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Increased plasma nucleosomes are associated with severe sepsis in foals

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ABSTRACT

Sepsis is a main cause of death in neonatal foals. While the syndrome is not completely understood, sepsis is a dysregulated inflammatory response of the host to infection. It can be difficult to diagnose because of varying and non-specific clinical signs and imperfect diagnostic tests. Increased circulating nucleosome levels have been detected in humans and animals with sepsis, but this has not been documented in foals. Nucleosomes are released extracellularly during neutrophil extracellular trap formation, as well as from damaged and dead cells. We analysed plasma samples from clinically healthy (n = 16), sick non-septic (n = 31) and septic (n = 36) foals using an enzyme-linked immunosorbent assay (ELISA) that targeted nucleosomes. Septic foals with evidence of hypoperfusion and/or organ dysfunction were classified as severe sepsis (n = 24). The main objective was to determine if nucleosome levels were increased in foals with sepsis, particularly those with severe sepsis. Our data identified that nucleosome levels in foals with severe sepsis on the day of study entry were increased significantly compared to all other foals. There was not a significant difference in nucleosome levels between sick non-septic or clinically healthy foals. Foal groups were not age-matched and factors associated with the clinical nature of the study may have affected the results. Further research with larger numbers of foals of similar ages, would be necessary to determine if the analysis of nucleosomes and related biomarkers are helpful adjuncts for the assessment and understanding of equine neonatal sepsis.

Introduction

Sepsis is a primary reason for neonatal foals requiring veterinary treatment, and a main cause of death during the first week of life (Cohen, 1994; Bohlin et al., 2019). Sepsis is defined as a systemic, dysregulated inflammatory response of the host to infection (Sheats, 2019; Blangy-Letheule et al., 2023; Eaton, 2023). However, the syndrome varies across individuals and the pathobiology of sepsis is not fully understood (Singer et al., 2016; López-Martínez et al., 2022). It can be difficult to detect sepsis in neonatal foals because of variable and non-specific clinical signs, and lack of a highly sensitive and specific diagnostic test (Wilkins, 2018; Eaton, 2023).

Nucleosomes consist of DNA and histone complexes residing in the nucleus of cells that are released extracellularly during neutrophil extracellular trap (NET) formation, and with other forms of cell death and damage (Marsman et al., 2016). NETs are web-like structures comprised of DNA strands, histones and other antimicrobial proteins that are produced by neutrophils to capture microorganisms and prevent infection (Goggs et al., 2020). When the NET mechanism is out of balance, due to excessive formation or inadequate clearance, there is an uncontrolled release of proteases and production of reactive oxygen species and resultant tissue damage (Schoen et al., 2022).

Increased circulating nucleosome levels have been identified in samples from other species with sepsis, including in humans (Zeerleder et al., 2003; Chen et al., 2012; de Jong et al., 2014; Kaufman et al., 2017; Duplessis et al., 2018) and in dogs (Letendre and Goggs, 2018b; Goggs et al., 2022). Higher nucleosome levels in humans have been detected in those with severe sepsis (Zeerleder et al., 2003), septic shock (Zeerleder et al., 2003; Beltrán-García et al., 2021), and in non-survivors (Zeerleder et al., 2003; Chen et al., 2012; Zeerleder et al., 2012; de Jong et al., 2014; Kaufman et al., 2017; Duplessis et al., 2018). It is not known if circulating nucleosome levels increase in horses with sepsis, and whether they may be useful for diagnostic and prognostic purposes. Increased plasma nucleosome levels have been identified in adult horses with strangulating and inflammatory gastrointestinal lesions (Bauquier et al., 2016), but not in horses with confirmed sepsis, to the authors' knowledge.

The main objective of this paper was to evaluate nucleosome levels in

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plasma samples from clinically healthy, sick non-septic and septic foals using the Cell Death Detection ELISA^{PLUS}. This assay has been used in numerous NET and nucleosome studies, including in human (Kessenbrock et al., 2009; Chen et al., 2012; Kaufman et al., 2017; Ebrahimi et al., 2018; Kim et al., 2020; Yang et al., 2022), canine (Jeffery et al., 2015; Smith et al., 2017; Lawson et al., 2018; Letendre and Goggs, 2018a; Martiny and Goggs, 2019; Goggs et al., 2022) and equine research (Bauquier et al., 2016; Fingerhut et al., 2022). We hypothesized that median nucleosome levels would be higher in septic foals, particularly those with severe sepsis. Serial analysis of nucleosome levels was performed in plasma samples collected during hospitalisation to determine temporal trends. Plasma nucleosome levels were also normalised to circulating neutrophil numbers to assess if this affected results.

