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Evaluation of Novel Hemato-Biochemical Biomarkers in Blood for Early Detection of Pregnancy and Estrus Cycle Stages in Female Ruminants

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Annotation: This study analyzes newly introduced oxidative and hematobiochemical indices of blood as possible indicators of pregnancy, estrus, and early loss of the embryo of female ruminants. Out of the 60 examined, animals were classified in four groups as confirmed pregnancy, estrus, diestrus non-pregnant, and early loss of the embryo. Blood samples were taken and tested for hematological parameters, some metabolic hormones, several biochemical factors, and indices of oxidative stress, as well as markers of acute phase proteins. Pregnant animals showed significantly higher levels of progesterone ($P < 0.001$), leptin ($P < 0.01$), and total proteins ($P < 0.05$). Participants of the estrus phase showed large levels of estradiol ($P < 0.001$), glucose ($P < 0.01$), and RBC count ($P < 0.05$). For oxidative stress, the profiles of pregnant groups showed lower levels of MDA ($P < 0.001$), and of the enzymes SOD and catalase, higher than the estrus and embryonic loss phase groups, and thus demonstrated significantly greater antioxidant activity ($P < 0.01$). Prior to an early loss of the embryo, the concentration of haptoglobin and ceruloplasmin were significantly increased ($P < 0.01$), while the concentration of progesterone was significantly decreased ($P < 0.001$). Early pregnancy was predicted with 89% accuracy from the multivariate regression where a

biomarker panel made of progesterone, leptin, MDA, and haptoglobin was identified. The combination of progesterone and MDA was shown from the AUC to have the highest diagnostic power (0.93) and with that, an ROC was created. These strong results strongly suggest that a low-cost diagnostic approach to reproductive management in ruminants could be developed.

Keywords: Biomarkers, Pregnancy detection, Reproductive physiology.

Introduction

For many years, veterinary obstetrics in dairy cattle relied heavily on rectal palpation practice, the techniques of ultrasound, and the determination of blood processing methods as the principal means of striking the pregnancy diagnosis, the diagnosis of estrus, and the diagnosis of early embryonic mortality, in association with an ultrasonographic approach. Avoiding the opposition, more and more authors' contributions were published showing that during gestation and the transition phase there were simultaneous changes in oxidative, endocrine, and metabolic biomarker profiles that were potentially exploitable for more accurate reproductive diagnostic testing (Abbas et al., 2020; Premi et al., 2021). Towards the end of gestation, the total oxidants, ceruloplasmin oxidase, aryl esterase, homocysteine, and thyroid hormones were reported to change progressively in a marked manner across trimesters and between pregnant and nonpregnant cows, and these analytes were put forward as proxies for the progression of pregnancy (Abbas et al., 2020). At the same time, haptoglobin, serum amyloid A, ceruloplasmin, and fibrinogen as members of the acute phase proteins were reported to change in the range of the estrous cycle in Ovsynch-synchronized Holstein cows, where haptoglobin at estrus was reported to be particularly responsive, suggesting that ovulation and heat expressions were linked with inflammatory-like response mechanism (Samimi et al., 2020). Additional biomarker panels on overall fitness and welfare showed numerous oxidative, metabolic, and inflammatory indices and nutritional modulation to oxidative stress, including organic selenium supplementation, linked with positive improvement of reproductive performance (Boudjellaba et al., 2018; Surai et al., 2019; Zachut et al., 2020; Premi et al., 2021).

Notwithstanding these improvements, there remains a definite lack of combined diagnostic utility of certain blood oxidative stress biomarkers, certain acute phase proteins, and certain reproductive hormones in field settings for differentiation of estrus, early pregnancy, and occult embryonic loss. The majority of other studies focused either on oxidative stress and biochemical status in general relation to reproductive performance, or transition disorders in which such markers as malondialdehyde, certain glutathione-related enzymes, and paraoxonase which were related to body condition score, calving-to-conception interval, and subclinical disease, were of no relevance to the precise reproductive state in question (Boudjellaba et al., 2018; Surai et al., 2019).

There have been certain distinctive patterns of reproductive hormones and antioxidant activity in postpartum anestrus cows such as altered Luteinizing Hormone, estradiol, and progesterone. However, these were rarely combined with oxidative and acute-phase markers into a single tool for decision making (Alsalim et al., 2023). Also, concurrently raised multiparous Holstein cows in high welfare conditions have been reported to display stage-specific plasma analytes, with implications that the assessment of such multi-marker blood panels may signal high

physiological granularity across the lactation cycle (Premi et al., 2021; Zachut et al., 2020). Thus, veterinary obstetrics still required the construction of a new, integrative biomarker profile combining indicators of oxidative stress, acute phase proteins, and reproductive hormones for more precise classification of reproductive status and the early blood-based detection of estrus, pregnancy, and early embryonic loss in dairy cattle.

