Effect of a high cornstarch diet on hepatic cytochrome P450 2C and 3A activity and progesterone half-life in dairy cows¹

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ABSTRACT

In the cow, inadequate concentrations of progesterone during gestation may lead to an abrupt termination of pregnancy. The primary organ involved in progesterone catabolism is the liver, which contains an abundance of cytochrome P450 isozymes (EC 1.14.14.1; mixed-function monooxygenases). The objectives of the current experiment were to determine the effect of feeding 2 isoenergetic and isonitrogenous diets, formulated to cause divergent insulin secretion, on hepatic cytochrome P450 2C (CYP2C) and 3A (CYP3A) activity as well as the resulting biological half-life of progesterone. Twenty-two Holstein cows averaging 80 ± 7 d in milk were randomly assigned to either a high cornstarch diet or a high fiber diet in a crossover experimental design consisting of two 14-d periods. Dry matter intake, milk yield, milk lactose yield, and milk lactose percentage were similar between the 2 diets. Milk fat yield and milk fat percentage were higher in cows fed the high fiber diet, whereas milk protein yield tended to be higher and milk protein percentage was higher in cows fed the high cornstarch diet. Energy balance tended to be improved by 57% in cows consuming the high cornstarch diet. Insulin concentrations at the time of liver biopsy (3.16 ± 0.04 h post-feeding) were increased by 44% in cows consuming the high cornstarch diet compared with cows consuming the high fiber diet. Cytochrome P450 2C activity was decreased by 45%, whereas CYP3A activity tended to be lowered by 34% in cows consuming the high cornstarch diet. Cytochrome P450 2C mRNA expression tended to be decreased by 21% in cows fed the high cornstarch diet, whereas CYP3A mRNA expression was not different between

the dietary treatments. The fractional rate constant of progesterone decay was not different between the 2 diets; however, the half-life of progesterone tended to be longer in cows fed the high cornstarch diet compared with cows fed the high fiber diet (85 vs. 64 min, respectively). In summary, cows consuming the high cornstarch diet had increased insulin concentrations and lower hepatic CYP2C and CYP3A activity and tended to have a longer progesterone half-life compared with cows consuming the high fiber diet. Feeding diets that stimulate insulin secretion could alter progesterone clearance during lactation, when dairy cows have increased rates of progesterone inactivation because of high energy demands and increased DMI.

Key words: progesterone, insulin, cytochrome P450, dairy cow

INTRODUCTION

Several researchers have reviewed the importance of nutritional status in relation to reproductive performance in lactating dairy cows (Van Knegsel et al., 2005; Roche, 2006; Garnsworthy et al., 2008a). The severe negative energy balance (EB) following calving has been correlated with several reproductive dysfunctions; attempts to improve EB during early lactation can cause a faster restoration of cyclicity and increase peripheral concentrations of steroids (Butler et al., 2004). Resumption of normal endocrine signaling in the early postpartum cow has been associated with high energy diets that promote insulin secretion (Chagas et al., 2007). High starch diets have been implicated in improving EB as well as stimulating production of steroids and secretion of growth factors (Leroy et al., 2008). Added energy in the form of a once-daily propylene glycol drench has been reported to increase peripheral concentrations of insulin, concentrations of progesterone, and pregnancy rates in dairy cows (Hidalgo et al., 2004).

A portion of early embryonic and fetal loss may be accounted for by inadequate concentrations of progesterone caused by increased rates of steroid inactivation, deficiencies in luteal function, or a combination of both

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(Inskeep and Dailey, 2005). Some researchers have found no change or increased first-service conception rates after supplementing cows with progesterone in the form of a controlled internal drug-releasing device (CIDR), which can increase peripheral concentrations of progesterone by approximately 1 ng/mL (Larson et al., 2007; Stevenson et al., 2007). However, supplementation with progesterone partially masks the high rates of progesterone clearance in dairy cows and fails to address the issue of excessive steroid clearance caused by elevated feed intake during early lactation. Steroid inactivation occurs primarily in the liver, and independent reports in sheep and cattle have shown a positive relationship between metabolic clearance rate of progesterone and DMI (Parr et al., 1993a; Sangsritavong et al., 2002). The relationship between DMI and excessive steroid clearance may be attributed to an increase in steroid delivery to the liver via an equal and proportionate increase in liver blood flow (Parr et al., 1993b).

