	1318	gi 83638561	Haptoglobin (<i>Bos taurus</i>)	81	44831	HP	-	↓	-
13	1357	gi 83638561	Haptoglobin (<i>Bos taurus</i>)	204	44831				

¹⁾ CH, clinical hypocalcemia group; SH, subclinical hypocalcemia group; C, healthy group.

²⁾ ↑, up-regulation; ↓, down-regulation; -, no change.

Bioinformatics analysis of differentially expressed proteins

Gene ontology analysis: All differentially expressed proteins were analyzed using the BinGO plugin of the Cytoscape software. As shown in Supplementary Table S4, there were 150 and 24 annotations to the molecular function and biological process, respectively. Considering that a given protein can be attributed to several functions and/or processes, the annotations of biological

processes and molecular functions were filtrated, which were related with hypocalcemia (Figure 3). “Steroid metabolic process”, and “Vitamin D metabolic process” were the most represented biological processes. “Vitamin D binding”, “Vitamin transporter activity”, and “Protein binding” were the most represented molecular functions.

Pathway analysis: The KEGG Pathway database was used to search for all differentially expressed proteins. Four proteins, A2M,

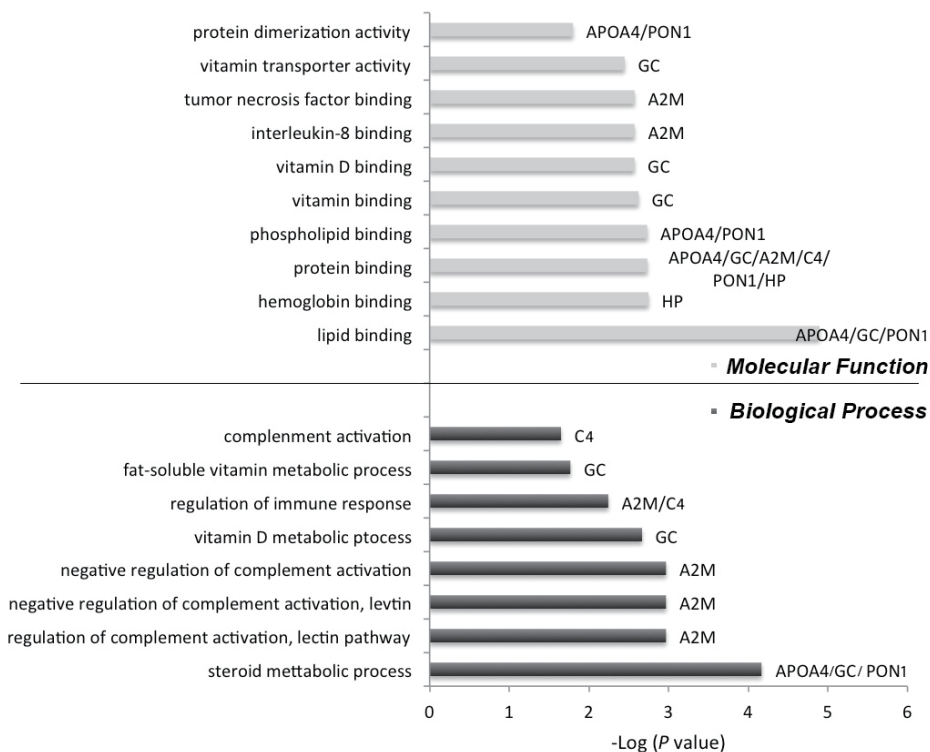


Figure 3. Gene ontology analysis. The abscissa was the $-\text{Log}_{10}(\text{p value})$ to shown the significance between proteins and annotation. The ordinate was the annotation of molecular function (above) and biological process (below). Protein abbreviations are included in the bar graph. APOA4, apolipoprotein A-IV; PON1, Paraoxonase 1; GC, vitamin D-binding protein; A2M, A2M protein; C4, complement C4; HP, haptoglobin.

