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Dissertation

Redblood Cell Distribution Width to Platelet Ratio (RPR): Novel diagnostic possibilities for perinatal disease in newborn foals

Rebeca Scalco

Pelotas, 2023

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If you can see a thing whole, it seems that it's always beautiful. Planet, lives…
But close up, a world's all dirt and rocks. And day to day, life's a hard job, you get tired, you lose the pattern. You need distance, interval. The way to see how beautiful the earth is, is to see it as the moon. The way to see how beautiful life is, is from the vantage point of death.

— Ursula K. Le Guin, The Dispossessed

Abstract

SCALCO, Rebeca. **Redblood Cell Distribution Width to Platelet Ratio (RPR): Novel diagnostic possibilities for perinatal disease in newborn foals.** 2022. 60f. Dissertation (Master's degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2022.

Perinatal diseases are the leading cause of death among foals up to 7 days of age. Early clinical signs of diseases in neonatal foals are often non-specific, and untreated foals deteriorate rapidly. Therefore, early detection of the disease onset is essential. The development of rapid and accurate markers to aid in diagnosing neonatal foals is needed. The CBC parameters redblood cell distribution width (RDW) and RDW to platelet ratio (RPR) have been used in human medicine as markers of inflammation in many diseases. Therefore, the purpose of this work was to report the values of RDW and RPR in neonatal foals and to investigate their possible correlation with a risk score based on obstetric and clinical data. In this retrospective case-control study, 309 full-term Thoroughbred foals less than 24h old were clinically evaluated within 15 minutes after birth, and blood samples were collected within 24h of life. Foals showing an unremarkable physical examination, normal gestational length (320 to 365 days), adequate righting reflexes, and CBC values within the normal range were considered healthy. Foals that were born through dystocia, displayed delayed adaptative milestones (sternal recumbence > 5 minutes, suckling reflex > 20 minutes, stand >1 hour), gestational length > 365 days, and/or displayed physical/hematological characteristics of dysmaturity (silky hair-coat, domed head, floppy ears, abnormal granulocyte:lymphocyte ratio), were categorized in the group at-risk. Extracted data were assessed for normal distribution using the Shapiro-Wilk test. The Student's T-test was used to evaluate the influence of groups and foal sex on hematological variables (RBC, hematocrit, hemoglobin, MCV, MCH, MCHC, RDW, platelets, WBC). Pearson's coefficient test was used to analyze the associations between gestational length and RDW, and RPR. Continuous variables are presented as mean ± SD. Categorical variables are presented as frequencies and percentages. All statistical analysis was conducted in the statistical package R Studio, and significance was set at $P < 0.05$. Based on the risk score, 221 (71.6%) foals were healthy, and 88 (28.4%) were considered at risk of developing perinatal disease (at-risk group). The mean gestational age for all the foals was 346.31 ± 9.69 days. RDW values did not differ between groups. Gestational length demonstrated to have a negative correlation with RDW ($r = -0.156$, $P = 0.005$) and MCV ($r = -0.135$, $P = 0.01$), indicating a link of these variables to foal maturity. RPR was higher for at-risk (0.073 ± 0.018) than for healthy foals (0.068 ± 0.014, $P = 0.01$). Therefore, the RPR ratio is a low-cost, readily accessible index and might be a promising early indicator of disease for the field triage of neonatal foals and rapidly estimate possible systemic disorders. For further expanding

the applicability of RPR in neonatal foals, multicenter longitudinal studies with a larger number of healthy and critically ill neonates are necessary.

Keywords: equine neonatology; hematology; RDW; RDW to platelet ratio; sepsis.

Resumo

SCALCO, Rebeca. **Razão Amplitude de Distribuição dos Glóbulos Vermelhos (RDW) – Plaquetas (RPR): Novas possibilidades diagnósticas para doenças perinatais em potros neonatos.** 2022. 60f. Dissertation (Master's degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2022.

As doenças perinatais são a principal causa de morte em potros de até 7 dias de idade. Os sinais clínicos de enfermidades em potros frequentemente são inespecíficos e animais não tratados agravam rapidamente o quadro clínico. Portanto, a detecção precoce do início do processo é essencial. É necessário o desenvolvimento de marcadores rápidos e sensíveis para auxiliar no diagnóstico de doenças perinatais em potros. Os parâmetros RDW e RPR são derivados do hemograma e estão sendo estudados em medicina humana como marcadores de inflamação em diversas enfermidades. Portanto, o objetivo deste trabalho foi reportar os valores de RDW e RPR e investigar a possível aplicação destes índices em potros neonatos classificados como saudáveis ou de risco, baseado em informações obstétricas e clínicas. Realizou-se um estudo caso-controle retrospectivo, em que 309 potros Puro Sangue Inglês ≤ 24h nascidos a termo foram avaliados 15 minutos após o parto e amostras de sangue foram coletadas dentro das primeiras 24 horas de vida. Um escore de risco foi calculado baseado em dados clínicos e obstétricos e os potros foram categorizados em dois grupos: saudáveis e de risco. Os potros que apresentaram reflexos comportamentais, exame físico e hemograma dentro dos parâmetros de referência e tempo de gestação entre 320 e 365 dias foram considerados saudáveis. Potros que nasceram de parto distócico, apresentaram reflexos comportamentais alterados (decúbito esternal > 5 minutos, reflexo de sucção > 20 minutos, permanecer em estação > 1 hora), tempo gestacional > 365 dias, e/ou apresentando características físicas/hematológicas de dismaturidade (pelagem fina, abaulamento de cabeça, flacidez de orelhas, desproporção na razão granulócito:linfócito) foram categorizados no grupo de risco. A normalidade dos dados foi avaliada utilizando o teste Shapiro-Wilk. A influência dos grupos e do sexo dos potros nas variáveis hematológicas (hemácias, hemoglobina, VCM, HCM, HCMC, RDW, plaquetas e contagem total de leucócitos) foi verificada por meio do teste T. A correlação entre tempo gestacional e RDW e RPR foi analisada por meio do teste de coeficiente de Pearson. Variáveis contínuas estão apresentadas como média ± desvio padrão. Variáveis categóricas estão apresentadas como frequências e porcentagens. As análises estatísticas foram conduzidas no pacote estatístico R, e o valor de significância estabelecido em $P < 0.05$. Com base no escore de risco, 221 (71,6%) dos potros foram classificados como saudáveis e 88 potros (28,4%) foram considerados em risco de desenvolvimento de doenças perinatais (grupo de risco). O tempo de gestação médio de todos os potros foi 346,31 ± 9,69 dias. Os valores de RDW não foram diferentes entre os grupos. O tempo gestacional demonstrou ter correlação negativa com RDW (r = -0,156,

P = 0,005) e VCM (r = -0,135, P = 0,01), indicando uma relação destas variáveis com maturidade dos potros. Os valores de RPR foram mais altos para os potros de risco (0,073 ± 0,018) do que nos potros saudáveis (0.068 ± 0,014, P = 0.01). Portanto, o RPR é um índice de baixo custo, acessível, e pode ser um indicador promissor de enfermidades na triagem a campo de potros neonatos, e rapidamente estimar possíveis alterações sistêmicas. Mais estudos longitudinais multicêntricos são necessários para expandir a aplicabilidade do RPR em potros neonatos.

