Effects of Urea Infusion on the Uterine Luminal Environment of Dairy Cows

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ABSTRACT

Previous research indicates that high plasma urea nitrogen (PUN) concentrations are associated with decreased fertility in lactating dairy cows. The objective of this study was to monitor changes in the uterine environment during acute elevation of PUN. Lactating dairy cows ($n = 8$) were infused with saline or urea (0.01 g of urea/h per kg of body weight) through jugular vein catheters on d 7 after estrus. After 24 h, cows were switched to the opposite treatment for a second 24-h infusion period. Blood samples were collected every 2 h, and the pH within the lumen of the uterine horn ipsilateral to the corpus luteum was recorded every 6 h. At the end of each 24-h infusion period, 30 mL of sterile saline was flushed into the uterine lumen and immediately retrieved. Mean PUN concentration increased from 16.6 ± 1.3 mg/dL during saline infusion to 22.6 ± 1.3 mg/dL during urea infusion. Uterine pH decreased during urea infusion from 7.08 ± 0.07 at 6 h to 6.88 ± 0.08 at 18 h, but was unchanged during saline infusion (7.01 ± 0.08 at 6 h to 7.06 ± 0.07 at 18 h). Protein concentration, $\text{PGF}_{2\alpha}$, and prostaglandin E_2 concentrations in uterine lavage samples were not different between treatments. The results of this study indicate that a short-term increase in PUN can exert direct effects on the uterine environment by decreasing uterine pH.

(**Key words:** urea, uterus, reproduction, dairy cow)

Abbreviation key: CA = carbonic anhydrase, PGE_2 = prostaglandin E_2 , PUN = plasma urea nitrogen.

INTRODUCTION

Dairy farmers commonly feed diets that contain high levels of protein to maximize milk production. Feeding

excess protein to dairy cows increases blood urea nitrogen concentrations, and high blood urea nitrogen has been linked to low fertility (Blanchard et al., 1990; Canfield et al., 1990; Elrod and Butler, 1993; Ferguson et al., 1993; Butler et al., 1996). Conception rates of lactating dairy cows decreased approximately 20 percentage points when plasma urea nitrogen (PUN) or MUN concentrations exceeded 19 mg/dL (Butler et al., 1996). Similarly, Ferguson et al. (1988, 1993) reported a decrease in conception rate when serum urea nitrogen concentrations were greater than 20 mg/dL. Rajala-Schultz et al. (2001) found that cows with MUN concentrations below 10 mg/dL were nearly two and a half times more likely to be confirmed pregnant than cows with MUN concentrations above 15.4 mg/dL.

Composition of the uterine luminal fluid in cows fed high protein diets has been examined to elucidate the mechanisms responsible for reduced conception rates. Jordan et al. (1983) found that cows fed a high CP diet had decreased concentrations of P, Mg, and K in uterine fluid during the luteal phase, but uterine P, Mg, and K were similar at estrus. Elrod and Butler (1993) and Elrod et al. (1993) fed diets differing in RDP and RUP content to dairy cows and heifers, resulting in elevated PUN. Uterine luminal pH was measured on the day of estrus and 7 d after estrus. Uterine pH of control and high PUN cattle was similar at estrus. On d 7 after estrus, however, high PUN concentrations were associated with low uterine pH. Uterine pH on d 7 after estrus is of interest because if the oocyte had been fertilized, the embryo would be within the uterine lumen. Because placentation has not yet occurred, the d 7 embryo is dependent on uterine secretions for survival. Changes in the uterine environment during this period could compromise early development or viability of the embryo.

The results of previous research showed that elevated PUN concentrations caused by feeding high protein diets change the uterine environment of dairy cattle. However, previous studies have not tested the effects of urea separately from other metabolites of protein

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digestion. Therefore, the objective of this experiment was to measure the characteristics of the uterine luminal environment during acute elevation of PUN initiated by intravenous urea infusion.

