Thiobarbituric Reactive Substances as a marker of oxidative stress to detect sepsis and predict outcome in hospitalized neonatal foals: a multicenter study

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Abstract

Background: Sepsis is diagnosed frequently in ill neonatal foals and therapy is often intensive. In human neonates it has been observed that oxidative stress level is associated with patient survival and several diseases including sepsis, however this has not been investigated on a large scale in hospitalized neonatal foals. One way of evaluating oxidative stress is by measuring Thiobarbituric Acid Reactive Substances (TBARS).

Objectives: To compare TBARS concentration in septic and sick non-septic neonatal foals in relation to survival.

Animals: 129 sick neonatal foals admitted to four different equine clinics and 16 healthy foals were included in the study.

Type of study: Retrospective multicenter study

Methods: Heparin plasma was collected from healthy foals within 24 hours after birth and from hospitalized foals <14 days old at day 1 (n=84), day 2 (n=88) and day 3 (n=42) of hospital admission. TBARS concentrations were measured using a specific assay and compared between septic and sick non-septic foals and surviving and non-surviving foals. Differences between sample day and length of hospitalization were also analyzed. Comparisons were performed using ANOVA followed by Tukey's post-hoc test for multiple comparisons.

Results: No differences in TBARS concentration were found between septic and sick non-septic foals or surviving and non-surviving foals. A non-significant trend in association between TBARS concentration and sample day was visible, with higher values at day 1 compared to day 2 and day 3. Furthermore, TBARS concentration at hospital admission seemed to be associated with length of hospitalization, although non-significant.

Conclusions: TBARS concentrations are not different between septic and sick non-septic foals or surviving and non-surviving foals. A trend was seen in correlations between sample day and length of hospitalization. Additional studies evaluating other oxidative stress parameters such as antioxidants are warranted to assess which hospitalized foals have increased oxidative stress.

1. Introduction

Every year, a considerable amount of sick neonatal foals is presented to equine hospitals for various diseases, which might result in admission to intensive care units. These conditions include, but are not limited to, sepsis, prematurity and neonatal maladjustment syndrome (also referred to as "dummy foals", perinatal asphyxia syndrome (PAS) and hypoxic ischemic encephalopathy) [1]. Since therapy is often both intensive and expensive, research regarding diagnosis, prognosis and therapy is needed. The field of oxidative stress in equine neonates is

a promising subject, since oxidative stress is associated with inflammation, sepsis, prematurity and hypoxic ischemic encephalopathy in newborn human babies [2–5].

Oxidative stress reflects a state in which there is an excess of free radicals or reactive oxygen species (ROS). This is caused by an imbalance between the production or accumulation of ROS and antioxidant defenses. Excess of ROS damages proteins and DNA and causes lipid peroxidation, thereby damaging membranes and threatening cell function. Oxidative stress is prevalent in healthy neonates, because after birth the newborn is exposed to relatively hyperoxic conditions compared to intra-uterine conditions and they still have an immature antioxidant system [6].

In equine medicine, several studies that focus on oxidative stress in healthy and sick neonates have been performed recently. The course of various antioxidants in the first days after birth of healthy foals are well described, among which the enzymatic antioxidants superoxidase dismutase (SOD) and glutathione peroxidase (GPx) and non-enzymatic antioxidants bilirubin and ascorbic acid [7–9]. One study examined levels of the antioxidant ascorbic acid in septic foals and non-septic foals, which demonstrated that septic foals have lower levels of ascorbic acid than non-septic foals [10]. Other research found no evidence of oxidative stress in sick foals, which was evaluated using antioxidants selenium and vitamin E, and GPx as a marker for oxidative stress [11]. However, it must be stressed that this study only consisted of fourteen sick foals with diverse diagnoses [11]. Another study suggested that oxidative stress might relate to the respiratory health status of foals, since H_2O_2 concentrations in exhaled breath condensates and advanced oxidation protein products (AOPP) in blood were elevated, while thiol antioxidant barrier was decreased in a foal with upper respiratory tract infection [12].

