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Plasma and Milk Urea Nitrogen in Relation to Pregnancy Rate in Lactating Dairy Cattle

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ABSTRACT: The objectives of this study were to relate concentrations of plasma (PUN) and milk (MUN) urea nitrogen to pregnancy rate in dairy cows and compare various methods of analysis and preparation of milk for measuring MUN. In two experiments, blood or milk samples were collected on the day of AI from Holstein cows (n = 160 and n = 155, respectively). Three methods of MUN analysis were compared. Two laboratory chemical procedures yielded similar results, whereas a quick dipstick method overestimated chemical analyses. Before and after milking strip samples had MUN concentrations equivalent to those in composite milk. Concentrations of PUN or MUN greater than 19 mg/dL were associated with decreased ($P < .02$) pregnancy rates (18 and 21 percentage point reduction in the two experiments).

In two subset groups of cows (n = 51 and n = 23, respectively), plasma progesterone or MUN concentrations were monitored during the 5-d period after AI. Plasma progesterone concentrations increased similarly during the period for cows divided into low vs high PUN but were greater in pregnant than in nonpregnant cows on d 4 and 5 ($P < .04$). The MUN concentrations showed low within-cow variation (CV = 8%) but were lower in pregnant cows and had a decreasing trend over time compared with nonpregnant cows ($P < .05$). Based on this study, plasma and milk will yield similar results for monitoring urea nitrogen in dairy cows; PUN and MUN concentrations > 19 mg/dL were associated with approximately a 20 percentage point decrease in pregnancy rate after AI in lactating dairy cattle.

Introduction

As the genetic capacity for milk production has increased, conception rate in dairy cattle has decreased (Butler and Smith, 1989). High dietary protein intake stimulates milk production (Grings et al. 1991) but also has been associated with decreased fertility (Jordan and Swanson 1979a; Kaim et al. 1983; Canfield et al. 1990). Cows fed excess dietary protein had increased blood urea, altered uterine fluid composition, decreased uterine pH, and reduced conception rates (Jordan et al., 1983; Elrod and Butler, 1993; Elrod et al., 1993). Plasma progesterone concentrations were reportedly lower in cows fed high dietary protein (Jordan and Swanson, 1979b; Sonderman and Larson, 1989). In relating dietary protein degradability to fertility, Ferguson et al. (1988, 1993) reported that blood urea nitrogen concentrations exceeding 20 mg/dL were associated with reduced conception rates in lactating cows.

Plasma urea nitrogen (**PUN**) and milk urea nitrogen (**MUN**) are useful indicators of protein metabolism and status in cows (Roseler et al., 1993); milk is particularly advantageous for field monitoring purposes. The objectives of this study were to interrelate plasma and milk urea nitrogen concentrations following AI with progesterone concentrations and pregnancy rate in dairy cattle fed normal diets formulated for high milk production.

Materials and Methods

Experiment 1: Relationship of Plasma Urea Nitrogen and Progesterone Concentrations Near the Time of Artificial Insemination to Pregnancy Outcome in Dairy Cattle

Normal lactating Holstein cows (n = 160, multiparous) from the Teaching and Research Center at Cornell University were used for this experiment. Cows were fed total mixed diets (50% forage and 50% concentrate) that were formulated to provide at least 1.62 Mcal/kg NE$_l$ and contained 17.5 to 19% crude protein. On the day of AI (first estrus after 60 d postpartum), blood samples were taken from the

coccygeal vein or artery into heparinized, evacuated tubes. In a subset of the cows (n = 51), daily blood samples were continued until d 5 after AI. Blood samples were centrifuged at $1,200 \times g$ for 20 min, and plasma aliquots were frozen at –20°C until the time of analysis. The PUN on the day of AI was determined using an automated diacetylmonoxamine method (**Auto method**, Technicon Industrial Method 339-01, Technicon Industries, Tarrytown, NY). Plasma samples collected during the 5-d period after AI were analyzed for progesterone in a single RIA (Elrod and Butler, 1993) with an intraassay CV of 7%. Cows were determined to be either pregnant or not pregnant to AI by veterinary palpation of the reproductive tract 40 to 50 d after breeding. All cattle (entire study) were housed in AAALAC-accredited facilities. All animal experimentation (entire study) was performed in compliance with regulations set by the Center for Research Animal Resources, Institutional Animal Care and Use Committee, Cornell University, Ithaca, New York.