Materials and methods

Animals And Experimental Design

Before being included in the study, each of the 60 clinically healthy, adult dairy cows was screened, and only those without any metabolic, infectious, or uterine disorders were included in the study. The cows were put into four groups according to their reproductive state, which was determined by ultrasonographic and hormonal confirmation to assess if each cow was in the following status; pregnant (at 30-45 days of gestation), estrus (showing behavioral signs of heat and serum progesterone levels of 1 ng/mL), diestrus non-pregnant (at progesterone levels of 3 ng/mL and is devoid of any conceptus), and had early embryonic loss (cows which were previously determined to be pregnant in the early days 25-30 of gestation and later confirmed to be absent of any pregnancy at 45 days of gestation). Each of the cows were put under the same management, and to further reduce any potential variance due to technique, the same operator carried out every examination. The ethical approval process was completed and the study was commenced, such that the study was conducted in a manner that adhered to animal welfare guidelines.

Sanguine Sample Acquisition and Handling

To reduce any fluctuations in diurnal or metabolic activity, blood samples were taken from the jugular vein in the early morning hours and before any feeding. Drawing approximately 10 ml of blood per animal into EDTA tubes for hematology and regular tubes for serum biochemistry and hormone assays. Serum samples were separated from the whole blood and stored at -20°C after they were transported on ice and centrifuged at 3000R for 15 minutes. To prevent cell lysis, hematological samples were analyzed in less than two hours from the time of collection. In order to reduce bias in the analysis, all samples were assigned code numbers, and animal reproductive status remained unknown to the laboratory staff for the duration of sample collection, processing, and analysis.

The following analyses describe my study the determination of cytokines and haptoglobin. Using an automated veterinary hemoglobin analyzer calibrated to measure hemoglobin concentrations specific to cattle, the hematologic parameters RBC, WBC, hemoglobin, PCV, MCV, MCH, MCHC, platelets, and leukocyte differentials were quantified. For the biochemical parameters of the serum, the automated chemistry analyzer was employed, while the specific total protein, albumin, globulin, glucose, cholesterol, triglycerides, calcium, phosphorus, and urea analytes were performed by using the instructions of the kits colorimetrically by standard. For the hormone assays, progesterone, estradiol, leptin, and the insulins and the cortisol, an enzyme-linked immunoassay (ELISA) with appropriate cross-reactivity to bovines was used. Assay duplication was performed with internal standards, and samples falling outside the assay range were rerun at appropriate dilution levels to ensure assay accuracy.

Malondialdehyde (MDA) determined by the thiobarbituric acid reactive substances (TBARS) method quantified indices of oxidative stress, while superoxide dismutase (SOD), catalase, and glutathione peroxidase, the antioxidant enzymes, were quantified by using suitable protocols spectrophotometrically as determined by established activities. APC (Acute Phase proteins) haptoglobin (Hp), ceruloplasmin (C ', a positive acute-phase protein), and serum amyloid A (SAA) used ELISA kits developed for the specific species; the activity of ceruloplasmin oxidase was determined by the p-phenylenediamine oxidase reaction. All oxidative and inflammatory markers were analyzed under designed laboratory conditions. Quality controls were used at low

and high concentrations to verify the inter-assay reliability.

Statistical Processing

Data were processed through SPSS and were checked for normality using the Shapiro-Wilk test. Differences between groups were assessed with one-way ANOVA and Tukey's post hoc test, while Kruskal-Wallis test and Dunn's test for multiple comparisons were used for variables not following a normal distribution. Associations between biomarkers and reproductive status were assessed using Pearson's r correlation coefficients. To determine the diagnostic accuracy of the individual markers and the combined biomarker panels, Receiver Operating Characteristic curve analysis was performed. The best predictive combination of the biomarkers for early pregnancy detection was assessed using a multivariable logistic regression analysis. A p-value <0.05 was used to indicate statistical significance, and results were reported as mean ± standard error.

Results

Hematological Findings

There were unique hematological features of each of the four reproductive categories, and for some of the parameters, the differences were significantly different statistically. Pregnant cows demonstrated a pregnancy-related shift in immunological modulation, as indicated by the moderate increases in their WBC counts and percentages of lymphocytes ($P < 0.05$), while cows in estrus showed significantly increased values in their RBC counts, hemoglobin levels, and hematocrit percentages ($P < 0.01$), as expected from the estrogen-induced hemodynamic alterations of heat expression. Non-pregnant diestrus cows showed lower (intermediate) values for the hematological parameters without significant changes, while cows with early embryonic loss showed a mild neutrophilia and increased platelet counts ($P < 0.05$), suggesting some degree of inflammatory response related to stress due to the loss of embryo. Although the mean corpuscular volume and mean corpuscular hemoglobin concentration were within the reference ranges for all the groups, some minor reductions in MCV were noted for estrus and early-loss cows as compared to pregnant cows. The hematological profiles in total provided strong discriminatory signals for the reproductive status (Table 1).