Palavras-chave: hematologia, neonatologia equina, razão RDW-plaquetas**,** RDW, sepse.

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Abbreviation List

bpm	beats per minute
CBC	complete blood count
LAC	lactate
h	hour
HGB	hemoglobin
RBC	red blood cell
RDW	red cell distribution width
rpm	respirations per minute
RPR	red cell distribution width to platelet ratio
s	seconds
SAA	serum amyloid A

Symbols List

< Less than

> Greater than

≤ Less than or equal to

≥ Greater than or equal to

°C Celsius degree

$ dollar

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1 Introduction

The neonatal period in foals comprises the first 7 days of life, and it's considered critical, as it encompasses a succession of physiological challenges to adapt to the extrauterine environment (ROSSDALE, 1993). Several events during the gestation, birth and immediate post-partum periods can predispose foals to develop perinatal diseases. Mainly, maternal, environmental, and immune factors account for clinical outcomes and pose a substantial risk to the development of the disease process (ROSSDALE, 1972; KOTERBA; DRUMMOND; KOSCH, 1990). The adequate development of the foal entirely depends on the uterine milieu conditions, specifically through the placenta, that fulfills the metabolic, nutritional, and endocrine needs during most of the gestation (ROSSDALE, 1993). Most foals successfully overcome the physiological challenges of perinatal life; however, infectious and stressor events can adversely affect the foal's maturation and development, placing the foal at risk of developing perinatal diseases (FOWDEN; FORHEAD; OUSEY, 2012). Foals with a history of maternal illness, dystocia, or difficult delivery, suspect or proven placental alteration, abnormal gestational length, and altered hemogram should be monitored intensively and treated as high-risk foals until proven contrary (KOTERBA; DRUMMOND; KOSCH, 1990). The most frequent clinical processes affecting foals in the newborn foal are prematurity, dysmaturity, equine neonatal encephalopathy (before known as hypoxic-ischemic encephalopathy or "dummy foal"), failure in the passive transfer of immunity, and, finally, sepsis (STONEHAM, 2006; HAHN, 2008; TORIBIO, 2019; O'FALLON, 2021).

Sepsis is a systemic condition caused by bacterial, viral, or fungal agents that can cause hemodynamic changes, resulting in substantial morbidity and mortality rates (SHANE, 2017). Worldwide, sepsis is a significant disease process with profound clinical and economic repercussions (CECCONI, 2018). Despite advancements in equine husbandry and neonatal care in the last decades, bacterial sepsis remains the leading cause of death among foals up to 7 days old (SANCHEZ, 2005; TAYLOR, 2015; FLOYD; EASTON-JONES; THEELEN, 2021). The patient's outcome heavily depends on the rapid

assessment of foals and the prompt establishment of adequate antimicrobial therapy and support treatment (FURR; MCKENZIE, 2020). Because clinical signs of sepsis in foals might be unspecific, early diagnosis challenges veterinarians. Blood culture is the gold standard for sepsis diagnosis (RUSSELL *et al.,* 2008; TOWNS; JARVIS; HSUE, 2010). Results of the culture usually take around 24 to 48h to report bacterial growth of an organism with a low percentage of positive and a high of false negative results, and often the presence of bacteria unrelated to the sepsis is reported, indicating sample contamination (RUSSELL *et al.,* 2008; TOWNS; JARVIS; HSUE, 2010). Furthermore, performing a blood culture is not feasible for field clinicians working in remote areas, demonstrating the importance of exploring more accessible markers.

Red cell distribution width (RDW) represents a coefficient of variation of the size of the circulating erythrocyte (SALVAGNO; SANCHIS-GOMAR; LIPPI, 2015). There is increasing evidence that high RDW values indicate active systemic inflammation and determine the prognosis for a wide range of inflammatory and infectious diseases (LIPPI *et al.,* 2013; PEDRAZZANI *et al.*, 2020; ZHANG *et al.,* 2020; SARKAR *et al*., 2022). A correlation between RDW to cardiovascular conditions in dogs and cats has been demonstrated (STANZANI *et al.*, 2015; MAZZOTTA *et al.*, 2016). To date, a limited number of studies have explored the role of RDW in healthy adult horses (BALARIN *et al.*, 2001; BALARIN *et al.*, 2006; CARVALHO *et al.*, 2016). A recent study reported higher RDW values in hospitalized Quarter Horses compared to matched healthy equine population, indicating that RDW may serve as a marker and predictor of the prognosis of diseases in horses (RAMIRES *et al.*, 2019). Currently, no studies specifically evaluate RDW levels in newborn foals and their correlation with disease status.

Similarly, recent studies in human patients demonstrated that the RDW to platelet ratio (RPR) is a valuable novel indicator of systemic inflammation and correlates with prognosis in many conditions, such as liver fibrosis, acute pancreatitis, and septic shock (ÇETINKAYA *et al.,* 2014; CAI *et al.*, 2019; GE *et al.*, 2020). RPR has also been suggested to be a helpful marker in diagnosing early-onset sepsis and predicting mortality in human infants (KARABULUT; ARCAGOK, 2020). RPR has not been investigated in horses. Therefore, this work aimed to describe the values of RDW and calculate RDW to-platelet-ratio (RPR) in neonatal foals in the first 24h of life. The secondary aim was to

investigate the correlation of RDW and RPR to a foal risk score (high- vs. low-risk) based on obstetrical and clinical information. We hypothesize that higher RDW and RPR index might be associated with foals considered to be at a higher risk of systemic perinatal disease.