One marker of oxidative stress is thiobarbituric acid reactive substances (TBARS)[7]. TBARS like malondialdehyde (MDA) are a byproduct of lipid peroxidation, which process is initiated by reactive oxygen species[7]. Because TBARS are a byproduct of lipid peroxidation, it (indirectly) represents oxidative damage more closely than the levels of antioxidants, because the latter only shows capacity to deal with oxidative stress.

In neonatal foals, TBARS concentration is only described in healthy individuals [7]. TBARS concentration was high at 5 minutes after birth in healthy neonatal foals and stabilized from 12 to 168 hours of age, while the antioxidant systems (SOD, GPx, bilirubin and ascorbid acid) were activated [7]. This resulted in the conclusion that healthy newborn foals have a dynamic and pro-oxidant state, meaning that there are initially not enough antioxidants available to neutralize the oxidants [7]. Activation of antioxidant systems is necessary to prevent excessive oxidative damage and thereby prevent failure of cell function. More studies have been performed regarding TBARS concentrations in human neonates. It has been shown that TBARS concentrations is independently associated with neonatal sepsis in infants, with higher TBARS concentration in septic neonates then control infants [4,13]. The TBARS assay was also performed on serum samples of human neonates with hypoxic-ischaemic encephalopathy (HIE), which showed that TBARS concentration correlates with severity of HIE [14,15]. In addition, TBARS correlated with mortality in human neonates diagnosed with HIE [14,15]. TBARS was higher in preterm human neonates then term infants at birth and four days after birth [16].

The objective of the present study was to characterize oxidative stress by measuring TBARS concentration in hospitalized neonatal foals and to evaluate whether TBARS can be correlated to type of disease and patient outcome. It was hypothesized that, similar to human research, foals with sepsis and non survivors show higher levels of TBARS compared to healthy neonates.

2. Materials and methods

2.1 Study design

The study was designed as a multicentric, retrospective study performed with data collected from the 2022 breeding season at 5 equine hospitals in western Europe. Clinical data was retrieved from data records and only excess plasma from blood samples collected for clinically relevant tests were used for analysis.

2.2 Animals

Sick foals - 129 sick foals (all < 14 days of age) presented to four different specialized equine clinics (Veterinary Centre Someren (NL), Equine veterinary teaching hospital of Utrecht University (NL), Equine Clinic Meslay du Maine (FR) and Rossdales Equine Hospital (UK)) were included in this study. For each case, clinical data on age, normality parturition, rectal temperature, scleral injection, petechial hemorrhage, anterior uveitis, diarrhea, respiratory distress, neurological signs, joint swelling, diagnosis and outcome (survivor/non survivor) were collected retrospectively from data records as well as the following test results: white blood cell (WBC) count, IgG concentration, serum amyloid A (SAA) concentration, plasma lactate concentration, blood culture, the presence of band neutrophils, the morphology of neutrophils, fibrinogen concentration, glucose concentration, pH, PaCO2, $HCO3^-$.

Healthy foals - Healthy foals were included in this research as a control group. Foals could be included if they had a normal physical examination as evaluated by an equine veterinarian, had evidence of adequate passive transfer of immunity measured by a serum immunoglobulin G (IgG) concentration >800 mg/dL and if a blood sample was collected within 36 hours after birth. Sixteen warmblood foals were included in this group, of which twelve foals were born in a clinic located in Emmeloord, the Netherlands and four healthy foals were admitted at the clinic of Utrecht University, the Netherlands, due to hospitalization of their dam.

2.3 Groups

Sick foals were divided into two groups based on their clinical condition: septic and sick non-septic (SNS) groups. A foal was included in the septic group if it fulfilled one of the following criteria: (1) positive blood culture on day 1, 2 or 3, (2) more than one site of infection or (3) post mortem evidence of more than one septic processes [17]. If a foal did not fulfill the criteria above, the foal would be classified as SNS. Foals were divided according to outcome into survivors and non-survivors. Foals discharged from the hospital were considered survivors and non-survivors either died of were euthanized during hospitalization.