Materials and methods

Foals

Blood samples collected from unwell foals ≤ 35 days of age that presented to the Veterinary Clinical Centre (VCC), Charles Sturt University (CSU) were utilised in this study. The study was exploratory in nature and sample size was dependent on the number of foals coming into the hospital for treatment during the research period. Samples were collected, in August-December 2020 and September to November 2021, by clinical staff for diagnostic and monitoring purposes during hospitalisation, and they were stored for later nucleosome analysis. The aim was to analyse samples collected from unwell foals at 0 h (hospital admission or first assessment for non-hospitalised foals), 12 h later if the foal was very ill, and at 24 h, 48 h, 4 d and 7 d, and then approximately every 3 d afterwards, depending on how long the foal was hospitalised. However, timing of blood collection was not strictly controlled, and was dependent on when foals were admitted and on clinical priorities (i.e., animal requirements, judgment of attending veterinarians). Non-hospitalised foals had single blood samples collected, unless a repeat examination was performed at the VCC. Samples from clinically healthy foals aged ≤ 37 days owned by CSU and from a local stud, were used as controls. Collection of additional blood samples with owner consent was approved by the Animal Care and Ethics Committee, CSU (Approval number, A20250; Approval date 13 August 2020).

Foals were classified retrospectively as septic, sick non-septic or clinically healthy based on analysis of data collected on the day of study entry. A number of classification systems and criteria for sepsis were used to make the diagnosis as robust as possible. Septic foals had to fulfill a minimum of two sepsis classification systems: modified sepsis score ≥ 11 (Brewer and Koterba, 1988; Brewer et al., 1988), neonatal systemic inflammatory response syndrome (NSIRS) with a clinical concern for infection (Wong and Wilkins, 2015) or five clinical or pathologic signs of systemic sepsis (Theelen et al., 2020). Minor modifications were made to the classification systems for the purposes of this study (Appendix: Supplementary Tables S1-S3). Foals meeting one of the sepsis classification systems were defined as septic if they also met one of the following criteria: positive blood culture result (bacterial growth); site of infection in an area that is normally sterile based on a positive microbial culture or the presence of bacteria on cytological or histopathological examination (Wong et al., 2018; Theelen et al., 2020); or serum amyloid A (SAA) > 100 mg/L (Stoneham et al., 2001; Barr and Nieman, 2022). The modified sepsis score was only used to assess foals that were ≤ 12 d old, as it is inaccurate beyond this age (Brewer and Koterba, 1988). Septic foals were further classified into a severe sepsis group based on evidence of tissue hypoperfusion and/or organ dysfunction (Fielding and Magdesian, 2015; Hoeberg et al., 2022) (Appendix: Supplementary Table S4). Sick non-septic foals did not meet the diagnostic threshold for sepsis; they included foals presenting for inadequate nursing, colic, localised infection, and/or diarrhoea. Foals were defined as clinically healthy if they had adequate passive transfer (immunoglobulin G >8 g/L), unremarkable physical examination findings, SAA < 27 mg/L (Stoneham et al., 2001), and did not meet criteria for NSIRS (Wong and Wilkins, 2015). For a more detailed discussion of sick foals and how sepsis classification was used in this study, see Birckhead (2024).

Foals discharged from hospital following a full recovery or receiving ongoing care at home, were defined as survivors. Foals that died naturally, that were euthanased in hospital or those discharged for home euthanasia due to poor prognoses, were all classified as non-survivors. Foals euthanased due to owner financial constraints, those that re-presented to the hospital ≤ 3 d because of ongoing illness, and those that were not admitted to hospital due to subclinical illness were not included in this analysis.