Experiment 2: Comparison of Plasma Urea Nitrogen and Milk Urea Nitrogen Concentrations and Methods of Analysis

Blood and milk samples were taken on the same day from 22 randomly selected cows fed diets as described in Exp. 1. The milk samples were obtained from the milk weigh jar at the end of regular milking. In addition, from 10 of the cows, strip samples of milk were taken from the same quarter before and after milking. Preservative (Broad Spectrum Microtabs, D & F Control Systems, San Ramon CA) was added to half of the bulk milk sample from all cows, leaving the other half as control. Within 30 to 45 min after milking, blood was sampled from all cows from the coccygeal vein into heparinized, evacuated tubes, and plasma was processed as in Exp. 1. Milk samples were centrifuged at $1,120 \times g$ for 20 min. The fat layer was aspirated, and aliquots of supernatant were frozen at –20°C until the time of analysis. The PUN and MUN samples were analyzed with the Auto method described for Exp. 1. In preliminary trials, defatting the milk samples was necessary to prolong the functional lifespan of the osmotic membrane integral to the Auto instrument. The milk samples were also analyzed with either a manual urease/Berthelot determination (**Sigma**, urea nitrogen procedure no. 640, Sigma Diagnostics, St. Louis, MO) or a dipstick urease/pH method (**Azotest**, Compagnie Chimique d'Aquitaine, Lalande de Pomerol, France). For the Azotest, analyses were performed with whole milk samples (not defatted) as directed by the manufacturer. Using specifications from the manufacturer, results of the Azotest were converted to rank and range values to allow comparison with results of the Auto procedure (Table 1). The comparison of the Azotest and Auto methods was repeated on additional samples (n = 22).

Table 1. Azotest rank, color, and urea concentrations as provided by the manufacturer and converted to milk urea nitrogen (MUN)

Rank	Color	MU, g/L	MUN, mg/dL[a]
1	Orange	.10	4.7
2	Very light green	.20	9.4
3	Light green	.30	14.1
4	Green	.35	16.5
5	Dark green	.40	18.8
6	Very dark green	.50	23.5

[a]MUN (mg/dL) = MU(g/L) × 47. On a weight basis, urea is 47% nitrogen.

Experiment 3: Comparison of Milk Urea Nitrogen Concentrations on the Day of Artificial Insemination to Pregnancy Rate in Dairy Cattle

Lactating Holstein cows (n = 155; 50 were primiparous) were selected for this experiment from the Cornell University herd. Cows were fed diets similar to those described for Exp. 1, but Exp. 3 began approximately 18 mo later. Milk samples were collected on the day of AI (first estrus after 60 d of lactation). A subset of cows (n = 23) continued to be sampled at each milking (twice a day) over the next 5 d. Milk samples were prepared as described for Exp. 2 and analyzed for MUN using the Auto method. Cows were determined to be either pregnant or not pregnant to AI by veterinary palpation of the reproductive tract 40 to 50 d after breeding.

Statistical Analysis

Experiment 1. To relate pregnancy outcome to PUN, chi-square analysis (Number Cruncher Statistical Systems, NCSS, Kaysville, UT) was used to test pregnancy rates for cows with PUN concentrations greater than and less than the mean of the sample population. To further discriminate the relationship between PUN and fertility, cow data were categorized into incremental ranges (3 mg/dL) of PUN for calculation of pregnancy rate likelihood ratios (Ferguson et al., 1993). Only information for the first AI in each cow was used to avoid confounding effects of repeated measures and repeat breeding. Repeated measures ANOVA was used to determine the effects of PUN category (above or below the mean), pregnancy status, and days on progesterone concentrations.