2.4 Sample collection

Blood samples (1-2 ml) of the healthy foals were collected within 36 hours of age. Blood samples of the hospitalized foals (n=129) were taken at hospital admission (n=86), day 2 (n=89) and day 3 (n=45). Samples were collected by jugular or cephalic venipuncture using a syringe with 23G needle into tubes containing lithium heparin as anti-coagulant and were centrifuged for 15 minutes at 1000x g within 60 minutes of sampling. Plasma was retained and stored at -20 °C until further analysis.

2.5 Thiobarbituric Acid Reactive Substances assay

A commercially available TBARS assay kit (R&D Systems, Minnesota, USA) was used to measure TBARS concentration. Plasma samples were thawed at room temperature (RT). 300 µL of plasma was added to 300 µL of TBARS Acid Reagent and mixed using a pipette tip. After 15 minutes of incubation at RT, the mixture was centrifuged at 12.000g for 4 minutes, after which

supernatant is removed. A standard dilution series was made by diluting TBARS Standard of 167 µM into a series ranging from 16.7 µM to 0.26 µM, using deionized water. 150 µL of acidified sample or standard dilution was pipetted in duplicate into a flat transparent 96-wells plate and 75 µL of TBA reagent was added to each well. Optical density was pre-read with spectrophotometry at 532 nm using a microplate reader (FLUOstar Omega, BMG Labtech). The plate was incubated at 45-50 ºC. After 2-3 hours, optical density was evaluated again using the microplate reader at 532 nm. Optical densities prior to incubation were subtracted by optical densities post incubation and duplicate readings were averaged. The intensity of the color corresponds to the amount of TBARS and a standard curve was created with the optical densities of the standard dilution series.

A pilot study was performed one year earlier with the same commercially available kit prior to this study to evaluate efficacy, accuracy and feasibility. For the pilot, 19 samples from neonatal foals of the 2021 breeding season were used and 12 samples from healthy adult horses. The standard curve created was similar to the typical data of the manufacturer and repeated measurements of the samples demonstrated that the median intra-assay difference was 0.06 µM and the median inter-assay difference was 0.07 µM. Therefore we concluded that this kit was suitable for this study.

2.6 Statistical analysis

A commercial statistics software program (Graph Pad Prism version 9, GraphPad Software, California) was used. A power analysis based on a previous study regarding sepsis in human neonates suggested that 7 foals per group afforded satisfactory power (>0.8, α=.05) to discriminate differences in TBARS (mean group 1 = 10 nmol/mg; mean group 2 = 4.2 nmol/mg; SD of 3.0 nmol/mg and sampling ratio of 40/80) [4]. A data point was considered an outlier if the Z-score exceeded 3 and was removed from the analysis. The difference in TBARS concentration between sampling day, disease group (sepsis, SNS, healthy), survivors vs non-survivors and length of hospital day was evaluated using an ANOVA-analysis with Tukey post hoc test for multiple comparisons. Pearson´s correlation coefficient was used to determine correlations between TBARS and blood parameters lactate, pH, pCO_2, SAA, WBC, HCO_3^- and glucose. Lactate was expressed as mean ± SD and differences between lactate and patient outcome and diagnosis were assessed using an unpaired T-test. The results of TBARS concentration are expressed in mean ± SEM and $p<0.05$ was considered statistically significant.

