TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2016) 40: 206-216

doi:10.3906/biy-1501-30

Analysis of protein expression changes in the blood plasma of cows during the last month before parturition and 2 months after calving

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Received: 11.01.2015 • **Accepted/Published Online:** 26.06.2015 • **Final Version:** 05.01.2016

Abstract: The organism undergoes tremendous changes at the end of pregnancy and beginning of lactation. The aim of the study was to analyze the physiological changes in cows during the last month of a first pregnancy and the first 2 months of lactation, based on the protein expression in peripheral blood plasma. Changes were observed in the expression of proteins involved in lipid metabolism (apolipoprotein A-I, apolipoprotein E, apolipoprotein J, and apolipoprotein A-IV); immune defense (complement C4 protein and mannose-binding protein C); blood clotting (fibrinogen alpha chain, fibrinogen gamma B-chain, and fibrinogen-like protein 1); as well as reconstruction, regeneration, and defense of cells and tissues (fibrinogen-like protein 1, glutathione peroxidase 3, endopin-1, Kelch-like ECH-associated protein 1, 72 kDa type IV collagenase, and pigment epithelium-derived factor). Multidimensional changes in the cow organism observed on the protein level at the end of pregnancy and during the first months of lactation highlight the immense challenges the organism must overcome to maintain homeostasis.

Key words: Proteomics, blood plasma, cows, pregnancy, lactation

1. Introduction

At the end of pregnancy and during early lactation, the body undergoes great changes in order to adapt and maintain homeostasis. This difficult period abounds in challenges for the mother and affects the health of the fetus/calf. Monitoring tools that allow for a multidimensional screening and application of the least invasive methods should be used during this time. Analysis of blood plasma with the aid of proteomic tools enables a comprehensive description of animal health status and indicates potential health problems, making them available for therapeutic solutions. Proteomic studies in cows during the periparturient period are sparse in context of their high potential and may provide valuable information for veterinary practice (Kupczyński and Chudoba-Drozdowska, 2002; Kindahl et al., 2004; Lippolis and Reinhardt, 2008; Chen et al., 2014; Kurpińska et al., 2014; Ożgo et al., 2015).

Therefore, the aim of the present study was to analyze physiological changes in cows during the last month of the first pregnancy and during the first 2 months of lactation on the basis of changes in protein expression in peripheral blood plasma. This analysis may contribute to a better understanding of the changes occurring in the bodies of pregnant and lactating young cows.

2. Materials and methods

2.1. Sample collection and preparation

The study was conducted in 10 primiparous cows of Polish Holstein–Friesian Black-and-White variety (95%–100% HF, half-sisters, clinically healthy) in the last month of pregnancy and the first 2 months of lactation. Animals were bred in a free-stall housing system with a TMR system of feeding (INRA dietary standards, 2001) (more details of feeding shown in Table 1).

Blood, the material for analysis, was drawn from the external jugular vein and collected using sterile needles and tubes with anticoagulant (EDTA K 3). Collection of samples was performed at the following points: 30, 14, and 7 days before parturition and 1, 7, 14, 30, and 60 days after calving. Blood was collected each time from the same animals, and groups were formed according to the sampling date; thus, each experimental group consisted of 10 animals. The total number of samples was 80. Blood samples were centrifuged (3000 rpm, 4 °C), and the resulting plasma samples were stored at –80 °C until analysis. The local ethics committee for experiments on animals in Szczecin approved the design of the experiment (resolution no.: 22/2009, 10.07.2009).

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Table 1. Nutrient composition of the feed for cows during the last month of pregnancy and lactation.

Ingredients	Daily dose (kg)		Dry matter (kg)	
	Pregnancy	Lactation	Pregnancy	Lactation
Haylage	4.0	-	2.16	-
Alfalfa silage	3.5	6.0	1.6	2.34
Maize silage	10.0	19.0	3.0	7.95
Straw	1.0	1.0	0.92	0.92
Coarse fodder: sum	18.5	26.0	7.68	11.22
Nutritive mixes*	4.0	8.0	3.52	7.08
Wet beet pulp	1.0	-	0.2	-
Maize cob silage	-	2.0	-	1.15
Dried sugar beet pulp	-	0.5	-	0.45
Salt	-	0.07	-	0.07
Molasses	-	0.8	-	0.63
Brewers' spent grains	-	3.0	-	0.79
Concentrated feed: sum	5.0	14.37	3.72	10.16
Dose: sum	23.5	40.37	11.41	21.38
Nutrients	Feed (kg)		Concentrate	
	Pregnancy	Lactation	Pregnancy	Lactation
Crude protein	1.703 kg	3.862 kg	7.245%	18.065%
Neutral deterg fiber	4.266 kg	6.586 kg	18.154%	30.808%
Nonfiber carbohydrt	4.206 kg	8.436 kg	17.899%	39.460%
Fat	0.281 kg	0.780 kg	1.194%	3.648%
Ne lact 3×	16.874 mcal	35.547 mcal	32.571 mcal/cwt	75.421 mcal/cwt
Sol prot, % of CP	36.725 ratio	30.489 ratio	36.725 ratio	30.489 ratio
RDP, % of CP	68.853 ratio	66.620 ratio	68.853 ratio	66.620 ratio
RUP, % of CP	31.136 ratio	33.374 ratio	31.136 ratio	33.374 ratio
Calcium	0.120 kg	0.198 kg	0.513%	0.928%
Phosphorus	0.051 kg	0.101 kg	0.216%	0.473%
Potassium	0.147 kg	0.274 kg	0.624%	1.283%

