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Protein profile of uterine washings of cyclic and pregnant cows from Days 14 to 18 post-estrus

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Abstract

In cattle, successful recognition of pregnancy depends on a biochemical interplay between endometrial and conceptus secretions. Because failure of pregnancy recognition accounts for 30-40% gestation losses, understanding mechanisms regulating this event may lead to increasing profits in the cattle industry. The goal of the present study was to characterize the protein profile of uterine washings collected from cyclic and pregnant cows during the critical period for pregnancy recognition. In Experiment 1, uterine flushings from cyclic (n = 15) and inseminated cows (n = 30) were collected *in vivo*, through a Foley catheter, on Days 14, 16, or 18 post-estrus. Protein composition of washings was evaluated by SDS-PAGE, coomassie staining, and densitometry. Overall, there was no effect of reproductive status or day post-estrus on optical density of protein bands in washings ($P > 0.10$). In Experiment 2, uterine flushings from cyclic (n = 12) and inseminated cows (n = 36) on Day 17 post-ovulation (i.e., Day 18 post-estrus) were collected either *in vivo* or *post-mortem*. Protein composition of washings was evaluated as in Experiment 1. Of the 20 protein bands analyzed, 5 had higher and 7 had lower optical densities in washings obtained *in vivo* in comparison to washings obtained *post-mortem* ($P < 0.05$). Optical densities of protein bands were similar between reproductive statuses ($P > 0.10$). In conclusion, sensitivity of analytical techniques was not sufficient to discriminate temporal nor reproductive status-specific proteins, neither in *in vivo* nor *post-mortem* flushings. Moreover, protein composition of washings depended on the method used for collection. It is speculated that a high relative abundance of serum proteins in washings diluted conceptus- and uterine-specific proteins to concentrations below the sensitivity of the methods used for protein detection.

Keywords: cattle, electrophoresis, histotroph, maternal recognition of pregnancy, uterus.

Introduction

Bovine females have epitheliochorial placentation, in which there are six tissue layers between the fetal and maternal circulation, and there is no erosion of the maternal uterine epithelium throughout pregnancy. This is especially important during the pre-implantation period of gestation (i.e., before the formation of placentomes) because conceptus nutrition and growth depend exclusively on secretions contained in the uterine lumen, the histotroph. The histotroph is conditioned by proteins, carbohydrates, lipids, and ions secreted by the conceptus and endometrial glands or diffused from the maternal circulation (Roberts and Bazer, 1988; Gray *et al*., 2001; Burghardt *et al*., 2002; Spencer and Bazer, 2004; Alavi-Shoushtari *et al*., 2006).

In cattle, embryonic mortality occurring between Days 15 to 19 after estrus (i.e., the "critical period" for maternal recognition of pregnancy) accounts for 20% to 40% of pregnancy losses (Diskin and Sreenan, 1980; Kunz *et al*., 2002). Such mortality is associated with poorly developed conceptuses and inadequate secretion of antiluteolytic factors such as interferon-tau (Thatcher *et al*., 2001). Conceptus elongation and secretion of interferon-tau are modulated by molecules in the histotroph. For example, Hernandez-Ledezma *et al*. (1992) reported greater *in vitro* secretion of interferon-tau by conceptuses exposed previously to the maternal uterine environment as compared to unexposed conceptuses. Also, cows with higher plasma concentrations of progesterone, a pregnancy hormone known to affect endometrial gland activity, yielded conceptuses that were larger and had a greater capacity to secrete interferon-tau (Mann *et al*., 1999). Collectively, it is hypothesized that an optimal composition of the histotroph is required for a successful gestation. However, specific molecules in the histotroph regulating conceptus growth and antiluteolytic capacity have received little study (Wolf *et al*., 2003). Consequently, to understand the biology of early pregnancy, focusing on the identification of causes for early pregnancy failure, it is critical to define the

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composition of the histotroph.

Early studies applied endometrium (Bartol *et al*., 1985b) or conceptus (Bartol *et al*., 1985a) tissue cultures and two-dimensional electrophoresis to characterize secretory proteins synthesized de novo by these tissues. More recently, Berendt *et al*. (2005) used an elegant monozygotic-twin model to detect differences in the endometrial proteome of cyclic and pregnant cows. However, limited information is available about the global protein composition of the whole histotroph, especially of cows from Zebu genetics.

To characterize histotroph protein composition, it is necessary to obtain a representative sample. One possibility is to obtain uterine flushings and perform protein characterization by electrophoresis, staining, and sequencing. Uterine flushing consists of infusion and recovery of specific medium from uterine horns *in vivo* or *in vitro. In vivo* flushings are more convenient because they can be performed in the live, standing animal. *Post-mortem* flushing decreases the probability of contamination of histotroph by infiltration of serum proteins resulting from manipulation of the reproductive tract which occurs *in vivo*. The hypothesis of the present study was that there are differences in histotroph protein profiles of uterine flushings obtained both *in vivo* and *in vitro* between cyclic and pregnant cows. Specific objectives were: (1) to compare protein composition of uterine flushings collected *in vivo* between cyclic and pregnant cows on Days 14, 16 or 18 post-estrus and (2) to analyze the effects of two different methods of uterine flushing on protein composition of flushings obtained from cyclic and pregnant cows on Day 18 after estrus.

