Comparison between endometrial protein profile in Holstein-Friesian heifers and female prepubertal calves

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Keywords
Calves,
Cows,
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Proteome.

Summary
The protein profile of each tissue depends on the expression of genes that are regulated, among others, by sex steroids. Comparing the profiles of sexually immature and mature females should therefore define markers of age-related changes. The aim of this study is to compare the pattern of proteins in the endometrium of heifers and pre-pubertal female calves by using difference in gel electrophoresis (DIGE), and to identify the presence and amount of similar or significantly different proteins. Endometrial samples were collected in slaughterhouse from heifers aged between 14-27 months (n = 6; sexually mature) and calves between 0.5-2 months (n = 6; sexually immature). All animals were healthy, Holstein-Friesian bred animals. Samples were subjected to fluorescent staining and 2D electrophoresis. Out of more than 900 spots detected in the endometrium of heifers, 73% were similar to calves. Selected spots were identified. Angiopoietin-2 and dynamin-like protein were detected only in heifers. These proteins are involved in angiogenesis and cell membrane remodelling, respectively. Aldose reductase, and phospholipase, which are important for prostaglandin metabolism, were present in different amounts in both sources. These results help to further understand the mechanism of steroid hormone action and look for markers of bovine endometrium status. Moreover, fluorescent staining appeared to be an useful tool when comparing 2 samples from different sources.

Comparazione tra profili proteici endometriali di giovenche e di vitelle di razza Holstein-Friesian

Parole chiave
DIGE,
Endometrio,
Mucche,
Proteome,
Vitelli.

Riassunto
Il profilo proteico di ciascun tessuto dipende dall'espressione di geni che sono regolati anche da steroidi sessuali. Confrontare i profili delle femmine sessualmente mature e immature dovrebbe quindi definire i marcatori delle variazioni legate all'età. Scopo di questo studio è confrontare il pattern di proteine nell'endometrio di giovenche e vitelli pre-puberali utilizzando l'elettroforesi differenziale su gel (DIGE) e identificare la presenza e la quantità di proteine simili o significativamente differenti. In mattatoio sono stati raccolti campioni endometriali da giovenche di età compresa tra 14 e 27 mesi (n = 6, sessualmente mature) e vitelle tra 0,5 e 2 mesi (n = 6, sessualmente immaturi). Gli animali, tutti di razza Holstein-Friesian, erano sani. I campioni sono stati sottoposti a colorazione fluorescente e elettroforesi 2D. Il 73% degli oltre 900 prelievi era simile sia nell'endometrio delle giovenche che in quello dei vitelli. I punti selezionati sono stati identificati. L'angiopoietina-2 e la dinamina-like protein, invece, coinvolte rispettivamente nell'angiogenesi e nel rimodellamento della membrana cellulare, sono state rilevate solo nelle giovenche. La aldoso reduttasi e la fosfolipasi, importante per il metabolismo delle prostaglandine, erano presenti in quantità diverse in entrambe le fonti. I risultati dovrebbero aiutare a comprendere meglio il meccanismo d'azione degli ormoni steroidei e cercare i marcatori dello stato dell'endometrio bovino. La colorazione fluorescente, inoltre, si è rivelata uno strumento utile nel confronto tra 2 campioni esaminati.

Introduction

Uterine endometrium is a very dynamic tissue. Its appearance and function in bovine species is determined by the phase of oestrus cycle, which is regulated, among other compounds, by the concentration of the sex steroid hormones: estrogens and progesterone (Spencer *et al.* 2004). During pregnancy, the endometrium is responsible for creating the interplay between the mother and developing fetus.

Bauersachs and colleagues (Bauersachs *et al.* 2005) examined the profile of gene expression in bovine endometrium during the late oestrus (Day 0, low progesterone) and dioestrus phases (Day 12, high progesterone) of the cycle using a combination of subtracted cDNA libraries and cDNA array hybridisation. Different functional groups of proteins dominated during oestrus (day 0, low progesterone), while in dioestrus (day 12, high progesterone), the genes of several groups of enzymes and proteins increased.

Forde and colleagues (Forde *et al.* 2011) focused on the early (D7) and late luteal (D13) phases in their study, which describes the occurrence of significant differences in endometrial gene expression in the bovine endometrium. Faulkner and colleagues (Faulkner *et al.* 2013) described the impact of the phase of cycle and progesterone plasma level on the protein profile of the bovine endometrium.

