

Comparative proteomics analysis of plasma proteins during the transition period in dairy cows with or without subclinical mastitis after calving

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ABSTRACT: The transition period is the most critical time of the cow's lactation cycle that is associated with the onset of mastitis. In this study, changes of plasma proteins in cows ($n = 12$) with or without subclinical mastitis after calving were determined by two-dimensional electrophoresis (2-DE), which detected 18 spots with variations in protein spots abundance. These spots were identified by liquid chromatography coupled with tandem mass spectrometry. The changes in protein profile from day 21 before calving to day 1 after calving were similar in cows with or without subclinical mastitis. Abundance of $\alpha 1$ acid glycoprotein (AGP) and haptoglobin was dramatically increased at parturition, while transthyretin was down-regulated at parturition, and apolipoprotein E and immunoglobulin gamma 1 were up-regulated at postpartum compared with prepartum in periparturient dairy cows. In cows infected with subclinical mastitis, AGP, haptoglobin, and serum amyloid A were dramatically increased and continued to be elevated in plasma from day 1 to day 21 after calving compared with cows free of mastitis. Changes of protein in plasma at parturition may serve as an immune system response to parturition and lactation process at the protein level and suggest that these altered proteins would not serve as a potential marker for predicting if the periparturient dairy cows are susceptible to subclinical mastitis.

Keywords: dairy cow; plasma proteome; periparturient; mass spectrometry; mastitis

The transition period is traditionally considered to last for approximately 6 weeks – 3 weeks before and 3 weeks after calving (Drackley, 1999). During this period the dairy cow's immune system including neutrophil and lymphocyte function is suppressed (Kehrli et al., 1989), which is associated with increases in the incidence of infectious diseases, especially mastitis (Valde et al., 2004; Pyörälä, 2008). Thus, the transition period is the most critical time of the lactation cycle and can determine to a large measure how profitable the entire lactation will be. For this reason, many researchers have attempted to reveal complex physiological adaptive

mechanisms using transcriptomic and proteomic methodologies (Madsen et al., 2002; Loores et al., 2005; Cairoli et al., 2006), the latter of which is a powerful tool for comparative analysis of complex protein mixtures based on high-resolution two-dimensional electrophoresis (2-DE) coupled with mass spectrometry. This proteomic approach can provide useful information on temporal and dynamic processes occurring in the proteome.

Plasma proteins originate from all tissues in the body. The species and concentrations of these proteins may change as the host body undergoes various physiological or pathological changes. Thus,

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alteration of plasma proteins can serve as potential diagnostic markers for abnormal physiological or pathological conditions.

To investigate the host immune system response to parturition and lactation, neutrophil functionality was first investigated in cows using amine-reactive isobaric tagging reagents. Over 40 proteins in neutrophils were differentially expressed at parturition as compared to prepartum, including a bovine myeloid antimicrobial peptide of 28 residues with antimicrobial activity and several proteins involved in arachidonic acid metabolism and transport (Lippolis et al., 2006). Additionally, several serum proteins were shown to change during the last phase of pregnancy and early postpartum, which included a decrease of α 2-HS-glycoprotein, an increase of α 1-antichymotrypsin, and a dramatic variation in orosomucoid and haptoglobin (Cairolì et al., 2006). These results indicated that parturition and lactation induced changes in protein synthesis of the neutrophil and liver. However, few data are currently available on the plasma proteome during the periparturient period in dairy cows with or without subclinical mastitis after calving.

The objective of this study was to identify temporal changes of proteins during the transition of cows using 2-DE and liquid chromatography coupled with tandem mass spectrometry. The findings of this study may provide additional insight into the regulation of plasma proteins during this period and potentially discover markers that can be used to identify cows that are susceptible to mastitis.

MATERIAL AND METHODS

Sample collection and preparation

The procedures used for caring for the dairy cows in this experiment were reviewed and approved by the Animal Care Advisory Committee of the Chinese Academy of Agricultural Sciences. Blood samples (9 ml) were collected from the tail vein of sixty Chinese Holstein primiparous cows from a farm near Beijing, using BD vacutainer tubes containing ethylenediaminetetraacetic acid-anticoagulant 20.4 ± 1.6 days before the expected date of calving, and 1 and 21 days postpartum. The cows were housed in free-stalls, fed a total mixed ration, and milked three times daily. Twelve healthy cows considered free of mastitis (somatic cell count less than 500 000 cells/ml) as measured by

Fossomatic 5000 (Foss Electric, Hilerød, Denmark) on day 14 and 21 after calving and 12 cows with subclinical mastitis (somatic cell counts > 500 000 cells/ml) (Liu et al., 2010; Mollenhorst et al., 2010) were selected for proteomic analysis. Cows with any other diseases or disorders according to veterinarian records, such as retained placenta and metritis, were excluded from analysis in agreement with this record. Blood samples were centrifuged at 4°C and 3000 g for 15 min, plasma was collected and then stored at –80°C until analysis.