Materials and Methods

Experiments were carried out in strict accordance with the Brazilian Law of Animal Treatment and Protection Guidelines. All animal procedures were approved previously by the Bioethics Committee of the College of Veterinary Medicine and Animal Science of the University of São Paulo.

Experiment 1

Forty-five cyclic, multiparous, non-lactating, at least ¾-Zebu crossbred cows were maintained in grazing conditions (*Brachiaria decumbens*) and received a mineral supplement and water *ad libitum*. Ultrasound examinations were performed to verify follicular activity and presence of a corpus luteum (CL). Animals with a CL were treated with prostaglandin-F2α (PGF; 530μg sodium cloprostenol; Centro Paulista de Desenvolvimento Farmacotécnico – São Paulo, Brazil) and observed subsequently for estrus behavior twice daily. Animals were then divided randomly to remain non-inseminated (n = 15) or to receive artificial insemination 12 hours after estrus (n = 30). Insemination was conducted using frozen/thawed semen from a single ejaculate from a Simmental bull of good fertility (40% motility, 30 x 10^6 spermatozoa/straw, and 20% total abnormal spermatozoa).

Within each reproductive status (i.e., cyclic or inseminated), cows were further divided randomly to receive *in vivo* uterine washings on Days 14, 16, or 18 after estrus. While standing in a chute, animals were desensitized with a 2-ml epidural injection of 2% lidocaine (Centro Paulista de Desenvolvimento Farmacotécnico, Brazil). Next, the uterine lumen was washed with fresh 50 ml Ringer solution at 37ºC for five consecutive times (250 ml total volume). Through a Foley catheter (24 fr; Rush®, Brazil) and a Y-type tubing, the Ringer was infused and recovered by gravity. Special care was taken so that uterine massage was kept to a minimum. During recovery, the Ringer was stored in a glass container which was kept on ice. After recovery, the 50-ml washings from each cow were pooled, centrifuged at 3000 x g for 30 minutes at 4°C to remove tissue debris, and stored at -20ºC.

Inseminated cows were diagnosed pregnant when the conceptus or its fragments were visualized in the Ringer solution during the uterine flushing. For each day (14, 16 or 18) within each reproductive status, at least four washings were processed for further analyses.

Experiment 2

Forty-eight cows, similar to those used in Experiment 1 and managed in the same conditions, were synchronized with an ovulation synchronization protocol. On Day 0, cows were injected with 2 mg estradiol benzoate (Centro Paulista de Desenvolvimento Farmacotécnico, Brazil) and received an ear implant containing Norgestomet (CRESTAR® - Intervet, México). On Day 8, ear implants were removed and animals received PGF. On Day 9, animals received 1 mg estradiol benzoate and on Day 10 were divided randomly to receive artificial insemination (n = 36) or not (n = 12). Cows were inseminated with the same batch of semen used in Experiment 1. Only animals that ovulated to the protocol were maintained in this experiment. To confirm ovulations, ovaries from all animals were examined by ultrasonography on Days 9 and 11 (Aloka SSD-500 equipped with a 7.5-MHz linear-array probe). Animals with a follicle greater than 9 mm on Day 9 that disappeared on Day 11 were considered to have ovulated.

On Day 18 after estrus, the uterine flushing procedure was performed on all animals. Eighteen animals were submitted to *in vivo* uterine flushing using a Foley catheter (13 animals were inseminated and five animals were not) and 28 animals were submitted to *post-mortem* uterine flushing (23 animals were inseminated and five animals were not).

The *in vivo* flushings were divided into two portions. An initial portion was obtained as described

for Experiment 1 and called "Foley Flushing A". Subsequently, 5 extra portions of 50 ml of Ringer solution were sequentially infused and recovered from the uterus, but recovery was aided by a soft massage of uterine horns from cranial to caudal direction of flow. These 5 extra portions were pooled, and the compounded flushing was called "Foley Flushing B". According to Bartol *et al*. (1981), excessive manipulation of the female reproductive tract may alter composition of the histotroph, mainly by causing local edema and influx of macromolecules from the peripheral circulation. In the procedure described here, Foley Flushing A was expected to have minimum contamination of macromolecules from the peripheral circulation. However, because positive pregnancy diagnosis depended on visualization of the conceptus or its fragments, flushing the uterus of pregnant cows a second time (Foley Flushing B) provided an extra opportunity to detect pregnancies.

Post-mortem uterine flushings were obtained after slaughtering animals and recovering their reproductive tracts. Twenty milliliters of Ringer solution kept at 37ºC was injected through the cranial extremity of the uterine horn contra-lateral to the ovary containing the CL into the uterine lumen with a needle and a 20-ml syringe. The cranial extremity of the opposite uterine horn was cut off to facilitate collection of the uterine flushing and conceptus. Then, the uterus was massaged gently and the uterine flushing collected in a Petri dish. After recovery, flushings were maintained on ice, centrifuged at 3000 x g for 30 minutes at 4°C to remove tissue debris, aliquoted, and stored at -20°C.

