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Metabolic indicators for early pregnancy in zebu and crossbred dairy cows reared in a subtropical climate

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Abstract: To identify metabolic indicators in blood and urine for early pregnancy in dairy cows (32 crossbred and 12 purebred zebu cows), blood and urine samples were collected on days 0, 15, 20, 24, and 30 postbreeding. The plasma progesterone (P4) concentration was significantly ($P < 0.05$) higher in pregnant cows than in nonpregnant cows after day 15. The plasma blood urea nitrogen (BUN) concentration remained significantly ($P < 0.05$) higher in pregnant cows on day 15. The plasma concentrations of cholesterol, glucose, protein, and nonesterified fatty acid (NEFA) did not differ between pregnant and nonpregnant cows. The urine protein concentration was significantly higher ($P < 0.05$) in pregnant zebu cows compared to nonpregnant zebu cows. Plasma P4 was significantly ($P < 0.001$) and positively associated with urine glucose levels in nonpregnant crossbred cows. In nonpregnant zebu cows, the correlation between P4 was positive ($P < 0.05$) with plasma cholesterol, glucose, urine glucose, and protein. In pregnant zebu cows, P4 was negatively associated with plasma BUN and positively ($P < 0.05$) associated with both plasma and urine glucose levels. Thus, urinary proteins can be used as a noninvasive tool for early nonpregnancy confirmation in cows with very high accuracy at around 24 days after artificial insemination.

Key words: Cow, metabolic indicators, pregnancy, urine

1. Introduction

Early identification of nonpregnant cows with maximum accuracy is an important factor for optimizing the reproductive performance of dairy cattle. In order to attain economical dairy farming, cows must calve every year. In order to maintain this sequence, identifying nonpregnant animals at an early date and preparing them for subsequent artificial insemination (AI) at the proper time is important (1). Proper reproduction management increases the economic value of dairy cows (2). Relying only on the history of the cows not coming into estrus again after insemination often not only produces misleading results but also affects the farm's economy. It also results in time wasted on the maintenance of nonpregnant cows. Progesterone (P4) and glucose help in maintaining the optimum growth of the embryo or fetus and prevent pregnancy loss (3,4). In addition, the negative energy balance during the breeding period alters the proliferation and differentiation of uterine epithelial cells and subsequently leads to pregnancy failure (4). Proteins, the building blocks of living organisms, help in the growth of the fetus, and this is why pregnancy significantly influences the proteinogram of serum proteins in cattle (5). The common metabolites that are positive indicators of a body energy balance are plasma glucose, cholesterol, and urea; however, nonesterified fatty acids (NEFAs) are negative indicators of energy balance (6). A recent study revealed the presence of several proteins in cow urine, and this could be helpful for early pregnancy diagnosis (7). Biomolecules associated with pregnancy can be used for the diagnosis of factors affecting pregnancy. Thus, the relative presence of biomolecules such as P4, blood urea nitrogen (BUN), NEFAs, cholesterol, protein, and glucose is important in establishing and maintaining pregnancy in dairy cows (5,8,9). A better knowledge of these factors expressed during early or late pregnancy will make it possible to define new protocols for the study of the relationship between mother and fetus during gestation for overall economic benefit (10). Keeping in mind these biochemical indicators, the objective of the experiment was formulated to identify metabolic indicators in blood and urine as an early pregnancy marker in dairy cattle.

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2. Materials and methods

2.1. Experimental animals

The study was conducted on zebu (Sahiwal) and crossbred (Holstein Friesian × Tharparkar) cows kept at the ICAR-National Dairy Research Institute (NDRI) in Karnal, India, over 3 months (February–April). About 49 healthy cows were selected based on parity (1st–4th), body condition (3.5–4.5 on a 6-point scale), body weight (400–450 kg for the zebus and 450–550 kg for crossbred cows), and milk yield (8–12 kg for the zebus and 15–20 kg for the crossbred cows). After a voluntary period of 50 days postparturition, AI was carried out on the farm by a veterinary expert after the animals were detected for heat with a scheduled 2-time heat detection, confirmation of a heat and health status by rectal palpation of the reproductive organ, and analysis of cervical mucus with the blue sheath method. Of 49 cows, 4 came into heat before the 24 days of AI and, in one cow, the P4 concentration was observed to be ≥3 ng/mL, which confirmed a cystic condition using ultrasonography. These 4 animals were eliminated from the study. The final study comprised 44 cows, 32 of which were crossbred and 12 were zebu cows. Transrectal ultrasonography was done by rectal linear probe at 6.5 MHz (Aloka UST-5820-5, Aloka, China) on the 30th and 45thday postbreeding for a pregnancy check. The cows were kept in a loose housing system to facilitate their free movement and sufficient exercise. Feeding was done as per the NRC standard (11).