Enrichment of minor abundance proteins

After thawing, the plasma samples from twelve healthy cows were pooled as three samples, and from twelve cows with subclinical mastitis the plasma samples were also pooled as three samples collected at the same time during the transition period. These pooled samples were then enriched using a ProteoMiner kit (Bio-Rad Laboratories, Hercules, USA) per manufacturer's protocols. A 1 ml plasma sample was mixed with the beads after centrifuging and the storage solution was decanted off. The column containing the beads was rotated end-to-end at room temperature for 2 h and centrifuged at 1000 g to remove any remaining storage solution. Then the beads were rinsed with a buffer solution and then re-suspended in the elution buffer and mixed by gently vortexing. Following centrifugation at 1000 g at room temperature, the supernatant containing the released proteins was collected. Fresh elution buffer was added to the bead and the above process was repeated twice to ensure maximum protein recovery. The released proteins were pooled and the resulting enriched protein samples were analyzed for protein content using the bicinchoninic acid method (Kao et al., 2008).

Two-dimensional gel electrophoresis and software analysis

Protein samples (600 μ g) were mixed with 10% sodium dodecyl sulfate/2.3% dithiothreitol (DTT) at 95°C for 5 min (Steel et al., 2003) and diluted in 350 μ l rehydration solution containing 8 mol/l urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 2.5 mmol/l tributyl phosphate, and 0.5% immobilized pH gradient (IPG) buffer and incubated at room temperature

for 10 min with gentle mixing. Plasma samples enriched with ProteoMiner were diluted with rehydration solution to 350 μ l. The resulting solution then was rehydrated on 17 cm, pH 4–7 IPG strips (Bio-Rad Laboratories, Hercules, USA). After 12 h of passive rehydration, isoelectric focusing (IEF) was performed at 80 kVh using a Protean IEF Cell (Bio-Rad Laboratories, Hercules, USA) at 20°C as follows: 30 min at 250 V, 1 h at 1000 V, then increased from 1000 V to 10 000 V over a period of 5 h, and finally 10 000 V for 8 h. Subsequently, IPG strips were equilibrated with an equilibration solution (6 mol/l urea, 2% SDS, 20% glycerol, 50 mmol/l Tris-HCl, 0.01% w/v bromophenol blue) containing 2% DTT for 12 min with shaking and followed by equilibration solution containing 2.5% w/v iodoacetamide for an additional 12 min with shaking. Strips were transferred onto a 12% polyacrylamide gel for second dimension separation using an Ettan DALTsix Electrophoresis Unit (GE Healthcare, Waukesha, USA). The gels were run at 1 W for the first 30 min and then at 46 W until the bromophenol blue reached the bottom of the gels. Then the gels were stained with Coomassie Brilliant Blue G-250 as described by Candiano et al. (2004). Gel images were analyzed using the ImageMaster 2-D Platinum software, Version 6.0 (GE Healthcare, Waukesha, USA). For comparisons of relative abundance of proteins among gels, protein spots were automatically detected and manually confirmed. Three biological replicate gels were superimposed and a master gel was obtained. In each master gel protein spot position, shape and optical density were averaged, and individual spot intensity volume was normalized with total intensity volume. Protein spots were considered to be differentially expressed when there was at least a two-fold increase or decrease in spot stains density, and values were then analyzed for statistical significance (Gonçalves et al., 2010).

In-gel digestion and extraction of peptides

Protein spots that showed differential expression were excised manually using pipette tips. Each spot was placed into a 1.5 ml microtube and washed twice with MilliQ water for 15 min. After washing, gel pieces were incubated in 50% acetonitrile at room temperature for 15 min, and then shrunk in acetonitrile. The protein spots were digested in a buffer containing 50 mmol/l of ammonium bicar-

bonate and 10 ng/ml of sequence-grade trypsin by incubating at 37°C overnight. Then, peptides were extracted twice with 50% acetonitrile and 0.2% formic acid, with each extraction being followed by vortexing and sonication (10 min each).