GC, C4, and APOA4, were identified in five pathways which may be related with hypocalcemia, including the “Endocrine and other factor-regulated Ca reabsorption” (ID: 04961), “Mineral absorption” (ID: 04978), “Complement and coagulation cascades” (ID: 04611), “Fat digestion and absorption” (ID: 04975) and “Vitamin digestion and absorption” (ID: 04977) (Table 3).

Since so many pathways were found related to different proteins, all results were analyzed, filtered, and integrated. An integrated pathway to explain the relationship between the differentially expressed proteins and serum Ca regulation is shown in Figure 4. In summary, GC is an important factor in many pathways. GC specifically binds with vitamin D₃ (VD₃) in small intestinal epithelial cell and distal convoluted tubular (DCT) cells, and then promotes Ca²⁺ binding with the Ca-binding protein (CBP) in order to transfer Ca²⁺ to the blood. In addition, if there is an insufficient amount of GC specific binding with VD₃, in small intestinal epithelial cells, VD₃ with APOA4 enter the chylomicron, then the chylomicron is exocytosed to the extracellular space and finally enters the blood via lymph transfer.

Western blot analysis

Table 3. Pathway analysis

No.	Map ID.	Name of map	Proteins ¹⁾
1	04961	Endocrine and other factor-regulated calcium reabsorption	GC
2	04978	Mineral absorption	GC
3	04610	Complement and coagulation cascades	A2M/C4
4	04975	Fat digestion and absorption	APOA4
5	04977	Vitamin digestion and absorption	APOA4

¹⁾ GC, vitamin D-binding protein precursor; A2M, α-2-macroglobulin; C4, complement C4 precursor; APOA4, apolipoprotein A-IV precursor.

The expression levels shown in Figure 5 were calculated as the ratio among the three groups. All data are shown in Supplementary Table S5. A2M expression was increased (CH/C: 6.10%) in the CH group and reduced (SH/C: 15.53%) in the SH group. The WB analysis strongly supported the results of 2D-DIGE combined with MALDI-TOF-MS.