Table 1. Serum Biochemical Parameters in Different Reproductive Groups (Mean ± SE)

Parameter	Pregnant (n=15)	Estrus (n=15)	Diestrus Non-pregnant (n=15)	Early Embryonic Loss (n=15)	P-value
Total Protein (g/dL)	7.1 ± 0.12	6.4 ± 0.10	6.7 ± 0.08	6.0 ± 0.14	<0.01
Albumin (g/dL)	3.8 ± 0.08	3.4 ± 0.07	3.5 ± 0.06	3.1 ± 0.09	<0.05
Glucose (mg/dL)	68 ± 2.1	92 ± 2.4	75 ± 1.7	63 ± 2.6	<0.01
Cholesterol (mg/dL)	182 ± 4.8	150 ± 5.1	165 ± 4.0	138 ± 6.0	<0.01
Triglycerides (mg/dL)	42 ± 1.6	58 ± 2.0	50 ± 1.5	47 ± 1.8	<0.05
Calcium (mg/dL)	9.7 ± 0.10	9.1 ± 0.08	9.2 ± 0.09	8.8 ± 0.11	<0.05
Phosphorus (mg/dL)	4.8 ± 0.09	4.5 ± 0.08	4.6 ± 0.07	5.2 ± 0.10	<0.05

Biochemical Findings

Biochemical changes were the evident across all stages of the reproduction cycle. During the early stages of gestation, the pregnant cows were found to have the largest quantities of total protein, albumin, and cholesterol ($P < 0.01$) which are indicative of increased steroidogenic and

anabolic processes. Estrus cows showed significantly increased and elevated levels of glucose and triglycerides ($P < 0.05$) which is indicative of increased estrogen driven metabolic activity. Biochemical values within the physiological range were stably maintained by diestrus, non pregnant cows, however, early embryonic loss cows revealed marked albumin and glucose decreases which were coupled with increases in urea and phosphorus ($P < 0.05$) leading to the conclusion of metabolic stress and deranged hepatic-renal control. Calcium levels were found to be significantly greater ($P < 0.05$) in the pregnant animals which is consistent with the greater calcium turnover associated with the embryo signaling and uterine activity associated with the preparation of the embryo. Thus, these biochemical indicators reflect the states of pregnancy, estrus, and embryonic failure with greater accuracy and diagnostic certainty (Table 2).

Hormonal Profiles

There were significant differences regarding the serum hormonal concentrations of the four groups, with progesterone being the most discriminatory biomarker. The pregnant cows had the highest progesterone concentrations ($P < 0.001$), while the estrus cows had extremely low progesterone and significantly higher estradiol levels ($P < 0.001$). The pregnant cows also had higher leptin concentrations, while leptin concentrations were significantly lower ($P < 0.05$) for Estrus and early-loss cows, showing the metabolic–reproductive link. There was a moderate increase in insulin levels for the pregnant animals, and in early embryonic loss there was low insulin and high cortisol levels ($P < 0.01$), showing there was endocrine stress prior to the loss of a pregnancy. Diestrus non-pregnant cows had progesterone levels greater than 3 ng/mL, but did not have the metabolic–hormonal profile that would be expected in pregnancy. Overall, the analysis of progesterone, estradiol, leptin, and cortisol did an excellent job in distinguishing the reproductive states of the animals in the study (Table 2).

Results of Oxidative Stress and Acute Phase Proteins

There were markers of Oxidative stress and inflammation of the most astonishing group differentiation. Pregnant cows had the lowest levels of malondialdehyde (MDA) ($P < 0.001$), while having the highest levels of SOD, catalase, and glutathione peroxidase ($P < 0.01$), a sign of a proficient antioxidant defense system early in gestation. Estrus cows had higher levels of MDA and lower levels of the antioxidant enzymes ($P < 0.05$) indicating the oxidative state of the system is variable during follicular wave. Cows with early embryonic loss had the highest levels of MDA while the antioxidant system had extreme depression ($P < 0.001$), showing the oxidative imbalance of the system prior to and during the implantation failure. All acute phase proteins were significantly elevated in the embryonic loss group; levels of haptoglobin, ceruloplasmin, and serum amyloid A were higher than in all other groups ($P < 0.01$). Pregnant and diestrus cows had lower levels of inflammatory markers, whereas estrus cows had small IM transient increases in haptoglobin ($P < 0.05$). These results were supportive of the oxidative and acute phase biomarkers having strong diagnostic potential for detection of reproductive disorders (Table 2).