Flushings from inseminated cows that did not contain a conceptus were excluded from this experiment. In addition to the presence of a conceptus in flushings, plasma concentration of progesterone was measured on the same day as flushing collection to confirm the presence of a functional CL.

Sample preparation

To remove salt, eliminate macromolecules smaller than 10kD (e.g., peptides and products of protein degradation), and concentrate proteins, washings were ultra-filtered using Centricon Plus-20 (Millipore Corporation - EUA) apparatuses as per manufacturer's instructions. After ultra-filtration, samples were re-suspended in ultrapure water (Milli-Q) and ultra-filtration was performed again. Protein concentration of filtrates (fraction of flushings enriched with macromolecules > 10kD) was measured by a miniaturized Bradford method (Bradford, 1976), using 96-well plates (Falcon®, USA) and Bradford reagent (BioRad®, UK). Next, samples were aliquoted, lyophilized, and stored at -20°C.

Electrophoresis and densitometry

Lyophilized samples were reconstituted in 1x Laemmli solution and one-dimensional electrophoresis in 10% polyacrilamide gels was performed. Gels were stained by Commassie Stain Solution (BioRad, USA) for 2 hours and subsequently destained for 24 hours in a 10% acetic acid, 4% methanol solution. Destained gels were scanned by LabScan v5.0 (Labcrew™) and digitalized by Image Scanner™II (GE Healthcare, UK).

Protein bands with a similar molecular weight and relative position inter- and intra-gels were detected with specific software (Image Master 1D v.4.01, GE Healthcare, UK). Each protein band was named according to its mean molecular weight measured in all gels it was present. For each sample, the absolute optical density (AOD) of each protein band was measured at the point of peak pixel intensity. The AOD of all bands in a sample was added to yield the total optical density of each sample in the gel. Then, for each sample, the relative optical density (ROD) of each protein band was calculated by dividing its AOD by the total optical density of the sample and multiplying the result by 100.

Although the duration, voltage, and amperage of electrophoretic runs were standard to all gels, there was variation in protein band separation and it was difficult to differentiate bands in the high molecular weight region of gels. In such cases, bands were grouped as a band complex. The AOD of bands in a complex were added and the ROD of the complex calculated as described for individual protein bands.

Blood and radioimmunoassay

Blood samples were collected from the coccygeal vein of each animal on the same day of uterine washing in 16 x 100 borosilicate tubes containing 30% sodium citrate solution. Blood was centrifuged (3000 x g for 30 minutes at 4°C) and plasma stored at -20C. Plasma progesterone concentration was measured by radioimmunoassay as described by Badinga *et al*. (1992) and modified by Carriere and Lee (1994). Coefficients of variation for low (1.25 ng/ml), medium (6.46 ng/ml), and high (9.68 ng/ml) standards were 4.6%, 3.0% and 3.9%, respectively.

Statistical analysis

Only samples from animals with plasma concentrations of progesterone greater than 1 ng/ml, which is indicative of a functional CL, were analyzed. After an initial verification of the protein band array of each animal, only protein bands present in at least half the animals or absent in all animals of a given group were analyzed. In Experiment 1, the ROD of each protein band or complex was analyzed by analysis of variance using the PROC GLM from SAS. Effects of day after estrus, reproductive status (cyclic or pregnant), and the status by day interaction were considered as independent variables. In Experiment 2, it was verified initially that the ROD of each band or complex was

similar between Foley Flushings A and B ($P > 0.10$; data not shown). Therefore, the Foley Flushing A and B average ROD of each protein band was analyzed by analysis of variance considering the effects of method of washing (Foley or post-mortem), reproductive status, and the method by status interaction as independent variables. The results were shown as mean $\pm$ standard error of the mean (SEM). Effects were considered significant when $P \leq 0.05$ and considered a trend when $P \leq 0.10$.