The first phase of steroid biotransformation involves hydroxylation of the steroid nucleus by the mixed-function monooxygenases cytochrome P450 2C (CYP2C) and cytochrome P450 3A (CYP3A), as well as contributions from the aldo-keto reductase family (Penning et al., 2000; You, 2004). A relationship between cytochrome P450 expression and insulin was proposed when Barnett et al. (1990) and Shimojo et al. (1993) observed an increased expression of CYP3A in insulin-dependent diabetic rats (i.e., low insulin), which was reversed by insulin replacement. Smith et al. (2006) found a dose-dependent decrease in the fractional rate constant of progesterone decay in a murine hepatocyte cell-line challenged with increasing physiological concentrations of insulin. Using the same cell-line and experimental conditions, Lemley et al. (2009) reported a dose-dependent decrease in the activities of both CYP2C and CYP3A after challenging hepatocytes with increasing physiological concentrations of insulin. Similar to the *in vitro* data, Smith et al. (2006) found a decrease in progesterone clearance in anestrous ewes orally gavaged (drenched) with sodium propionate (gluconeogenic substrate) compared with those orally gavaged with sodium acetate (energy control group). In a follow-up experiment, Lemley et al. (2008a) found an approximately 50% decrease in both hepatic CYP2C and CYP3A activity at 1 h post-feeding in ovariectomized ewes supplemented with sodium propionate compared with those supplemented with sodium acetate. The *in vitro* and *in vivo* experiments helped illustrate a model for decreasing steroid clearance by altering cytochrome P450 activity via insulin signaling.

Recent observations in dairy cows have also linked elevated insulin concentrations with lower CYP2C and CYP3A mRNA expression (Lemley et al., 2008b);

however, we were unable to detect enzyme activity in frozen liver tissue and, because of the use of early postpartum, anestrous cows, we could not detect peripheral concentrations of progesterone. Therefore, the objective of the current experiment was to determine the effect of feeding 2 isoenergetic diets formulated to cause divergent insulin secretion on hepatic CYP2C and CYP3A activity as well as the resulting biological half-life of progesterone.

MATERIALS AND METHODS

Animals and Feeding

Twenty-two Holstein dairy cows (8 primiparous and 14 multiparous) were trained to acquire feed from the Calan Broadbent Feeding System (American Calan, Northwood, NH) at the West Virginia University Animal Science Farm (Morgantown). Three separate replicates were conducted beginning in the autumn of 2007 ($n = 6$), the spring of 2008 ($n = 8$), and the autumn of 2008 ($n = 8$). Experimental procedures and protocols were approved by the West Virginia University Animal Care and Use Committee #07-0503. Cows were randomly assigned to a high cornstarch diet or an isoenergetic and isonitrogenous high fiber diet (Table 1). Cows were provided each diet as a TMR, with 50% of the diet fed at each feeding. The crossover experimental design comprised two 14-d periods, with 10 d of diet acclimation and the remaining 4 d used for sampling (Figure 1). Cows were 80 ± 7 DIM at the beginning of the experiment and were milked twice daily at 0800 and 2000 h; feed was provided directly after each milking. Dry matter intake was kept constant for each cow across treatments to avoid confounding effects of DMI with differences caused by dietary treatment. The feed allocated to each cow was fixed across dietary treatments and was based on her voluntary intake shortly after completing her training to the Calan gate system and during the first 7 d preceding the experiment. On d 7, cows received a CIDR (Pfizer Inc., New York, NY) containing 1.38 g of progesterone, which was removed 3 h post-feeding on d 14. After removal of CIDR between periods 1 and 2, cows were monitored twice daily for estrous behavior, and all cows used in the present study were determined to be cycling. On d 13, 25 mg of Lutalyse (Pfizer Inc.) was administered to each cow. Average milk production was recorded and milk samples were collected at each milking on d 11, 12, 13, and 14, and samples were analyzed for lactose, protein, and fat percentage (Dairy One Inc., Ithaca, NY). Two TMR samples were collected during each sampling period. Average BW (579 ± 7 kg) was determined by weighing cows before milking, after milking, and halfway

Table 1. Experimental diet composition and average ($\pm$ SEM) nutrient composition from all 3 replicates