*Barley meal, triticate meal, corn meal, extruded soy meal, extruded canola meal.

2.2. Two-dimensional electrophoresis and image analysis
Preparation of blood plasma samples started with the concentration and enrichment of low- and medium-abundance proteins by using the ProteoMiner protein enrichment kit (Bio-Rad, USA). Selected steps for further sample preparation differed depending on the purpose of sample preparation, either for statistical analysis or identification of proteins [samples for statistical analysis – SAS (n = 80); samples for protein identification – PIS (n = 6)]. SAS (70 µL of ProteoMiner kit eluate) were precipitated in chilled (–20 °C) acetone (1:4 ratio) after a 2-h incubation and centrifuged at 14,000 rpm and 0 °C for 0.5 h and subsequently run on 2DE gels stained with silver.

PIS (300 µL of ProteoMiner kit eluate) were additionally purified using ReadyPrep 2-D cleanup kit (Bio-Rad, USA), run on 2DE gels, and stained with Coomassie brilliant blue.

Before electrophoresis, protein pellets were dissolved in lysis buffer to a final volume of 500 µL [5 M urea (Sigma-Aldrich, USA), 2 M thiourea (Sigma-Aldrich, USA), 2 mM TBP (Sigma-Aldrich, USA), 40 mM Tris (Sigma-Aldrich, USA), ampholyte (pH 3–10) (0.2% w/v; Bio-Rad, USA), and CHAPS (4% w/v; Bio-Rad, USA)]. Next, protein concentration was determined using a modified Bradford method (2D-QuantKit, Amersham BioSciences, UK). Then the samples containing either 140 µg of total protein (SAS) or 1.5 mg of total protein (PIS) were mixed with rehydration buffer [9 M urea, CHAPS 4% (w/v), 100 mM DTT (Sigma-Aldrich, USA), ampholyte (pH 3–10) 0.2% (w/v), bromophenol blue traces (Poch, Poland)] and loaded on IPG strips (24 cm, pH 4–7; Bio-Rad, USA) with 2 mL of mineral oil (Bio-Rad, USA). IPG strips were actively rehydrated for 17 h, at 20 °C and 50 V (Protean IEF, Bio-Rad, USA) and then isoelectrically focused (R: rapid voltage ramping mode, L: linear voltage ramping mode) [SAS: 250 V, 300 Vh (R) → 500 V, 750 Vh (R) → 1000 V, 1500 Vh (R) → 5000 V, 2 h (L) → 5000 V, 90,000 Vh (R): total 92,550 Vh; PIS: 150 V, 150 Vh (R) → 250 V, 500 Vh (R) → 500 V, 1000 Vh (R) → 1500 V, 2000 Vh (R) → 5000 V, 2.5 h → 5000 V, 90,000 Vh: total 93,650 Vh] (Protean IEF, Bio-Rad, USA). Subsequently, proteins were reduced by incubating each IPG strip for 15 min in DTT [1% (w/v)] in 10 mL of equilibration buffer: 0.5 M Tris/HCl, pH 6.8 (Bio-Rad, USA); 6 M urea, SDS 2% (w/v) (Sigma-Aldrich, USA); and 30% glycerol (w/v) (Sigma-Aldrich, Poland). Next, proteins were alkylated for 20 min in 2.5% (w/v) iodoacetamide (Sigma-Aldrich, USA) in 10 mL of equilibration buffer with bromophenol blue traces. To perform SDS-PAGE, 12% polyacrylamide gels were used [1.5 M Tris-HCl, pH 8.8 (Bio-Rad, USA); 30% acrylamide/bis 37, 5:1 (2.6% C) (Bio-Rad, USA); 10% (w/v) SDS 10% (w/v) ammonium persulfate (Sigma-Aldrich, USA); 1% TEMED (Sigma-Aldrich, USA); deionized water]. First, IPG strips were embedded in 0.5% molten agarose (Sigma-Aldrich, USA) on polyacrylamide gels, and migration buffer [25 mM Tris; 192 mM glycine (Sigma-Aldrich, USA); SDS 0.1% (w/v)] was added to the SDS-PAGE chamber PROTEAN plus Dodeca cell (Bio-Rad, USA), and molecular mass marker, Precision Plus Protein Kaleidoscope standard (Bio-Rad, USA), was applied (10 µL/gel). SDS-PAGE was run at 10 °C at a stable parameter of 15 mA/gel, 40 V for 1 h, and 120 V for 19 h. SAS gels were silver-stained in Dodeca stainer, large (Bio-Rad, USA). PIS gels for protein identification were stained with Coomassie brilliant blue G-250 (Fluka, USA). Images were scanned using a GS 800 calibrated densitometer (Bio-Rad, USA). Image analysis of gels was carried out at selected experimental time points: 30 and 14 days before calving and on days 1, 7, 30, and 60 after calving using the PDQuest 8.0 Advanced software (Bio-Rad, USA). Selection of time-points for further analysis was based on preliminary analysis of 2-D patterns. Preparation of 10 gels per group enabled a well-defined view on the proteome pattern characteristic for the particular analysis time-point. SAS gels were used for statistical analysis using Student's t-test integrated with the PDQuest software. The following parameters were applied: defined landmarks, smallest spot, faintest spot, size of the largest spot, and local regression model for normalization.