2 Literature review

As prey animals, horses (*Equus caballus*) have developed several strategies to ensure their survival throughout evolution. A clear example is the newborn foal's readiness within a few minutes of life. In nature, the neonatal foal should be readily adapted to stand up and quickly follow the herd, therefore considered a precocious specie (HOUPT *et al.*, 1986). The neonatal period in foals, which comprehends the first 7 days after birth, is considered critical and characterizes a transition between intra to extrauterine life (ROSSDALE, 1972; CURCIO; NOGUEIRA, 2018). These physiological changes should occur in a well-orchestrated fashion to ensure adequate adaptation of the foal to the extrauterine environment. Several homeostatic functions, such as thermoregulation, glucose metabolism, and pulmonary function, are not active before birth (FOWDEN; FORHEAD; OUSEY, 2012). During the final stages of gestation, the foal is prepared for extrauterine life. This involves structural changes in the endocrine system (*e.g*., activation of the hypothalamic-pituitary-adrenal axis, alterations in the neurosteroid dynamics) that determine the level of maturation of critical systems necessary for immediate survival following birth (FOWDEN; FORHEAD; OUSEY, 2012; TORIBIO, 2019).

In the equine species, the gestational length varies considerably due to individual and environmental factors (SATUÉ *et al*., 2011). Generally, the average gestational period in mares is 330 days. However, in Thoroughbreds, the literature reports a longer gestation, with an average of 345 days, but the experience in the field reveals pregnancies overpassing 365 days (MOREL; NEWCOMBE; HOLLAND, 2002). In this case, the foal is considered postmature, and the prolonged gestation could be caused by placental insufficiency or nutritional deficiency of the broodmare during pregnancy (KOTERBA; DRUMMOND; KOSCH, 1990). Stress factors, such as illness or drastic environmental changes, especially in the final third of pregnancy, have a direct correlation with a decrease in the gestational length and increase in the expulsive phase of the parturition in mares (MELCHERT, 2019; NAGEL, 2020). Foals born before 320 days are considered premature and commonly have altered metabolic, neurologic, and musculoskeletal

development. Dysmature foals are the ones that are born to term but display physical and/or hematological characteristics of immaturity (silky hair-coat, domed head, floppy ears, abnormal granulocyte:lymphocyte ratio (KOTERBA; DRUMMOND; KOSCH, 1990).

Foals with alterations in the maturation process are at higher risk of developing diseases in the neonatal period (ROSSDALE, 1973). The main processes that affect neonatal foals and may lead to perinatal disorders include equine neonatal encephalopathy (before known as perinatal asphyxia syndrome, or hypoxic-ischemic encephalopathy, or “dummy foal”), prematurity, dysmaturity, and immune-mediated disorders (*e.g.*, neonatal isoerythrolysis, immunomediated thrombocytopenia, severe combined immunodeficiency), and sepsis. Performing the differential diagnosis between these processes can be challenging for the clinician; therefore, early recognition of the risk foal based on clinical and obstetric data is essential.

Several sentinel events during the peripartum can predispose foals to develop perinatal diseases: maternal, environmental, and immune factors. Maternal factors include illnesses (*e.g*., colic, colitis, pneumonia), premature placental separation, placentitis, dystocia, and abnormal gestational length. Environmental factors include inadequate sanitary conditions during the peripartum and insufficient care of umbilical remnants. Immune factors primarily comprehend the failure of passive transfer of colostrum antibodies. However, immune-mediated disorders may also play a part in the establishment and progression of the disease process (ROSSDALE, 1972; KOTERBA; DRUMMOND; KOSCH, 1990; SANCHEZ, 2005; CURCIO; NOGUEIRA, 2018). The foal is relatively immune naïve at birth and mainly depends on the colostrum IgGs since no significant transplacental transfer of antibodies occurs *in utero*. Due to the progressively decreasing mucosal absorption of immunoglobulins, it is of utmost importance that the foal ingests at least 1 liter of high-quality colostrum (> 20 BRIX) within the first 6 hours of life (JEFFCOTT, 1971; SELLON, 2006). Fundamentally, rigorous gestational monitoring, adequate and clean delivery setting, and early foal assessment are the most effective strategies for preventing and/or detecting systemic processes during the neonatal period. Prenatal versus intranatal infection origin distinction has a significant impact on prognosis. Infectious processes originating during pregnancy have a significantly worse prognosis

than those originating in the postpartum period (ROSSDALES, 1972; KOTERBA; DRUMMOND; KOSCH, 1990; SANCHEZ, 2005; CURCIO; NOGUEIRA, 2018).

Sepsis is a clinical syndrome caused by the exaggerated host response to infection due to the release of many cytokines and other inflammatory mediators, often leading to multiple organ dysfunction, severe hypovolemia, and hypotension (VINCENT *et al.*, 2013). Sepsis poses a substantial social and economic burden in healthcare worldwide. In human medicine, Systemic Inflammatory Response Syndrome (SIRS) is characterized by alterations in two of the following: body temperature, heart rate, respiratory function, and peripheral white cell count (BONE *et al*., 1992; VINCENT *et al.*, 2013). The same parameters extrapolate to define SIRS in neonatal foals (WONG *et al.*, 2018). The progression of sepsis manifests its severity associated with organ dysfunction, hypotension, and/or hypoperfusion. Septic shock denotes the severe hypotension state caused by the progression of the septic process and organ dysfunction and represents the leading cause of death in neonatal foals (KOTERBA; DRUMMOND; KOSCH, 1990; SANCHEZ, 2005; TAYLOR, 2015; FLOYD; EASTON-JONES; THEELEN, 2021).

The portals of entry for infectious organisms in postpartum in neonatal foals are the umbilicus, the gastrointestinal tract, the respiratory tract, as well as any wound present. Initial clinical signs of sepsis in foals can be quite subtle, and the onset depends on the immune status of the neonate and the etiologic agent involved (KOTERBA; DRUMMOND; KOSCH, 1990; SANCHEZ, 2005). Gram-negative bacteria are the most frequent isolates from septic foals, with *Escherichia coli* being the most common bacterium isolated (MARSH; PALMER, 2001; RUSSELL *et al*., 2008; THEELEN *et al.*, 2014). Therefore, endotoxins play an important role in sepsis pathogenesis, and this information must be considered when selecting a therapeutic approach (FIELDING; MAGDESIAN, 2015).