Diet composition	High cornstarch	High fiber
Ingredient, % of DM		
Corn silage	31.1	36.7
Chopped alfalfa hay	17.8	15.3
Ground corn	12.5	6.1
Corn gluten meal	1.8	1.5
Corn gluten feed	5.4	5.1
Wheat middlings	7.3	5.2
Soybean meal (47% CP)	8.5	5.0
Soybean hulls	—	23.1
Refined cornstarch ¹	15.1	—
Mega-Lac ²	—	1.9
Dicalcium phosphate	0.29	0.08
Limestone	0.1	—
Magnesium oxide	0.04	0.04
Trace mineral salt	0.13	0.13
Vitamin premix ³	0.08	0.08
Nutrient composition, % of DM unless noted		
DM, %	65 $\pm$ 1	61 $\pm$ 1
NDF	28.9 $\pm$ 0.5	39.0 $\pm$ 0.5
ADF	16.1 $\pm$ 0.3	25.2 $\pm$ 0.3
NFC ⁴	50.3 $\pm$ 0.5	38.5 $\pm$ 0.5
Starch	35.3 $\pm$ 1.0	19.2 $\pm$ 0.4
CP	13.9 $\pm$ 0.2	13.5 $\pm$ 0.1
Ether extract	2.0 $\pm$ 0.1	2.8 $\pm$ 0.1
Ash	4.8 $\pm$ 0.2	5.6 $\pm$ 0.1
NE _L , ⁵ Mcal/kg of DM	1.58	1.58

¹Grain Processing Corporation (Muscatine, IA). Moisture <12.5%, pH 5.0–7.0, ash <0.5%, protein <0.35%; based on a 100-g sample (as fed), 89.9 g of starch, 0 g of fat, 0.3 g of protein.

²Ca-soaps of fatty acids; Church & Dwight Inc. (Princeton, NJ).

³Vitamin ADE premix (North American Nutrition Companies Inc., Brookville, OH); 9,920.9 IU/g of vitamin A; 2,204.6 IU/g of vitamin D₃; 4.4 IU/g of vitamin E.

⁴NFC = 100 – (% NDF + % CP + % ether extract + % ash).

⁵Based on tabular values (NRC, 2001).

between milkings on d 11, 12, and 13 of each period, respectively. Feed intake, milk composition, and BW were used to estimate EB over the 4-d sampling period using the equation $EB = NE_C - (NE_L + NE_M)$, where NE_C (net energy consumed) = $1.58 \text{ Mcal/kg} \times \text{DMI}$; NE_L = milk yield $\times [(0.0929 \times \% \text{ fat}) + (0.0563 \times \% \text{ protein}) + (0.0395 \times \% \text{ lactose})]$; and $NE_M = (\text{BW}^{0.75}) \times 0.08$ (NRC, 2001).

Microsomal Preparation and Enzymatic Activity

On d 12, the 0800 h milking and feeding was staggered for pairs of cows by 15 min to ensure similar liver biopsy times post-feeding (3.16 ± 0.04 h post-feeding). Briefly, hair was removed from each animal's right side and the skin was scrubbed twice with betadine (Columbus Serum, Columbus, OH). After determining the location of the tenth intercostal space, 2% lidocaine hydrochloride (Columbus Serum) was administered as a local anesthetic. The skin was punctured using a scalpel and the liver samples were collected using a biopsy needle machined at the West Virginia University Physics Department (Morgantown) following the

specifications of Swanson et al. (2000). Immediately after the liver biopsy, a blood sample was collected via jugular venipuncture into prechilled EDTA-containing tubes and used to determine insulin concentrations by RIA (Lemley et al., 2008a). Enzymatic activity was assessed in fresh liver samples following our previous protocol (Lemley et al., 2008a). Briefly, liver samples were submerged in phosphate buffer and homogenized using a dounce homogenizer. Microsomes were collected and concentrated using differential centrifugation techniques (modified from Nelson et al., 2001). Homogenized tissue was centrifuged at $10,000 \times g$ for 10 min. Pellets were discarded and the supernatants were centrifuged at $100,000 \times g$ for 60 min. The microsomal pellets were resuspended in phosphate buffer and the activity of cytochrome c reductase (product number CY0100; Sigma Chemical Co., St. Louis, MO) was determined to standardize samples. Cytochrome P450 2C activity was measured as the non-ketoconazole-inhibitable, omeprazole-dependent oxidation of NADPH. Microsomes were preincubated for 15 min with $250 \mu\text{M}$ ketoconazole. Enzymatic reactions contained CYP3A-inhibited microsomes, 2.5 mM omeprazole, and $250 \mu\text{M}$ NADPH.

Figure 1. The crossover experimental design consisted of two 14-d periods. Cows were acclimated to the new diets during the first 10 d of the experiment. Samples were collected on d 11, 12, 13, and 14. On d 14, plasma insulin ($\blacktriangle$) concentrations were determined at 0, 1, 2, 3, 4, 5, 6, 8, and 10 h post-feeding and plasma progesterone (P4; $\circ$) concentrations were determined after controlled internal drug release (CIDR) removal at 3, 3.5, 4, 5, and 6 h post-feeding.