2.3. MS analysis and protein identification

First, protein spots were manually excised from PIS gels (each spot was analyzed twice) and destained for 20 min in 40 mM NH_4HCO_3 (95%) (Sigma-Aldrich, USA) and acetonitrile (ACN; 5% v/v) (Sigma-Aldrich, USA). Next, they were washed for 20 min with 40 mM NH_4HCO_3 (50%) and ACN (50% v/v), dehydrated for 10 min in 100% ACN, and vacuum-dried in a Concentrator 5301 (Eppendorf, Germany). Proteins were digested with trypsin (12.5 µg/mL in 40 mM NH_4HCO_3 for 16 h at 37 (C). Then peptides were extracted in 100% ACN for 15 min. The dried droplet technique was used to prepare the samples and a steel target (MALDI-MSP AnchorChip 600/96, Bruker Daltonics, USA). Peptide and matrix solutions [5 mg/mL CHCA (Bruker Daltonics, USA); 0.1% (v/v) trifluoroacetic acid (Sigma-Aldrich, USA); 50% (v/v) ACN; and deionized water] were premixed before spotting (1 µL each). Protein identification was performed using a Microflex MALDI TOF (Bruker Daltonics, USA) mass spectrometer calibrated with a Peptide Mass Standard II, 700–4000 Da (Bruker Daltonics, USA). Acquisition and analysis of mass spectra were carried out using FlexControl and FlexAnalysis software (Bruker, Daltonics, Germany). The following search parameters were applied: trypsin as cleavage enzyme, one missed cleavage, fixed modification (carbamidomethylation of cysteine residues), variable modification (methionine oxidation), and mass tolerance <150 ppm. The mass spectra were compared with those at Swiss-Prot database using Mascot search engine. If no cow protein was identified, but an orthologous protein was assigned to the mass spectra, the protein was considered identified. If not otherwise stated, proteins identified were bovine.

3. Results

In SAS samples the average spot number was 283 with mass values between 15 and 250 kDa and pI range from 4 to 7. Seventy protein spots showed changes in expression (50 protein spots: $P < 0.01$; 20 protein spots: $P < 0.05$) (Figure). The whole profile determination in PIS gels enabled the