Experiment 2. For evaluating procedures for measuring urea nitrogen, the Auto method was considered the basis for standardization. Paired *t*-test was used to compare PUN and MUN concentrations in the same cows; Sigma and Auto methods were used to compare MUN in the same samples and the effects of preservative. The GLM ANOVA (NCSS) was used to compare the Azotest and Auto methods across test
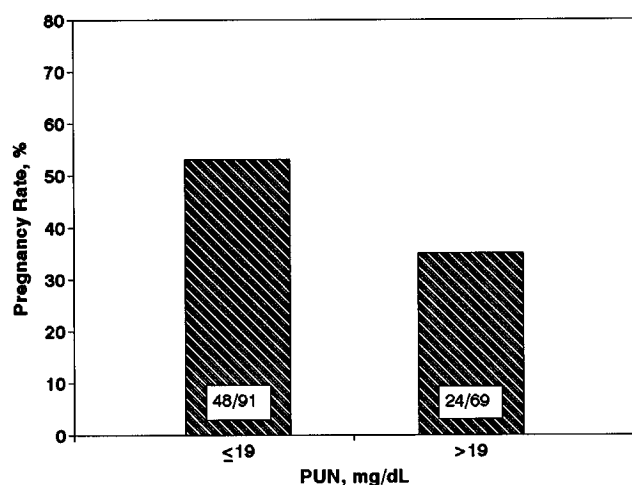
Figure 1. The relationship of plasma urea nitrogen (PUN) to pregnancy rate for first AI in lactating dairy cows (n = 160). Pregnancy rate was reduced ($P < .02$) in cows with PUN ≥ 19 mg/dL. The number of cows that became pregnant to AI is indicated in each PUN category.

Figure 2. Differences in rate of increase ($P < .04$) in plasma progesterone concentrations after AI of cows later diagnosed as pregnant (n = 29) or nonpregnant (n = 22). Progesterone was greater (*$P < .05$) in pregnant cows by d 4 after AI.

ranks. When the interaction term was significant, *t*-tests were performed within each rank.

Experiment 3. To relate MUN to pregnancy outcome after AI, cow data were categorized into incremental ranges (3 mg/dL) of MUN for calculation of pregnancy rate likelihood ratios as in Exp. 1. Chi-square analysis was used to evaluate pregnancy rates for cows with MUN concentrations greater than and less than 19 mg/dL and only information for the first AI was used, as described for Exp. 1. Mean MUN concentrations at AI for pregnant and nonpregnant cows were compared with Student's *t*-test. The variation in MUN over 10 milkings in relation to pregnancy status was evaluated using a repeated measures ANOVA and further analyzed with regression procedures.

Results

Experiment 1. The PUN concentrations in lactating cows (n = 160) averaged $18.9 \pm .3$ mg/dL on the day of AI. The pregnancy rate of cows with PUN concentrations greater than the mean was less than that for cows with PUN less than the mean (18 percentage point difference, $P < .02$, Figure 1). The relationship between PUN and pregnancy rate was discriminated further with likelihood ratio tests based on increments of 3 mg/dL of PUN (Table 2). As PUN increased to greater than 19 mg/dL, the likelihood ratio for pregnancy markedly decreased. The likelihood ratio seemed to increase again for the greatest PUN category, but there were few observations.

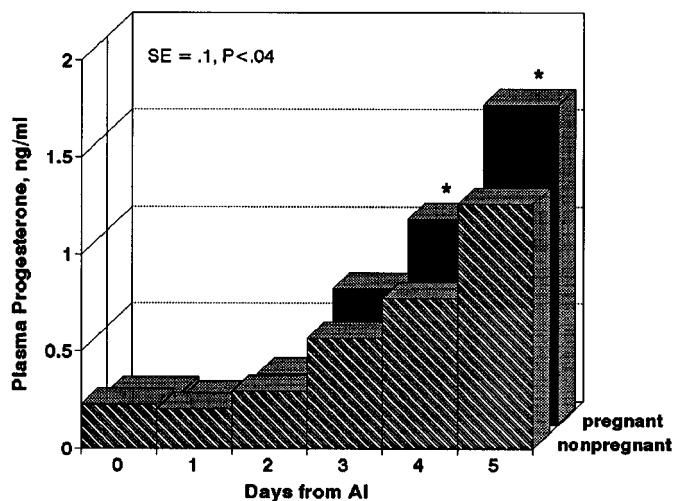For the subset of cows sampled daily (n = 51), plasma progesterone concentrations increased during the 5-d period after estrus and AI. Neither the profile of daily progesterone concentrations nor the rate of increase differed ($P = .94$) between cows with PUN concentrations greater than or less than the mean (data not shown). When cows were compared by subsequent pregnancy status, plasma progesterone concentrations were greater in pregnant than in nonpregnant cows on d 4 and 5 ($P < .04$, Figure 2). For the subset of cows, the PUN concentrations on the day of AI were $18.7 \pm .6$ and $20.7 \pm .6$ mg/dL ($P < .02$) for cows subsequently diagnosed as pregnant and nonpregnant, respectively.