Cytochrome P450 3A activity was measured as the nifedipine-dependent oxidation of NADPH. Enzymatic reactions contained fresh microsomes, 200 μ M nifedipine, and 250 μ M NADPH. All solutions were added to UV star 96-well plates (PGC Scientifics, Frederick, MD), and the oxidation of NADPH was determined by measuring the amount of light absorbed at 340 nm for 5 min. The extinction coefficient for NADPH (6,220 L/mol/cm) was used to calculate oxidized NADPH per unit of time.

CYP2C and CYP3A mRNA Expression

Liver samples were stored at -80°C until total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and precipitated with 2-propanol following the manufacturer's protocol. Concentrations of RNA in each sample were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). One microgram of RNA was electrophoresed through a 1.5% agarose gel to determine sample purity and for visualization of 28S and 18S rRNA bands. Real-time reverse transcriptase-PCR

was performed as described previously (Costine et al., 2007). Briefly, samples were diluted to 1 μ g of RNA/ μ L and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) following the manufacturer's protocol. β -Actin was used as a reference gene, and bovine CYP2C and CYP3A genes were chosen because of their homology to the human progesterone catabolic enzymes CYP2C19 and CYP3A4 (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>; Sonstegard et al., 2002; Kijas et al., 2006; Pruitt et al., 2007). Primers for β -actin (accession no. NM_001009784; forward: 5'-ATGAGCT-GCCCGATGGTC-3'; reverse: 5'-GGATGTCCACGT-CACACTTC-3'), CYP2C (accession no. XM_587518; forward: 5'-TATGGACTCCTGCTCCTGCT-3'; reverse: 5'-CATCTGTGTAGGGCATGCAG-3'), and CYP3A (accession no. BT030557; forward: 5'-GTGC-CAATCTCTGTGCTTCA-3'; reverse: 5'-CCAGTTC-CAAAAGGCAGGTA-3') were synthesized (Integrated DNA Technologies Inc., Coralville, IA). Amplification was optimal at an annealing temperature of 63.1°C and efficiencies for β -actin, CYP2C, and CYP3A were 1.89, 1.95, and 1.85 (theoretical yields of 94.5, 97.5, and

92.5%, respectively). The relative abundance of mRNA for CYP2C and CYP3A were corrected for PCR efficiency, standardized using β -actin, and expressed relative to a pooled sample, as described by Costine et al. (2007).

Progesterone and Insulin Concentrations

On d 14 at 0700 h, a jugular vein was catheterized with Tygon tubing (Thermo Fisher Scientifics, Waltham, MA; 1.02 mm i.d., 1.78 mm o.d.). Catheters were gas sterilized with Anprolene gas ampules (Anderesen Products Inc., Haw River, NC) before use and kept patent by flushing with 10 U/mL of heparin (Columbus Serum). A blood sample was collected at the time of catheterization and used as h 0. Following the 0800 h milking and feeding on d 14, blood samples were collected at 1, 2, 3, 3.5, 4, 5, 6, 8, and 10 h post-feeding. Exogenous progesterone in the form of a CIDR was removed following the 3-h blood collection. Jugular blood samples were collected into prechilled EDTA-containing tubes. Blood samples from h 3, 3.5, 4, 5, and 6 were used to determine progesterone decay. Briefly, the fractional rate constants of progesterone decay (k) were calculated for each individual cow using SigmaPlot 8.0 (Aspire Software International, Ashburn, VA) using the equation $P_t = P_0 e^{-kt}$ (P_t = progesterone concentrations at any given time; P_0 = initial progesterone concentrations; t = time), which describes a first-order exponential decay curve (Lemley et al., 2008a). The fractional rate constant of k was then used to calculate the half-life of progesterone using the equation $t_{1/2} = \ln(2)/k$. Plasma progesterone concentrations were determined using RIA (Sheffel et al., 1982) with a sensitivity of 100 pg/mL and intra and interassay coefficients of variation of 4.2 and 7.1%, respectively. Insulin concentrations over the 10-h sampling period were determined using RIA (Lemley et al., 2008a) with a sensitivity of 0.05 nM and intra- and interassay coefficients of variation of 3.9 and 6.5%, respectively. Insulin response over the 10-h sampling period was further assessed by calculating insulin area under the curve using SigmaPlot 8.0 (Aspire Software International).