MATERIALS AND METHODS

Experimental Study

Multiparous lactating Holstein dairy cows ($n = 8$; 40 to 100 DIM) were used. Cattle were housed in AAALAC-accredited facilities; protocols involving animals were approved by the Institutional Animal Care and Use Committee of Cornell University (Ithaca, NY). Estrous cycles of the cows were synchronized with one injection of GnRH (0.05 mg; Cystorelin, Rhone Merieux, Inc., Athens, GA) followed 7 d later by an injection of PGF_{2 α} (25 mg; Lutalyse Sterile Solution, Pharmacia Animal Health, Kalamazoo, MI). Three days after standing estrus was observed, cows were moved to monitor rooms in the Large Animal Research and Teaching Unit. Transrectal ultrasound examination of ovarian follicles was conducted daily from the time of the PGF_{2 α} injection until ovulation. Both jugular veins of each cow were then catheterized in preparation for treatment and blood sampling.

Cows were fed a TMR for ad libitum consumption (31% concentrate: 69% forage). The TMR had a moderate concentration of CP (15.2%) and energy content was formulated for early lactation (1.61 Mcal NE_L per kg of DM). Cows were given fresh feed every 6 h to encourage frequent feed consumption for maintaining a constant amount of endogenous urea production throughout the study. Cows were milked twice daily at 0600 and 1800 h.

Cows were assigned randomly to receive the saline (control; $n = 4$) or urea ($n = 4$) infusion treatment beginning on d 7 after estrus. For the urea treatment, an initial loading dose of urea (Sigma-Aldrich Company, St. Louis, MO) was administered through the jugular catheters to rapidly elevate circulating concentrations of urea and expedite equilibration of urea in tissue fluids to concentrations that would be maintained by the continuous infusion. The loading dose of urea (15 mg of urea per 100 mL of blood volume estimated as 7% of BW) was dissolved in 100 mL of saline and was determined from results of a preliminary trial. The infusion dose of urea (0.01 g of urea/h per kg of BW in saline) immediately followed the loading dose, and was delivered by a syringe pump at a rate of 0.5 mL/min. Control cows received comparable infusions of sterile saline. After 24 h of infusion, cows were switched to the opposite treatment, and received loading and infusion doses as described above. The second infusion period lasted 24 h, for a total treatment period of 48 h.

Blood samples were collected from a jugular vein catheter every hour for the first 12 h of treatment and thereafter collected every 2 h. The blood samples were centrifuged at $1200 \times g$ and the plasma fraction was collected and stored at -20°C . Plasma urea nitrogen concentrations were determined using an automated diacetylmonoxamine method (Technicon Industrial Method 339-01, Technicon Industries, Tarrytown, NY). Plasma progesterone concentrations were analyzed in samples collected at 22 and 24 h of the infusion periods in a single validated radioimmunoassay (Elrod and Butler, 1993). The intraassay coefficient of variation for the progesterone assay was 11%.

Uterine luminal pH was measured every 6 h during the treatment period. An adhesive EKG electrode was fixed to a shaved area of skin on the rump of each cow as the pH reference electrode. A micro pH electrode (MI-508 Esophageal pH electrode, Microelectrode Inc., Bedford, NH) attached to a Fisher Accumet pH meter (model 630) was used to measure uterine luminal pH. The pH electrode was standardized by covering the tip of the pH electrode in a gauze pad soaked in a pH 7 standard buffer and holding the pH electrode on the hide near, but not touching, the reference EKG electrode. A sheathed steel cannula (custom-made, 56-cm long, 5-mm outside diameter) was inserted through the vaginal orifice. The cannula was pushed through the protective sheath before entering the cervical canal. Finally, the cannula was positioned approximately 8 cm beyond the uterine bifurcation in the horn ipsilateral to the corpus luteum (method developed in the preliminary trial described below). After rinsing with sterile saline, the micro pH electrode was then inserted through the cannula into the uterine lumen and extended 2.5 cm beyond the tip of the cannula. After a brief equilibration period, the pH reading stabilized and was recorded. The cannula and electrode were then gently retracted from the uterus and disinfected before each subsequent use. The electrode was standardized for each cow prior to each pH measurement.