2.2. Blood and urine analysis

Blood and urine samples were collected early in the morning on days 0, 15, 20, 24, and 30 after AI blood samples were collected from the jugular vein using a sterile needle attached with 9-mL vacutainer tubes with heparin as the anticoagulant. The tubes were centrifuged at 3000 rpm at 4 °C for 20 min to separate the plasma. Plasma samples were stored at –20 °C in cryovials until analysis. Urine was collected by perinial hand massage in 50-mL sterilized test tubes and stored at –20 °C until analysis. Plasma P4 estimation was done using a Bovine Progesterone ELISA test kit (Endocrine Technologies Inc., Newark, CA, USA). Plasma glucose, total protein, and BUN concentrations were determined using kits (Span Diagnostic Ltd., Gujarat, India) as per the instructions given by the manufacturer. A Non-Esterified Fatty Acids Detection 500 Point kit (Zen-Bio, Inc., Research Triangle Park, NC, USA) was used to assess plasma NEFA concentrations. Urine total protein and glucose were determined using kits (Span Diagnostic Ltd., Gujarat, India). Experimental procedures were approved by the Institutional Animal Ethics Committee.

2.3. Statistical analysis

Statistical analysis was done using a SigmaPlot 11 software package (Systat Software Inc., San Jose, CA, USA). Data sets were analyzed with a general linear model to see the effects of the group type (pregnant and nonpregnant), the period (the day's effect), and their interaction. Pair-wise differences in means were compared by Tukey post hoc test, and the differences in mean were considered as significant if $P \leq 0.05$. The sensitivity and specificity of the progesterone test were calculated as per Broaddus and de Vries (12). The correlation of plasma/urine metabolites with plasma P4 was also analyzed to identify the metabolites for early pregnancy diagnosis.

3. Results

In the present study, of the 44 experimental cows, 7 crossbred and 5 zebu cows were confirmed as pregnant on day 30 postinsemination using ultrasonography. Upon reexamination on day 45, ultrasonography indicated one pregnancy loss indicating embryonic mortality.

3.1. Plasma progesterone

The P4 concentration was similar between pregnant and nonpregnant cows up to day 15 but differed significantly after this period ($P < 0.05$) (Figure). The plasma P4 concentration in pregnant crossbred (KF) and zebu (SW) cows reached a maximum level on day 24 andday 30, respectively, after AI. However, in the case of the nonpregnant group, peak concentration was achieved on day 15 post-AI and remained below 1.5 ng/mL throughout the experiment. The sensitivity and specificity of the progesterone test on day 24 was 92.31% and 100%, respectively (Figure).

3.2. Plasma BUN

The plasma BUN concentration remained at a significantly ($P < 0.05$) higher level during day 0 to day 20 post-AI in pregnant crossbred cows than in the nonpregnant group, and maximum concentration was observed on day 20 post-AI (38.34 ± 2.70 mg/dL). In the cases of pregnant zebu cattle, BUN concentration remained significantly ($P < 0.05$) higher on day 0 andday 15 post-AI than in the nonpregnant group (Table 1).

3.3. Plasma cholesterol

In crossbred pregnant cows, plasma cholesterol concentrations remained higher during day 15 to day 24 post-AI; however, the difference was not significant in pregnant zebu cows (Table 1). A maximum concentration of plasma cholesterol was observed on day 15 andday 24 post-AI in zebu cattle and crossbred cows, respectively.

3.4. Plasma glucose

Glucose concentrations remained slightly higher in the pregnant group than in the nonpregnant group from day 15 to day 24 in crossbred cows, with peak concentration on day 24, which was significantly higher than in the nonpregnant cow group (Table 1).