Early signs of sepsis include: increased recumbency, weakness, decreased suckle reflex, depression, dysphagia, and/or swelling, pain, or hyperemia in the umbilical remnants. With the advancement of the disease process, and the infection overburdening the foal's immune and compensatory responses, septic shock ensues, and clinical signs mirror the breadth of organ dysfunction. The foal then progressively becomes depressed, frequently recumbent, hyper- or hypothermic (<37.0°C or >38.9°C), brady- or tachypneic (<40 rpm. or >80 rpm), brady- or, tachycardic (<60 bpm and >100 bpm.). An increased

capillary refill time (> 2s), injected or hyperemic mucous membranes, and/or petechiation, evidence of uveitis, hyperemic coronary bands, effusive joints, lameness, and diarrhea are also signs that should alert the clinician. In advanced stages of the disease, decreased pulse in the digital artery and cold extremities indicate the severe stages of hypovolemia (KOTERBA; DRUMMOND; KOSCH, 1990; SANCHEZ, 2005; HAHN, 2008). Rapid identification of high-risk foals and promptly establishing adequate antimicrobial and supportive treatment is decisive to the patient outcome. Ultimately, performing nurse care for severely ill foals is a laborious and strenuous process that can be challenging to veterinarians working in the field (especially during the reproductive season), emphasizing the importance of early clinical assessment.

To date, no ideal tool has been developed for the early diagnosis of perinatal diseases. An ideal marker should have high sensitivity and specificity, ease of use, and low cost and indicate the disease's stages and the patient's prognosis. Recognizing infection and sepsis in neonatal foals is crucial for reducing mortality and preventing sequelae, particularly those related to the musculoskeletal, neurological, and respiratory systems. Since untreated foals deteriorate rapidly, empirical use of broad-spectrum antibiotics remains the cornerstone of treatment guidelines, as it reduces mortality and optimizes outcomes (SHARMA *et al.*, 2018; THEELEN *et al.*, 2019).

The definitive diagnosis of the septic process relies on identifying the causal agent through blood culture. However, delay in reporting organism growth, poor sensitivity, and high rates of false-negative results are shortcomings of the use of this test; therefore, negative results alone cannot exclude the septic process. In addition, culture results frequently report the presence of bacteria unrelated to sepsis, which indicates sample contamination. Previous foal exposure to antibiotics during peripartum can also impact blood culture's sensitivity and results. Nonetheless, performing a blood culture is not a practical option for field veterinarians working in remote areas, demonstrating the need to investigate more accessible markers (MARSH; PALMER, 2001; RUSSELL *et al*., 2008; TOWNS; JARVIS; HSUE, 2010; THEELEN *et al.*, 2014).

In addition, developing a scoring system to predict the probability of infection aimed to provide clinicians with a practical and rapid tool to predict sepsis in neonatal foals and identify the septic process at a treatable stage. The "sepsis score" is derived using a

combination of subjective clinical criteria and objective clinicopathological variables, assigning a number for each criterion. Points are summated and compared to a cutoff. Initially, foals with sepsis scores>11 were at high risk of developing the septic process. Updated scores have been published, and Wong *et al*. (2018) have proposed including SIRS elements to enhance the sepsis score's predictive power. However, no difference in its predictive power was observed. Sepsis scores can sometimes be limited by a lack of information available, leading to misclassification of likely septic foals below the cutoff point (BREWER; KOTERBA, 1988; WONG *et al*., 2018). An alternative was proposed by Koterba, Drummond and Kosch (1990), indicating that using a risk score may help assess the clinical status of foals presented with missing information or with limited means of treatment.

Hematological evaluation of neonatal foals is considered a standard of care and is generally performed around 12h of life to assess passive transfer status or earlier if clinically indicated. A significant difference is observed in the hematological intervals of newborn foals compared to those of adult horses, reflecting physiological changes that occur throughout the last trimester of pregnancy (BAZZANO *et al.*, 2014). The fetal liver is the main hematopoietic organ, as the bone marrow's function is limited until the final gestation. In healthy neonatal foals, the concentration of RBC (9.3–12.9 $x10^{12}$/L), hematocrit (0.40–0. 52 L/L), and hemoglobin (134–199 g/L) are higher in the first hours of life, reflecting the residual blood derived from the placenta. Hematocrit values decrease by approximately 10% during the first 24h of life due to the hemodilution caused by colostrum ingestion. RBC and HGB reach values similar to adults around 2 weeks of age. Compared to adults, foals initially appear mildly anemic, with a reduced Hgb per cell and smaller RBCs. This "physiologic anemia" is likely related to a reduced stimulus for erythrogenesis and decreased iron availability. In newborn foals, 2,3-diphosphoglycerate levels are higher than in adults, which is characteristic of immature RBCs. Because 2,3-diphosphoglycerate facilitates the release of oxygen into tissues, greater concentrations in the neonate may inhibit the erythrogenesis process (KOTERBA; DRUMMOND; KOSCH, 1990; AXON; PALMER, 2008; BARTON; HART, 2020).

Leukocyte counts in healthy foals also differ from those of adults, and WBC count increases significantly due to the increase of circulating neutrophils. At birth, the

neutrophil: lymphocyte ratio is 2:5 but rapidly increases to 3.5-4:1 after 3 hours of life as a result of the peak level of cortisol in the fetal circulation during this period. Systemically ill neonatal foals generally present leukopenia and alteration in the neutrophil: lymphocyte ratio (> 4:1) (AXON; PALMER, 2008; BARTON; HART, 2020). It has also been demonstrated that other biomarkers, such as lactate (LAC), fibrinogen, and serum amyloid A (SAA), are associated with perinatal diseases. In neonatal foals with systemic illness, hyperfibrinogenemia, hyperlactatemia, and elevated levels of SAA have been reported. However, these parameters may be unspecific and influenced by various factors (KOTERBA; DRUMMOND; KOSCH, 1990; CASTAGNETTI *et al*., 2012; TAYLOR, 2015). Another significant limitation is the financial cost of these point-of-care tests. In Brazil, the use of stall-side SAA or LAC testing is progressively growing. In comparison with the CBC, which averages $ 3 to 8 per test, SAA stall-side costs between $ 30 and 40 per test; LAC costs approximately $ 9 when performed in the laboratory, and stall-side tests prices vary between $ 20 to 40; prices can fluctuate across the country (personal communication with Drs. Ana Gorino and Fernanda Timbó, who practice equine medicine in 2 different states of Brazil: SP and BA, respectively). Due to the limited availability of these point-of-care diagnostics in remote areas, identifying other, more accessible markers is needed to assist in the triage, diagnosis, and treatment of neonatal foals. Nowadays, many equine farms have on-site automated hematology analyzers, and field veterinarians frequently have access to laboratories.