3. Results

3.1 Study population

Six TBARS values from the sick foals were identified as outliers and therefore removed from the data analysis, leading to 84 samples on day 1, 88 samples on day 2 and 42 samples on day 3. The percentage of foals that was categorized as septic or SNS and survivor or non-survivor is shown in **table 1.** The median age at admission was 24 hours and the mean age at admission

Table 1 *Study population divided in survivors and non-survivors and septic and sick non-septic (SNS) foals*

	Sample day 1	**Sample day 2**	**Sample day 3**
	(n=84)	(n=88)	(n=42)
Outcome			
Survivor	76%	83%	88%
Non-survivor	24%	17%	12%
Diagnosis			
Septic	24%	23%	29%
Sick non-septic	76%	77%	71%

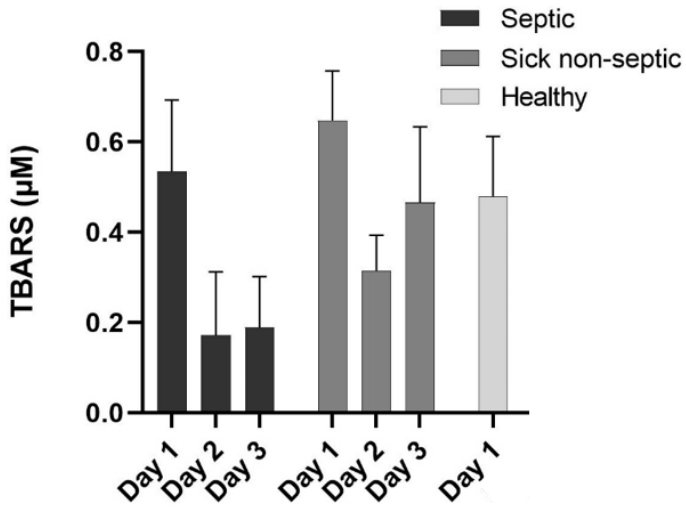

Figure 1 *TBARS in septic (n=52), sick non-septic (n=168) and healthy controls (n=17) on hospitalization day 1, 2 and 3. Results are expressed as mean +- SEM.*

was 91 ± 153 hours. The mean length of hospital stay was 5 days. Warmbloods, Thoroughbreds, Standardbreds, draft horses, coldblooded horses and mixed breeds were represented in this population and the majority (46%) of the foals belonged to the Dutch warmblood studbook 'KWPN'.

3.2 TBARS in septic and sick non-septic foals

TBARS concentrations in healthy foals and of septic and SNS foals at day 1, 2 and 3 of hospital admission are presented in **figure 1**. No significant differences were found between septic, SNS and healthy foals. Although not statistically significant, TBARS concentration in septic foals was higher in samples from day 1 (0.53 ± 0.16 µM) compared to day 2 (0.17 ± 0.14 µM, P = 0.072) and 3 (0.19 ± 0.11 µM, P = 0.797). This observation is most clear in septic foals, but also visible in SNS foals, where TBARS concentration at day 1 (0.65 ± 0.11 µM) was higher than day 2 (0.31 ± 0.08 µM, P = 0.072) and day 3 (0.47 ± 0.17 µM, P= 0.797).

3.3 TBARS in surviving and non-surviving foals

TBARS concentration in surviving and non-surviving foals at day 1, 2 and 3 of hospital admission are shown in **figure 2**. No significant differences were found, however, samples taken from surviving foals on day 1 (0.63 ± 0.11 µM) have a trend to higher TBARS concentrations compared to day 2 (0.27 ± 0.07 µM, P=0.074) and 3 (0.39 ± 0.14 µM, P=0.662). The same trend is visible in non-surviving foals, were day 1 (0.57 ± 0.18 µM) is higher than day 2 (0.32 ± 0.20 µM, P=0.937) and 3 (0.35 ± 0.29 µM, P=0.992). There were no differences between surviving and non-surviving foals.