Protein identification

Peptide mass was determined by ion trap mass spectrometry (LCQ Deca XP, Thermo Finnigan, San Jose, USA) equipped with a Surveyor high performance liquid chromatography system. A BioBasic-18 column (50 \pm 0.18 mm) (Thermo Electron, San Jose, USA) was equilibrated with mobile phase A consisting of MilliQ water and 0.1% formic acid. A 20 μ l aliquot was injected onto the BioBasic-18 column and the proteins were eluted using mobile phase B consisting of 0.1% formic acid in acetonitrile at a flow rate of 120 μ l/min with a linear gradient over 120 min. Using the full scan mode m/z 400–2000, tandem mass spectrometry was performed in a data-dependent mode. After acquisition of a full scan, three tandem mass spectrometry scans were acquired for the next three most intense ions using dynamic exclusion. Peptides were identified using the SEQUEST software (Bioworks 2.0, Thermo Finnigan, San Jose, USA), which uses the mass spectrometry and tandem mass spectrometry spectra of peptide ions to search the NCBI nr database. The following search parameters were used with trypsin as the cleavage enzyme, one missed cleavage, carbamidomethylation of cysteine residues as a fixed modification, and methionine oxidation selected as a variable modification. The protein identification criteria were based on Delta CN \geq 0.1 and Xcorr (+ 1 \geq 1.9, + 2 \geq 2.2, + 3 \geq 3.75).

Western blot analysis

The recombinant protein of haptoglobin was expressed in *Escherichia coli* BL21 strain after IPTG inducement and purified. Polyclonal antibodies against haptoglobin were produced to immunize rabbits and then the antiserum was collected and purified with protein A affinity resin for use.

The plasma samples were heated at 95°C for 5 min and equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membrane. Subsequently, the

membrane was blocked with tris-buffered saline tween-20 (TBST) containing 1% ovalbumin for 1 h at room temperature and then incubated with antibody for 1 h at 37°C. The membrane was then washed with TBST and visualized with horseradish peroxidase-coupled secondary antibody using diaminobenzidine as substrate. The relative quantity of the protein was defined as the gray scale of the gels. The resulting values were tested for significance using the statistic software SPSS, Version 16.0 (SPSS Inc., Chicago, USA), statistically significant of group and day relative to calving for relative quantity of the protein were performed by two-way analysis of variance (General Linear Model) followed by Tukey's test; two groups were the transition cows with or without subclinical mastitis after calving, and the three levels of the repeated measurement of time were on day 21 before calving, and on days 1 and 21 after calving. *P* values lower than 0.05 were considered as statistically significant.

RESULTS

Identification of changes in plasma proteins

Each pooled sample analysis was repeated three times in order to evaluate gel reproducibility and improve the reliability of the qualitative and quantitative changes in protein expression measured by

means of electrophoresis. The protein profiles either before or after calving were similar in periparturient dairy cows and representative gels were chosen to illustrate the protein profiles generated in the current study (Figure 1). After the software analysis, fourteen of the protein spots increased at parturition when compared with day 21 before calving in healthy cows and eighteen of the protein spots increased on days 1 and 21 after calving in cows with subclinical mastitis (Figure 1). Protein profile from day 21 before calving to day 1 after calving was similar in cows with or without subclinical mastitis, and protein profile on day 21 after calving exhibited a significant difference. Figure 1 presents relative abundance of protein spots from transition dairy cows with or without subclinical mastitis after calving. These protein spots were selected for follow-up identification. Proteins were identified in plasma samples from dairy cows with or without subclinical mastitis by the sequencing of at least 2 peptides as shown in Table 1.