Clinical and laboratory data

Clinical histories, physical examination findings and diagnostic test results were analysed to assess foals for sepsis. These data were obtained from the veterinary practice management software program (VisionVPM; Covetrus) and from the laboratory information management system (SampleManager LIMS 11.2 SPI; Thermo Scientific). Laboratory data from the VCC included immunoglobulin G concentrations (Gamma Check E; Plasvacc) and blood-gas analysis (Gem Premier 4000 or Gem Premier 3500; Werfen). Blood glucose and lactate concentrations were measured using the blood-gas machines (Gem Premier 4000 or Gem Premier 3500; Werfen) and hand analysers (Accutrend Plus; Cobas-Roche). Laboratory data from the Veterinary Diagnostic Laboratory, CSU included complete blood counts (XN-1000 analyser; Sysmex), serum biochemistry (AU480; Beckman-Coulter) including SAA (RUO LZ TEST EIKEN SAA; Eiken Chemical Co.), plasma fibrinogen (STA Compact Max2; Stago) and blood culture (BACTEC Peds Plus™/F culture bottles, BACTEC 9050 automated blood culture system; Becton Dickinson). Wright-Giemsa-stained blood smears (Hematek automated slide stainer; Siemens) were examined by experienced technical officers as part of the haematology analysis to assess for platelet clumps, band neutrophils and toxic change and to perform manual 100-cell differential counts.

Nucleosome analysis

EDTA blood samples were centrifuged at 1500 × *g* for 10 min using the Multifuge X1 Pro (Thermo Fisher Scientific) or Centrifuge 5702 (Eppendorf). Plasma was separated and aliquoted into at least four Eppendorf tubes (Sarstedt) to avoid repeat freezing and thawing. Foal plasma samples were frozen in a median of 1.6 h (range 0.6–3.8) from blood collection, and analysed for nucleosomes after being stored at −80°C (MDF-DU702VX TwinGuard Ultra-Low Temperature Freezer; PHC Cooperation) for a median of 124 d (range 24–453).

The Cell Death Detection ELISA^{PLUS} (Roche Diagnostics GmbH) was performed according to the manufacturer's instructions, and the ELISA was validated for analysis of foal plasma as described (Birckhead, 2024). This included analysing the limit of the blank, assay linearity, intra- and inter-assay imprecision, and stability. Controls provided with the ELISA kit, including DNA-histone complex (positive control) and incubation buffer (background control) were tested with each run. Pooled low nucleosome plasma from foals that were clinically healthy or had minimal illness were also used as plate controls. These samples were collected during the 2020 (6 foals) and 2021 (14 foals) breeding seasons to form two low nucleosome pools (see Birckhead, 2024 for further details). In addition, the nucleosome levels of the 16 clinically healthy foals were analysed individually and used as study controls. Plasma and control samples were analysed in duplicate. The microplate containing samples was put on a shaker until a sufficient colour change had developed, which was approximately 7 min. The optical density was measured at 405 nm and 490 nm using a microplate reader (BioTek ELx800UV; BioTek Instruments) with Gen5 2.07 data analysis software (Biotek Instruments).

Nucleosome levels were compared on the day of study entry. If more

than one sample was collected during the first 24 h of hospitalisation, the first sample with a nucleosome measurement was used, except for foals that clearly fulfilled sepsis criteria at the time of the second sample. Sequential samples from sick non-septic and septic foals collected during hospitalisation were analysed, to assess general trends in nucleosome levels. Changes in nucleosome levels were analysed statistically in foals that had repeat samples collected at 0 h, 24 h and 48 h of hospitalisation. The following equation was used for nucleosome normalisation to circulating neutrophil number:

plasma nucleosome levels/total neutrophil count (segmented and bands).

Statistical analysis

Data were analysed with GraphPad Prism 9.4.1 (GraphPad Software, LLC). A P value < 0.05 was considered statistically significant. The Shapiro-Wilk test was used to determine if the data were normally distributed. Data were not normally distributed and were presented as median (interquartile range or range) and were analysed using non-parametric methods. The chi-square test was used to evaluate for differences in sex of foals in clinically healthy, sick non-septic and septic groups; and between the number of surviving septic and sick non-septic foals. The Kruskal-Wallis test with Dunn's multiple comparisons, was used for the analysis of foal ages and plasma nucleosome levels (including nucleosome levels normalised for circulating neutrophil count) between septic, sick non-septic and clinically healthy foals. The Mann-Whitney test (two-tailed analysis) was used to compare nucleosome levels in surviving and non-surviving septic foals on the day of study entry. Spearman correlation r was calculated to investigate for relationships between plasma nucleosome levels on the day of study entry with time in hospital in surviving foals. The Friedman test and Dunn's multiple comparisons post-hoc test was used for serial analysis of nucleosome levels in plasma samples collected at 0 h, 24 h and 48 h.