The RDW is a measure of the variation in the size of circulating RBC. It is an easily accessible and inexpensive quantitative measurement of anisocytosis, automatically calculated by most modern hematologic analyzers and part of routine complete blood counts. The calculation of RDW represents the standard deviation of the Mean Corpuscular Volume (MCV) multiplied by 100 to yield a percentage value. RDW has been traditionally used as a differential diagnostic tool in human and veterinary medicine to characterize regenerative anemia, iron deficiency anemia, and thalassemia minor. A correlation between RDW and diseases in dogs and cats has been demonstrated, but results vary. Martinez *et al.* (2019) reported high RDW values in dogs with various conditions, including hyperadrenocorticism, hypothyroidism, pneumonia, chronic kidney disease, multi-centric lymphoma, and myxomatous mitral valve degeneration. Swann *et*

al. (2014) and Mazzotta *et al.* (2016) found a correlation between high RDW and pulmonary hypertension. However, Guglielmini *et al.* (2013) evaluated the correlation of RDW to chronic degenerative valvular disease and did not find an association between high RDW values and heart failure. In cats, similar findings have been reported. While Stanzani *et al.* (2015) reported higher RDW values in cats with hypertrophic cardiomyopathy, Roderik et al. (2016) demonstrated that RDW is helpful in indicating congestive heart failure but failed to predict mortality.

There have been few studies investigating the role of RDW and athletic training in healthy horses, and even fewer have examined the role of RDW in diseases in adult horses (BALARIN *et al.*, 2001; BALARIN *et al.*, 2006; CARVALHO *et al.*, 2016). Recently, Ramires *et al*. (2019) reported significantly higher RDW values in diseased Quarter Horses compared to a healthy equine population, indicating that RDW might be applicable as a potential marker of diseases. In human medicine, the potential of RDW as a sepsis biomarker has been investigated in critically ill adult and newborn patients (LORENTE *et al.*, 2014; MARTIN *et al.*, 2018). During the recent global pandemic of COVID-19, the use and applicability of RDW as a marker of disease severity and its relationship to prognosis, morbidity, and mortality have been evidenced (SARKAR *et al.*, 2022). In adult septic patients, for example, higher values of RDW on admission to the ICU are correlated with poor outcomes (SADAKA; O'BRIEN; PRAKASH, 2012). Elevated RDW in human neonates was associated with prematurity, intrauterine growth restriction (IUGR), and severe infection. It has not been completely established how the inflammatory process influences the increase in RDW. However, it has been suggested that increased inflammatory mediators such as tumor necrosis factor, interleukin-6, and interleukin-1 may suppress erythropoiesis and red blood cell maturation, decreasing their half-life and thereby increase RDW values (GAROFOLI *et al.*, 2013). Currently, no studies have investigated RDW in neonatal foals and its potential clinical correlation with disease processes.

Similarly, the RDW to platelet ratio (RPR) is a novel, inexpensive, noninvasive algorithm derived from RDW and PLT, two parameters easily derived from routine CBCs. Recent studies have reported that RPR may indicate inflammation and correlate with prognostic in human patients. Originally, the calculation of RPR was suggested by CHEN

et al. (2013) when evaluating individuals with hepatic fibrosis and cirrhosis in chronic hepatitis B. The suggested formula is RPR = RDW (%) / Platelet count (10^9 /L). It was then observed that RPR had a strong correlation with stage of liver fibrosis (r = 0.58, P = < 0,001), and high prognostic power for fibrosis (AUC = 0.825) and cirrhosis (AUC = 0.884). The rationale behind RPR calculation is that as a result of the inflammatory response, platelets are deployed more frequently as it plays a role in fibrogenesis and regeneration. Concomitantly, the release of inflammatory cytokines also increases heterogeneity in RBC maturation and impairment, characterized by an elevated RDW, as a consequence (CHEN *et al.*, 2013). Several other studies have been performed in adult humans proposing the correlation of a high RPR with multiple conditions, including acute pancreatitis, severe burn, acute kidney injury, and different types of neoplasia (ÇETYNKAYA *et al.*, 2014; QIU *et al.*, 2017; TAKEUCHI *et al.*, 2019; SCHNEIDER *et al.*, 2021; WU *et al.*, 2022). RPR has also been evaluated in newborn infants. Karabulut and Arcagok (2020) investigated the use of RPR as a diagnostic tool for early onset of sepsis. The study evaluated 6539 infants admitted to the ICU and indicated significant differences between healthy neonates and infants with suspected or proven early onset of sepsis. Infants with confirmed sepsis had higher RPR values than the ones with suspect sepsis and healthy infants. RPR at a cutoff of 0.052 had an AUC of 0.816 for sepsis prediction. In another study, Özer Bekmez *et al.* (2018) demonstrated RPR to be linked to the closure of patent ductus arteriosus and to predict patient evolution and response to therapy. As a matter of importance, across all studies, non-survivors demonstrated higher RPR values than survivors; in light of this, RPR is proposed to be correlated with inflammation severity, organ damage, and mortality. No study has been conducted to date evaluating the performance of RPR as an inflammatory index in horses or other domestic species.

3 **Article**

Red Cell Distribution Width values and Red Cell Distribution Width to Platelet Ratio in Thoroughbred foals in the first 24 hours of life

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Red cell distribution width values and red cell distribution width to platelet ratio in thoroughbred foals in the first 24 hours of life

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ABSTRACT

Objectives: To report red cell distribution width (RDW) values, to calculate RDW to-platelet-ratio (RPR), and to investigate a possible correlation of RDW and RPR index values in neonatal foals classified as healthy or at-risk based on clinical information from a population of foals up to 24 hours of life.

Design: Retrospective study conducted from records and complete cell blood counts (CBCs) of foals born between June and November from 2018 to 2020 foaling seasons.

Setting: Breeding farm.

Animals: Three-hundred nine neonatal full-term Thoroughbred foals.

Interventions: None.

Measurements and main results: Foals were evaluated by a veterinarian within 15 minutes after birth, and blood sample was collected within 24h of life. Based on clinical information, 88 of 309 foals (28.4%) were considered at risk of perinatal disease, and 201 were healthy. Mean gestational age for the foals was 346.31 ± 9.69 days. RDW values did not differ between groups. Gestational length demonstrated to have a negative correlation with RDW (r = -0.156, $P = 0.005$) and MCV (r = -0.135, $P = 0.01$), indicating a link of these variables to foal maturity. RPR index was higher for at-risk (0.073 ± 0.018) than for healthy foals (0.068 ± 0.014, $P = 0.01$).

Conclusion: Red cell distribution width-to-platelet ratio might be a promising early indicator of disease for the field triage of neonatal foals.