The α 1 acid glycoprotein (AGP) and haptoglobin were abruptly increased at parturition and then decreased on day 21, while transthyretin was down-regulated at parturition and then increased on day 21 after calving in healthy dairy cows. After plasma enrichment with ProteoMiner, serum amyloid A was increased before calving and down-regulated on day 21 after calving, while apolipoprotein E was up-regulated after calving in healthy cows. AGP and haptoglobin were abruptly increased,

Table 1. Changes of plasma proteins in the transition cows identified by ion trap mass spectrometry

Spot	Protein name	Accession No.	Isoelectric point	Molecular weight	No. of peptides matched	Coverage (%)
a1, b1	transthyretin	gi:27806789	5.90	15 726.98	4	46.26
a2, b2	haptoglobin	gi:156739654	7.25	43 383	7	20.20
a3, b3	haptoglobin	gi:94966763	7.83	45 629	6	17.96
a4, a5 b4, b5	α 1 acid glycoprotein	gi:94966811	5.62	23 182.5	9	43.56
a6, b6	α 1 acid glycoprotein	gi:94966811	5.62	23 182.5	11	53.96
a7, b7	α 1 acid glycoprotein	gi:94966811	5.62	23 182.5	7	37.13
a8, a9 b8, b9	α 1 acid glycoprotein	gi:94966811	5.62	23 182.5	6	32.67
a10, b10	immunoglobulin gamma 1 heavy chain constant region	gi:91982959	6.49	35 900.36	3	11.55
a11, b11	serum amyloid A	gi:245184	7.85	14 516.12	2	30.63
a12, b12	serum amyloid A	gi:245184	7.85	14 516.12	3	22.52
a13, b13	apolipoprotein E	gi:27806739	5.55	35 979.85	5	23.42
a14, b14	apolipoprotein E	gi:27806739	5.55	35 979.85	4	17.09
1, 2, 3	α 1 acid glycoprotein	gi:94966811	5.62	23 182.5	7	42.08
4	α 1 acid glycoprotein	gi:94966811	5.62	23 182.5	6	33.17