Experiment 2. The PUN and MUN concentrations measured in the same cows were not different ($P = .16$) and were significantly correlated ($20.9 \pm .7$ and $22.1 \pm .6$ mg/dL, respectively, n = 22; r = .82, $P < .001$). The equation describing the relationship between MUN (y) and PUN (x) was: y = .76(x) + 6.3; $R^2 = .69$. The results from the Sigma and Auto methods of MUN analysis on defatted samples were not different (Sigma = $17.2 \pm .9$ vs Auto = $16.9 \pm .8$ mg/dL; $P = .36$, r = .93; n = 22). The Azotest consistently overestimated the Auto values for samples within each rank ($P < .05$, Table 3), except for those in rank 6, which were underestimated. The CV for the Azotest, within each rank, were less than 15%, demonstrating acceptable repeatability of the Azotest within a rank. The MUN concentrations from the Auto method were not affected by preservative (unpreserved = $17.9 \pm .7$ vs preserved = $17.9 \pm .7$ mg/dL; $P = .48$; r = .99, n = 22). Preservative also had no significant effect on results with the other methods. Comparison of strip samples from before and after milking with composite milk samples showed no significant variation across milk fractions within the mammary gland; the average CV

Table 5. Pregnancy rate (PR) likelihood ratios for cows categorized by
milk urea nitrogen (MUN) concentration on the day of AI

| MUN category, mg/dL | Cows, n | PR, % | Percentage[a] | | Likelihood ratio[b] |
			Not pregnant	Pregnant	
< 16	16	75	5.5	14.6	2.65
16–18.9	28	64	13.7	22.0	1.61
19–21.9	46	48	32.9	26.8	.81
22–24.9	36	47	20.7	26.0	.80
≥ 25	29	45	15.9	21.9	.73

[a]Percentage of total cows within each MUN category.
[2]Percentage of cows pregnant divided by the percentage of cows not pregnant.

was 1.6% (Table 4, Auto method). The average CV among MUN concentrations for cows (n = 10) in the same stage of lactation fed the same diet was 8% (Table 4).

Experiment 3. The mean MUN concentrations on the day of AI averaged 22.3 ± .4 mg/dL (n = 155). For cows later determined to be pregnant (n = 82) and nonpregnant (n = 73), MUN concentrations were different (21.3 ± .4 and 22.8 ± .5 mg/dL, respectively; $P < .02$). The overall relationship between MUN and pregnancy rate was discriminated using likelihood ratio tests based on increments of 3 mg/dL of MUN (Table 5). As MUN increased to greater than 19 mg/dL, the likelihood ratio for pregnancy decreased markedly and similarly to the results for Exp. 1. The pregnancy rate of cows with MUN concentrations greater than 19 mg/dL was lower (21 percentage point difference, $P < .02$) than in cows with less MUN (Figure 3).

In a subset group of 23 cows, MUN concentrations within cow across 10 sequential regular milkings were relatively consistent (mean CV = 8.3%, individual cow data not shown). However, when this subset group was divided by subsequent pregnancy status, mean MUN values over time were greater in nonpregnant cows (24.9 ± .9 vs 21.1 ± .9 mg/dL; $P < .01$) and the interaction between pregnancy status and MUN over time was significant ($P < .05$). From regression analysis (Figure 4), MUN concentrations were lower at AI and decreased ($P < .01$), thereafter, in cows that were palpated pregnant. In contrast, MUN concentrations were consistently greater in nonpregnant cows (slope of regression line was not significantly different from zero).

Discussion

High dietary protein resulting in high concentrations of urea nitrogen in plasma and milk have been associated with decreased fertility in dairy cattle (Jordan et al., 1983; Kaim et al., 1983; Ropstad and Refsdal, 1987; Canfield et al., 1990; Elrod and Butler, 1993). In the present study, PUN concentrations greater than 19 mg/dL were associated with decreased pregnancy rate (18 percentage point reduction).