Results

Experiment 1

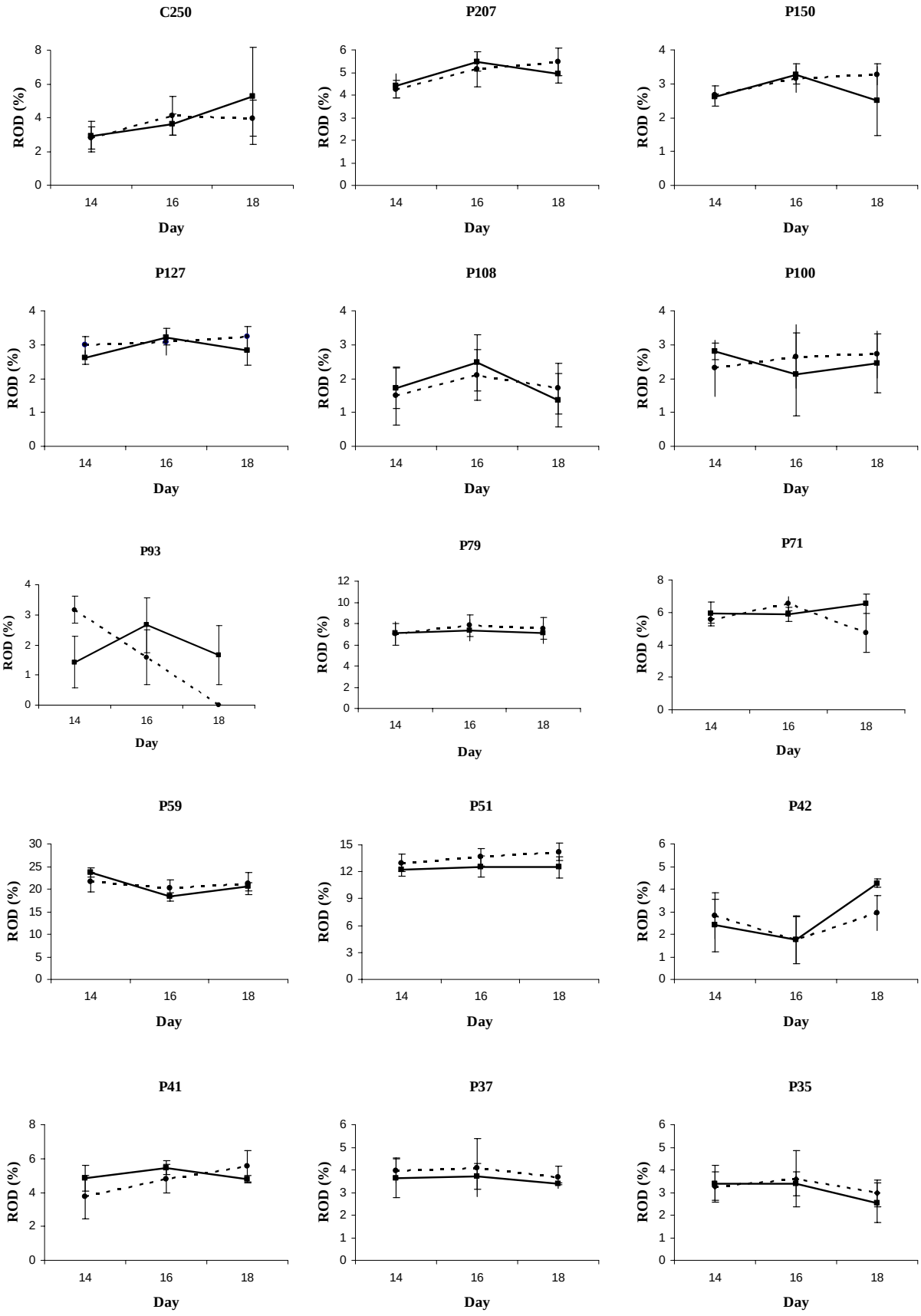
A 47% conceptus-presence rate (number of washings containing a conceptus/number of cows inseminated) was observed. On the day of flushing, plasma progesterone concentrations were above 1 ng/ml for all experimental animals.

Thirty-one different protein bands were identified in uterine flushings from cyclic and pregnant cows on Days 14, 16 and 18 after estrus (Table 1). However, only 21 protein bands met the criteria for analysis. In general, ROD of bands was similar, independent of day after estrus or reproductive status ($P > 0.1$; Table 2 and Fig. 1). The only exception was the ROD of P93, which tended to be affected by the day x status interaction ($P < 0.07$). The ROD of P93 decreased near the end of the estrous cycle but increased and remained elevated in flushings from pregnant cows.

Table 1. Frequency of protein bands in flushings collected from pregnant (P) or cyclic (C) cows on Days 14, 16, or 18 post-estrus.

ID	MW (kD)[a]	P14 (n = 4)[b]	C14 (n = 4)	P16 (n = 4)	C16 (n = 4)	P18 (n = 4)	C18 (n = 5)
C250	250	4	4	4	4	4	5
P214	214	0	0	0	0	0	1
P207	207	4	4	4	4	4	5
P184	184	2	1	1	0	0	0
P150	150	4	4	4	4	3	5
P127	127	4	4	4	4	4	5
P110	110	0	0	0	0	0	1
P108	108	3	2	3	3	2	3
P100	100	4	3	2	3	3	4
P93	93	2	4	3	2	2	0
P86	86	1	2	0	1	3	1
P84	84	0	0	0	0	0	1
P79	79	4	4	4	4	4	5
P71	71	4	4	4	4	4	4
P59	59	4	4	4	4	4	5
P51	51	4	4	4	4	4	5
P42	42	3	3	2	2	4	4
P41	41	4	3	4	4	4	5
P38	38	2	0	1	0	0	0
P37	37	4	4	4	4	4	5
P35	35	4	4	4	4	3	5
P33	33	4	4	4	4	4	4
P32	32	4	4	4	3	4	5
P31	31	4	4	4	3	4	5
P28	28	4	4	4	4	4	5
P22	22	3	4	3	3	3	2
P20a	20	0	1	0	0	1	0
P20	20	2	2	3	2	2	3
P17	17	1	2	2	2	2	3
P16	16	0	0	1	0	1	1
P15	15	0	0	0	0	0	1

[a]Average molecular weight.
[b]Number of flushings containing a given protein band.