3.4 TBARS in relation to length of hospital stay

The relationship between TBARS at hospital admission (day 1) and length of hospital stay of surviving foals is shown in **figure 3**. Although no statistical differences were found, a trend towards a higher TBARS concentration in longer hospital stays is visible. Foals that only stayed in the hospital for 0-2 days had a mean TBARS concentration of 0.41 ± 0.19 µM, which is lower

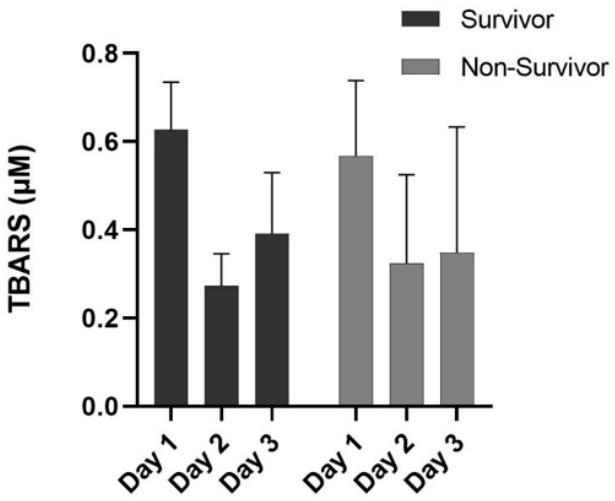

Figure 2 *TBARS in samples of hospital day 1, 2 and 3 in relation to outcome (survivor (n=183) or non-survivor (n=41)) of hospitalized neonatal foals. Results are expressed as mean +- SEM.*

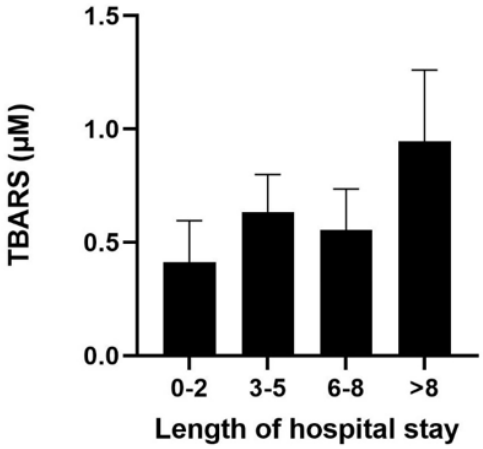

Figure 3 *TBARS of hospital day 1 in surviving hospitalized foals (n=64) in relation to the length of hospital stay. Results are expressed as mean +- SEM.*

than TBARS of foals that stayed in the hospital for 3-5 days (0.63 ± 0.17 µM, P=0.924), 6-8 days (0.55 ± 0.19 µM, P=0.984) or more than 8 days (0.95 ± 0.32 µM, P=0.520).

3.5 Correlations between TBARS and blood parameters

No correlations were found between TBARS concentration and plasma lactate concentration (r=0.105), pH (r=-0.160), pCO2 (r=0.112), SAA (r=-0,082) and WBC (r=-0,031), HCO_3^- (r=-0,145), glucose (r=-0,090).

3.6 Lactate concentrations in relation to survival and sepsis

Plasma lactate concentration of the hospitalized foals ranged from 0.33 mmol/L to 13.6 mmol/L. Mean lactate concentrations between surviving and non-surviving foals and septic and SNS foals were compared, which is shown in **table 2**. Here it is demonstrated that surviving foals had statistically significant lower plasma lactate levels than non-surviving foals on day 1 and day 2. There were no differences found in plasma lactate levels between septic and SNS foals.

Table 2 *Lactate concentration (mean ± SD) of hospitalized neonatal foals by sample day, outcome (survivor/nonsurvivor) and diagnosis (septic or sick non-septic (SNS)).*

Sample day	Outcome	Mean lactate (mmol/L)	P-value (outcome)	Diagnosis	Median lactate (mmol/L)	P-value (diagnosis)
Day 1	Survivor (n= 57)	3.3 ± 2.7	<0.0001	Septic (n=17)	3.9 ± 3.1	0.716
	Non-survivor (n=18)	6.9 ± 4.2		SNS (n=58)	4.2 ± 3.6	
Day 2	Survivor (n=70)	2.4 ± 1.4	<0.0001	Septic (n=19)	2.8 ± 1.3	0.891
	Non-survivor (n=10)	5.5 ± 3.8		SNS (n=61)	2.9 ± 2.5	
Day 3	Survivor (n=29)	2.0 ± 1.3	0.928	Septic (n=12)	1.9 ± 0.95	0.706
	Non-survivor (n=4)	2.1 ± 0.8		SNS (n=21)	2.1 ± 1.4	