Feed Analysis

Dry matter of diets was determined by oven drying at 60°C for 48 h. Ether extraction of diets was performed according to AOAC (1995) using a Soxtec Foss Tecator (Foss Analytical, Hillerød, Denmark). Ash content was determined by ashing at 500°C for 16 h (AOAC, 1995). Neutral detergent fiber and ADF contents were determined using an Ankom 200 Fiber Analyzer (Ankom Technology Corp, Macedon, NY). Total nitrogen and

CP were analyzed according to AOAC (1995) using an automated Tecator digestion system (Tecator Inc., Herndon, VA). Sugars in the diet were determined by the extraction procedure adapted from Deriaz (1961). Reducing sugars were determined spectrophotometrically with potassium ferricyanide. Starch content of the diets was determined by the procedure of Smith (1969).

Statistical Analysis

The effects of diet on dependent variables were tested with the MIXED procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). The model statement included replicate, period (period that dietary treatment was applied), order (order of dietary treatments), parity, and diet, and DIM was used as a covariate. Cow within order was considered random and means were separated using the PDIF option of the LSMEANS statement. Insulin concentrations were analyzed as repeated measurements using an autoregressive covariance structure, and means were separated using the PDIF option of the LSMEANS statement. Pearson correlation coefficients were determined using the CORR procedure of SAS. Least squares means and standard errors of the mean are reported. Trends were declared at $P < 0.10$ and $P > 0.05$. Statistical significance was declared at $P \leq 0.05$.

RESULTS

Dry matter intake, milk yield, milk lactose percentage, and milk lactose yield were similar (Table 2) between the 2 experimental diets. Milk fat percentage was decreased by 11% ($P < 0.05$) whereas milk protein percentage was increased by 7% ($P < 0.001$) in cows consuming the high cornstarch diet compared with cows consuming the high fiber diet (Table 2). Milk fat yield was decreased by 10% ($P < 0.05$), whereas milk protein yield showed a trend ($P = 0.06$) for a 7% increase in cows consuming the high cornstarch diet compared with cows consuming the high fiber diet (Table 2). Energy balance tended ($P = 0.07$) to be improved in cows consuming the high cornstarch diet compared with the high fiber diet (Table 2). Effect of parity was not significant for all variables measured.

Average insulin area under the curve, generated from a 10-h blood sampling period, increased ($P < 0.005$) by 20% in cows consuming the high cornstarch diet compared with cows consuming the high fiber diet (Figure 2). Average plasma insulin concentrations at the time of liver biopsy (3.16 ± 0.04 h post-feeding) were elevated ($P < 0.005$) by 44% in cows consuming the high cornstarch diet compared with cows consum-

Figure 2. Insulin area under the curve (AUC; left 2 bars) and average insulin concentrations (right 2 bars) at the time of liver biopsy (3.16 ± 0.04 h post-feeding) in cows consuming a high cornstarch diet (open bars) or a high fiber diet (closed bars). Asterisk indicates $P < 0.005$.

Figure 3. Hepatic cytochrome P450 2C (CYP2C; left 2 bars) and P450 3A (CYP3A; right 2 bars) activity for cows consuming a high cornstarch diet (open bars) or a high fiber diet (closed bars). Asterisk indicates $P < 0.05$; double dagger indicates $P = 0.09$.

ing the high fiber diet (Figure 2). Cytochrome P450 2C activity was decreased ($P < 0.05$) by 45% in cows fed the high cornstarch diet compared with cows fed the high fiber diet (Figure 3). Cytochrome P450 3A activity showed a tendency ($P = 0.09$) for a 34% lower enzyme activity in cows consuming the high cornstarch diet compared with cows consuming the high fiber diet (Figure 3). The fractional rate constant of progesterone decay was not significantly different (Figure 4) between the 2 diets; however, the half-life of progesterone tended to be increased ($P = 0.08$; Figure 4) in cows consuming the high cornstarch diet compared with cows consuming the high fiber diet and averaged 85 versus 64 min, respectively. Cytochrome P450 2C mRNA expression tended ($P = 0.09$) to be decreased by 21% in cows fed the high cornstarch diet (0.89 ± 0.09) compared with cows fed the high fiber diet (1.12 ± 0.09). Cytochrome P450 3A mRNA expression was not different between the 2 experimental diets.

Correlation analysis revealed a positive association of CYP2C activity with both DMI and milk yield (Table 3). Cytochrome P450 3A activity was positively correlated with both EB and DMI, whereas CYP3A mRNA

expression was negatively correlated with EB and DMI. There was a tendency for CYP2C activity to be positively correlated with CYP3A activity.