Results

Eighty-three foals were enrolled in the study, including 67 unwell foals and 16 clinically healthy foals. Thirty-six foals met the sepsis definition and 31 foals were classified as sick non-septic. The septic foals (median age 1 d, range 0–33) were significantly younger compared to clinically healthy (median age 15 d, range 0–31) and to sick non-septic foals (median age 2 d, range 0–35). There was a higher proportion of male foals in the sick non-septic group compared to the clinically healthy and septic groups (Table 1). The septic group included 24 foals with severe sepsis. Two of 31 sick non-septic foals showed evidence of decreased peripheral perfusion (n = 1) or organ dysfunction (n = 1).

Hospitalisation duration ranged from 0 to 22 d (sick non-septic) and 0–16 d (septic). Survival was 23 of 27 (85 %) for sick non-septic foals, and 22 of 32 (69 %) for septic foals (P = ns). Four sick non-septic foals were excluded from the survival analysis; 3 had subclinical or mild illness and were not admitted to hospital, and in 1 the outcome was affected by financial constraints. Four septic foals were excluded from the survival analysis; in 3 the outcome was affected by financial constraints, and 1 re-presented ≤ 3 d of discharge due to ongoing illness. All 10 non-surviving septic foals were euthanased: 3 during the first 12 h of hospitalisation, 1 at 12–24 h, 4 at 24–48 h, and 2 at 48–72 h.

Nucleosome levels were measured in a median of 2 plasma samples (range 1–7) per sick non-septic foal, and 4 plasma samples (range 1–6) per septic foal collected during hospitalisation. On the day of study entry, there was a significant difference between median nucleosome levels in clinically healthy, sick non-septic and septic foals. Levels were increased the most in septic foals ($P = 0.037$); however, post-hoc analyses were not significant (Fig. 1). When septic foals were classified according to severity, median nucleosome levels increased significantly in foals with severe sepsis (n = 24) compared to non-severe sepsis (n = 12), and to sick non-septic (n = 31) and clinically healthy (n = 16) foal groups (Fig. 2; Appendix: Supplementary Figs. S1-S3). Results

Table 1
Signalment of 83 foals that had plasma nucleosomes levels individually measured using the Cell Death Detection ELISA[PLUS].

Foal details	Clinically healthy (n = 16)	Sick non-septic (n = 31)	Septic (n = 36)
Age (d)[a]	15 (range 0–31)[b]	2 (range 0–35)[c]	1 (range 0–33)
Sex (F/M)	9/7[d]	8/23[e]	17/19
Breed:			
TB	0	13	10
SB	8	9	11
WB	0	3	3
ASH	0	1	3
QH	0	1	0
Appaloosa	0	0	1
Arab	0	0	1
Pony/ crossbreed	8 [f]	4 [g]	7 [h]

ASH, Australian stock horse; F, Female (filly); M, Male (colt); NA, Not applicable; QH, Quarter horse; SB, Standardbred; TB, Thoroughbred; WB, Warmblood.
[a] Age at hospital admission, or first assessment for non-hospitalised foals.
[b] Comparison of the median age of clinically healthy and septic foals (P = <0.001).
[c] Comparison of the median age of sick non-septic and septic foals ($P = 0.040$).
[d] Comparison of sex of clinically healthy and sick non-septic foals ($P = 0.040$).
[e] Comparison of sex of sick non-septic foals and septic foals ($P = 0.071$).
[f] Connemara cross TB (n = 7), Connemara cross unknown (n = 1).
[g] Connemara cross TB (n = 3), Riding pony (n = 1).
[h] Connemara (n = 1), Riding pony cross (n = 1), Anglo Arab (n = 1), ASH cross (n = 1), Polo pony (n = 1), Roughstock (n = 1), Miniature Shetland pony (n = 1).
Figure legends