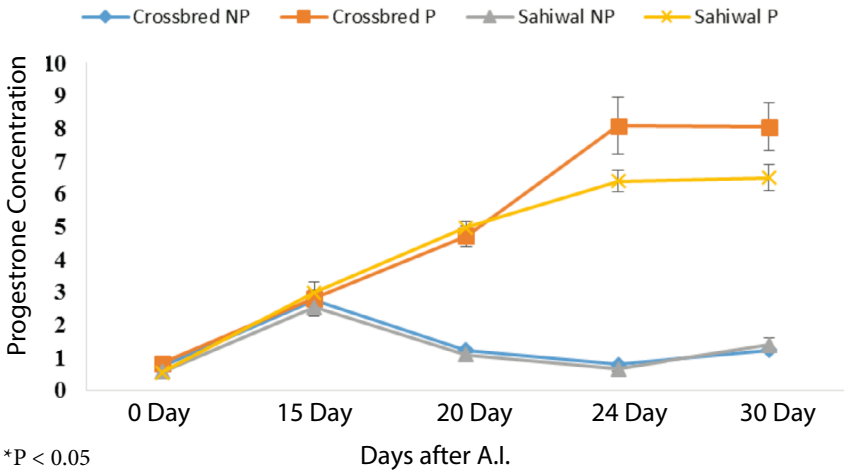

Figure. Blood progesterone concentration (ng/mL) during different periods in crossbred and Sahiwal cows; NP: nonpregnant; P: pregnant.

Table 1. Least square (mean ± SE) of certain blood metabolites in nonpregnant and pregnant crossbred (KF) and zebu (SW) cows during different time intervals after AI.

Parameter	Period	Crossbred		Zebu	
		NP (N = 25)	P (N = 7)	NP (N = 7)	P (N = 5)
BUN (mg/dL)	day 0	$28.18^{aA} \pm 1.8$	$35.53^{bA} \pm 2.2$	$26.68^{aA} \pm 1.89$	$39.32^{bA} \pm 3.20$
	day 15	$29.24^{aA} \pm 1.9$	$37.45^{bA} \pm 1.4$	$28.46^{aA} \pm 1.83$	$35.97^{bAB} \pm 4.91$
	day 20	$29.66^{aA} \pm 1.4$	$38.34^{bA} \pm 2.7$	$28.15^{aA} \pm 1.0$	$26.06^{aB} \pm 1.80$
	day 24	$31.12^{aA} \pm 0.0$	$33.46^{aA} \pm 2.8$	$30.05^{aA} \pm 2.59$	$28.40^{aAB} \pm 4.33$
	day 30	$29.60^{aA} \pm 1.4$	$30.65^{aA} \pm 2.6$	$27.42^{aA} \pm 1.49$	$30.67^{aAB} \pm 2.08$
Cholesterol (mg/dL)	day 0	$205.03^{aA} \pm 5.7$	$200.16^{aA} \pm 5.67$	$208.77^{aA} \pm 4.79$	$207.01^{aA} \pm 6.81$
	day 15	$204.27^{aA} \pm 4.46$	$209.13^{aA} \pm 5.47$	$211.40^{aA} \pm 6.17$	$231.15^{aA} \pm 8.06$
	day 20	$204.72^{aA} \pm 3.74$	$214.94^{aA} \pm 8.12$	$205.23^{aA} \pm 4.53$	$215.06^{aA} \pm 7.96$
	day 24	$208.39^{aA} \pm 5.99$	$218.56^{aA} \pm 7.02$	$205.10^{aA} \pm 5.01$	$206.34^{aA} \pm 4.80$
	day 30	$208.46_{aA} \pm 4.18$	$206.46^{aA} \pm 4.23$	$201.11^{aA} \pm 3.80$	$211.41^{aA} \pm 5.94$
Glucose (mg/dL)	day 0	$49.66^{aA} \pm 1.56$	$48.81^{aA} \pm 3.09$	$51.76^{aA} \pm 3.54$	$52.55^{aA} \pm 2.61$
	day 15	$49.71^{aA} \pm 1.48$	$54.67^{aAB} \pm 2.3$	$50.78^{aA} \pm 1.74$	$48.23^{aA} \pm 5.91$
	day 20	$54.20^{aAB} \pm 1.66$	$54.92^{aAB} \pm 2.39$	$56.02^{aA} \pm 3.58$	$60.97^{aA} \pm 2.58$
	day 24	$56.52^{aB} \pm 1.39$	$63.95^{bB} \pm 4.07$	$54.62^{aA} \pm 4.87$	$59.47^{aA} \pm 6.22$
	day 30	$54.80^{aAB} \pm 1.85$	$53.67^{aAB} \pm 3.03$	$57.02^{aA} \pm 4.27$	$57.99^{aA} \pm 1.37$
Protein (g/dL)	day 0	$6.48^{aA} \pm 0.12$	$6.12^{aA} \pm 0.26$	$6.61^{aA} \pm 0.23$	$6.42^{aA} \pm 0.21$
	day 15	$6.30^{aA} \pm 0.09$	$6.41^{aA} \pm 0.30$	$6.26^{aA} \pm 0.09$	$6.75^{aA} \pm 0.09$
	day 20	$6.26^{aA} \pm 0.10$	$6.35^{aA} \pm 0.25$	$5.65^{aA} \pm 0.25$	$6.27^{aA} \pm 0.60$
	day 24	$6.16^{aA} \pm 0.11$	$6.45^{aA} \pm 0.21$	$5.86^{aA} \pm 0.14$	$6.59^{aA} \pm 0.06$
	day 30	$6.39^{aA} \pm 0.07$	$6.59^{aA} \pm 0.21$	$6.22^{aA} \pm 0.18$	$6.95^{aA} \pm 0.32$
NEFA (µmol/L)	day 0	$373.75^{aA} \pm 6.5$	$375.65^{aA} \pm 5.46$	$369.35^{aA} \pm 7.38$	$373.28^{aA} \pm 4.49$
	day 15	$369.72^{aA} \pm 6.65$	$366.18^{aA} \pm 5.96$	$373.21^{aA} \pm 14.9$	$371.33^{aA} \pm 4.19$
	day 20	$361.77^{aA} \pm 5.98$	$361.49^{aA} \pm 4.99$	$378.39^{aA} \pm 5.59$	$369.62^{aA} \pm 4.00$
	day 24	$367.20^{aA} \pm 4.44$	$358.07^{aA} \pm 4.64$	$373.81^{aA} \pm 4.19$	$367.55^{aA} \pm 2.76$
	day 30	$374.83^{aA} \pm 5.16$	$356.02^{aA} \pm 4.31$	$376.78^{aA} \pm 6.07$	$365.07^{aA} \pm 2.98$