DISCUSSION

The current study in lactating dairy cows reveals a role for added dietary starch in decreasing progesterone clearance without affecting lactational performance. Pereira et al. (1999) found an increase in DMI, milk yield, milk fat yield, and milk fat percentage when replacing dry, cracked corn with different nonforage fiber sources. In the current experiment, cows received a diet containing 15.1% refined cornstarch (DM basis), which decreased milk fat yield and tended to increase milk protein yield compared with a high fiber diet (containing 23.1% soybean hulls). The increase in milk protein percentage in the current study may be because of an increase in starch digestion in the lower gut or increased propionate production, which could cause sparing of AA. Several researchers have found that replacing corn with soybean hulls increases the ratio of ruminal acetate to propionate, with propionate concentration

Table 2. Effect of a high cornstarch versus a high fiber diet on DMI, milk production, and energy balance

Dependent variable	High cornstarch	High fiber	SEM	P-value
DMI, kg/d	19.75	18.81	0.54	0.21
Milk yield, kg/d	27.53	27.10	1.36	0.66
Fat, %	3.44	3.86	0.15	0.03
Fat yield, kg/d	0.94	1.05	0.06	0.04
Protein, %	3.00	2.80	0.07	<0.001
Protein yield, kg/d	0.82	0.76	0.04	0.06
Lactose, %	4.75	4.76	0.05	0.47
Lactose yield, kg/d	1.31	1.30	0.07	0.82
Energy balance, Mcal/d	3.44	1.49	0.91	0.07

Table 3. Pearson correlation coefficients for energy balance (EB), DMI, milk yield (MY), insulin concentrations (Insulin), progesterone half-life (Half-life), hepatic cytochrome P450 2C activity (2C Act), hepatic cytochrome P450 3A activity (3A Act), hepatic cytochrome P450 2C mRNA expression (2C mRNA), and hepatic cytochrome P450 3A mRNA expression (3A mRNA)¹

Item	Insulin ²	Half-life	2C Act	3A Act	2C mRNA	3A mRNA
EB	0.25 (0.11)	0.32 (0.16)	0.06 (0.71)	0.36 (0.02)	0.04 (0.80)	-0.35 (0.02)
DMI	0.14 (0.38)	0.32 (0.17)	0.38 (0.01)	0.35 (0.02)	0.14 (0.35)	-0.43 (0.003)
MY	0.03 (0.85)	0.03 (0.90)	0.30 (0.05)	0.04 (0.78)	0.04 (0.81)	-0.25 (0.11)
Insulin		0.31 (0.17)	-0.15 (0.33)	-0.12 (0.45)	-0.15 (0.32)	-0.09 (0.57)
Half-life			-0.08 (0.73)	0.33 (0.16)	-0.07 (0.76)	-0.12 (0.40)
2C Act				0.26 (0.10)	0.10 (0.50)	-0.23 (0.14)
3A Act					0.25 (0.10)	-0.10 (0.52)
2C mRNA						0.60 (<0.001)

¹P-values are shown in parentheses.

²Insulin concentrations at the time of liver biopsy (d 12) versus EB, DMI, MY, 2C Act, 3A Act, 2C mRNA, and 3A mRNA. Insulin concentrations on d 14 at 3 h post-feeding were analyzed against Half-life.

either decreasing (Sarwar et al., 1992; Cunningham et al., 1993) or remaining constant (Sievert and Shaver, 1993; Mansfield and Stern, 1994). Providing 15.1% refined cornstarch in the current experiment caused a 20% increase in insulin area under the curve, which would be expected after increasing propionate availability for hepatic gluconeogenesis.

Garnsworthy et al. (2008a) altered energy source in isoenergetic and isonitrogenous dairy rations (5 dietary levels of starch: 9, 14, 16, 18, and 23% starch). They reported increasing insulin concentrations in cows fed starch from 9 to 16% in the form of wheat; however, the postovulatory rise in progesterone was delayed in cows fed the diet containing highest starch (23% starch) even though insulin concentrations (~0.07 nM) had already reached a plateau at 16% dietary starch. Their second highest level of starch was below the dietary treatments fed in the current study (35 vs. 19% starch), where we found a 44% increase in insulin concentrations at the time of liver biopsy (0.16 vs. 0.08 nM), which might be explained by the differences in the type of carbohydrate provided (wheat vs. refined cornstarch) or the level of feed intake. Gong et al. (2002) found that high insulin diets increased the percentage of cows ovulating by d 50 postpartum. Garnsworthy et al. (2009) altered dietary treatments at the time of the first postpartum ovulation from a low insulin diet (9.8% starch) to a high insulin diet (18.2% starch) or a high insulin diet to a low insulin diet, or continued feeding a low or high insulin diet throughout the experiment. Average plasma insulin concentrations were comparable to our high fiber diet and ranged from 0.06 to 0.09 nM insulin in all treatment groups at approximately 10 wk postpartum. Pregnancy rates were highest in cows fed the high insulin diet until the first postpartum ovulation and then switched to the low insulin diet during early pregnancy compared with the 3 other dietary variations. This may be because of the beneficial effect of insulin