C250
P207
P150
P127
P108
P100
P93
P79
P71
P59
P51
P42
P41
P37
P35
ROD (%)
Day
14
16
18

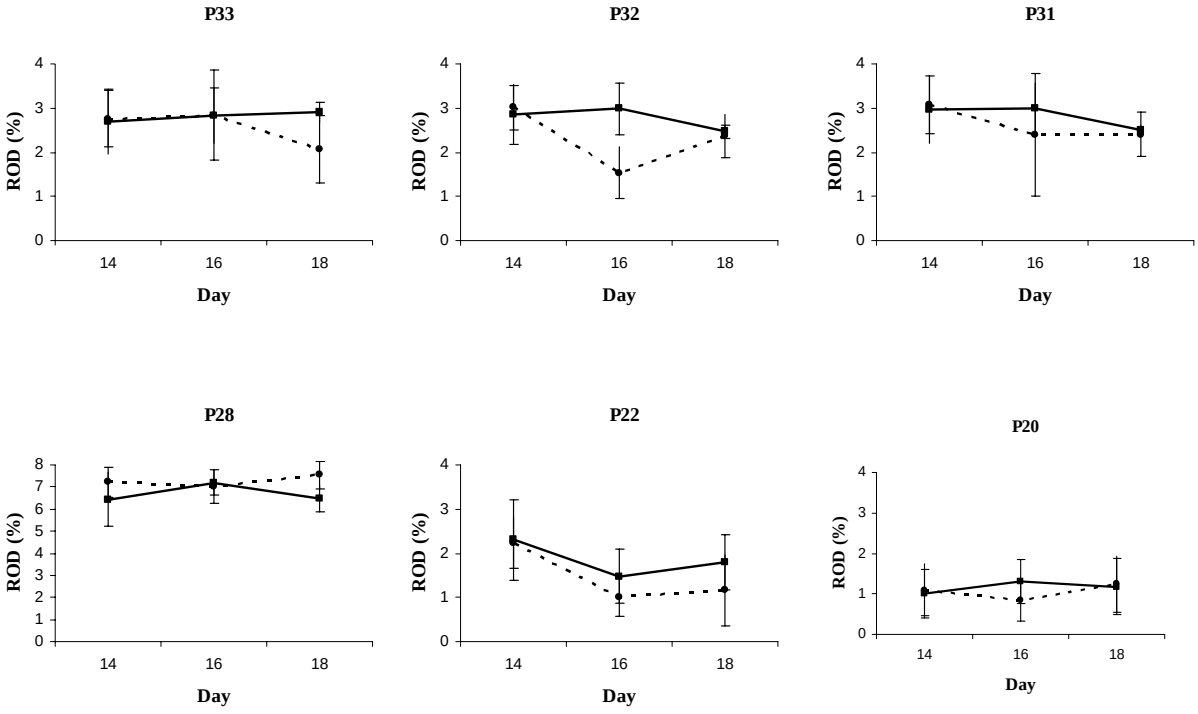


Figure 1. Means (± SEM) of the relative optical density (ROD) of protein bands in flushings collected from pregnant (solid lines) or cyclic (dotted lines) cows on Days 14, 16, or 18 post-estrus.

Table 2. Analysis of variance of the relative optical density (ROD) of protein bands in flushings collected from pregnant or cyclic cows on Days 14, 16, or 18 post-estrus.

ID	P < F		
	Status[a]	Day[b]	Status x by Day
C250	0.79	0.45	0.80
P207	0.94	0.18	0.73
P150	0.54	0.53	0.64
P127	0.41	0.57	0.67
P108	0.90	0.57	0.87
P100	0.90	0.96	0.82
P93	0.58	0.11	0.07
P79	0.67	0.76	0.89
P71	0.43	0.73	0.29
P59	0.93	0.61	0.83
P51	0.27	0.84	0.92
P42	0.69	0.16	0.61
P41	0.63	0.51	0.49
P37	0.59	0.86	0.99
P35	0.80	0.62	0.94
P33	0.67	0.88	0.77
P32	0.30	0.43	0.29
P31	0.74	0.74	0.89
P28	0.38	0.93	0.71
P22	0.50	0.33	0.91
P20	0.83	0.96	0.89

[a] Pregnant or cyclic.
[b] Day 14, 16, or 18 post-estrus.