The activity of the endometrium is reflected by concert action of different proteins that are synthesised as the response to stimuli coming from hormones and their receptors. The endometrium undergoes temporal remodelling during the cycle, but also develops during pregnancy. The profile of endometrial proteins is responsible for the appropriate function of the endometrium. Cytological changes related to the sequence of sex steroid activity are well described, but proteins that are directly involved in morphological changes have not yet been fully established.

The available literature about protein profile in the bovine endometrium is focused on the differences between pregnant and non-pregnant animals and the proteins that are characteristic for pregnancy (Berendt *et al.* 2005, Ledgard *et al.* 2009, Forde *et al.* 2014). However, there is still a lack of information about the protein profile in non-pregnant animals and the associated changes in this profile during ageing or sexual maturation. It is well-known that the influence of sex steroids differentiates immature from mature organisms, which is furthermore reflected in their different protein profiles. Protein profile dependent on sex steroids was not elucidated yet.

Analysis of mRNA often does not reflect the real amount/biological activity of certain proteins due to, for example, the post-transcriptional modification of protein or catalytic capacity of enzymes (Fu *et al.* 2009).

Two-dimensional electrophoresis is a useful tool to determine protein molecules in a particular biological sample. Each run, however, may differ slightly. Difference in gel electrophoresis (DIGE) overcomes this disadvantage by allowing 2 samples to be labelled with fluorescent dye whilst also allowing for their separation in the same run. This way of comparison between 2 samples can reveal the details of which proteins are characteristic for one or another or both biological sources. This technique is used in human medicine to search for markers of diseases (Gharbi *et al.* 2002, Tian *et al.* 2008) but could also be used to search for markers of physiological status in animals.

The aim of the present investigation is to describe the endometrial pattern of proteins and to compare the presence as well as the amount of selected proteins between heifers (sexually mature) and female calves (sexually immature) by using fluorescent staining.

Materials and methods

An experimental protocol was approved by the appropriate Ethics Committee appointed at the University of Life Sciences in Lublin (decision no 60/2011).

The uterus samples were sourced from a slaughter-house. In particular, uteri were collected from heifers in their luteal phase, aged between 14-27 months (n = 6), and pre-pubertal female calves, aged between 2 weeks-2 months (n = 6). The classification of luteal phase was based on gross detection of a corpus luteum, which was mature in all the heifers included in the experiment (Ireland *et al.* 1980). All animals were healthy, neither inseminated nor pregnant, and of Holstein-Friesian breed.

Tissues were collected immediately after the animals were slaughtered, washed in 0.9 % NaCl, portioned, and frozen. Uteri were separated manually into myometrium and endometrium, samples were taken consistently from the same region of uterus. The endometrium was used for further determinations. Tissues were homogenised in phosphate buffer (0.05 mol/dm^3, pH 7.2), centrifuged at 4 ^{o}C for 20 minutes at 6000 x g. Supernatant was used for protein precipitation, which was performed with Ready Prep 2-D Clean-up Kit (Bio-Rad, Warszawa, Poland) according to the manufacturer's instructions.

DIGE labelling

Fifty micrograms of resolubilised protein preparations were labelled with 333 pmol of CyDye

DIGE Fluor minimal dyes (GE, Warsaw, Poland). Pre-electrophoretic labelling was performed according to the manufacturer's instructions. Samples from cows were stained with Cy 3 and samples from calves with Cy 5. In order to avoid non-specific labelling, dye swap was made. The internal standard was created as a pool from equal amounts of all samples in particular experiment and was stained with Cy 2.

Electrophoresis

Isoelectric focusing was performed by loading 10 µg proteins by in-gel rehydration in a volume of 250 µl; denaturating the 2D buffer (8 M urea, 4 % CHAPS, 70 mM DTT, 0.5% Ampholyte pH 4-7) onto 11 cm IPG-Ready Strip linear pH 4-7 NL (Bio-Rad, Warsaw, Poland) and focused for 30 kVh, using a PROTEAN® IEF system (Bio-Rad, Warsaw, Poland). The same amount of protein from both examined sources was loaded.

Before loading IPG strips onto SDS-polyacrylamide gels, they were incubated for 15 minutes in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% DTT, and then for another 15 minutes in equilibration buffer containing 2.5% iodoacetamide.