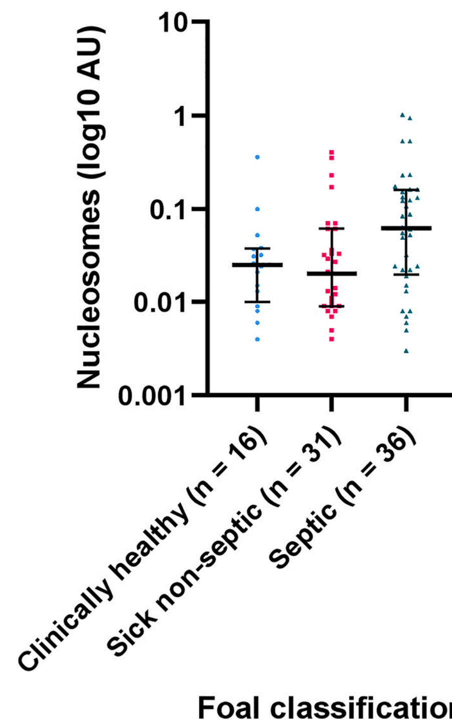

Fig. 1. Nucleosome levels in plasma samples from clinically healthy, sick non-septic and septic foals on the day of study entry. Median nucleosome levels differed significantly between the foal groups ($P = 0.037$); however, post-hoc analyses were not significant. Nucleosome data are expressed as log10-transformed arbitrary units (AU) and the medians and interquartile ranges are depicted as solid lines. The circles, squares and triangles represent individual nucleosome measurements in clinical healthy, sick non-septic, and septic foals, respectively.

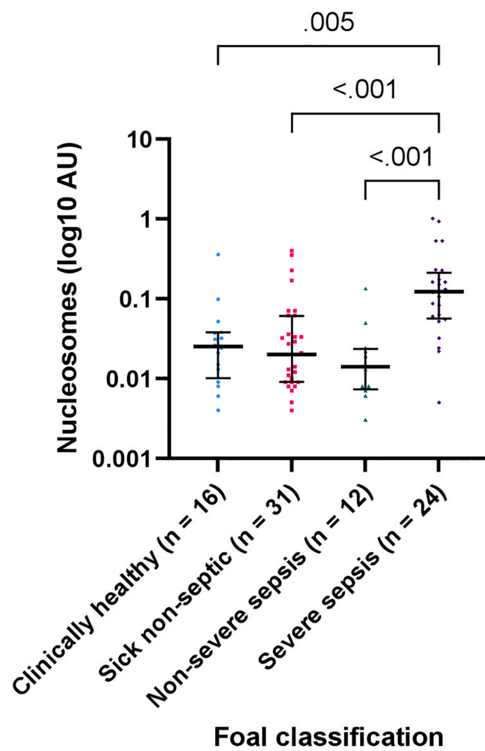

Fig. 2. Nucleosome levels in plasma samples from foals on the day of study entry, with separation of septic foals based on severity. Significant differences in median nucleosome levels were detected in foals with severe sepsis compared to all the other groups. There were no significant differences when the other foal groups were compared to each other. Nucleosome data are expressed as log10-transformed arbitrary units (AU) and the medians and interquartile ranges are depicted as solid lines. The circles, squares, triangles, and diamond shaped boxes represent individual nucleosome measurements in clinical healthy, sick non-septic, non-severe sepsis, and severe sepsis foals, respectively.

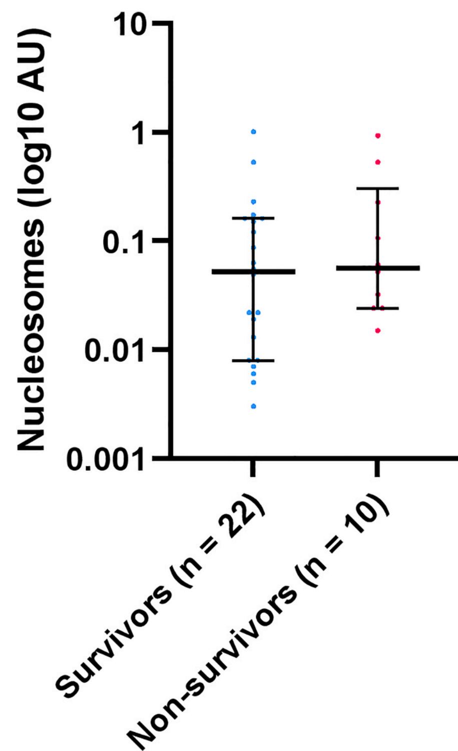

Fig. 3. Median plasma nucleosome levels did not differ significantly in surviving and non-surviving septic foals on the day of study entry. Nucleosome data are expressed as log10-transformed arbitrary units (AU) and the medians and interquartile ranges are depicted as solid lines. The circles represent individual nucleosome measurements in septic foals.