Means with different superscripts in the same row (a, b) and in the same column (A, B, C, D, and E) differ significantly ($P < 0.05$).

3.5. Plasma protein

The plasma protein concentration did not show any significant difference between the pregnant and nonpregnant groups. However, there were higher levels of protein concentration in the pregnant group after day 15 than in the nonpregnant group. The mean peak plasma protein concentration of pregnant crossbred and zebu cows on day 30 was 6.59 and 6.95 g/dL, respectively (Table 1).

3.6. Plasma NEFA

Although the plasma NEFA concentration gradually decreased in pregnant crossbred and zebu cows, there was no significant difference. The NEFA concentrations of pregnant cows were found to be slightly lower than in nonpregnant cows from day 15 onwards (Table 1).

3.7. Urine glucose

The pregnant crossbred and zebu cows showed an increase in urine glucose concentration after day 15, reaching a peak concentration on day 24, which is significantly higher ($P < 0.05$) than in nonpregnant cows. However, after day 24, the concentration decreased (Table 2).

3.8. Urine protein

Pregnant crossbred cows showed an increasing trend of urine protein concentration from day 15 to day 24 with significantly ($P < 0.05$) higher concentrations on day 24. The pregnant crossbred and zebu cows had significantly ($P < 0.05$) higher peak concentrations of urine protein on day 24 and on day 30, respectively, than nonpregnant cows (Table 2).

3.9. Correlation of plasma P4 with plasma and urine metabolites

The correlation of P4 with different plasma and urine metabolites is depicted in Table 3. In nonpregnant crossbred cows, the plasma P4 was significantly and positively associated with urine glucose ($P < 0.001$); however, in pregnant crossbred cows, no such association was observed. In nonpregnant zebu cows, plasma P4 was associated positively with plasma cholesterol, glucose, urine glucose, and protein ($P < 0.05$). On the other hand, plasma P4 was negatively associated with plasma BUN but positively associated with both plasma and urine glucose levels ($P < 0.05$) in pregnant zebu cows.

4. Discussion

The results regarding progesterone concentrations are in line with those presented in previous studies (13,14) that observed higher progesterone concentrations in pregnant than in nonpregnant cows by day 20 post-AI. P4 in pregnant and nonpregnant cows was reported as 2.3–4.0 ng/mL and 0.1–2.2 ng/mL (8). Lucy and Poock (15) recorded data on nonpregnant cows with progesterone concentrations on day 21 of <1 ng/mL, which is similar to our results. Waldman (16) found a specificity of progesterone tests conducted between 18 and 24 days postbreeding of about 98%, which is slightly higher than our results. Similarly, Otava et al. (17) and Muhamad et al. (8) both reported 100% sensitivity for nonpregnancy diagnosis. The P4 hormone is essential for implantation of the embryo, optimum growth, and maintenance of pregnancy (18).