We proceeded with the SDS-polyacrylamide gels (20 x 20 cm, 1.5 mm, T = 11%, C = 2.6%) in accordance to Laemmli (Laemmli 1970). The second dimension was performed using a Protean II XI (Bio-Rad, Warsaw, Poland) according to the manufacturer's instructions.

The gels with separated labelled proteins were scanned using the Typhoon® 9410 imager (GE Warsaw, Poland) and the protein patterns were displayed with IQ Tools software. All sample gel images were processed by the Image Master Platinum DIGE 7.0 software (GE, Warsaw, Poland) in order to co-detect and differentially quantify the protein spots.

In order to extract manually spots of interest, gels were stained using protein silver nitrate, according to Mass Spectrometry compatible protocol (Shevchenko *et al.* 1996).

Selected spots of interest were excised from the gels, chopped into pieces, and transferred into 0.5 mL tubes. The gel pieces were washed 3 times with 100 µL of 100 mM NH_4HCO_3 buffer (pH 8.5) (Sigma, Poznań, Poland) for 5 minutes. The gel pieces were subsequently dehydrated by adding 100 µL Acetonitrile (ACN) and dried in CentriVap (Labconco, local seller A.G.A Analitical, Warsaw, Poland) (room temperature, 15 minutes). They were then allowed to re-swell in 100 µL of 10 mM DTT in 50 mM NH_4HCO_3 of buffer in order to perform reduction (56 °C, 60 minutes). After cooling to room temperature, the solution was replaced by 100 µL of 50 mM iodoacetamide in 50 mM of NH_4HCO_3 buffer. The gel pieces were incubated in this solution in the dark for 45 minutes at room temperature. The gel pieces were then washed 3 times with 100 mL of a 100 mM NH_4HCO_3 buffer for 5 minutes at room temperature, dehydrated with 100 µL ACN, and dried in the CentriVap (Labconco, local seller A.G.A Analitical, Warsaw, Poland) for 15 minutes.

The enzymatic digestion of the proteins was carried out on ice by a stepwise addition of 10 µl of 12.5 ng/mL trypsin (Trypsin Gold, mass spectrometry grade, Promega, Madison, USA) in a 50 mM NH_4HCO_3 buffer, until they were totally rehydrated. Finally, 30 µl 50 mM NH_4HCO_3 buffer was added to keep the gel pieces covered during digestion at 37 °C overnight. After digestion, the supernatant was collected and the peptides were extracted 3 times with 50 µl 70% ACN with 1.5% trifluoroaceticacid (TFA) by sonification for 15 minutes at room temperature in an ultrasonic water bath (Ultron U-507, Ultron, Dywity, Poland). The supernatant was collected and dried in the CentriVap (Labconco, local seller A.G.A Analitical, Warsaw, Poland) for 45 minutes at 40 °C.

Peptide pellet was allowed to re-swell in 10 µL of 0.1% TFA and purified with µC18 ZipTip (Eppendorf, Poznań, Poland) according to the manufacturer's instructions. The 1 µl of cleaning peptide mixture was picked to a pre-spotted HCCA-PAC (with 3,5-dimethoxy-4-hydroxycinnamic acid) frame (Bruker, Poznań, Poland) and allowed to dry at room temperature. Mass spectra were acquired with an Ultraflex III MALDI TOF/TOF spectrometer (Bruker, Poznań, Poland). Acquisition was performed in positive ion reflector mode with a 25-kV acceleration voltage. External calibrations were performed using the peptide calibration standard (Bruker, Poznań, Poland). Flex analysis 3.0 software Bruker-Daltonics was used for the selection of the monoisotopic peptide masses.

The identification of peptides and proteins from mass spectrometry data was achieved by MASCOT algorithm interrogating, the UniProtKB database was restricted to the 'other Mammalia' taxonomy. Search parameters were set as follows: enzyme – trypsin; modification obligatory – carboamoinodmethylacion cysteine; possible modification – oxidation of methionine; error of 50 ppm.

Statistical analysis

The spots were selected for further examination and identification based on the statistical analysis of gels [Anova, Wilcoxon (1945) and Kolmogorov (1933) tests]. The selection was based on the presence of spots and their intensity, which reflects the amount

of protein. Statistical analyses allowed us to select spots that were most different and similar between heifers and pre-pubertal female calves. P-value was defined as < 0.05.