remained statistically significant when nucleosome levels were normalised to circulating neutrophil numbers (Appendix: Supplementary Fig. S4). Median plasma nucleosome levels on the day of study entry, were similar in both surviving and non-surviving septic foals and did not differ significantly (Fig. 3). Median plasma nucleosomes on the day of study entry did not significantly correlate with length of time in hospital in surviving foals.

Changes in nucleosome levels during hospitalisation were compared for 18 sick non-septic foals and 30 (9 non-severe and 21 severe) septic foals. All foals had a minimum of two plasma samples collected $\geq$ 12–24 h apart. Nucleosome measurements tended to be highest during the first 24 h of hospitalisation, which was most evident in foals with severe sepsis (Fig. 4). There were 11 sick non-septic and 14 septic foals that had nucleosomes levels measured in repeat samples collected at 0 h, 24 h and 48 h of hospitalisation. Nucleosome levels in the septic foals were significantly decreased at 48 h compared to samples collected at 0 h and 24 h. The difference in nucleosome levels between 0 h and 24 h was not significant (Fig. 5). Results were similar when nucleosome levels were normalised to circulating neutrophil numbers (Appendix: Supplementary Fig. S5). There were no significant differences in nucleosomes levels in the sick non-septic foals at these timepoints (Appendix: Supplementary Fig. S6).

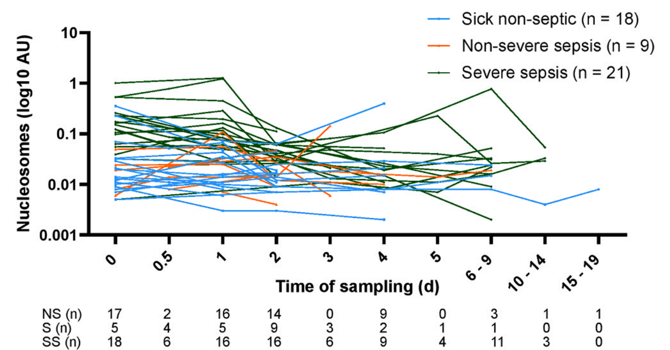

Fig. 4. Changes in nucleosome measurements in sick non-septic and septic foals in plasma samples collected during hospitalisation, from admission (0 d) and up to 19 d post-admission. Septic foals have been subclassified based on severity. Nucleosome data are expressed as log10-transformed arbitrary units (AU). The squares, triangles and diamond shaped boxes represent individual nucleosome levels in sick non-septic, non-severe sepsis, and severe sepsis foals, respectively. The connecting lines depict repeat nucleosome measurements over time. Below the x-axis: NS (n) = number of sick non-septic foals that had samples at the specified time points; s (n) = number of non-severe sepsis foals that had samples at the specified time points; SS (n) = number of severe sepsis foals that had samples at the specified time points.

Discussion

This study aimed to assess whether plasma nucleosome levels would be increased in septic compared to sick non-septic and clinically healthy foals on the day of study entry, and whether there was an association with severity of sepsis. Plasma nucleosome levels did not significantly differ in septic foals generally. However, there appeared to be an association with sepsis severity.

The nucleosome results in foals with severe sepsis are similar to human studies where nucleosome levels are highest in patients with more advanced sepsis or concurrent complications (Zeerleder et al., 2003; Patel et al., 2019; Beltrán-García et al., 2021), and correlated with severity of immune response as well as organ dysfunction (Chen et al.,