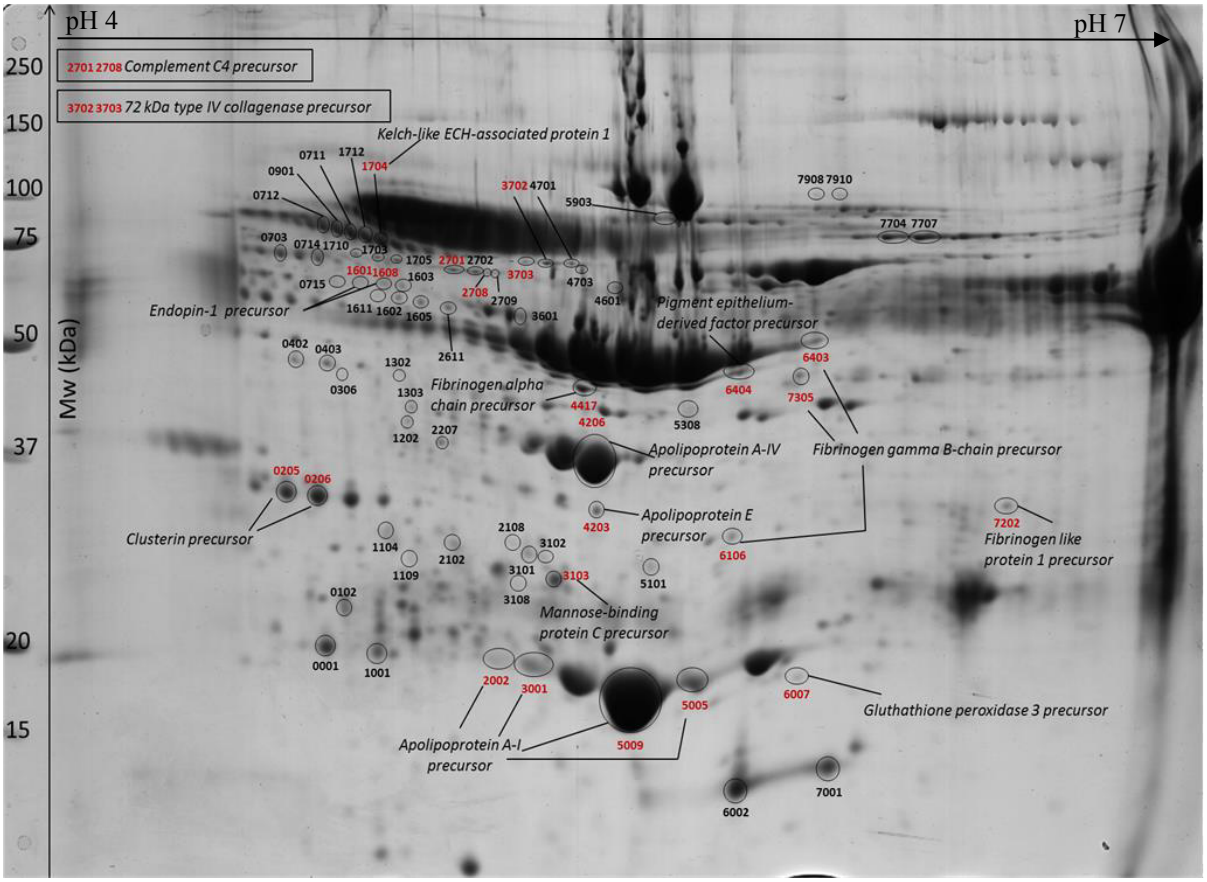


Figure. Blood plasma protein profile (1 month after calving) with differentially expressed protein spots stained with Coomassie brilliant blue (PIS gel); spot numbers in red denote identified spots; spot numbers were assigned by PDQuest software. High (1.5 mg) protein load resulted in higher number of resolved spots but also greater high abundant protein content.

naming of 128 spots; however, among the 70 spots showing a different expression pattern identification was successful in only 23. The 23 identified spots represented 13 different proteins: complement C4 precursor, mannose-binding protein C precursor, fibrinogen alpha chain precursor, fibrinogen gamma B-chain precursor, fibrinogen-like protein 1 precursor, glutathione peroxidase 3 precursor, endopin-1 precursor, Kelch-like ECH-associated protein 1 (identified in *Sus scrofa*), 72 kDa type IV collagenase precursor, pigment epithelium-derived factor precursor, apolipoprotein A IV precursor, apolipoprotein E precursor, apolipoprotein A-I precursor, and apolipoprotein J.

Differences in expression levels during the period analyzed are presented below. Table 2 shows the level of statistically significant changes in the expression and selected protein spots of the identified proteins. Identification details are presented in Table 3.

The highest expression of complement C4 precursor was observed 1 month before calving. A statistically significant lower level of this protein was observed 14 days before calving, on day 1 after calving, and day 7 of lactation. A slight increase was observed on days 30 and 60 of lactation; however, these changes were not statistically significant.

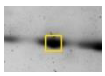
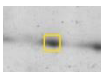
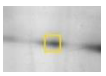
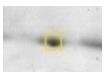
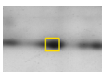
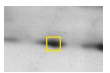
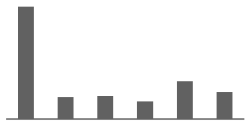
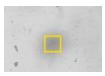
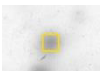
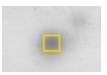
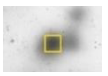
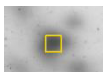
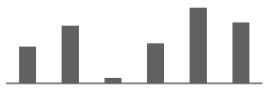
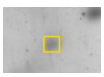
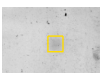
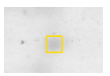
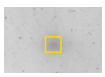
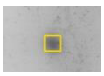
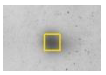
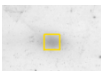
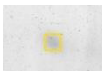
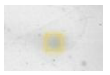
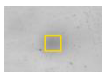
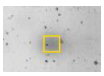
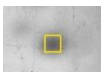
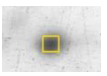
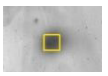
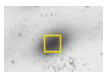
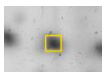
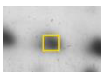
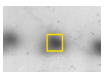
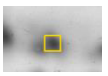
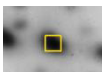
The expression of mannose-binding protein C precursor was stable during the prepartum period. The lowest expression was recorded on the 1st day after calving. Statistically significant higher expression was noted on lactation days 7, 30, and 60.