Uterine lavage samples were collected after the 24- and 48-h pH measurements. The micro pH electrode was withdrawn from the steel cannula. Sterile saline (30 mL) was flushed into the uterine lumen and immediately aspirated back through the cannula. Recovery for the uterine lavages varied from 2 to 9 mL. A proteinase inhibitor, phenylmethylsulfonyl fluoride (0.5% in ethanol; wt/vol), was added to the lavage samples to a final concentration of 0.005%. The prostaglandin E₂ (PGE₂) and PGF_{2 α} content of the lavage samples were evaluated by ELISA (PGE₂ ELISA kit EA 02 and PGF_{2 α} ELISA kit EA 05, Oxford Biomedical Research Inc., Oxford, MI). Each prostaglandin was measured in a single assay according to the protocol provided by the

Figure 1. Plasma urea nitrogen (PUN) concentrations and uterine pH values of 2 cows (5928 and 5919) during the preliminary study.

supplier. In each system, serial dilutions of uterine lavage yielded inhibition lines that were parallel with the standard. Intraassay coefficients of variation among sample replicates were 5.9 and 5.8% for $\text{PGF}_{2\alpha}$ and PGE_2 , respectively. Total protein content of the lavage samples was measured by a phenol reagent method for biological fluids (micro protein determination procedure no. 690, Sigma Diagnostics, St. Louis, MO). The $\text{PGF}_{2\alpha}$ and PGE_2 concentrations were reported per microgram of uterine lavage protein to account for variable dilution and recovery of the uterine fluid in lavages.

Statistical Analysis for Experimental Study

Data were analyzed using the mixed model procedure of SAS (SAS Inst., Inc., Cary, NC). The main effect of treatment and the interaction of treatment $\times$ time were tested using treatment by cow nested within group as

the error term, where group represented the order in which treatments were administered. When the effect of treatment or treatment $\times$ time was significant, means separation procedures were carried out using the Tukey procedure of SAS. Data are presented as least square means and standard errors of the least square means.

Preliminary Study

Before the experimental study, additional dairy cows ($n = 8$) were used in a preliminary study to establish the feasibility of repetitive uterine pH measurements over time. Stability and reliability of uterine pH measurements taken with an indwelling uterine Foley catheter (Elrod and Butler, 1993) were compared with those using a metal cannula. Estrus synchronization, blood sampling, and other management conditions were similar to the experimental study, except that cows were fed twice daily and no intravenous infusions were made. Cows were held without feed for 6 h and then fed 30 min before the first measurements at 0 h, and fed every 12 h after that.

The PUN profiles of 2 cows, with uterine pH measurements every 6 h are shown in Figure 1. Uterine pH was depressed when PUN was elevated. The metal cannula approach provided more consistent pH recordings after 24 h compared with the Foley catheter. Therefore, this technique was adopted for the experimental study.

RESULTS

Experimental Study

Uterine pH before infusion was similar for the saline- and urea-infused cows. At 24 h, cows were switched immediately from saline to urea or from urea to saline infusion. There was no significant effect of treatment sequence on PUN, uterine pH, or uterine lavage concentrations of prostaglandin. Plasma urea nitrogen increased or decreased rapidly following initiation or termination of urea infusion. Therefore, treatment sequence was ignored in subsequent analyses.

Because cows were immediately switched from one treatment to the other at 24 h, no 0 h measurements for the second infusion period were collected. Therefore, data are presented at 6, 12, 18, and 24 h of infusion. Mean PUN concentrations for saline- and urea-infused cows are shown in Figures 2 and 3, respectively. Infusion of urea increased PUN ($P < 0.001$) relative to control treatment (22.6 ± 1.3 vs. 16.6 ± 1.3 mg/dL for urea and saline infusion, respectively). A treatment $\times$ time interaction ($P < 0.05$) was detected for uterine pH. Uterine pH in control cows was neutral or higher and did not significantly change during the infusion period (Figure 2).

Figure 2. Least square means and standard errors for plasma urea nitrogen (PUN) concentrations and uterine pH during intravenous infusion of saline in lactating dairy cows ($n = 8$). Uterine pH was not significantly affected by saline infusion.