Experiment 2

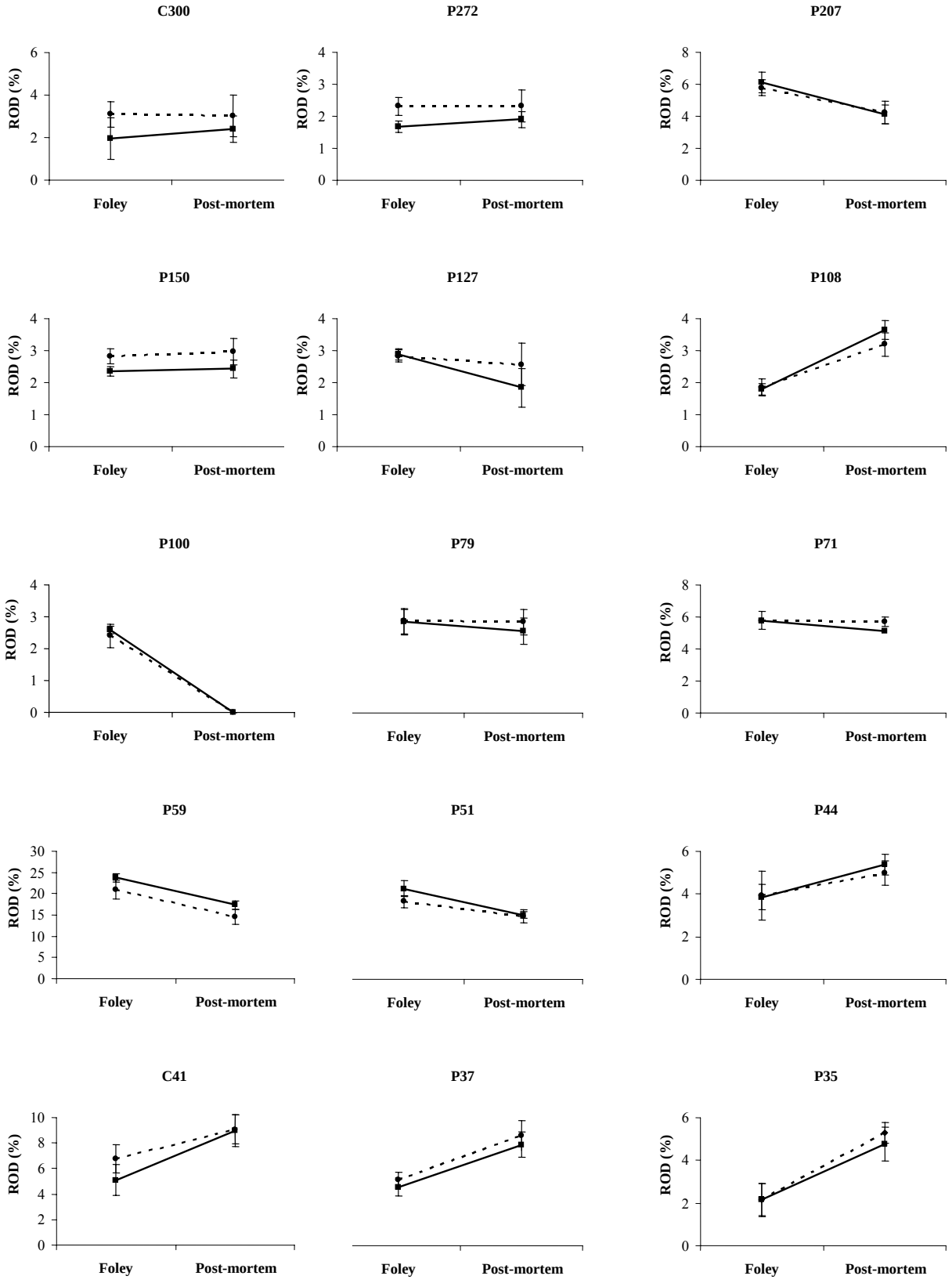
Ovulation rate (number of cows ovulated/total number of cows) was 73%. Pregnancy rate (number of washings containing a conceptus/number of inseminated cows) was 27%. Plasma progesterone concentrations were above 1 ng/ml in all experimental animals.

Thirty-two different protein bands were identified in *in-vivo* and *post-mortem* uterine flushing from cyclic and pregnant cows on Day 18 after estrus (Table 3). However, only 20 protein bands met the criteria for analysis. Mean ROD and results from the analysis of variance are shown in Fig. 2 and Table 4, respectively. There was a significant effect ($P \leq 0.05$) of flushing method on the ROD of 11 of the 20 protein bands (P207, P108, P100, P59, P51, C41, P37, P35, P31, P28, P22) and a trend for P44 ($P \leq 0.1$). The ROD of protein bands was not affected by status nor by status by flushing technique interaction ($P > 0.1$). Among protein bands affected by method of washing, the P100 was the only one found in washings obtained by the Foley catheter exclusively. The ROD from P207, P59, P51, and P28 were greater in washings obtained in vivo, while ROD from P108, P44, C41, P37, P35, P31, and P22 were greater in washings obtained *post-mortem*.

Table 3. Frequency of protein bands in flushings collected through a Foley catheter or *post-mortem* from pregnant or cyclic cows on Day 18 post-estrus.

ID	MW (kD)[a]	FC (n = 4)[b,c]	FP (n = 3)	PMC (n = 5)	PMP (n = 6)
C300	300	4	3	4	6
P272	272	4	3	5	6
P223	223	4	1	1	0
P207	207	4	3	5	6
P172	172	1	0	1	0
P150	150	4	3	5	6
P127	127	4	3	4	4
P119	119	2	0	1	1
P108	108	4	3	5	6
P100	100	4	3	0	0
P93	93	1	1	5	6
P87	87	1	0	4	4
P79	79	4	3	5	6
P78	78	0	0	0	1
P71	71	4	3	5	5
P59	59	4	3	5	6
P51	51	4	3	5	6
P46	46	1	1	0	1
P44	44	4	3	5	6
C41	41	4	3	5	6
P37	37	4	3	5	6
P35B	35	1	0	1	1
P35	35	3	3	5	6
P33	33	4	3	5	4
P32	32	4	3	4	6
P31	31	4	2	5	6
P28	28	4	3	5	6
P22B	22	0	0	1	0
P22	22	4	3	5	6
P20	20	3	3	2	3
P17	17	3	3	1	2
P15	15	0	1	3	1

[a]Average molecular weight.
[b]FC: Foley cyclic; FP: Foley pregnant; PMC*:* *post-mortem* cyclic; PMP: *post-mortem* pregnant.
[c]Number of flushings containing a given protein band.