Table 2. Least square (mean ± SE) of certain metabolites of urine glucose and urine protein in nonpregnant and pregnant crossbred (KF) and zebu (SW) cows during different time intervals after AI.

Parameter	Period	Crossbred		Zebu	
		NP (N = 25)	P (N = 7)	NP (N = 7)	P (N = 5)
Glucose (mg/dL)	day 0	$0.49^{aA} \pm 0.05$	$0.53^{aAD} \pm 0.08$	$0.60^{aA} \pm 0.10$	$0.60^{aA} \pm 0.24$
	day 15	$0.91^{aB} \pm 0.04$	$0.71^{aAC} \pm 0.10$	$0.95^{aA} \pm 0.22$	$0.67^{aA} \pm 0.17$
	day 20	$0.66^{aAC} \pm 0.06$	$0.94^{bBC} \pm 0.11$	$0.66^{aA} \pm 0.12$	$0.87^{aA} \pm 0.12$
	day 24	$0.56^{aAC} \pm 0.06$	$1.13^{bB} \pm 0.11$	$0.57^{aA} \pm 0.13$	$1.25^{aA} \pm 0.09$
	day 30	$0.71^{aBC} \pm 0.06$	$0.93^{aBD} \pm 0.05$	$0.83^{aA} \pm 0.07$	$1.01^{aA} \pm 0.13$
Protein (g/dL)	day 0	$2.31^{aA} \pm 0.31$	$2.01^{aA} \pm 0.41$	$1.62^{aA} \pm 0.32$	$3.44^{bA} \pm 0.31$
	day 15	$2.50^{aA} \pm 0.27$	$2.68^{aAB} \pm 0.40$	$2.14^{aA} \pm 0.17$	$3.14^{aA} \pm 0.54$
	day 20	$2.14^{aA} \pm 0.33$	$3.09^{aAB} \pm 0.48$	$1.69^{aA} \pm 0.47$	$2.38^{aA} \pm 0.86$
	day 24	$2.44^{aA} \pm 0.26$	$4.10^{bB} \pm 0.18$	$2.25^{aA} \pm 0.28$	$3.98^{bA} \pm 0.28$
	day 30	$2.60^{aA} \pm 0.20$	$3.19^{aAB} \pm 0.27$	$2.81^{aA} \pm 0.70$	$4.14^{aA} \pm 0.32$

Means with different superscripts in the same row (a, b) and in the same column (A, B, C, D, and E) differ significantly ($P < 0.05$).

Table 3. Correlation of plasma progesterone (P4) with different plasma and urine metabolites.

Pregnancy status	Parameters							
	Plasma P4	Plasma BUN	Plasma cholesterol	Plasma glucose	Plasma protein	Plasma NEFA	Urine glucose	Urine protein
Crossbred-NP	1	0.05	–0.06	–0.15	–0.03	0.04	0.35***	0.08
Crossbred-P	1	0.05	0.06	–0.02	0.09	–0.15	0.21	0.18
Zebu-NP	1	–0.19	0.37*	0.44**	0.31	–0.24	0.51**	0.57***
Zebu-P	1	–0.53**	–0.09	0.55**	0.21	0.33	0.69***	0.21

*P <0.05 **, P < 0.01 ***, P < 0.001 NP: nonpregnant; P: pregnant

Alameen and Abdelatif (9) found that during early pregnancy the blood urea level was 32.13 mg/dL, which is lower than our value, but the overall trend during the early pregnancy period was similar. Higher protein intake may lead to an elevated plasma urea nitrogen concentration (19) since during the experimental period the animals were fed with high protein legumes, which might have contributed to high BUN. BUN concentration is an indicator of an energy protein balance (20) in dairy animals. The high BUN in animals might be due to physiological changes during early pregnancy. Under the influence of various hormonal changes during this stage, the blood urea level might have risen due to increased protein degradation.