Keywords:

equine, red cell distribution width-to-platelet ratio, risk score, biomarker, perinatal disease

Abbreviations:

RDW	Red cell distribution width
RPR	Red cell distribution width-to-platelet ratio
MCV	Mean corpuscular volume

INTRODUCTION

Red blood cell distribution width (RDW) represents a coefficient of variation of the size of circulating erythrocytes.[1] RDW is a readily accessible and inexpensive quantitative measurement of anisocytosis, automatically calculated by most modern hematologic analyzers as part of routine complete cell blood counts.[1,2] The calculation of RDW represents the standard deviation of the mean corpuscular volume (MCV) multiplied by 100 to yield a percentage value.[1] This variable has been used to characterize anemia in human and veterinary medicine.[3–6] A correlation of RDW to cardiovascular conditions in dogs and cats has been demonstrated.[7–12] In human medicine, RDW has been studied as a potential biomarker for sepsis in critically ill adults and newborn patients.[13–16] During the recent global pandemic of COVID-19, RDW has served as a marker of disease severity, prognosis, morbidity and mortality.[17] Higher values of RDW on admission in the ICU have been correlated with poor outcome in humans.[18–20] Elevated RDW in human neonates was observed to have an association with prematurity, intrauterine growth restriction, and severe infection.[14,21–23]

Similarly, the RDW to platelet ratio (RPR) is a novel index with high predictability of inflammatory processes.[24] A high RPR is thought to be a predictor of increased fibrosis, reflection of the severity of systemic inflammation and organ damage, and independent prognostic marker, as non-survival patients had a higher RPR than survivors.[25,26] High RPR values have been shown to correlate with prognosis in adult human patients with liver fibrosis, acute pancreatitis, and septic shock.[24,26,27] RPR has also been proposed to be a useful marker in the diagnosis of patent ductus arteriosus, early onset of sepsis and prediction of mortality in newborn humans. Thus, RPR is considered to be a marker that reflects the status of systemic inflammatory severity and organ damage in vivo. [28]

Sepsis remains the leading cause of death among foals up to 7 days of age.[29] Early recognition, referral and treatment are essential to patient outcome.[29,30] Blood culture is considered the gold

standard for the detection of bacteria in presumed septic foals.[31] Other biomarkers such as lactate, fibrinogen and serum amyloid A have been shown to correlate with perinatal disease in foals.[32,33] Although hyperfibrinogenemia, hyperlactatemia, and elevated concentrations of SAA have been described in foals with systemic illness,[34–36] these parameters can be influenced by a variety of conditions. As a result, the lack of availability of these point-of-care diagnostics in the field in remote areas urge the identification of other, more accessible markers to aid in triage, diagnosis and treatment of neonatal foals.[37]

Currently, there are a limited number of studies evaluating the role of RDW in adult horses.[38–42] A recent publication has reported significantly higher RDW values in diseased Quarter Horses compared to a matched healthy equine population, suggesting that RDW might have clinical application as a potential marker of disease.[41] Neonatal hematological intervals differ significantly from those of adult horses,[43] reflecting physiological changes occurring throughout the last trimester of pregnancy.[44] However, we are not aware of studies specifically evaluating RDW and RPR values in neonatal foals. Therefore, the objectives of this study were to report the values of RDW and RPR and investigate its possible correlation to clinical status of neonatal Thoroughbred foals up to 24 hours of life. We hypothesize that higher RDW and RPR index values might be associated with foals considered to be at risk of development of perinatal disease.

MATERIAL AND METHODS

Animals and Study Design

The study was based on retrospective data records of the parturition of 309 full-term Thoroughbred foals born spontaneously (not induced) between the months of July and November during three foaling seasons (2018 to 2020) from a breeding farm in the Southern hemisphere located at Aceguá – Rio Grande do Sul, Brazil. All deliveries were assisted by a veterinarian, and foals were checked

daily for health and normal development. Physical examination and blood sampling were routinely performed in all the foals after birth as per farm standard of care by experienced personnel. Blood samples were routinely collected around 12 hours of life to evaluate passive transfer of immunity, or earlier if there was clinical indication. Parturition record sheets and CBC results performed in the first 24 hours of life were reviewed for all foals. The study was approved by the blinded for review.

Clinical assessment of the foals

Within 24h after birth, foals had blood collected by jugular venipuncture (2 ml) in a vacutainer tube with 10% EDTA anticoagulant for CBC, using an automatic analyzer[a]. Blood samples were stored at room temperature and analyzed within two hours after the collection. The calculation of RDW to platelet index (RPR) was performed as suggested by Chen *et al.*[24], using the formula RPR = RDW (%) / Platelet count (10^3).

Within 15 min after parturition, foals underwent physical examination as farm routine protocol (data not showed). Foals with a normal physical examination, normal gestational length (320 to 365 days),[36] adaptive responses and reflexes, and CBC were considered healthy.[45] Foals that were born through dystocia, showing delayed adaptative milestones (sternal recumbence > 5 minutes, suckling reflex > 20 minutes, stand >1 hour), gestational length > 365 days, and displayed physical characteristics of dysmaturity (silky hair-coat, domed head, floppy ears, abnormal granulocyte:lymphocyte ratio) as described elsewhere,[46] were classified as at-risk foals.

Statistical Methods

Extracted data were assessed for normality using the Shapiro-Wilk test. The Student T-test was used to evaluate influence of sex and at-risk group on hematology variables (RBC, hematocrit,

hemoglobin, MCV, MCH, MCHC, RDW, platelets, WBC). Correlations among gestational length with RDW and RPR were evaluated by means Pearson's coefficient. Continuous variables were presented as the mean ± SD. Categorical variables were presented as frequencies and percentages. All statistical analysis was conducted in the statistical package R Studio[b] and significance was set at $P < 0.05$.

RESULTS

A total of 309 parturition records and hemograms were reviewed. The study population was composed of 169 fillies and 140 colts. Animals in the at-risk group represented 28.47% (n = 88/309) of the foals, those being 49 (55.68%) fillies and 39 (44.31%) colts. Of the 88 foals in the at-risk group, 5 (5.68%) were born from dystocia, 7 (7.95%) had gestational length > 365 days, 24 (27.27%) had slower righting reflexes, and 64 (72.72%) had abnormal granulocyte:lymphocyte ratio. Eight foals (9.09%) presented more than one risk factor. Mean gestational age for all foals was 346.31 ± 9.69 days. Healthy foals had a shorter gestation than at-risk foals (345.26 days ± 8.3532 and 348.95 days ± 12.092, respectively, $P = 0.009$). A single blood sample per foal was collected between 6 minutes and 24 hours after delivery; CBCs were performed within the first 12 hours of life for 70% of all the foals.