Table 3. Comparison of the Auto and Azotest methods for measurement of milk urea nitrogen (MUN, mg/dL). Milk samples (n = 44) with MUN values determined with the Auto method were classified into expected ranks for the Azotest comparison

| Method | Expected rank[a] | | | |
	3	4	5	6
Auto[b]	14.7	16.5	19.3	24
Azotest[c]	16.5	18.2	22.6	22.6
n	5	10	15	14
CV, %[d]	0	14	8	7

[a]Expected rank for Azotest based on Auto MUN values.
[b]Mean MUN for samples classified within an expected rank.
[c]MUN values calculated from Azotest rank described in Table 1.
[d]CV = coefficient of variation among the Azotest results within each rank.

Table 4. Mean (mg/dL) and coefficient of variation (CV) for milk urea nitrogen (MUN) among samples collected during milking. Composite, fore-strip, and post-strip milk fractions are represented by bulk MUN, first MUN, and last MUN, respectively. Cows (n = 10) were fed the same diet and were at the same stage of lactation

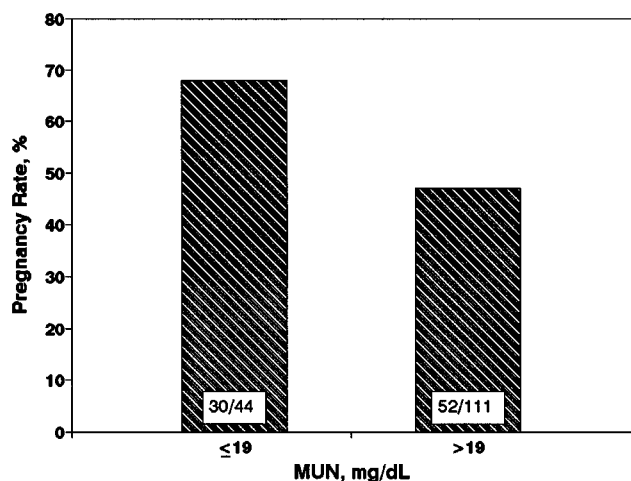| Cow #	Bulk MUN	First MUN	Last MUN	CV, %
5237	24	24.5	24	1.0
5209	21	25	21	1.0
5214	21	20	20	2.3
5068	24.5	24.5	23.5	1.9
5278	19	19	19	0
4931	21	20	25	2.0
5156	24	23.5	23	1.7
4602	23	22	22	2.1
5250	24	23	23.5	1.7
4799	21	21	22	2.2
Mean	22.3	21.8	21.9	1.6
CV, %	8.0	8.6	7.3	—

Figure 3. The relationship of MUN to pregnancy rate for first AI in lactating dairy cows (n = 155). Pregnancy rate was reduced ($P < .02$) in cows with MUN > 19 mg/dL. The number of cows becoming pregnant to AI is indicated within each MUN category.
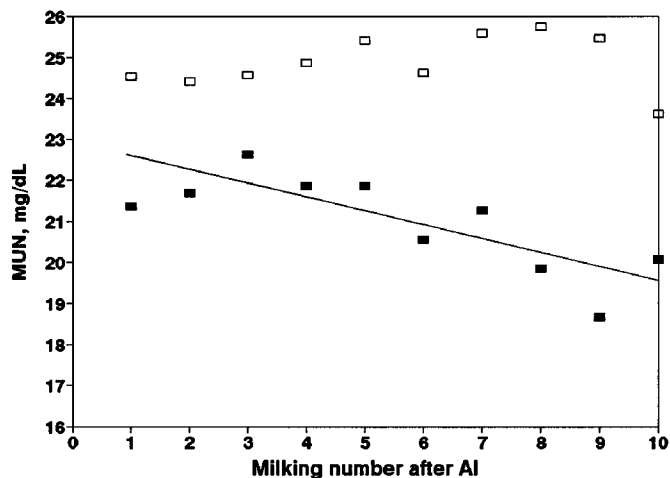

Figure 4. Mean MUN concentrations for 10 consecutive milkings starting from the day of AI. From regression analysis, the equation for the regression line in pregnant cows (■; n = 11) was $Y = -.30X + 22.6$ ($P < .01$, $R^2 = .59$, SE = .6); for nonpregnant cows (□; n = 12) the slope was not significantly different from zero.