Results

The statistical analysis of data is presented in Figure 1 (scatter plot). Gel analysis detected 947 spots in the endometrium of heifers. Out of this number, 73% of spots (689) were similar to the endometrium of pre-pubertal female calves. The endometrium of calves showed 816 spots. Out of this number, 34% of spots were specific to calves, while 538 spots (66%) were similar to the endometrium of heifers.

The protein profile of bovine endometrium from heifers and pre-pubertal female calves is shown in Figure 2.

Proteins were selected for further analysis based on the statistical analysis of gels (Anova test, Wilcoxon and Kolmogorov). Statistical tests allowed us to select the most different and the most similar spots between heifers and pre-pubertal female calves. Eight spots were selected for preliminary identification (Figure 1). Spots 1, 6, 7, and 8 were detected only in heifers while 2, 3, 4, and 5 were present in both sources. Spot number 5 showed a similar intensity of staining (similar amount of protein) both in heifers and pre-pubertal female calves, while spots 2, 3, and 4 showed a higher intensity of staining (higher amount of protein) in heifers than in pre-pubertal female calves. The results of the identification of proteins (shown in Figure 1) are presented in Table I.

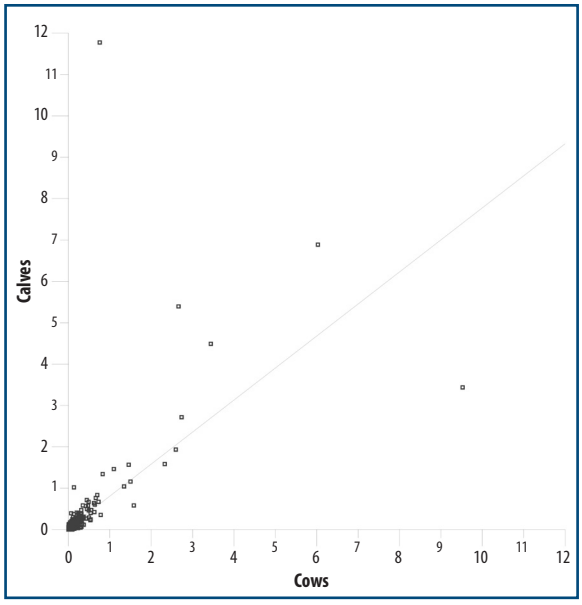

Figure 1. *Scater plot of spots from endometrium of heifers and pre-pubertal female calves.*

Spots 4 and 7 were identified as similar proteins, however they do differ in molecular weight. This may suggest that one of them is simply a fragment.

Further analysis of the spots is planned.

Discussion

In this study we performed a preliminary examination of protein profiles from bovine endometrium and conducted a comparison between heifers (sexually mature) and female calves (sexually immature). To our knowledge this is the first time such an experiment has been performed. The 2-D DIGE analysis allowed for the detailed detection of spots that belonged to either 1 of 2 compared samples, or both examined sources.

The elucidation of protein profiles in this particular tissue should help further understanding about the relationship between their biological role and the mechanism of action of sex steroids. Moreover, any differences related to age and the same status of prepuberty-puberty may help to establish possible markers of alterations.

The gene profiles of uteri in humans (Borthwick *et al.* 2003), mice (Tan *et al.* 2003), rhesus monkeys (Ace and Okulicz 2004), and bovines (Bauersachs *et al.* 2008) were determined and described. Steroid hormones, using genomic way of action, are responsible for the expression of genes leading to the synthesis of the particular proteins necessary for

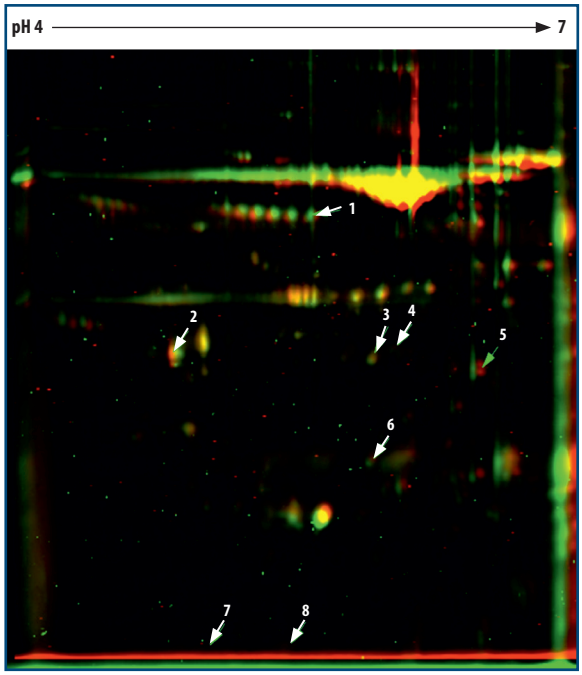

Figure 2. *2-D DIGE image of gel with endometrial proteins from heifers and pre-pubertal female calves.* Identified proteins are marked with numbers.