on early follicular development, leading to a shorter postpartum anestrous interval, and the negative effects of insulin on oocyte competence, leading to higher embryonic loss (Chagas et al., 2007). However, insulin may have an embryo protective role if concentrations are high enough to alter endogenous concentrations of progesterone during early pregnancy. In beef heifers fed insulin-stimulating diets, Mann et al. (2003) reported an increase in early embryonic development compared with low insulin diets; however, this result was not accompanied by any changes in plasma progesterone or insulin concentrations even though these diets were similar to the diets fed by Gong et al. (2002). Hawkins et al. (1995) found an increase in progesterone half-life in beef heifers fed a diet supplemented with 7.5% Mega-Lac (170 min; Church & Dwight Inc., Princeton, NJ) compared with those fed a diet supplemented with an energy control (113 min). In the current study, our high fiber diet was supplemented with 1.9% Mega-Lac,

Figure 4. Fractional rate constant of progesterone decay (k ; left 2 bars) and average half-life of progesterone ($T_{1/2}$; right 2 bars) in cows consuming a high cornstarch diet (open bars) or a high fiber diet (closed bars). The equation $T_{1/2} = \ln(2)/k$ was used to calculate the half-life of progesterone. The double dagger ($\ddagger$) indicates $P = 0.08$.

which resulted in a 40% increase in fat percentage compared with the high cornstarch diet. In the Hawkins et al. (1995) study, supplementation with 7.5% Mega-Lac resulted in a 150% increase in dietary fat percentage. The lower amounts of Mega-Lac in the current study may explain the lack of a fat supplementation response in progesterone half-life compared with the high cornstarch diet. Moreover, the insulin response in beef heifers is marginal compared with dairy cows because they have not been predisposed to a severe negative EB and insulin is already increased. In our current study, the inclusion of 1.9% Mega-Lac in the high fiber diet may have resulted in decreased insulin concentrations, contributing to the divergent secretion of insulin between the 2 dietary treatments. Garnsworthy et al. (2008b) found decreased insulin concentrations in dairy cows supplemented with Mega-Lac at 23 and 30 g/kg of DM compared with 0, 8, and 15 g/kg of DM, whereas starch was held constant across dietary treatments. Progesterone concentrations from d 3 to 5 postovulation were increased in cows supplemented with Mega-Lac at 23 and 30 g/kg of DM, which may be because of increased production, decreased clearance of progesterone, or both (Garnsworthy et al., 2008b).

Selvaraju et al. (2002) found increased peripheral concentrations of progesterone in crossbred, repeat-breeding cows ($n = 21$) treated with long-acting bovine insulin. Those cows showed a 46% increase in plasma insulin concentrations after injection as well as a 17 and 15% increase in plasma progesterone on d 8 and 10 of the estrus cycle, respectively; however, pregnancy rates were not different between the insulin-treated (63.6%) and saline-treated (40.0%) groups (Selvaraju et al., 2002). These higher concentrations of progesterone may be caused by a decrease in hepatic clearance brought about by increased insulin concentrations, which is in agreement with our current findings. Miyoshi et al. (2001) reported an increase in days to first ovulation in cows ($n = 36$) drenched daily with 500 mL of propylene glycol. Cumulative plasma progesterone concentrations during the first estrus cycle in the propylene glycol (42.9 ng) and control (29.4 ng) cows were not different, nor were the first-service conception rates between the propylene glycol (57.1%) and control (33.0%) groups (Miyoshi et al., 2001). In a larger propylene glycol drenching experiment, Hidalgo et al. (2004) found a 33% increase in insulin concentrations, a 27% increase in progesterone concentrations, and a 33% increase in pregnancy rates in cattle ($n = 286$) drenched daily with 250 mL of propylene glycol before embryo transfer. In nonlactating, ovariectomized cows divided into groups of high and low plasma concentrations of insulin before feeding, Moriel et al. (2008) reported increased plasma progesterone (CIDR) concentrations at 1 h post-feeding

in the high insulin group compared with the low insulin group, which would suggest a protective role for insulin in decreasing progesterone clearance *in vivo*. However, these effects were reversed at 5 h post-feeding, where the low insulin group had the highest concentrations of progesterone (Moriel et al., 2008), which may be an indicator of progesterone clearance, progesterone absorption into the periphery, or both.