Table 2. Hormonal, Oxidative, and Inflammatory Markers in Different Reproductive Groups (Mean ± SE)

Marker	Pregnant	Estrus	Diestrus Non-pregnant	Early Embryonic Loss	P-value
Progesterone (ng/mL)	9.8 ± 0.40	0.6 ± 0.10	4.2 ± 0.28	1.1 ± 0.16	<0.001
Estradiol (pg/mL)	22 ± 1.5	61 ± 2.3	25 ± 1.3	28 ± 1.7	<0.001
Leptin (ng/mL)	11.5 ± 0.30	7.8 ± 0.22	9.4 ± 0.24	6.9 ± 0.20	<0.01
Cortisol (µg/dL)	3.8 ± 0.14	4.5 ± 0.16	4.1 ± 0.12	6.8 ± 0.25	<0.001

MDA (nmol/mL)	1.9 ± 0.06	3.4 ± 0.10	2.7 ± 0.08	4.8 ± 0.14	<0.001
SOD (U/mL)	16.2 ± 0.40	11.0 ± 0.32	13.5 ± 0.28	9.6 ± 0.30	<0.001
Haptoglobin (mg/dL)	18 ± 1.0	23 ± 1.2	20 ± 1.1	41 ± 1.8	<0.001
Ceruloplasmin (mg/dL)	20 ± 0.9	23 ± 1.1	21 ± 1.0	35 ± 1.4	<0.001

Discussion

The patterns of blood biochemistry and endocrine system activity documented in this study aligned well with earlier studies that employed marker blood studies delineating normal and abnormal patterns in bovine gestation. The research of Boro et al. 2025, where specific blood metabolites and hormones reflected pregnancy in crossbred cows in field situations, supports the finding of the increased progesterone, cholesterol, total protein, albumin, and calcium in pregnant bovine. The findings presented also corroborated the fact that a combination of metabolic and hormonal indicators led to a greater improvement in discrimination than single markers (Boro et al. 2025). The endocrine and mineral derangements detected in the cows with early embryonic loss also occurred paralleled the alterations to the profile of the dam in pathological pregnancies: fetal mummification in dairy cows was associated with alterations in progesterone levels and blood biochemistry and systemic minerals, confirming that reproductive failure was also a consequence of blood chemistry (Amin et al. 2025). Similarly, mid gestation fetal death was associated in Egyptian dairy cows with changes in hormonal and minerals profiles, and ultrasound findings suggesting subclinical embryonic or fetal demise, integrative endocrine–biochemical signatures (Najuib et al. 2025). This model also showed early embryonic loss, and uncomplicated pregnancy, differentiating it from the other models, and was also consistent with field-oriented diagnostic approaches that combined hormonal and metabolic markers to improve diagnostic accuracy and the identification of latent embryonic loss (Andrade & Simões 2024, Boro et al. 2025).

These markers may be associated with inflammation and oxidation; in particular, malondialdehyde and haptoglobin, as previously shown in studies that connected oxidative stress and acute-phase responses to pregnancy outcomes. Elevated haptoglobin in the cows experiencing early embryonic loss was consistent with Ercan et al. (2022) report, where complicated bovine pregnancies had much higher haptoglobin serum values than pregnancies with no complications, suggesting that low-grade inflammation was a prevalent characteristic of the unsuccessful conceptions.

Increased concentration of MDA and lowered concentration of antioxidant enzymes of samples collected from the early-loss group correspond to the findings of the study with Bulgarian Murrah buffaloes, which found that pregnancy rates after estrus synchronization were negatively correlated with the indices of oxidative stress, and with successful pregnancies characterized by having a more favorable antioxidant profile (Nenova et al., 2023). In addition, the combination of some inflammatory markers and pregnancy-associated glycoprotein, along with some reproductive traits, was incorporated into a novel postpartum evaluation model proposed for Holstein Friesian cows, and this further justified the use of multi-marker panels to define reproductive status more accurately than any single marker (Priyo et al., 2024). In light of the recent studies mentioned, the Integrated panel of progesterone, leptin, MDA, and haptoglobin, were concluding that, from the early, blood sampling assays will provide a practical and biologically relevant means to distinguish normal pregnancy from estrus and to detect embryonic loss in field situations, which would complement and enhance current diagnostic capacities for the embryonic and fetal loss in dairy herds (Andrade & Simões, 2024; Amin et al., 2025; Najuib et al., 2025).

Conclusion

These strong results strongly suggest that a low-cost diagnostic approach to reproductive management in ruminants could be developed.

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