In contrast, the uterine pH of urea-infused cows was similar at 6 and 12 h, but decreased to acidic levels by 18 h ($P < 0.05$), and tended ($P = 0.09$) to remain at that level through 24 h (Figure 3). Individual cows showed dynamic changes in uterine pH in response to PUN during the 48-h treatment period (Figure 4). The uterine pH of cow 6327 decreased during the urea treatment period, reached a nadir at the 36-h measurement, and remained stable thereafter despite a continued rise in PUN through 48 h. Likewise, the uterine pH of cow 6350 was low during urea infusion and increased with declining PUN concentrations after being switched from urea to saline infusion.

Plasma progesterone concentrations at 22 and 24 h of the infusion periods averaged 4.2 ± 0.2 and 4.4 ± 0.2

Figure 3. Least square means and standard errors for plasma urea nitrogen (PUN) concentrations and uterine pH during intravenous infusion of urea in lactating dairy cows ($n = 8$). A treatment $\times$ time interaction was detected for uterine pH. *Uterine pH at 18 h differed ($P < 0.05$) from that at 6 and 12 h. †Uterine pH at 24 h tended ($P = 0.09$) to differ from that at 12 h.

Figure 4. Plasma urea nitrogen (PUN) concentrations and uterine pH values from 2 cows (6327 and 6350) during intravenous infusion of saline and urea. Cow 6327 was infused with saline (0 to 24 h) followed by urea (24 to 48 h). Cow 6350 was infused with urea (0 to 24 h) followed by saline (24 to 48 h).

ng/mL, respectively, and were not affected by treatment ($P > 0.05$). Concentrations of $\text{PGF}_{2\alpha}$ (3.38 ± 1.49 pg/ μg of protein) and PGE_2 (1.95 ± 0.63 pg/ μg of protein) in uterine lavage samples did not differ between treatments ($P > 0.10$). The ratio of $\text{PGF}_{2\alpha}$ to PGE_2 was similar (0.83 ± 0.18 , $P > 0.10$).

DISCUSSION

Effects of PUN on conception rate of dairy cows have been studied. Most (Blanchard et al., 1990; Canfield et al., 1990; Elrod and Butler, 1993; Ferguson et al., 1993;

Butler et al., 1996), but not all (Howard et al., 1987; Carroll et al., 1988; Barton et al., 1996), studies have concluded that high PUN concentrations are associated with decreased fertility. The present study demonstrated that increasing PUN by intravenous infusion of urea reduces uterine luminal pH in lactating cows during the luteal phase of the estrous cycle. These results support those of previous reports for cows (Elrod et al., 1993) and heifers (Elrod and Butler, 1993), in which uterine luminal pH increased from estrus to d 7 in control cows, but remained low during the luteal phase in cows fed a high protein diet. Smith et al. (2000) infused urea for 2-h periods, increasing PUN in heifers, and found that uterine pH decreased in response to elevated PUN. Collectively, these studies provide strong evidence that PUN concentrations in lactating cows and heifers directly alter uterine pH.

Timing of the observed changes in uterine luminal pH in response to elevated PUN may be critical for understanding the effects of PUN on embryo survival. Elrod and Butler (1993) suggested that the decrease in uterine luminal pH during the luteal phase might reflect changes in uterine secretory activity in response to urea. Variations in the uterine environment caused by high PUN concentrations may therefore create a hostile or suboptimal environment for early embryo development. The interaction between diet-related increases in PUN and the inductive effects of progesterone on uterine luminal secretions, as well as other mechanisms for reproductive failure were previously reviewed (Butler, 1998; Westwood et al., 1998).

Uterine luminal pH is controlled by carbonic anhydrase (CA), which catalyzes the reversible reaction: $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$. This enzyme is present in many secretory epithelia including the reproductive tract (Rodriguez-Martinez et al., 1991), and functions in the selective transport of hydrogen and bicarbonate ions (Swenson, 1991). Depending on the permeability of apical vs. basolateral plasmalemmae, epithelial cells can export H^+ or HCO_3^- apically or basally in exchange for sodium, potassium, and chloride ions to affect cellular ion content and to modify the pH of the luminal fluid (Rodriguez-Martinez et al., 1991). Unique isozymes of CA are induced by progesterone in the human or rabbit uterus (Hodgen and Falk, 1971; Falk and Hodgen, 1972) and, therefore, abundance varies by stage of cycle. In preliminary unpublished studies, uterine endometrial biopsies were collected from lactating cows and CA increased between estrus and d 9 of the estrous cycle (4.6 ± 0.4 and 6.1 ± 0.5 units/mg of protein, respectively; $P = 0.02$; $n = 13$). The apparent steroid-dependency of CA is particularly intriguing because the observed changes in uterine pH in association with PUN occurred only during the luteal phase (Elrod