C300
P272
P207
P150
P127
P108
P100
P79
P71
P59
P51
P44
C41
P37
P35
ROD (%)
Foley
Post-mortem

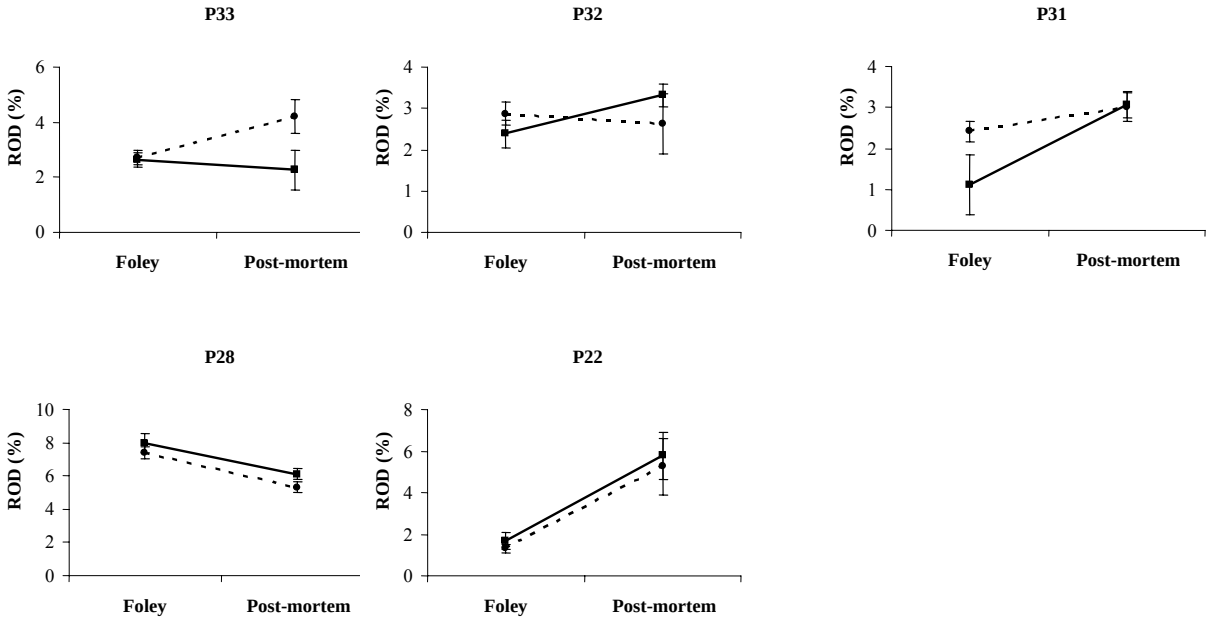


Figure 2. Means (±SEM) of the relative optical density (ROD) of protein bands in flushings collected using a Foley catheter or *post-mortem* from pregnant (solid lines) or cyclic (dotted lines) cows on Day 18 post-estrus.

Table 4. Analysis of variance of the relative optical density (ROD) of protein bands in flushings collected through a Foley catheter or *post-mortem* from pregnant or cyclic cows on Day 18 post-estrus.

ID	P < F		
	Status[a]	Flushing[b]	Status x by Flushing
C300	0.30	0.82	0.77
P272	0.18	0.78	0.75
P207	0.87	0.02	0.74
P150	0.16	0.76	0.91
P127	0.58	0.29	0.52
P108	0.40	0.00	0.31
P100	0.61	<0.01	0.61
P79	0.30	0.29	0.40
P71	0.72	0.66	0.71
P59	0.26	0.02	0.99
P51	0.15	0.00	0.25
P44	0.81	0.10	0.74
C41	0.51	0.03	0.55
P37	0.56	0.01	0.95
P35	0.64	0.00	0.65
P33	0.15	0.42	0.19
P32	0.83	0.51	0.26
P31	0.15	0.01	0.12
P28	0.11	0.00	0.77
P22	0.70	0.00	0.95