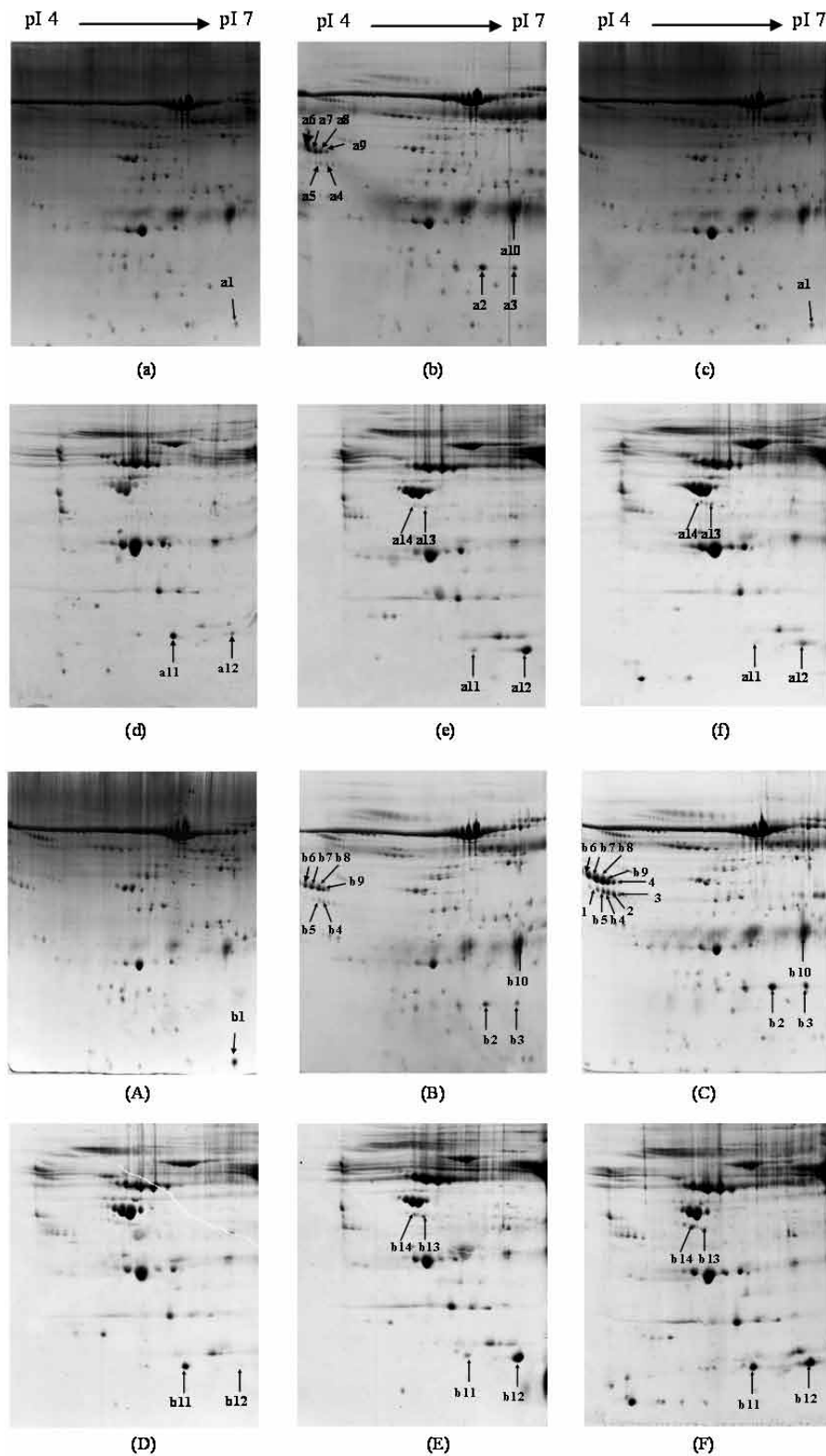


Figure 1. Analysis of expression protein patterns of plasma in transition dairy cows with or without subclinical mastitis 2-DE maps of plasma on day 21 before calving (a), on day 1 (b), and on day 21 (c) postpartum from healthy cows; 2-DE maps of plasma enrichment with ProteoMiner kit on day 21 before calving (d), on day 1 (e), and on day 21 (f) postpartum from healthy cows; 2-DE maps of plasma on day 21 before calving (A), on day 1 (B), and on day 21 (C) postpartum from cows with subclinical mastitis; 2-DE maps of plasma enrichment with ProteoMiner kit on day 21 before calving (D), on day 1 (E), and on day 21 (F) postpartum from cows with subclinical mastitis

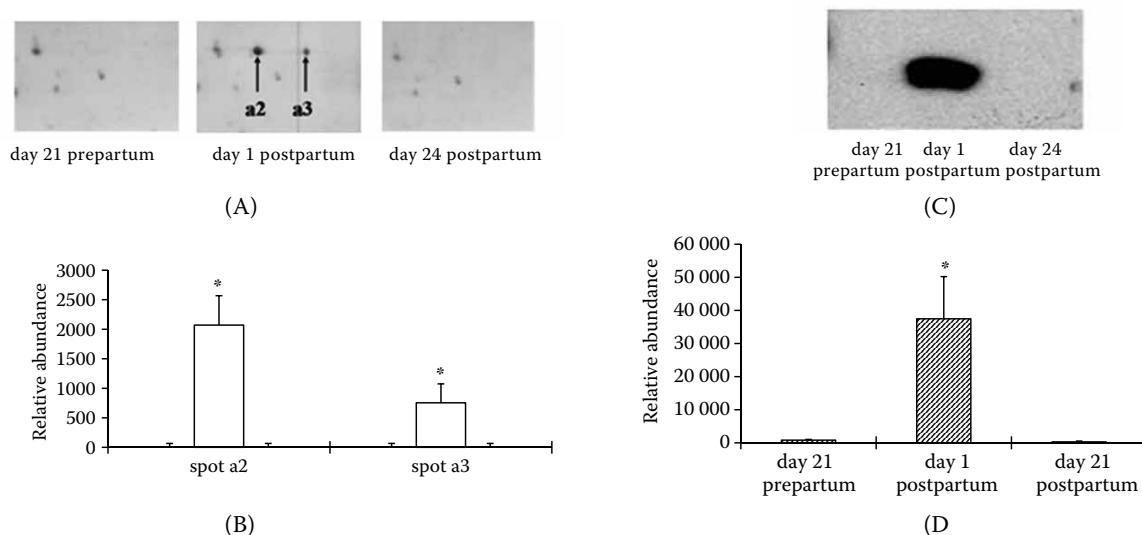


Figure 2. Changes of haptoglobin in plasma of healthy dairy cows during the transition period

(A) representative images of haptoglobin spots in the 2-DE map and (B) densitometric analysis; (C) Western blot analysis of haptoglobin and (D) densitometric analysis

* $P < 0.05$ ($n = 3$)

while transthyretin was down-regulated on days 1 and 21 after calving compared with day 21 before calving in cows with subclinical mastitis. Serum amyloid A in cows with subclinical mastitis was similar to that in healthy cows before calving, but sharply increased on day 21 after calving. In addition, there were four spots on day 21 after calving identified as AGP that were up-regulated in cows with subclinical mastitis when compared with healthy cows.