The expression of fibrinogen gamma B-chain precursor and fibrinogen alpha chain precursor was highest 14 days before calving. Statistically significant lower expressions of this protein were observed one month before calving and on the 7th, 30th, and 60th days of lactation.

The expression of fibrinogen-like protein 1 precursor increased up until the 1st day after calving (highest expression); during the postpartum period, a decrease in the level of this protein was observed. Statistically significant differences were observed between day 1 after calving and day 30 before calving and lactation days 7, 30, and 60.

The expression of glutathione peroxidase 3 precursor was low during the prepartum period. Higher expression

Table 2. Expression pattern of selected proteins on successive days of analysis with the scheme of their relative abundance. Row: I, C4 complement precursor; II, mannose-binding protein C precursor; III, fibrinogen gamma-B chain precursor; IV, fibrinogen-like protein 1 precursor; V, glutathione peroxidase 3 precursor; VI, endopin-1 precursor; VII, Kelch-like ECH-associated protein 1; VIII, 72 kDa type IV collagenase precursor; IX, pigment epithelium-derived factor precursor. Spots marked with the same letter in a single line represent statistically significant differences; capital letter denotes $P < 0.01$, small letter $P < 0.05$. Abbreviations used in the table: D, day; '-' indicates time points before parturition.

	−30D	−14D	1D	7D	30D	60D	Scheme of relative abundance (bars represent spots in the same order as in the table)
I	 Aab	 a	 A	 b			
II			 abc	 a	 b	 c	
III	 A	 ABCa		B	 C	 a	
IV	 A		 ABCa	 a	 B	 C	
V	 A					 A	
VI	 A			 b	 ABab	B	
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was measured in the postpartum period. Statistically significant changes were found between days 30 before calving and 60 of lactation.

The highest expression of endiopin-1 precursor was detected on day 30 of lactation. At other time points, expression of this protein was lower and remained at a

Table 3. List of differentially expressed protein spots with identification details. If not otherwise stated the proteins were identified in *Bos taurus*.

Protein name (species in which the protein was identified)	Spot number	Theoretical pI/ molecular mass (kDa)	Experimental molecular mass (kDa)	Score/ sequence coverage	Accession number	Peptides matched	Peptides searched
Complement C4 precursor (*Bos taurus*)	2701 2708	6.15/103.02	67.5 66.8	72/26% 64/23%	P01030	17 15	77 69
Mannose-binding protein C precursor (*Bos taurus*)	3103	5.11/26.91	28.5	75/26%	O02659	10	78
Fibrinogen gamma B-chain precursor (*Bos taurus*)	6403 7305 6106	5.54/50.84	50.8 45.9 31.2	83/33% 74/22% 85/31%	P12799	11 9 11	55 39 47
Fibrinogen alpha chain precursor (*Bos taurus*)	4417	6.73/67.48	44.2	103/30%	P02672	21	87
Fibrinogen like protein 1 precursor (*Bos taurus*)	7202	6.32/36.40	33.2	147/54%	Q3SZZ7	15	51
Gluthathione peroxidase 3 precursor (*Bos taurus*)	6007	6.84/25.89	23.1	64/29%	P37141	10	45
Endopin-1 precursor (*Bos taurus*)	1601 1608	5.57/46.29	64.5 64.2	66/28% 66/34%	Q9TTE1	10 12	55 71
Kelch-like ECH-associated protein 1 (*Sus scrofa*)	1704	6.13/71.14	76.5	61/28%	Q684M4	13	86
72 kDa type IV collagenase precursor (*Bos taurus*)	3703 3702	5.42/74.81	69.7 69.3	74/17% 187/41%	Q9GLE5	8 23	18 51
Pigment epithelium-derived factor precursor (*Bos taurus*)	6404	6.57/46.31	46.2	134/54%	Q95121	19	68
Apolipoprotein A-I precursor (*Bos taurus*)	2002 3001 5009 5005	5.71/30.26	24.1 23.7 21.8 22.8	121/56% 181/60% 237/62% 238/73%	P15497	17 19 20 25	91 66 75 80
Apolipoprotein A IV precursor (*Bos taurus*)	4206	5.30/42.99	36.4	190/65%	Q32PJ2	27	97
Apolipoprotein E precursor (*Bos taurus*)	4203	5.55/36.02	32.9	131/50%	Q03247	19	55
Clusterin precursor (*Bos taurus*)	0205 0206	5,73/51,65	34,2 33,8	69/18% 67/18%	P17697	11 14	54 67

similar level with slight, statistically insignificant differences. Hence, statistically significant differences were recorded between day 30 of lactation and 1 month before calving, on day 14 before calving, and on lactation days 7 and 60.