[a]Pregnant or cyclic.
[b]Foley catheter or *post-mortem.*

Discussion

In cattle, before placentome formation on around Day 42 of gestation, conceptus nutrition and growth rely entirely on secretions present in the uterine lumen (Spencer *et al*., 2006). Moreover, conceptus morphology and function change quickly during early pregnancy and probably so does its requirements for specific nutrients and growth factors (Hue *et al*., 2006). In addition, adequate signaling from the conceptus is required to modulate uterine function, prevent luteolysis, and maintain pregnancy (Binelli *et al*., 2000). Collectively, it is proposed that an optimum balance between uterine and conceptus molecules is required for successful establishment of pregnancy. However, studies to characterize temporal and reproductive status-dependent changes in uterine secretion composition are scarce. Understanding the biology of early pregnancy in cattle requires appropriate techniques to probe the uterine microenvironment and characterize its composition (Wolf *et al*., 2003). One convenient method to obtain uterine secretions is to infuse and recover medium through a Foley catheter. Macromolecules contained in medium recovered could be analyzed subsequently by appropriate techniques, including protein electrophoresis, purification, and sequencing. In the present paper, there was an attempt to identify temporal changes in the protein profile of the uterine lumen during early pregnancy and the late luteal phase of the estrous cycle (Experiment 1). With the exception of one 93kD protein band, composition of washings was similar across time and reproductive status. It was hypothesized that reproductive tract manipulation, necessary for medium infusion and recovery, although kept to a minimum, changed uterine composition acutely and prevented discrimination of proteins in the different washings studied. In

Experiment 2, *post-mortem* washings were tested as an alternative technique for uterine luminal content recovery. Although protein composition of washings obtained *in vivo* was distinct from that of washings obtained *post-mortem*, composition between reproductive statuses was still similar. Therefore, the two major findings of the present paper were that (1) stage- and reproductive status-non specific proteins predominate in the uterine environment, and (2) methods used to probe the uterine environment influence protein profiles.

One possible explanation for the similar composition among the uterine flushings studied is that the majority of proteins found in the uterine lumen are plasma proteins such as albumen and immunoglobulins (Lee *et al*., 1998; Alavi-Shoushtari *et al*., 2006). Therefore, uterus and conceptus secreted proteins were represented in the samples collected in amounts which were below the detection limit of the techniques used. The Image Master 1D v.4,01 software used in the present experiment detected protein bands with a relative density representing 0.15% of the flushings. This represented approximately 75 ng of the 50μg total protein loaded per well of gels. Thus, individual uterus- or conceptus-specific proteins, regulated in a time- or status-dependent manner, accounted for less than 0.15% of the flushings. This information is in agreement with Berendt *et al*. (2005), who stated that the excess albumen found in uterine flushings was serum-born, and crossed through highly-vascularized endometrial capillary walls into the uterine lumen.

In Experiment 1, reproductive tract manipulation, necessary to obtain uterine secretions using the Foley catheter technique, may have caused local inflammation, edema, and resulted in a further influx of serum protein to the uterine lumen. This may explain the similar protein profiles across time and reproductive statuses. However, techniques with greater discriminatory capability, such as two-dimension electrophoresis, or greater sensitivity of staining, such as silver staining or CyDyes^{tm} (GE Healthcare Bio Sciences, UK), may enable detection of low-concentration uterus- and conceptus-specific proteins (Westermeier, 2001; Berendt *et al*., 2005).

In Experiment 2, a *post-mortem* method of obtaining uterine secretions was used in an attempt to overcome possible changes in uterine secretion composition resulting from *in vivo* manipulation applied in Experiment 1. It was anticipated that flushings from pregnant cows would be enriched with early pregnancy specific proteins, detectable by the protein separation and staining techniques used. Furthermore, composition of flushings obtained *in vivo* and *post-mortem* were compared, and the effect of *in vivo* manipulation of the reproductive tract was determined. Similar to results in Experiment 1, there was no difference in the abundance of proteins detected in flushings obtained from pregnant and non-inseminated cows. This occurred despite major changes in the composition of washings recovered *post-mortem* compared to *in vivo* (Experiment 2). Indeed, the optical density of eleven out of twenty protein bands detected varied between flushings obtained by the two different methods. It is speculated that protein bands P207, P100, P59, P51, and P28, which presented a higher optical density in washings recovered by *in vivo*, represent serum proteins that penetrated the uterine lumen as a result of reproductive tract manipulations. For example, P59 presented the highest ROD of all protein bands analyzed. Band P59 corresponds to albumen, which is a known component of the histotroph (Alavi-Shoushtari *et al*., 2006). The average relative abundance of P59 increased from 16.0% in washings collected *post-mortem* to 22.4% in washings collected *in vivo* (i.e., a 39.8% increase). In contrast, proteins P108, P44, C41, P37, P35, P31, and P22 had a higher ROD in washings obtained *post-mortem*. This leads to the hypothesis that such proteins did not migrate from serum as an acute result from manipulation of the reproductive tract.

In order to study global temporal- and reproductive status-specific changes in protein composition in the uterine microenvironment, there remains the technical challenge of increasing the relative abundance of endometrial and conceptus-synthesized proteins in the sample. This may include pre-processing of the washings to remove major serum contaminants, such as albumen and immunoglobulins. It is important to mention that although differences in protein abundance were not detected in the present study, unpublished data from our laboratory indicate that the washings obtained from pregnant cows *post-mortem* contained both interferon-tau protein (detected by western blotting) and showed antiluteolytic activity (measured in a specific cell assay). This supports the idea that, similar to interferon-tau, pregnancy specific proteins were indeed present in washings, but in concentrations below the sensitivity of the techniques herein used.

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