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Table I. *The list of identified proteins from endometrium of heifers and prepubertal female calves selected based on similarities and significant differences in intensity of staining.*

Spot ID*	Protein name	Uniprot	Score	Anova	Wilcoxon	Kolmogorov
1	Angiopoietin-2	ANGP2_PIG	41	0.001	0	1
2	Phospholipase DDHD1	DDHD1_BOVIN	36	0.489	2	0.5
3	Putative RNA exonuclease NEF-sp	REXON_BOVIN	54	0.001	0	1
4	Dehydrogenase/reductase SDR family member 4	DHRS4_BOVIN	39	0.025	0	1
5	Aldose reductase	ALDR_BOVIN	59	0.982	1	0.5
6	Cytochrome P450 3A6	CP3A6_RABIT	38	0.001	0	1
7	Dehydrogenase/reductase SDR family member 4	DHRS4_BOVIN	39	0.013	0	1
8	Dynamin-1-like protein	DNM1L_BOVIN	60	0.016	0	1

* See Figure 2.

a cellular response to receptor-hormone interaction. Their biological activity may differ depending on age, sex, and the phase of cycle.

Bauersachs and colleagues (Bauersachs *et al.* 2005) suggested that proteins representing 'cytoskeleton', 'cell motility', 'cell adhesion', 'extracellular matrix structural proteins', 'ECM remodelling', and 'proliferation' dominate during oestrus, while in dioestrus, genes of several groups of enzymes and proteins connected with 'transport' are increased.

Berendt and colleagues (Berendt *et al.* 2005) observed higher amount of proteins such as Rho GDP dissociation inhibitor beta; 20 alpha-hydroxysteroid dehydrogenase; soluble NADP1-dependent isocitrate dehydrogenase 1; and acyl-CoA-binding protein in pregnant cows than in non-pregnant animals. These proteins are involved in redox reactions and cell signalling.

In the present study we identified angiopoietin-2 (Ang-2) in endometrium samples from heifers, which was absent in the endometria from female pre-pubertal calves. Angiopoietin-2 belongs to a family of vascular growth factors and plays a role in embryonic and postnatal angiogenesis. It influences angiogenesis through the activation of endothelial cell-specific receptor tyrosine kinase Tie-2. Ang-2, which is antagonist of Ang-1, takes part in regulation of vascular re-modelling together with vascular endothelial growth factor (VEGF). When VEGF is present, Ang-2 stimulates the proliferation and migration of endothelial cells, but while VEGF is absent, Ang-2 causes endothelial cell apoptosis and vessel regression (Holash *et al.* 1999). Ang-2 is very important in appropriate female reproduction related to uterine vascular permeability and angiogenesis, and its presence has been confirmed in the ovaries, uteri, and placentas of mice (Matsumoto *et al.* 2002) as well as in human endometrium (Hirchenhain *et al.* 2003), human placenta during normal pregnancy (Schiessl *et al.* 2009), and in uterine natural killer cells in the late secretory phase (Li *et al.* 2001). According to Bin *et al.* (2012), oestrogens increased relevant changes in Ang-2 concentration in uteri from mice, while progesterone inhibited it. The actions of steroid hormones were compared with the action of its antagonists ICI 182,780 and RU486, respectively.

On the contrary, Hirchenhain and colleagues (Hirchenhain *et al.* 2003) did not observe significant differences in the concentration of Ang-2 during the human menstrual cycle. This could suggest that Ang-2 might not be regulated by progesterone. Krikun and colleagues (Krikun *et al.* 2004) also did not notice major changes in the amount of Ang-2 after estradiol or medroxyprogesterone acetate treatment in cultured human endometrial endothelial cells.