In dairy cows chronically exposed to high insulin via a hyperinsulinemic-euglycemic clamp (96-h exposure) we found a decrease in both CYP2C and CYP3A mRNA expression (Lemley et al., 2008b). It is well established that chemically induced, insulin-dependent diabetes enhances the expression of cytochrome P450 2E1, 2B, 3A, and 4A and that insulin administration to diabetic rats lowers the expression of these cytochrome P450s to basal levels (Barnett et al., 1990). Both insulin and glucagon have been implicated in increasing or decreasing CYP3A expression and activity in rat hepatocytes (Saad et al., 1994). In these previous experiments, supraphysiological concentrations of insulin or glucagon modified cytochrome P450 mRNA expression after extended exposure (24, 48, or 96 h). Longer exposure times combined with high concentrations of hormone may be misleading because actual *in vivo* hepatic exposure to elevated insulin or glucagon could be as short as 2 h/d, which may be enough time to regulate enzyme activity posttranslationally while having little to no effect on mRNA expression (Berry and Skett, 1988). In the current experiment, CYP2C mRNA expression tended to be lower in cows fed the high cornstarch diet whereas CYP3A mRNA expression was unchanged. Correlation analysis revealed no associations between CYP2C mRNA with CYP2C activity or CYP3A mRNA with CYP3A activity. Furthermore, CYP3A activity was positively correlated with EB and DMI, whereas CYP3A mRNA expression was negatively correlated with EB and DMI. Similar to our own results, Zangar and Novak (1998) found a 50% decrease in CYP3A mRNA expression in hepatocytes treated with dimethyl sulfoxide for 48 h, whereas both CYP3A protein expression and activity increased almost 5-fold under the same culture conditions. These results are not surprising given the evidence associated with specific substrates that regulate cytochrome P450 activity by decreasing or increasing protein turnover, which is independent of mRNA expression (Zangar and Novak, 1998).

In the current experiment, we found a 20% increase in average insulin area under the curve and a 25% increase in progesterone half-life in cows consuming diets containing 15.1% refined cornstarch. Elevated endogenous concentrations of progesterone may decrease pregnancy loss during early gestation. Starbuck et al. (2004) found

increased pregnancy retention to wk 7 of gestation in dairy cows that were classified during wk 5 as having high compared with low concentrations of progesterone. Rhinehart et al. (2009) collected corpora lutea from pregnant (d 28–34) lactating dairy cows that were divided into low or high progesterone groups. A subcutaneous injection of 150 mg of progesterone was given every 12 h to maintain pregnancy, and those cows that were previously classified as having high concentrations of progesterone had a greater progesterone area under the curve compared with the low progesterone group post-lutectomy. Gene expression for components of the endothelin and prostaglandin systems were not different between the high and low progesterone groups, which may indicate a greater involvement in steroid clearance versus steroid production in regulating peripheral concentrations of progesterone in pregnant lactating dairy cows (Rhinehart et al., 2009).

CONCLUSIONS

Pregnancy loss continues to be a major economic concern to the dairy industry, and alleviating pregnancy wastage would improve the efficiency of all dairy operations. Several researchers have found differences in plasma concentrations of steroids during pregnancy or the mid-luteal phase of the estrus cycle after varying dietary treatments or after treatment with different metabolic hormones (i.e., growth hormone and insulin); however, a substantial portion of these studies have failed to distinguish between differences caused by steroid production, steroid clearance, or a combination of both. In the current study, we focused on clearance of progesterone from an exogenous source and found a trend for a longer half-life of progesterone after feeding a diet high in refined cornstarch compared with a high fiber diet. Cytochrome P450 2C activity was lower, whereas cytochrome P450 3A activity tended to be lower in cows consuming a high cornstarch diet compared with cows consuming a high fiber diet. Feeding diets that stimulate insulin secretion may modify progesterone clearance by inhibiting hepatic activity of cytochrome P450 2C and 3A. Decreasing steroid clearance during early pregnancy may lead to higher peripheral concentrations of progesterone, which could advance embryonic development during the early period of pregnancy when embryonic loss and pregnancy wastage are high.

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