Because these cows were fed typical diets for lactating cows, the measured urea concentrations should reflect normal values in the population. Previously, Ferguson et al. (1988, 1993) reported a similar range of urea concentrations in cows and also reported that serum urea nitrogen greater than 20 mg/dL resulted in decreased conception rates. The magnitude of the associated decrease in pregnancy rates with high blood urea nitrogen seems to be related to the underlying reproductive performance among herds (Ferguson et al., 1993). Carroll et al. (1988) found no detrimental effect of high dietary protein and proposed that the disagreement among studies in associating decreased fertility with the amount of dietary protein could be attributed to differences in reproductive management, rather than to protein intake or to urea production.

Because urea equilibrates within bodily fluids, MUN should be similar to PUN as an indicator of urea nitrogen status in dairy cows and is more conveniently monitored (Oltner and Wiktorsson, 1983; Oltner et al., 1985; Ropstad et al., 1989; Roseler et al., 1993). The association of increased concentrations of MUN with decreased pregnancy rate was consistent with comparison between PUN and conception rate. In Exp. 3, the pregnancy rate was 21 percentage points less among cows with MUN greater than 19 mg/dL as compared with the 18 percentage point reduction in Exp. 1. The differences in mean urea nitrogen concentrations (19 vs 22 mg/dL, respectively) in these experiments most likely reflect differences in protein quality and solubility in the forages being fed.

In the present study, PUN and MUN were significantly correlated. The MUN concentration was some-what greater than PUN when milk samples were collected 4 to 5 h after feeding and blood was collected after milking. Gustafsson and Palmquist (1993) and Elrod and Butler (1993) reported that PUN concentrations fluctuate throughout the day. Generally, the minimum PUN concentration is before feeding and the maximum is approximately 4 to 6 h after feeding. Because there is a lag of approximately 1 to 2 h between PUN and MUN peaks (Gustafsson and Palmquist, 1993), sampling time relative to feeding can be important in the interpretation of PUN and MUN measurements, especially when cows are fed dietary forages and concentrates separately rather than a total mixed ration. In the present study, in which MUN was somewhat greater than PUN, rather than equal, the difference probably was due to the differential timing of milk and blood collection after feeding. In a more recent study (Frajblat and Butler, unpublished data), milk and blood were collected simultaneously, and MUN was not statistically different from PUN.

Because urea equilibrates across the mammary epithelium (Gustafsson and Palmquist, 1993), we found little variation in MUN concentrations in different milk fractions collected during milking, as also noted by Carlsson and Bergstrom (1994). Thus, it should make little difference whether a milk sample intended for MUN analysis comes from a composite milk sample or from a quarter strip sample, before or after milking (Gustafsson and Palmquist, 1993; Carlsson and Bergstrom, 1994). In addition to the small variation among milk fractions, the variation in MUN among cows fed the same diet or across sequential milkings was also low (CV = 8% in this study). It is

important to note that this low variation most certainly reflects the feeding of total mixed rations, which results in rather small changes in blood urea during the day (Elrod et al., 1993). In turn, low variation indicates that MUN analysis from several cows may be representative of a group (i.e., receiving the same diet and at a similar stage of lactation). Urea concentrations in bulk tank milk have been used to predict protein supply (Refsdal et al., 1985; Ropstad et al., 1989) and fertility differences among herds (Ropstad and Refsdal, 1987).

We found no significant difference between the Auto and Sigma laboratory methods for MUN analysis of defatted milk. Choosing to use the Sigma or Auto method is a matter of convenience and availability of equipment. The presence of preservative had no effect on MUN analysis. That is expected, because the preservative does not contain chemicals that should interfere with these analyses. Being able to use preservative is beneficial in situations, such as field trials, when there may be a long period between the time of milk sampling and MUN analysis. Field trials or on-farm MUN analysis would be benefited by an easy and reliable test system. Although there was a positive association between the Azotest and Auto methods (r = .6), the two methods yielded significantly different results. The Azotest consistently overestimated the Auto values by approximately 2 mg/dL, except at the highest rank the Azotest value underestimated the Auto mean. This is because 23.5 mg/dL is the upper limit designed for the Azotest and any samples with greater concentrations will be underestimated by this method. Although the Azotest tended to overestimate the Auto method, the variation within each rank of Azotest values was within acceptable limits. This indicates that the overestimation of MUN is consistent and that the Azotest has use as a semiquantitative measure of MUN.