The expression of Kelch-like ECH-associated protein 1 showed a high degree of instability throughout the study period. The lowest expression was observed 14 days before calving and the highest 1 month after birth. Statistically significant changes in expression were found between the 14th day before calving and the 1st day after calving and the 30th day of lactation as well as between the 7th day of lactation (expression was low) and the 1st day after calving and the 30th day of lactation.

The highest expression of 72-kDa type IV collagenase precursor was noted on the 1st day after calving. The level of this protein was lower in both the last month of the pregnancy and during the first 2 months of lactation. Statistically significant differences in expression were demonstrated between the 1st day after calving and the 14th day before calving and the 7th day of lactation.

The lowest expression of pigment epithelium-derived factor precursor was detected 30 days before calving, and then an increase in the expression was observed, with the highest value on day 1 after calving. The expression of this protein during lactation was lower and showed negligible fluctuation.

Changes in the expression of apolipoprotein A IV precursor, apolipoprotein E precursor, apolipoprotein A-I precursor, and apolipoprotein J were characterized by the lowest values around parturition and higher values during the pre- and postpartum periods (Kurpińska et al., 2015).

4. Discussion

Analysis of the scientific literature shows few studies conducted on blood serum/plasma during pre- and postpartum periods. These studies were carried out by Cairoli et al. (2006), Li (2012), and Yang et al. (2012) using different proteomic techniques, i.e. 1- and 2-dimensional electrophoresis, high performance liquid chromatography (HPLC), isobaric tags for relative and absolute quantitation (iTRAQ), and liquid chromatography–tandem mass spectrometry (LC-MS/MS). When studying changes in protein expression the choice of analytical technique and the development of proteomic maps is extremely important for proper interpretation of the results of physiological and pathological changes in the bodies of animals and identification of potential molecular markers. In our analysis there were difficulties in assessing results of proteomic analysis of cattle plasma/serum due to the incomplete characterization of the genome, as noted by Talamo et al. (2003). Proteomic analysis of the blood plasma of cows investigated in this study characterized in detail the changes in protein profiles during the last month of pregnancy and the first 2 months of lactation. The selection of multiple time points for analysis and the discovery of differences in expression of 70 protein spots revealed the dynamic changes occurring in the bodies of cows during this period. However, from among 70 protein spots showing different expression in our study, only 23 were identified. Patterns of change in expression of identified proteins such as apolipoprotein E and apolipoprotein A-IV were similar in our studies and in the analysis of other authors; thus, proteins described by Cairoli et al. (2006), Li (2012), and Yang et al. (2012) might be among the unidentified spots in our studies.

The identified spots showing variable expression (23 out of 70) were represented by the 13 proteins characterized below.

The proteins of the complement system and mannose-binding protein C are important elements of the innate immune system (Ip et al., 2009). Complement C4 protein is a glycoprotein involved in the process of immune defense. It participates in the classical, lectin, and alternative pathways of complement activation leading to cell lysis (Klaska and Nowak, 2007). The high expression of this protein observed in cows in our study 1 month before calving was also noted by Gallery et al. (1981) in women. Richani et al. (2005) suggested that the physiologically high activity of the complement system during pregnancy is a compensatory mechanism to defend against potential infection. The lower and stable expression of this protein in the last 2 weeks of pregnancy and during the first week of lactation that was observed in our experiments was also reported by Kovar and Richez (1988); however, these authors reported significant differences in the concentration of this protein among individuals. The lower expression of this protein, observed in our experiment at the end of gestation and early lactation, may also be associated with its transfer into colostrum and milk (Trégoat et al., 1999). It should be noted that the expression of this protein is higher in the second month of lactation. Miura et al. (1987) argued that this protein was upregulated in response to inflammation or tissue damage and participated in the acute phase response.