DISCUSSION

In this study, eight types of proteins were found among the three

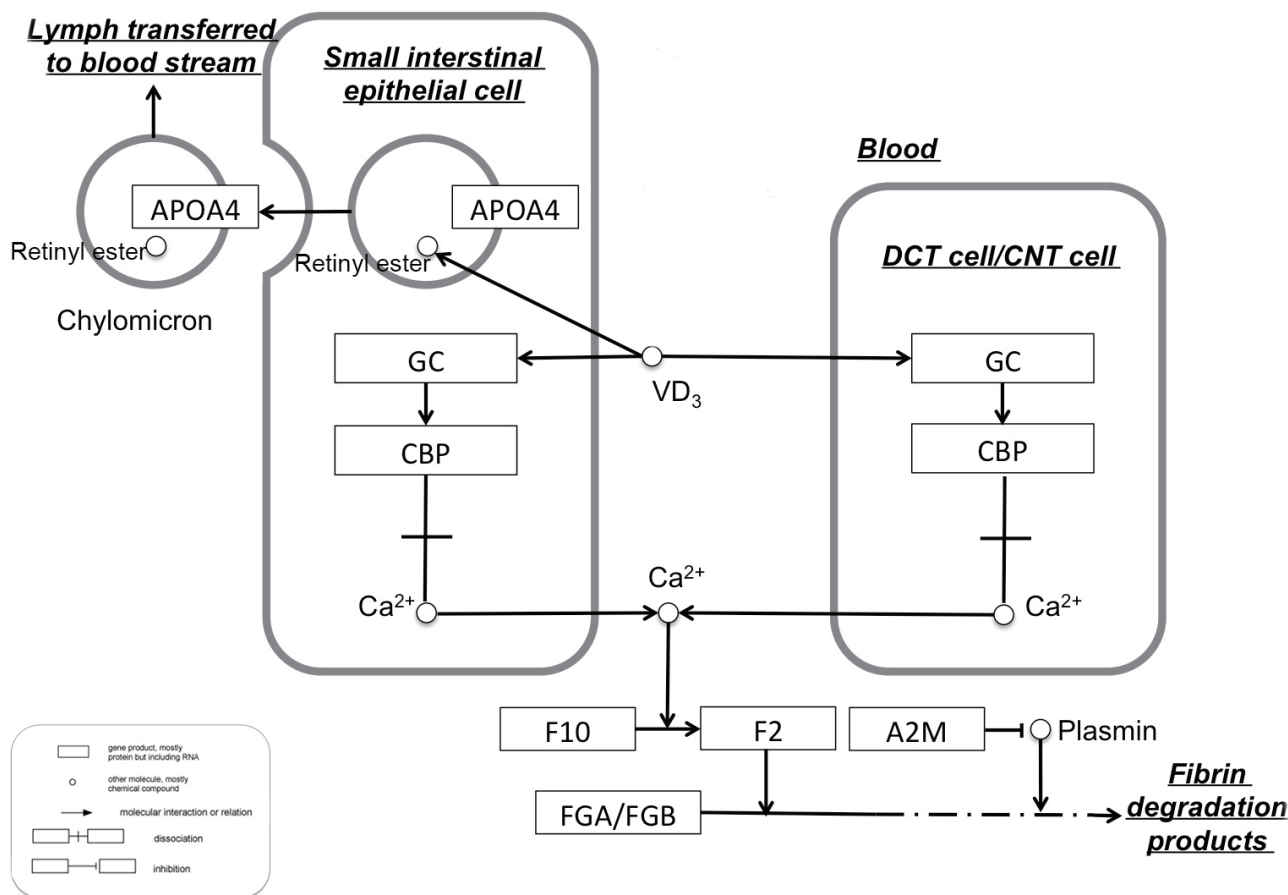


Figure 4. Pathway analysis. This is an integrated pathway to show the relationship between differentially expressed proteins and Ca regulation. The location and physiological process are indicated in italic and underlined font, respectively. APOA4, apolipoprotein A-IV; GC, vitamin D-binding protein; A2M, A2M protein; CBP, calcium binding protein; F2, blood coagulation factor 2; F10, blood coagulation factor 10; FGA, fibrinogen A chain; FGB, fibrinogen B chain. DCT cell, distal convoluted tubular cell; CNT cell, connecting tubular cell.

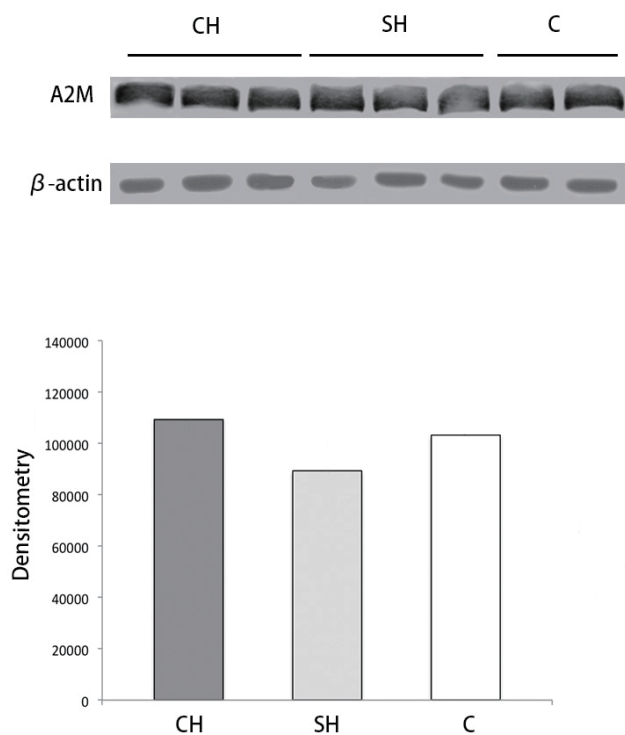


Figure 5. Alpha-2-macroglobulin (A2M) was compared among the three groups for validation by western blot (WB) analysis. Upper figure: Three clinical hypocalcemia (CH) group samples and three subclinical hypocalcemia (SH) group samples were subjected to western blotting analysis of A2M including two normal plasma controls. Lower figure: A2M protein expression in all cows.

groups via 2D-DIGE/MALDI-TOF-MS. Of these, bioinformatics analysis indicated that GC and A2M were relative to hypocalcemia. A2M was chosen for western blotting analysis based on the results of DIGE/MALDI-TOF-MS and bioinformatics analysis.