Western blot analysis

To confirm the proteomic data, expression of haptoglobin in plasma samples from transition dairy cows with or without subclinical mastitis after calving was further confirmed by Western blot analysis (Figures 2 and 3). The proteomic data showed an abrupt increase in level of haptoglobin at parturition in healthy dairy cows, and continued

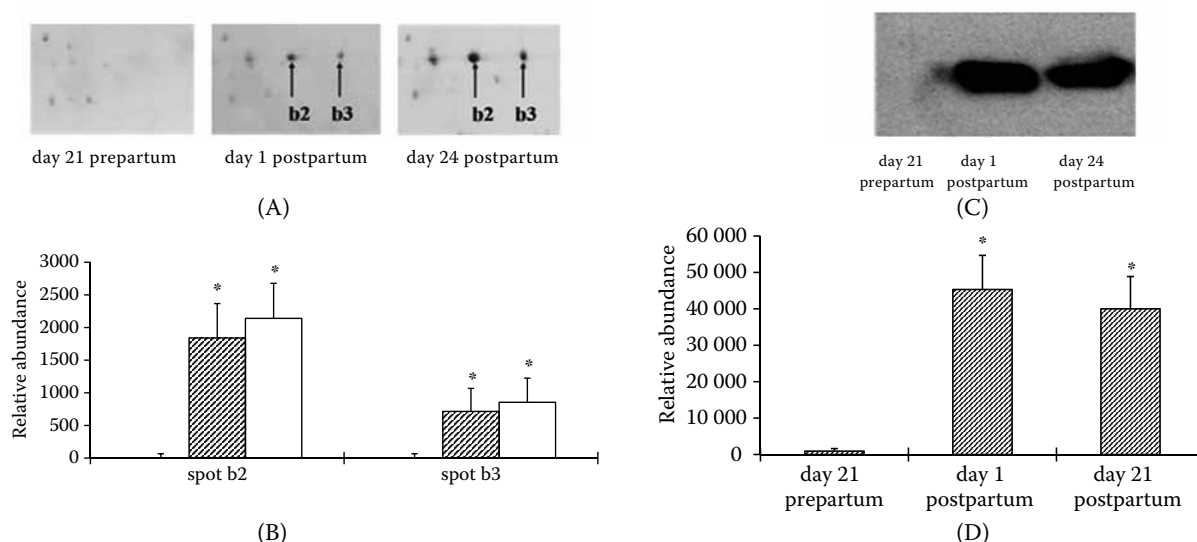


Figure 3. Changes of haptoglobin in plasma of dairy cows during the transition period associated with a new intramammary infection after calving

(A) representative images of haptoglobin spots in the 2-DE map and (B) densitometric analysis; (C) Western blot analysis of haptoglobin and (D) densitometric analysis

* $P < 0.05$ ($n = 3$)

to be up-regulated in plasma 21 day postpartum in cows with subclinical mastitis. Results from Western blot analysis were consistent with data from 2-DE followed by mass spectrometry.

One protein with multiple spots

There were multiple spots with varying masses and isoelectric points present on the gels of plasma collected after calving that revealed a marked increase in abundance of AGP. Two spots (spots a2 and a3, or spots b2 and b3) were detected on the gels with different isoelectric points but similar molecular weight, which were identified as haptoglobin, and two other spots (spots a11 and a12, or spots b11 and b12) were identified as serum amyloid A. Multiple spots may be due to the presence of genetic variants or post-translational modifications. These spots, identified by ion trap mass spectrometry as identical proteins, had similar peptide sequences (Table 1).

DISCUSSION

Results of the current study demonstrate that protein profiles change in transition dairy cows with or without subclinical mastitis after calving. The changes of AGP, haptoglobin, and serum amyloid A indicate that the current methods were successful in identifying differentially expressed plasma proteins in periparturient dairy cows.

Western blot analyses of haptoglobin in plasma in transition dairy cows with or without subclinical mastitis were found to be highly correlated and agree with the 2-DE in combination with mass spectrometry data.