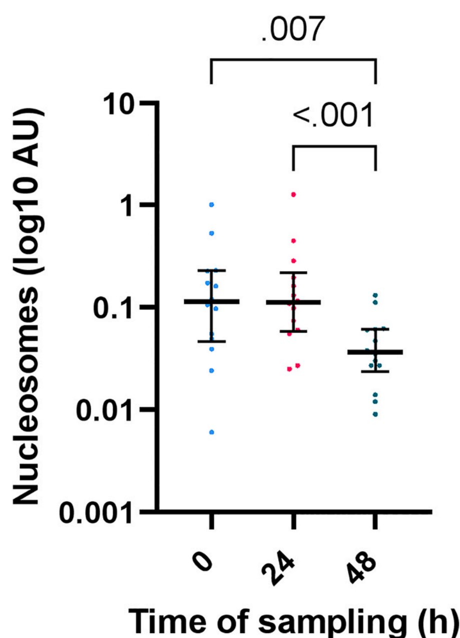

Fig. 5. Repeat analysis of plasma nucleosome levels in 14 septic foals in samples collected at hospital admission (0 h), 24 h and 48 h. Plasma nucleosome levels decreased significantly in the 48 h samples compared to both the 0 h and 24 h samples. Nucleosome levels did not differ significantly in the 0 h and 24 h samples. Nucleosome data are expressed as log10-transformed arbitrary units (AU) and the medians and interquartile ranges are depicted as solid lines. The circles represent individual nucleosome measurements in septic foals.

2012). Circulating nucleosomes have a short half-life (Holdenrieder and Stieber, 2009); most are rapidly metabolised by the liver, with 71–84.7 % of nucleosomes eliminated in 10 min in a murine model (Gauthier et al., 1996). Therefore, increases are only detected when the clearance capacity of the liver is exceeded due to severe illness (Iba et al., 2014). Elimination may also be delayed if nucleosomes are bound by acute phase proteins (Holdenrieder and Stieber, 2009), or if there is liver dysfunction (Yehya et al., 2021). With a mild insult, circulating nucleosome levels would be expected to remain low (Iba et al., 2014).

In this study, septic foals with high plasma nucleosomes had increased levels at hospital admission. In human studies, elevations in nucleosome levels have also been detected in newly admitted patients or on the day of sepsis onset (Chen et al., 2012; Miki and Iba, 2015). Nucleosome levels in septic foals tended to remain increased for the first 24 h, and to decrease by 48 h of hospitalisation. This probably reflected improvements in clinical condition in response to treatment. However, it was during this same time period when the majority (8 of 10) of non-surviving foals with sepsis were euthanased due to poor prognoses, including two foals with high nucleosome levels.

Higher nucleosome levels in a subset of septic foals may have been due to increased NET release. However, nucleosome normalisation to neutrophil numbers had a minimal impact on results, suggesting that other factors were contributing to increased nucleosome levels, rather than circulating neutrophils alone. During severe systemic inflammation, release of nucleosomes from multiple sources is more likely (van der Meer et al., 2019). Nucleosomes are not specific for NET release and can originate from various damaged or dying cells and tissues (Marsman et al., 2016; Goggs et al., 2020). NETs themselves may induce apoptosis or necrosis of tissue cells, including endothelium (Gupta et al., 2010; Saffarzadeh et al., 2012; Sun et al., 2021), and create a positive feedback loop with further increases in nucleosomes correlating with disease progression (Beltrán-García et al., 2021). Injury to endothelium has been associated with sepsis severity (Zhang et al., 2023; Gomez et al., 2024). Damaged muscle may also have contributed to increased nucleosome levels. A study established a strong correlation between plasma nucleosome and creatine kinase levels in rats after experimental induction of trauma. This was thought to be due to passive release from damaged parenchymal cells rather than from NET release (Hayakwa et al., 2020). Decreased clearance of circulating nucleosomes could be another explanation for increased levels in foals. Hepatic metabolism is immature in neonatal foals (Castagnetti and Mariella, 2015) and circulating nucleosomes may be cleared more slowly. Reduced renal elimination of nucleosomes could also be a factor (Lichtenstein et al., 2001; Phan et al., 2018).