Potential pathogenesis of clinical hypocalcemia

In this study, the serum Ca concentrations of all cattle decreased on day 0, which was consistent with the results of previous studies [11,12]. This decrease likely resulted from providing nutrition to the fetus and formation of the beestings. Mechanisms are in place to efficiently maintain normal serum Ca concentration most of the time, but occasionally these homeostatic mechanisms fail, which results in hypocalcemia [3]. Physiologically, hormones, including calcitonin (decreases Ca concentration), parathyroid hormone (PTH, increases Ca concentration), and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, increases Ca concentration), act to control Ca metabolism [1]. 1,25(OH)₂D₃ is required to stimulate the intestine to absorb dietary Ca. This hormone is produced in the kidney from vitamin D [13]. About 85% of serum 1,25(OH)₂D₃ binds with high affinity to GC [14]. GC is a 55-kDa protein that is a member of the albumin superfamily and is secreted by the liver. One of the physiological functions of the GC protein is vitamin D transport and storage [15]. According

to the results of this study, GC was down-regulated in the CH group, as compared with the C and SH groups, while there was no significant difference between the C and SH groups. As a potential pathogenesis of CH, GC was reduced before calving. When the serum Ca concentration was decreased on calving day, the amount of GC is insufficient to combine with vitamin D. Thus, the role of vitamin D was inhibited leading to a severe decrease in serum Ca concentration. Ca is continually consumed by providing nutrition to the fetus and forming the beestings, which prevents restoration of serum Ca to normal concentrations.

Another surmise was that APOA4, a 46-kDa high-density lipoprotein (HDL), is secreted from the small intestine in response to lipid absorption and chylomicron formation [16]. It is regulated by leptin, a major component of energy homeostasis [17]. The integrated pathway (Figure 5) shows that ApoA4 enters the circulation on lymph chylomicrons and is subsequently released as a lipid-free protein during lipolysis of chylomicrons [18]. Haas and Mooradian [19] reported that vitamin D levels were positively correlated with plasma HDL levels, but inversely with plasma low-density lipoprotein cholesterol levels. The result of this study showed that APOA4 was down-regulated in the CH group, as compared with the C and SH groups, while there was no significant difference between the C and SH groups. An additional physiological function of the GC protein is VD₃ storage. In summary, serum APOA4 levels may be affected by Ca regulation and may be positively correlated with GC expression.

Early detection index of hypocalcemia

In this study, the A2M protein was detected by 2D-DIGE/MALDI-TOF-MS and verified by WB analysis. A2M expression was up-regulated in the CH group and down-regulated in the SH group, as compared with controls. Wang et al [20] reported that A2M plays a role in osteoarthritis and suggested that A2M is a powerful inhibitor of many cartilage catabolic factors through protease activities. A2M inhibits cartilage catabolic factor through decreased gene expression to protect the cartilage. In addition, tartrate-resistant acid phosphatase (TRACP) 5b is derived from osteoclasts, which circulate in large complexes that contain A2M and Ca [21]. Many reports have suggested that serum TRACP 5b is a specific and sensitive marker of bone restoration that can be used for the diagnosis and follow-up of skeletal pathologies [22]. Bone-resorbing osteoclasts contain high amounts of TRACP 5b and secrete it into the bloodstream.

This evidence suggests that A2M expression is related to Ca concentration. Although the relationship between A2M and Ca remains unclear, A2M was useful as an early detection index to distinguish CH from SH from healthy dairy cows before calving. However, further studies are needed to elucidate the underlying mechanisms.