Previous studies have not reported any significant increases in serum cholesterol levels during early pregnancy, and the same result was observed in this study (21). Alameen and Abdelatif (9) observed a lower cholesterol concentration of 128.75 mg/dL in cows, and this is lower than our result during early pregnancy. This indicates that animals in the experiment were in a positive energy balance. In certain cases when the animal suffers from water deprivation and thirst or has diseases such as diarrhea, urinary diseases, pregnancy toxemia, and acidosis, the BUN level might increase (22). However, these conditions were not present in cows used in this study.

The results are similar in terms of glucose concentration during early pregnancy as reported by Mir et al. (23) and Alameen and Abdelatif (9). Higher values of glucose might be due to more demand of energy for embryonic development during early pregnancy. Westwood et al. (24) reported that the pregnancy rate was higher in cows with high plasma glucose concentrations. Since glucose is the main source of energy for the ovary, its deficiency may impair ovarian function and result in a poor quality of follicles and oocytes (25). Furthermore, like P4, glucose also helps in the optimum growth of the embryo or fetus and decreases the chance of early pregnancy losses (3,4). The nonsignificant increase of glucose in our experiment might be due to the homeostatic control of glucose concentrations in dairy cows as the nutrition partitioning towards milk production is higher. During early pregnancy, the demand of the fetus is also low, resulting in less glucose use (26).

Our results are similar to those presented by Padodara et al. (21), who found that serum total protein levels increased nonsignificantly from early pregnancy up to advanced pregnancy. Similarly, Mir et al. (23) observed a concentration of protein of 6.92 g/dL at 3 months pregnancy in crossbred cows, which was similar to our results. Different from our results, Bhoraniya et al. (13) found a lower total plasma protein concentration of 5.93 g/dL, and no significant difference was observed between sampling days. The higher levels of protein concentration in our study might be due to an increased demand for proteins as pregnancy significantly influences the proteinogram of serum proteins in cattle (5). Proteins are needed for growth, development of the embryo, reproduction, and various metabolic activities in living organisms. Changes in plasma protein concentrations might be due to hormonal changes in the body. During the experimental period, the animals were on high-protein legume supplementation, which might have affected their conception rate. The pregnancy rate in cows decreases because of a high protein diet (27,28). Feeding the animals meals high in dietary protein increases urea levels, which might reduce fertility by interfering with the normal inductive effects of progesterone on the uterine microenvironment, thereby providing nonfeasible conditions for embryonic development (29).

Decreased plasma NEFA concentrations during early pregnancy have also been found in goats (26), which is in agreement with our results. NEFA concentrations are assumed to be the best indicators of energy balance in cows (30) as there is a direct physiological relationship between NEFA concentrations and negative energy balance (31). The concentration of NEFA in the blood also reflects the degree of adipose tissue mobilization regulated

by endocrine hormonal function; therefore, the greater the extent of negative energy balance, the more NEFA gets released from body fat into the blood, indicating that our animals did not suffer from an extensive and prolonged negative energy balance.

In a similar way, Katiyar et al. (32) observed that urine glucose and protein concentrations in pregnant cows were higher than in nonpregnant cows, and there was a continuous increasing trend of urine glucose and protein concentrations in pregnant cows only. However, the authors observed higher urine glucose and protein concentrations in the pregnant group betweenday 0 andday 20 than those found in our results. It is therefore presumed that during early pregnancy many metabolic changes take place that may have some effect on kidney filtration, leading to more glucose concentration and protein concentrations in the urine. There is an insufficient amount of literature available to compare our data regarding urine glucose and urine protein in pregnant animals.

Previous studies have reported a negative energy balance associated with reduced circulating P4 in dairy cows during the breeding period, reducing the conception rate in dairy cows (33). Furthermore, both P4 and glucose enhance the optimum growth of the embryo or fetus and decrease the chance of pregnancy losses in dairy cows (3,4). Thus, the energy balance is positive when associated with plasma progesterone. In our study, both plasma and urine glucose levels were positively associated with pregnant and nonpregnant zebu cows. On the other hand, urine glucose was positively associated with plasma P4 in nonpregnant crossbred cows. This indicates that the association of plasma P4 with different metabolites is not consistent in these breeds. Davoudi and Mobaraki (34) reported a nonsignificant correlation between serum levels of NEFA in pregnant and nonpregnant Sarabian and Holstein cows, which is supported by our result.

Considering the aforementioned data, it was found that a progesterone test on day 24 is ideal for a negative pregnancy test. Urine protein is a noninvasive tool that can be used as an indicator for early pregnancy in zebu cows. However, for crossbred cows, further studies with larger sample sizes are required to improve accuracy.

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