Median plasma nucleosomes were not increased significantly in non-surviving compared to surviving septic foals and did not correlate with hospitalisation duration. Some studies have detected increased nucleosome levels in non-surviving septic human patients (Zeerleder et al., 2012; de Jong et al., 2014; Kaufman et al., 2017); in other studies, nucleosome levels did not differ in surviving and non-surviving septic human patients (Miki and Iba, 2015) or in canine patients (Letendre and Goggs, 2018b). Differing causes of sepsis, other complications, differences in treatment, and individual immune responses may all contribute to variations in nucleosome levels. The small number of non-surviving septic foals in this study limited the analysis, and a difference may have been seen if larger numbers of foals were analysed. Another limiting factor in this study was that all non-surviving septic foals were euthanased because of poor prognoses; none died naturally as a result of sepsis. This leaves untested the possibility that nucleosome levels may have increased with further deterioration to the point of death. There is also the possibility that foals classified as survivors may have developed complications related to their primary problems or died after hospital discharge.

Differences in plasma nucleosome levels in sick and septic foals have not previously been reported to the authors' knowledge. Cell-free DNA (cfDNA), another marker that increases with NET release and cell death, has been measured in septic foal plasma samples. Its concentrations did not differ significantly between septic, sick non-septic and healthy foals; although, sepsis was not subclassified based on severity (Colmer et al., 2021; Hobbs et al., 2024). Nucleosomes are also made up of DNA and can be derived from the same sources as cfDNA (Goggs et al., 2020). Despite their similarities, nucleosomes are a separate chromatin component to double stranded DNA and can activate the immune system in different ways (Marsman et al., 2016). In a canine study, plasma nucleosomes and cfDNA concentrations only had a limited association (Letendre and Goggs, 2018b). The increased susceptibility of free DNA to digestion by nucleases compared to nucleosomes, could also contribute to these differences (Holdenrieder and Stieber, 2009).

Limitations of the study included variations in timing of data collection, small sample sizes, age and sex differences between foal groups and only using one NET marker. This was a clinical study and all unwell foals were being treated at the VCC and clinical requirements were prioritised over the research, which may have introduced biases such as more frequent sampling and monitoring of critically unwell foals. Foals came from the general region and with differing clinical histories. Age of unwell foals was dependent on when foals arrived at the hospital, and septic foals were found to be significantly younger than clinically healthy and sick non-septic foals. Because clinically healthy foals were not hospitalised, blood collection from these foals was largely based on convenience. Storage of plasma at −80°C for batch analysis was necessary to minimise artificial increases or decreases of nucleosomes (Holdenrieder et al., 2005) whilst sufficient sample numbers were obtained for analysis. Nucleosome concentrations are known to decline with time in human samples, reportedly by about 7 % each year in serum stored at −70°C (Holdenrieder et al., 2010). Therefore, mild false decreases in nucleosome levels in older foal plasma samples may have occurred. To the authors' knowledge, the effects of freezing and storage on nucleosome levels in foals have not been reported. There were also limitations associated with the ELISA. Results are semiquantitative as there are no nucleosome standards provided with the assay (Bauquier et al., 2016). Each ELISA kit came with a 96 well-plate, limiting the

number of samples that could be analysed at once. This potentially introducing a degree of variation associated with using each new kit.

The source of the nucleosomes could not be identified in this study, so it was uncertain if NET release was a contributing factor in foals with increased levels. This could have been explored further by measuring markers of neutrophil activation (de Jong et al., 2014; van der Meer et al., 2019) or other markers of NET release, such as citrullinated histone H3 (Goggs et al., 2020; Thålin et al., 2020). Direct visualisation of NET release, such as using immunofluorescence (Brinkmann et al., 2016; Yang et al., 2017; Birckhead et al., 2023), would be the most confirmatory (Cox et al., 2020). It would be interesting also to compare plasma nucleosome levels in foals with severe non-septic illness and severe sepsis, to determine if increased levels are associated with disease severity or with sepsis. Studies into the effect of age on nucleosome levels in healthy and unwell foals would help clarify whether increases are due to physiological or disease factors.

Conclusions

This study is the first one to detect circulating nucleosomes in foal plasma. It found that a small number of foals with severe sepsis had increased plasma nucleosome levels, which may have reflected increased NET release or other forms of cell death. However, there was a large degree of overlap in nucleosome levels in septic and sick non-septic foals. Research involving larger numbers of foals studied over time could elucidate further the involvement of nucleosomes in sepsis.

CRediT authorship contribution statement

S. L. Raidal: Writing – review & editing, Supervision, Methodology, Formal analysis. **Emily M Birckhead:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **S. R. Raidal:** Writing – review & editing, Visualization, Supervision, Project administration, Conceptualization. **S. Das:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106297.

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