Mannose-binding protein C (MBL) is a lectin produced in the liver that takes part in the immune response, including the recognition of apoptotic and necrotic cells (Bouwman et al., 2006). Geijn et al. (2007) indicated that MBL is involved in the activation of the lectin pathway of the complement system. In blood plasma this protein is associated with serine proteases. Both low and high levels of MBL in the blood indicate a variety of diseases, e.g., a high level of MBL was observed during inflammation (Bouwman et al., 2006). Here we focused on alterations that might be connected with periparturient changes. In contrast to our results Geijn et al. (2007) observed a higher concentration of MBL during pregnancy in women in comparison to 6 weeks postpartum. According to these authors, a MBL concentration during pregnancy that is too low might lead to premature births and reduced birth weight of the newborn, while a higher concentration of MBL promotes proper completion of the pregnancy. In addition, these authors reported that a higher concentration of MBL might indicate a reduction in adaptive immunity and provide compensation in the form of increased activity of the innate immune response. It could also reflect the increased removal of apoptotic cells. Our study demonstrated a significant increase in the expression of this protein during lactation, while Geijn et al. (2007) observed a gradual increase in the concentration of MBL starting from 6 weeks after parturition. According to Kehrli et al. (2009) and Goff (2008), decreased expression of this protein observed in the pre- and postpartum period could also be associated with short-term immune suppression, perinatal maternity stress, lower food intake, and high concentrations of glucocorticoids, as well as reduced levels of calcium. Lower expression of this protein in the first week after calving in the analyzed cows may be associated with secretion of the protein into colostrum and milk, which is consistent with the results reported by Trégoat et al. (2002).

Proteins involved in blood clotting–fibrinogen and fibrinogen-like protein 1 also showed variations in expression.

Fibrinogen is a dimer linked by disulfide bonds; both subunits are composed of α, β, and γ chains. It is primarily involved in the processes related to hemostasis (Mosesson, 2005). A statistically significant increase in fibrinogen expression was observed at the end of pregnancy. This tendency was also observed by Heuwieser et al. (1990) in cows and women (Adler et al., 2000). The latter author reported its decrease in the postnatal period, which was also noted in our experiment. The changes might be connected to significant changes in the fibrinolytic system, which was suppressed during pregnancy, as observed by Nakamura et al. (1984).

Protein fibrinogen-like protein 1 is an acute phase protein produced in the liver. Liu and Ukomadu (2008) reported that this protein was involved in the structural and functional changes of the liver, including higher expression during liver regeneration. Hara et al. (2000) reported that the protein stimulated autocrine proliferation of liver cells. Moreover, Liu and Ukomadu (2008) suggested that it might be involved in the processes of hemostasis. Rijken et al. (2006) showed that this protein was involved, together with fibrin and fibrinogen, in the process of clot formation. In our experiment the biggest expression changes were noted in the last 2 weeks before parturition (increase in expression) and the first 2 weeks after calving (decrease in expression). It is worth noting that the expression of fibrinogen-like protein 1 and fibrinogen A and gamma was lower 4 weeks before calving and also during lactation compared to the period immediately before parturition and after calving. Hara et al. (2001) demonstrated that fibrinogen-like protein 1 had a similar structure to fibrinogen γ. Liu and Ukomadu (2008) indicated that the trend of changes in concentration of fibrinogen-like protein 1 and fibrinogen α were similar in response to the supply of cytokines (IL-6) (applied to induce acute inflammation).

The main function of glutathione peroxidase 3 is to protect cells against oxidation. This includes searching for the reactive oxygen species produced in metabolic processes and during oxidative stress and maintaining vasoactive and antithrombotic properties of the blood vessel endothelium. Expression of this protein occurs in many tissues, including kidney, lung, heart, and liver (Jin et al., 2011). The lower expression of this protein observed in the present experiments was consistent with findings of other authors, who observed a decrease in glutathione peroxidase activity during pregnancy in rats (Mistry et al., 2008) and women (Behene and Wolters, 1979). These changes might be associated with a reduction in the concentration of selenium, a component of glutathione peroxidase, in the blood due to increasing fetal demand for this element (Mistry et al., 2008). Contrary to our findings, Butler et al. (1982) observed an increase in glutathione peroxidase activity in women during pregnancy.

Endopin-1 is a serpin, an endogenous inhibitor of serine proteases. It is expressed in tissues such as neuroendocrine secretory vesicles of the adrenal medulla and the pituitary gland (Hwang et al., 1999). Muscle endopin-1 is widely distributed in cattle, and its presence was found, inter alia, in blood plasma, liver, spleen, thymus, and kidney (Tassyet al., 2005). This protein is related to alpha-1antichymotrypsin. It is an inhibitor of trypsin-like serine proteases and exhibits selective inhibition of trypsin (Hook and Hwang, 2002). Husna and Suhaimi (2009) analyzed changes in gene expression in the hypothalamus during the transition period in cows. They showed increased expression of the gene coding for endopin-1 during the development of ovarian follicles after calving. The authors suggested a significant role of this protein in the regulation of follicular growth and ovulation (Husna and Suhaimi, 2009). In the context of these studies, the peak of endopin-1 expression was observed 1 month after calving in our analysis, which might be connected with the onset of reproductive cycles after calving.