Genes for Ang-2 like ANGPTL2 and endothelial tyrosine kinase (TEK, angiopoietin receptor TIE-2) were expressed during oestrus in bovine endometrium (Bauersachs *et al.*2008).

Another protein that was identified in examined samples is dynamin-1-like, which was present only in heifers. The protein belongs to a superfamily of large Guanosine triphosphatase GTPase proteins.

The main function of this particular protein is membrane remodelling in a variety of cellular membranes. It is also associated with receptor-mediated endocytosis at the plasma membrane and is involved in the process of fission of mitochondria. The lack of dynamin-like protein, which is essential to the remodelling process described above, may lead to a defect in division (Bleazard *et al.* 1999, Sesaki and Jensen 1999). For more details about the dynamin-like protein, see Williams and Kim's (Williams and Kim 2014) review.

Among the identified proteins that were present in both groups of animals, and which also showed a similar intensity of staining (similar amount of protein), there was Aldose reductase (EC 1.1.1.21).

The Aldose reductase enzyme plays an important role in the endometrium because there is a strong

connection between AKR1B5 – recently renamed as *Bos taurus* – AKR1B1 (gene ID 317748), and prostaglandin F2α (PGF2α) production, and because it is involved in alternative pathway of PGF2α synthesis (Madore *et al.* 2003). It is moreover associated with additional enzymatic activity of 20α-HSD and glucose metabolism activities. The new activity has been confirmed in bovine (Madore *et al.* 2003) and human endometrium (Bresson *et al.* 2011). It is interesting that human and bovine AKR1B1 belong to the AKR1B family and share 86% identity or homology.

The same protein was identified in the peri-implantation uterine luminal fluid that was taken from pregnant sheep. According to Rita and colleagues (Rita *et al.* 1998), the protein was synthesised by the trophoblast and detected even before the formation of the placenta.

Bresson and colleagues (Bresson *et al.* 2011) studied the presence of both AKR1B1 and AKR1C3 at the mRNA and protein levels in human endometrium during the menstrual cycle. They observed that both mRNAs were present throughout the cycle without significant variation. The AKR1C3 protein immunostaining was constant during the cycle in epithelial cells, but completely absent in the stromal compartment. This has also been confirmed in other studies (Pelletier *et al.* 1999). At the same time, the AKR1B1 protein was present in luminal and glandular epithelial cells as well as in stromal cells of the endometrium. A higher amount of AKR1B1 protein was observed in the early proliferative and mid-late secretory phases compared with other phases of the menstrual cycle. The results were surprising taking into consideration that human endometrial stromal cells produce high concentrations of PGF2α (Chapdelaine *et al.* 2006, Kang *et al.* 2006). This leads to the hypothesis that the corresponding human enzyme AKR1B1 could possess PGFS activity in the human endometrium as similar to AKR1B5 in bovine endometrium (Madore *et al.* 2003).

The human AKR1B1 can metabolise PGH2 and form PGF2 with a high efficiency. According to Kabututu and colleagues *et al.* (Kabututu *et al.* 2009), AKR1B1 is 20 times more efficacious than AKR1C3 in producing PGF2 from its precursor PGH2.

Aldose reductase may require arachidonic acid for its activity. This is released from phospholipids by phospholipase. This enzyme was identified in both examined sources of tissues, but a higher intensity of staining (higher amount of protein) was detected in heifers than in pre-pubertal female calves.

Putative RNA exonuclease, which cleaves phosphate bonds in RNA, is necessary for appropriate cellular metabolism and in the present study was detected in higher amounts in heifers than in pre-pubertal female calves. Dehydrogenase/reductase SDR family member 4 known as NADPH-dependent retinol dehydrogenase/reductase (1.1.1.184) reduces all-trans-retinal and 9-cis retinal. It is well known that vitamin A is crucial for the proper functioning of epithelia not only in the reproductive tract.

The proteins mentioned above were identified in the present study and were known earlier. Moreover, their biological activity was described in different tissues and processes that were not limited to the reproductive tract. These proteins have not yet been considered with respect to age and hormone related alterations in concentrations in bovine endometrium.

Conclusions

In this preliminary study we used DIGE and partial identification to compare proteins that were absent or present in different amounts based on sex steroid influence in conditions of current experiment (i.e. the age of used animals and used tissues) in the endometrium of heifers and pre-pubertal female calves.

Obtained results demonstrate that differences in protein profile can be expected depending on pre-pubertal or pubertal status.

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