Enhanced detection of minor serum proteins can be achieved by selective removal of major proteins using specific immunoaffinity columns and/or protein fractionations based on charge, size or hydrophobicity (Fountoulakis et al., 2004; Echan et al., 2005). Recently, hexapeptides library was shown to enable detection of many new proteins and to improve detection of minor proteins as compared to the depletion technique (Sihlbom et al., 2008). This new method could lead to determining both major proteins that are reduced and less predominant proteins that are concentrated, thus enabling detection of all proteins within a single sample (Boschetti and Righetti, 2008; Hartwig et al., 2009). Recently, Marco-Ramell and Bassols (2010) have analyzed the

efficiency and reproducibility of the ProteoMiner with bovine and porcine serum samples enriched with low-abundance proteins.

Haptoglobin, AGP, and serum amyloid A are primarily synthesized and secreted by hepatocytes. Indeed, parturition is an inflammatory process (Norman et al., 2007). Haptoglobin, which acts as an acute phase protein, was shown by ELISA detection to be higher at week 1 postpartum, and then decreased in cows without a new subclinical intramammary infection (Piccinini et al., 2004; Rezamand et al., 2007). Using 2-DE and mass spectrometry to analyze serum in periparturient dairy cows, haptoglobin and orosomucoid were abruptly increased at parturition as compared with the last phase of pregnancy (Cairolì et al., 2006). In addition, haptoglobin has been shown to increase during inflammation (Humblet et al., 2006). This is associated with several non-specific immune parameter changes, such as decreased respiratory burst and decreased nitric oxide concentration after calving (Piccinini et al., 2004). In cows infected with mastitis, the concentrations of haptoglobin, AGP, and serum amyloid A in serum were significantly increased as compared to those of healthy cows (Eckersall et al., 2001; Lehtolainen et al., 2004). Our results were consistent with previous observations that haptoglobin in plasma was increased at parturition in cows with and without mastitis but haptoglobin continued to be elevated 21 days postpartum in cows with subclinical mastitis.

AGP belongs to the subfamily of immunocalins, a group of binding proteins that also have immunomodulatory functions. Glycosylation is one of the most important ways to modulate both protein function and lifespan. Glycosylation microheterogeneity of AGP can be modified during disease (Ceciliani and Pocacqua, 2007). The AGP can also be used to adjust inflammation-induced hyporetinolemia in vitamin A-sufficient animals (Gieng and Rosales, 2006).

In addition, serum amyloid A is a family of proteins encoded in a multigene complex and is one of the acute-phase reactants in response to infection, inflammation, and trauma (Jensen and Whitehead, 1998). Serum amyloid A in periparturient dairy cows was measured by ELISA and it was found to increase dramatically after calving (Meglia et al., 2005; Jafari et al., 2006). In our study, isoforms of serum amyloid A were found to be altered in plasma before calving in cows with or without new intramammary infection. Serum amyloid A concentrations were down-regulated after calving in healthy

cows and up-regulated in cows with mastitis. This phenomenon is probably caused by the inflammatory process that was occurring during this period (Norman et al., 2007), which altered the plasma proteins at parturition. These changes may serve as an acute and temporal regulatory mechanism in response to parturition- and lactation-induced immunosuppression. Thus, cows are more susceptible to an intramammary infection after calving.

Our results show that apolipoprotein E is up-regulated in plasma after calving in early lactation cows with and without mastitis. It was reported that apolipoprotein E increased lipid uptake by macrophages in lipoprotein lipase deficient cows during pregnancy (Steinberg et al., 1996). Other researchers have shown that apolipoprotein E would stimulate the phospholipid transfer protein-mediation of surface fragments of triglyceride-rich lipoproteins as mediated by high-density lipoproteins (HDL) and promote HDL remodelling (Dallinga-Thie et al., 2007), and also interfere with lipolysis by interacting with the lipoprotein lipase activator, apolipoprotein C2 (Chalas et al., 2002).

CONCLUSION

In the current research with cows during the transition period, the plasma proteome was separated by 2-DE and several proteins that were associated with immune function were identified by LC-MS/MS. Expression of serum amyloid A, AGP, and haptoglobin, which serve as positive acute phase reactant proteins in response to parturition and lactation stress, was found to be up-regulated at parturition. Proteins that were altered at parturition were acute and temporal regulatory mechanisms in response to stressors of parturition and lactation, and so cannot serve as potential markers for periparturient cows susceptible to subclinical mastitis. These findings may provide valuable insight on the changes occurring in plasma protein in periparturient dairy cows with or without subclinical mastitis after calving.

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