Kelch-like ECH-associated protein 1 is a cytoplasmic factor that binds to the actin cytoskeleton and the Nrf2, contributing to its storage within the cell. It also participates in ubiquitination and proteasome degradation of Nrf2. After the exposure of the cells to oxidative stress or chemopreventive molecules, Nrf2 dissociated from Keap-1 and contributed to increased gene expression (Zhang, 2006). The proteins of this signaling pathway play an important role in vascular homeostasis and protect epithelial and smooth muscle cells against oxidative stress in conditions such as preeclampsia or atherosclerosis (Mann et al., 2007). Noguchi (2008) reported that the stress in response to changes in the shape of the cells might activate the Nrf2/Keap-1 pathway. Sykiotis and Bohmann (2008) reported that Keap-1 was involved in protection against neurodegeneration, chronic inflammation, and neoplasia. The high variability in expression of Keap-1 may reflect its high involvement in prevention of damaging factors like oxidative stress and the occurrence of many challenges during the periparturient period.

The 72 kDa type IV collagenase is a metalloproteinase with collagen-degrading activity located in the basement membrane. Arthur et al. (1992) and Vartio and Baumann (1989) showed higher concentrations of this protein during liver damage in humans. These authors showed that the expression of this protein in normal human liver was low, and the increased expression was dependent, inter alia, on cytokines. In human blood plasma it was present in the form of proenzyme, and, according to the

above-mentioned authors, its role was more local than systemic. This protein was also expressed in various reproductive tissues during labor in humans and rats (Riley et al., 2000). In our experiment the most remarkable expression was observed on the first day after calving. Othmani-Mecif et al. (2006) also showed an increase in the concentration of gelatinase A in rabbit plasma shortly after parturition. This phenomenon might be connected with intensive reconstruction of these tissues. Othmani-Mecif et al. (2006) justified the increased concentration of gelatinase A through declining levels of progesterone in the blood before parturition; they also observed a positive correlation between the increase in concentrations of gelatinase A and an increase in leptin levels before parturition. Ricke et al. (2002) indicated that prostaglandin F2α stimulated the expression and activity of metalloproteinases, including gelatinase A, in the corpus luteum, suggesting the role of metalloproteinases in its regression. In contrast to our findings Oddsdottir et al. (2007) demonstrated that the activity of gelatinase A during labor in mares was significantly lower than in the last 3 days before parturition.

Pigment epithelium-derived factor (PEDF) is a serine protease inhibitor, a potent inhibitor of angiogenesis (Petersen et al., 2003). It has neurotrophic properties (Famullaet al., 2011), and depending on the interactions with other proteins and posttranslational modifications it can contribute to the induction or inhibition of apoptosis. It is also involved in cell differentiation. PEDF is present in blood plasma, can diffuse into the interstitial space, and binds collagen type I. This protein was expressed in many tissues (Petersen et al., 2003). These authors claimed that this protein, present in the blood, played a specific role in the termination of angiogenesis in wound healing. In fact, expression of this protein is related to the immune defense of the organism and is correlated with body mass. Umea et al. (2010) and Dong et al. (2008) indicated that the increased concentration of PEDF in the blood could protect against inflammation; thus, the high expression of PEDF observed in our experiment on day 1 after calving might be connected with intensive processes of regeneration. In contrast, Wang et al. (2008) showed that the concentration of PEDF was positively correlated with body fat, which was confirmed by Famulla et al. (2011), who showed that this protein is an adipokine produced in large quantities in the adipose tissue. Tshoneret et al. (2011) demonstrated that the reduction in the concentration of PEDF could be associated with weight and fat loss (low BMI) and the concentration of insulin and triglycerides. It is worth noting that Chen et al. (2012) found no differences in the concentration of PEDF in the serum of women at different phases of the sexual cycle. These observations are consistent with the results of our study, as we have not found statistically significant variations in the expression of this protein in the first 2 months of lactation.

Intensive changes in the expression of blood plasma proteins are observed during the last month of pregnancy and the first 2 months of lactation, especially associated with lipid metabolism; immune defense; and blood clotting; as well as reconstruction, regeneration, and defense of cells and tissues. The above indicates that intensive functional changes occurring in the body of pregnant and lactating cows are reflected in different protein levels. It should be noted that the analysis of blood plasma proteome in cattle is complex, as evidenced by the fact that, despite repeated spectrometric analysis, only 23 of the 70 spots showing differential expression have been identified. However, the identification of 70 spots showing differences in expression indicates the multidimensionality of changes in the body during this difficult period. The techniques applied allowed for comprehensive analysis and point to an area for further research on pregnancy and lactation in cattle.

Acknowledgment

This work was supported by a research grant from the Ministry of Science and Higher Education, Poland (project no.: N311 112538; 2010–2013).

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