Other proteins related to hypocalcemia

Other proteins identified in this study included PON1, C4, HP,

EN1, and AAT. PON1 is a 44-kDa glycoprotein that hydrolyzes organophosphates found in pesticides, neurotoxins, and arylesters [22,23]. PON1 is synthesized mainly in the liver, with lower expression found in the lungs, heart, brain, kidneys, and small intestine [24]. The results of 2D-DIGE/MALDI-TOF-MS showed that PON1 expression was down-regulated in the CH group, as compared with the SH and C groups, respectively. A possible mechanism for the effects of PON1 is the up-regulation of PTH receptor 1, as described by Lu et al [25]. PTH is an important hormone for the balance of Ca and P. Since the PTH receptor 1 specifically binds to PTH, the level of PTH receptor 1 is positively correlated with the level of PTH, suggesting that PON1 affects serum Ca concentration via the PON1 receptor 1 and available PTH.

C4 activation acts as immune surveillance system in healthy and altered host cells to detect the presence of foreign matter. C4 is a component of C3 convertase in the classical and lectin complement pathways, which is expressed in two polymorphic isotypes, C4A and C4B [26].

HP is primarily produced in the liver and is functionally important for binding free hemoglobin from lysed red cells *in*, preventing its toxic effects [27]. Decreased HP is a marker of hemolysis, as HP levels become depleted in the presence of large amounts of free hemoglobin [28].

EN1 is present in endocrine tissues, as specified by “endo” and its serpin-like characteristic indicated by “pin”, thus designating this new serpin as endopin 1. EN1 is present within isolated chromaffin granules, secreted from chromaffin cells in culture, and inhibits trypsin and plasmin via cleavage at basic residues [29].

AAT is a 52-kD glycoprotein produced mainly by hepatocytes and secreted into the blood. AAT deficiency is a rare genetic disorder associated with the development of liver and lung diseases [30].

In summary, the results of this study identified several proteins associated with hypocalcemia, although this evidence could also explain other diseases that often occur during and after calving caused by hypocalcemia, including fatty liver (AAT, C4) and endometritis (HP, C4).

In this study, 2D-DIGE/MALDI-TOF-MS, bioinformatics, and western blotting were performed to distinguish healthy cows from those with CH and SH. A total of eight differentially expressed proteins were identified among the three groups. Due to its specific expression, A2M was selected for validation, and results show that it was as an early detection index. The possible pathogenesis of CH caused by GC and APOA4 was speculated. Further evaluation of these findings is required for clinical translation.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

The authors thank the Beijing Proteome Research Center for providing technological services and International Science Editing for editing of this article. This study was supported by grants from the National Science and Technology Foundation of China (30972235) and Heilongjiang Province Nature Science and Technology Foundation of China (C200916), the Chinese National Science and Technology Project of China (2013BAD21B01-2), and Heilongjiang Bayi Agriculture University graduate student innovation fund project (YJSCX2015-Z03).

REFERENCES

1. DeGaris PJ, Lean IJ. Milk fever in dairy cows: A review of pathophysiology and control principles. *Vet J* 2008;176:58-69.
2. Kimura K, Reinhardt TA, Goff JP. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. *J Dairy Sci* 2006;89:2588-95.
3. Goff JP. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. *Vet J* 2008;176:50-7.
4. Shu S, Xia C, Zhang H, et al. Plasma proteomics analysis of dairy cows with milk fever using SELDI-TOF-MS. *Asian J Anim Vet Adv* 2014;9:1-12.
5. Wang PX, Shu S, Xia C, et al. Protein expression in dairy cows with and without subclinical hypocalcaemia. *N Z Vet J* 2016;64:101-6.
6. Liu W, Ma Y, Huang L, et al. Identification of HSP27 as a potential tumor marker for colorectal cancer by the two-dimensional polyacrylamide gel electrophoresis. *Mol Biol Rep* 2010;37:3207-16.
7. Kang G, Li G, Ma H, Wang C, Guo T. Proteomic analysis on the leaves of *TaBTf3* gene virus-induced silenced wheat plants may reveal its regulatory mechanism. *J Proteomics* 2013;83:130-43.
8. Jorrián JV, Maldonado AM, Castillejo MA. Plant proteome analysis: A 2006 update. *Proteomics* 2007;7:2947-62.
9. Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 1997;18:2071-7.
10. Tonge R, Shaw J, Middleton B, et al. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* 2001;1:377-96.
11. Bauman DE, Currie WB. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. *J Dairy Sci* 1980;63:1514-29.
12. Thilising-Hansen T, Jørgensen RJ, Østergaard S. Milk fever control principles: a review. *Acta Vet Scand* 2002;43:1-19.
13. Ebert R, Schütze N, Adamski J, Jakob F. Vitamin D signaling is modulated on multiple levels in health and disease. *Mol Cell Endocrinol* 2006;248:149-59.
14. White P, Cooke N. The multifunctional properties and characteristics of vitamin D-binding protein. *Trends Endocrinol Metab* 2000;11:320-7.
15. Nagasawa H, Uto Y, Sasaki H, et al. Gc protein (vitamin D-binding protein): Gc genotyping and GcMAF precursor activity. *Anticancer*