RPR mean value for risk foals was 7.35% higher than observed in healthy foals (0.073 ± 0.018 and 0.068 ± 0.014, respectively, $P = 0.01$, Figure 1). Total WBC and neutrophil:lymphocyte ratio values were also higher in the at-risk group (7.85 ± 2.82, and 13.42 ± 18.81, respectively) than in healthy foals (6.94 ± 2.94, and 2.17 ± 0.85, respectively) ($P < 0.05$). No significant difference in RDW values was observed between the two groups. Comparison of hematological variables is summarized in Table 1.

In our foal population, gestational length was found to be negatively correlated with RDW (r = -0.156, *P* = 0.005). RPR was not correlated with gestational length in both healthy and at-risk foals. No correlation was found between RDW and WBC, and RPR and WBC. Within each one of the groups, RDW, RPR, HCT, HGB and PLT values did not differ between gender.

DISCUSSION

The present study showed that RPR index was higher in at-risk foals, indicating that the calculation of this ratio might be a suitable early marker of perinatal disease for neonatal foals. Platelet count and RDW did not significantly differ between at-risk and healthy foals. Furthermore, RDW had a negative correlation with gestational age. This study evaluated for the first time an association between clinical status (healthy *vs.* at-risk) in neonatal foals up to 24 hours and RDW and RPR. Estimation of a sepsis score in these foals was not possible due to lack of access to medical records. Instead, we used perinatal events and physical and laboratory variables to classify neonatal foals at-risk of developing disease as previously suggested by Koterba.[36] The difference in hematological variables between groups, particularly the WBC and granulocyte:lymphocyte ratio, supports that the foals in the at-risk group had systemic disease. The use of CBC in an automatic analyzer lacks the systematic cytological and differential evaluation of a clinical pathologist, which unquestionably aggregates valuable information about morphologic cellular alterations, degeneration, and their association to systemic processes. Despite this, there was a significant difference in RPR values between healthy foals and those at-risk of developing systemic disease. These findings emphasized the importance and practicality of RPR in the field triage of neonatal foals, particularly in areas with limited access to referral centers and laboratories offering point-of-care tests. More research using data from neonatal foals from different breeds and hospitalized for

diverse reasons is required to investigate the accuracy of RDW and RPR as a reliable early indicator of disease.

The applicability of RPR as a novel indicator of systemic inflammation has been investigated in a variety of medical conditions in humans.[24,25,27,28] Our results support the findings reported for newborn infants diagnosed with early onset of sepsis, as we have encountered similar RPR values for at-risk foals (0.073 ± 0.018) to those described for neonates with suspected early onset of sepsis (0.073 ± 0.035),[23] and ill infants' RPR values were higher than healthy newborns, similar to our foal population.

Differences have been reported when evaluating the correlation of age in neonatal foals erythrogram and leukogram.[43,47] The fetal liver is the main hematopoietic organ for the equine fetus, as the bone marrow has limited activity until the end of gestation.[48] Additionally, it is well stablished that erythrocyte size of fetuses is less uniform than of newborn and adults.[21] Concentration of RBC, hematocrit, and hemoglobin are higher in the first hours of life.[43] Hematocrit values decrease approximately 10% during the first 24h of life, and RBC and HGB reach values similar to adults around two weeks of age.[43] The values of hematological variables reported in our healthy full-term foals were within reference ranges previously described in the literature.[43,47]

The lack of difference in RDW between healthy (16.69 ± 0.74, n = 221) and at-risk foals (16.66 ± 0.69, n = 88) in our study could be explained by the lower number of foals in the at-risk group. A study evaluating RDW in human neonates has demonstrated that these values are negatively correlated to gestational length in all infants and remarkably higher in intrauterine restricted growth and premature neonates when compared to healthy full-term newborns.[21] Higher MCV values have also been correlated to immaturity in neonatal foals born from mares with placentitis.[33] Here, we found that RDW had a negative correlation to gestational age, suggesting a link to foal maturity.

We presume that a stronger correlation might be observed when evaluating the hematology in a larger number of animals, including premature foals. Due to individual and environmental factors, gestational length in horses is known to be a variable.[49] Premature foals (born before 320 days of gestation)[50] commonly have altered metabolic, neurologic and musculoskeletal development, requiring intensive care. [36,50–52]

The mechanism of increased RDW during inflammatory processes is not yet fully elucidated, but it is proposed that an increase in inflammatory mediators such as tumor necrosis factor, interleukin-6, and interleukin-1 have a suppressive effect on both erythropoiesis and red blood cell maturation, as well as a decrease in its half-life, resulting in elevated values.[13] The increased destruction of RBCs during the acute phase inflammatory response is also influenced by the activation of the mononuclear phagocyte system.[53] Furthermore, acute hypoxic insults have been linked to increased variability in erythrocyte size.[54] The platelet role in sepsis, on the other hand, is well established.[55] Platelets aid in and modulate inflammatory and immune responses, in addition to hemostasis and thrombosis.[56] They are rapidly deployed and activated at the site of injury or infection. As a result, they serve as both defenders and signalers.[57] Platelet counts in healthy equine neonates are comparable to or slightly higher than the adult reference range.[43] However, coagulopathy and thrombocytopenia are commonly seen in critically ill foals.[37] Our study population's platelet count remained within the reference range, possibly reflecting the initial state of disease in the at-risk foals group. The authors speculate that the elevated values of RPR in at-risk foals may indicate the probability of increased RDW and decreased platelet count, representing an imbalance in the proportion of these cells in response to the inflammatory process.

The authors recognize the limitations of this study. The study's retrospective nature is a confounding factor. Furthermore, data analysis from a single farm using only Thoroughbreds may not apply to other breeds; thus, a study involving a larger number of foals from different breeds is

needed. As CBC was performed in a single automatic analyzer, with no morphologic evaluation of cells, selection bias cannot be ignored, and conclusions cannot be generalized. Additionally, platelet clumping was not evaluated in these samples, which might impact our study population's results. The at-risk foals evaluated in this study were in early stages of disease, and no information about the mares' health status before and during pregnancy was available to identify foals exposed to chronic processes throughout the gestation. Furthermore, specific disease processes were not fully determined, and short- and long-term follow-up was not performed.