and Butler, 1993; Elrod et al., 1993). Conceivably, high urea could alter uterine pH by changing CA activity during the luteal phase, but not at estrus.

Studies have addressed the effects of high PUN concentrations on early embryo development. Blanchard et al. (1990) found no differences in the average number of fertilized, unfertilized, transferable, or nontransferable ova collected from lactating cows fed 73 or 64% rumen-degradable intake protein. A greater percentage of fertilized ova were recovered, however, from cows fed less rumen-degradable intake protein. Cows fed smaller amounts of rumen-degradable intake protein also tended to have a higher mean percentage of transferable embryos. A higher percentage of cows fed greater amounts of rumen-degradable intake protein failed to yield transferable embryos. In a related experiment, we found that embryos from donor cows having elevated PUN concentrations were less likely to survive after transfer to recipient heifers than embryos from cows having moderate PUN concentrations (Bode et al., 2001). Taken together, these studies indicate that high PUN concentrations may affect oocyte health, embryo survival, or both.

Decreased uterine pH observed in the current study is a likely mechanism by which elevated PUN concentrations affect embryo survival. Culture of early bovine embryos at pH less than 7 reduced cleavage rates and development to the blastocyst stage (Ocon and Hansen, 2003). These *in vitro* results indicate that suboptimal uterine pH in response to high concentrations of PUN *in vivo* may inhibit early embryonic development.

In the current study, uterine pH during the luteal phase was consistently reduced after 6 to 12 h of elevated PUN resulting from intravenous urea infusion. Results from individual cows attest to the dynamic nature of uterine pH in response to increases or decreases in PUN. Uterine pH decreased in response to prolonged exposure to increased PUN, but quickly recovered when PUN decreased. Uterine pH often stabilized near the end of the urea infusion period despite further increases in PUN concentrations. The potential for adaptation to high concentrations of urea was suggested by Westwood et al. (1998) and merits further investigation.

Previous studies indicated an inverse relationship between PUN and progesterone concentrations during the luteal phase. Jordan and Swanson (1979) found that serum progesterone was lower on d 14 after estrus when cows were fed 16.3 or 19.3% CP than when fed 12.7% CP. Sonderman and Larson (1989) reported reduced progesterone concentrations on d 12 after estrus in cows fed 20% protein compared with cows fed 14% protein. In the present study, an acute elevation of PUN around d 7 after estrus did not affect plasma progester-

one concentrations, in contrast to earlier reports in which high protein diets were fed for many days.

Studies conducted by Buford et al. (1996) and Seals et al. (1998) showed that cows treated with PGF_{2α} 5 to 8 d after breeding had lower pregnancy rates than control cows, despite progesterone supplementation. Those authors suggested that PGF_{2α} or another product of the corpus luteum might contribute to embryonic loss. During the present study, concentrations of PGF_{2α}, PGE₂, or the ratio of PGF_{2α} to PGE₂ in uterine lavage samples were not altered by treatment. Therefore, changes in uterine pH may be the primary mechanism through which fertility is affected in cows having high PUN concentrations. Changes in uterine pH may indicate or reflect additional changes in the uterine environment that impact fertility.

CONCLUSIONS

Feeding high levels of dietary protein is associated with decreased fertility in dairy cows. The results of this study suggest a potential mechanism through which PUN can affect the uterine environment and embryo survival. A decrease in uterine luminal pH occurred in response to urea infusion. The uterus, therefore, can acutely respond to circulating PUN. Further study is necessary to determine how a change in uterine pH may influence the fertility of dairy cows through effects on embryo development or viability.

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