- Res 2005;25:3689-95.
16. Kohan AB, Wang F, Li X, et al. Apolipoprotein A-IV regulates chylomicron metabolism—mechanism and function. *Am J Physiol Gastrointest Liver Physiol* 2012;302:G628-G36.
 17. Doi T, Liu M, Seeley RJ, Woods SC, Tso P. Effect of leptin on intestinal apolipoprotein AIV in response to lipid feeding. *Am J Physiol Regul Integr Comp Physiol* 2001;281:R753-R9.
 18. Weinberg RB, Cook VR, Delozier JA, Shelness GS. Dynamic interfacial properties of human apolipoproteins A-IV and B-17 at the air/water and oil/water interface. *J Lipid Res* 2000;41:1419-27.
 19. Haas MJ, Mooradian AD. What evidence is there for the role of vitamin D and apoA-1 in atheroprotection? *Clin Lipidol* 2012;7:255-7.
 20. Wang S, Wei X, Zhou J, et al. Identification of α 2-macroglobulin as a master inhibitor of cartilage-degrading factors that attenuates the progression of posttraumatic osteoarthritis. *Arthritis Rheumatol* 2014;66:1843-53.
 21. Ylipahkala H, Halleen JM, Kaija H, Vihko P, Väänänen HK. Tartrate-resistant acid phosphatase 5B circulates in human serum in complex with α 2-macroglobulin and calcium. *Biochem Biophys Res Commun* 2003;308:320-4.
 22. Hong SH, Song J, Min WK, Kim JQ. Genetic variations of the paraoxonase gene in patients with coronary artery disease. *Clin Biochem* 2001;34:475-81.
 23. Mackness MI, Arrol S, Abbott C, Durrington PN. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis* 1993;104:129-35.
 24. Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. The human serum paraoxonase/arylesterase gene (*PON1*) is one member of a multigene family. *Genomics* 1996;33:498-507.
 25. Lu J, Cheng H, Atti E, et al. Role of paraoxonase-1 in bone anabolic effects of parathyroid hormone in hyperlipidemic mice. *Biochem Biophys Res Commun* 2013;431:19-24.
 26. Paakkanen R, Wennerström A, Liesmaa I, et al. 205-P Immunogenetics of complement C4 deficiencies. *Hum Immunol* 2011;72:S144.
 27. Shih AW, McFarlane A, Verhovsek M. Haptoglobin testing in hemolysis: measurement and interpretation. *Am J Hematol* 2014;89:443-7.
 28. Nielsen MJ, Moestrup SK. Receptor targeting of hemoglobin mediated by the haptoglobins: roles beyond heme scavenging. *Blood* 2009;114:764-71.
 29. Hwang S-R, Steineckert B, Yasothornsrikul S, et al. Molecular cloning of endopin 1, a novel serpin localized to neurosecretory vesicles of chromaffin cells. Inhibition of Basic Residue-Cleaving Proteases by Endopin 1. *J Biol Chem* 1999;274:34164-73.
 30. Silverman EK, Sandhaus RA. Alpha1-antitrypsin deficiency. *New Engl J Med* 2009;360:2749-57.