CONCLUSION

The RPR ratio is a low-cost, widely accessible index and easier to measure than traditional point-of-care tests, such as SAA, lactate, and fibrinogen. RPR was higher in neonatal foals at-risk of disease, suggesting that it could be useful to aid the field triage of foals, and rapidly estimate possible systemic disorders. RDW values did not differ between healthy full-term foals and at-risk foals. RDW was found to have an association with gestational in at-risk foals, suggesting a link to foal maturity. Multi-center longitudinal studies with a larger number of healthy and critically ill neonatal foals with a definitive diagnosis are needed to further expand the applicability of RPR in neonatal foals.

Footnotes

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[b]The R Foundation; http://www.r-project.org; version 1.3.1093© 2009-2020

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Table 1. Comparison of hematological variables of the groups (*P* = < 0.05).

Variables	Healthy Foals (n = 221) (mean ± SD)	Risk Foals (n = 88) (mean ± SD)	*P*-value
RBC	9.48 ± 1.34	9.24 ± 1.50	0.15
Hematocrit	39.34 ± 5.43	38.62 ± 7.61	0.40
MCV	42.08 ± 7.77	42.33 ± 2.52	0.66
Hemoglobin, g/dl	14.97 ± 1.73	14.75 ± 2.61	0.47
MCH g/dl	15.72 ± 2.11	15.95 ± 1.06	0.22
MCHC g/dl	37.63 ± 2.49	37.88 ± 1.68	0.54
RDW, %	16.69 ± 0.74	16.66 ± 0.69	0.78
WBC 10^9/L	**6.94 ± 2.94**	**7.85 ± 2.82**	**0.01**
Platelet count, 10^3/L	252.21 ± 42.07	240.93 ± 54.43	0.08
RPR	**0.0676 ± 0.014**	**0.0728 ± 0.018**	**0.01**
Granulocyte:Lymphocyte ratio	**2.17 ± 0.85**	**13.42 ± 18.81**	**< 0.001**

SD: standard deviation; RBC: red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: red cell distribution width; WBC: white blood cell; RPR: red cell distribution width to platelet ratio.

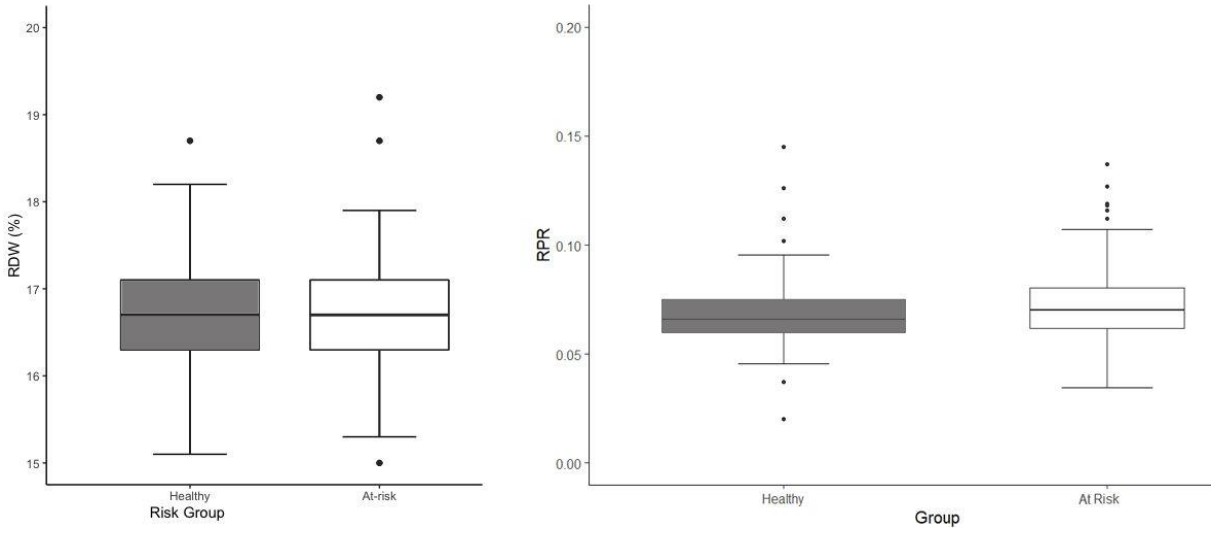

Figure 1. Box-and-whisker plot of RDW and RPR in at-risk (n = 88) and healthy (n = 221) neonatal Thoroughbred foals. Interquartile range and median are represented by horizontal lines. Error bars represent standard deviation, and dots represent individual outlier values.

4 Final considerations

Based on the results of the current study, we present novel information showing evidence that RPR might serve as an indicator of systemic diseases in neonatal foals up to 7 days of age. Using a risk score derived from clinical and obstetrical data allows the field clinician to reliably identify foals with an increased risk of developing systemic diseases. The values of RPR were greater in neonatal foals at-risk of developing perinatal diseases than in healthy foals, indicating a link between RPR and inflammatory response. In support of this, the difference in WBC count indicates that risk foals had a systemic disease. RDW values did not differ between healthy and at-risk foals; nevertheless, RDW was associated with gestational age in the at-risk group, indicating a correlation between RDW values and foal maturity. These findings also emphasize the importance of performing hemograms within the first hours of life to better assess the clinical status of foals and aid in early recognition of the disease process. Adding the RPR index to the CBC can be a useful tool to aid the field triage of neonatal foals and rapidly estimate systemic disorders. We can therefore conclude that RPR might be a suitable early marker of perinatal disease for neonatal foals. The calculation of RPR is simple and inexpensive and does not entail extra costs or additional intervention to the foal. To further expand the applicability of RPR in neonatal foals, multi-center longitudinal studies encompassing a larger number of healthy and critically ill neonates are necessary.

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Appendix

Appendix - Document from the Committee on Ethics and Animal Experimentation (CEFA)

Pelotas, 18 de dezembro de 2010.

De: Prof. Dr. Orlando Antonio Lucca Filho
Pres. da Comissão de Ética e Experimentação Animal (CEEA)

Para: Professores Carlos Eduardo Wayne Nogueira e Bruna da Rosa Curcio
Departamento de Clínicas Veterinária
Faculdade de Veterinária

Senhor Professor:

A CEEA analisou o projeto intitulado: **"Estudo reprodutivo retrospectivo e prospectivo e sua relação com o desenvolvimento gestacional e periodo neonatal em criatórios de eqüinos na região sul,"** processo nº 23110.005810/2010-91, sendo de parecer **FAVORÁVEL** a sua execução considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste Projeto ser cadastrado junto ao Departamento de Pesquisa para posterior registro no COCEPE (Código para Cadastro nº CEEA 5810).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Orlando Antonio Lucca Filho
Presidente da CEEA