Finally, the interaction of progesterone and urea nitrogen over time after AI deserves attention. Plasma progesterone concentrations during mid-diestrus were reported to be approximately 30 percentage points lower in cows with high PUN due to feeding high-protein diets (Jordan and Swanson, 1979b; Sonderman and Larson, 1989). In the present study, plasma progesterone concentrations did not differ during the 5-d period following AI (early diestrus) when cows were categorized into high and low PUN groups, but the differences in PUN concentrations between these groups were not as large as in the previous reports. However, when categorized by subsequent pregnancy status, the rate of increase in plasma progesterone concentrations during the several days following AI was significantly greater by d 4 in pregnant than in nonpregnant cows. This early increase in progesterone in pregnant cows confirms several previous reports (Randel et al., 1971; Erb et al., 1976; Lee et al., 1985). Shelton et al. (1990) reported that the

increase in postovulatory peripheral progesterone concentrations was delayed and occurred more slowly in subfertile cows than in heifers. Those authors suggested that luteal inadequacy, due to diminished responsiveness to luteotropic hormones, may contribute to embryo mortality in subfertile cows. In contrast, the presence of a viable embryo signalled an earlier increase in progesterone (by d 3) that was not seen in cases of embryo failure (Maurer and Echternkamp, 1982) and may indicate a luteotropic effect of an early embryo. Thus, the differential pattern of progesterone increase between pregnant and nonpregnant cows in the present study could reflect either differences in luteal function or luteotropic stimulation by the embryo or the combined effects. Regardless, it seems that greater progesterone availability 3 to 4 d after ovulation is conducive to increased survival of the early embryo and results in greater pregnancy rate.

Early embryonic development requires appropriate oviductal and uterine environments. Jordan et al. (1983) reported alterations in uterine secretions in high-producing cows fed diets high in crude protein and resulting in high PUN. When heifers were fed diets with high rumen degradable protein, PUN concentrations increased, uterine pH decreased, and pregnancy rate decreased (Elrod and Butler, 1993). When cows were fed either excess rumen degradable or undegradable protein, PUN was increased and uterine pH decreased (Elrod et al., 1993). Uterine pH was significantly affected when PUN exceeded 19 mg/dL. In sheep fed excess rumen degradable protein, PUN concentrations greater than 18 mg/dL were detrimental to the early development and survival of embryos (Bishonga et al., 1994). Excess rumen degradable protein has been reported to be deleterious to embryonic development in lactating cows (Blanchard et al.,1990), but not in nonlactating cows (Garcia-Bojalil et al., 1994). Although we found little variation within cow in MUN across time after AI, there was an interaction between pregnancy outcome to AI and MUN. Nonpregnant cows maintained greater MUN concentrations over time, whereas MUN values were lower at estrus and tended to decrease over the next 5 d in cows that were later palpated as being pregnant. The significance of the possible interaction between urea nitrogen and progesterone concentrations during the early days of embryonic development cannot be determined from results of the present study, but this deserves further research toward improving fertility in lactating cows.

Implications

Urea nitrogen concentrations greater than 19 mg/dL in plasma and milk were associated with decreased pregnancy rate in dairy cattle. Therefore, it may be beneficial to dairy producers to monitor urea concen-

Table 2. Pregnancy rate (PR) likelihood ratios for cows categorized by plasma urea nitrogen (PUN) concentration on the day of AI

| PUN category, mg/dL | Cows, n | PR, % | Percentage[a] | | Likelihood ratio[b] |
			Not pregnant	Pregnant	
< 16	51	49	29.5	34.7	1.18
16–18.9	44	57	21.6	34.7	1.61
19–21.9	40	32	30.7	18.1	.59
22–24.9	17	29	13.6	6.9	.51
≥ 25	8	50	4.5	5.6	1.24

[a]Percentage of total cows within each PUN category.
[b]Percentage of cows pregnant divided by the percentage of cows not pregnant.

trations in their herds in efforts to maintain or improve reproductive efficiency. If chemical tests are not available, MUN concentrations can be estimated quickly and easily with the Azotest. The Azotest results can indicate whether more precise, chemical procedures should be performed. Maintaining and monitoring MUN in a dairy herd provides an opportunity to formulate the dietary protein constituency that optimizes nitrogen utilization for milk production and avoids possible negative effects on herd fertility.

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