4. Discussion

To the best of our knowledge, this is the first multicenter study to examine oxidative stress in hospitalized neonatal foals using TBARS. Despite our relatively large sample size of ill foals, no correlations were found between septic, sick non-septic foals and healthy foals, or between surviving and non-surviving foals. These results are surprising, since human literature shows marked differences in TBARS between sick and healthy neonatal babies [4,13–16]. It has been demonstrated previously that septic foals have lower levels of antioxidants than non-septic foals, suggesting that TBARS concentrations might also differ [10]. However, the results of the present study are in line with Furr et al. (2012), who also did not find a difference between sick and healthy neonatal foals. But, it must be noted that this research used 3-nitrotyrosine instead of TBARS as a marker of oxidative stress and the study population consisted of 15 healthy foals and only 14 sick foals with various diseases, ranging from sepsis to meconium impaction [11].

It could be argued whether our population and/or study design is suitable to assess differences in oxidative stress between septic, non-septic and healthy foals, as well as between surviving and non-surviving foals. For example, our healthy control group consisted of only 16 foals and only one sample per foal was available. Furthermore, this study population consisted of only 24% (31/129) septic foals, while the study population of Wong et al. (2021) and Furr et al. (2012) had respectively 52% (14/27) and 35% (5/14) septic foals. Plasma lactate concentration has been shown to differ between surviving and non-surviving critically ill foals [18]. In the present study this difference was also demonstrated. This suggests that our study population is representative for a population of hospitalized neonatal foals.

Inclusion criteria for sepsis in foals differ between studies [19]. A positive blood culture is in many studies considered an inclusion criteria for sepsis, although the results of a blood culture can be false negative due to low numbers of circulating bacteria or low volume of blood for culture[20,21]. In the present study, foals were labeled as septic if they had (1) a positive blood culture on day 1, 2 or 3, (2) more than one site of infection or (3) post mortem evidence of more than one septic process. A blood culture was not carried-out routinely in two of the participating clinics and post-mortem exam was also not performed regularly. This could have attributed to an underestimation of the number of septic foals and some foals may have been falsely classified as sick-non-septic. Several other sepsis scoring systems have been described, which include clinical and blood parameters [19,22]. A scoring system for sepsis was not used in this study because various parameters were unknown in part of the study population. It is therefore important to note that different inclusion criteria for sepsis could lead to different results.

It can be questioned whether TBARS concentration is the appropriate parameter to measure oxidative stress in foals. The TBARS assay measures a byproduct of lipid peroxidation and lipid peroxidation has been described in neonatal foals previously [7]. However, in the present study, the TBARS assay failed to demonstrate the differences between septic, SNS and healthy foals that were expected based on human and equine studies [4,10]. The absence of variation could be explained by a questionable technical validity of our test. It was remarkable that there were several extreme outliers in the test, whereas the majority of the samples (59%) had a value of 0 µM TBARS. This raises the question whether the test was sensitive enough for this research. Moreover, we used six plates from three different kits to test all samples and the amount of samples that had a TBARS concentration of 0 µM differed per kit (respectively 27%, 65% and 68% negative test results per kit). This challenges the sensitivity and interassay precision,

although the sensitivity and interassay coefficient of variability of the kit has been tested by the manufacturer and in our pilot study.

The choice for a certain TBARS assay (kit) also might influence results, since TBARS concentrations differ greatly between this study and other studies. For instance, another study regarding TBARS in foals demonstrated concentrations ten times as large as our results [7], while there are also studies performed in healthy adult horses with TBARS concentrations in our range of results [23,24], but also more than two times as low [25] or more dan twenty times as high[26]. These differences might be explained by methodological differences, since not one study used the exact same method or kit. This causes difficulty in comparing and interpreting results.

We did see several non-significant trends. First of all, TBARS on hospital admission day seemed to be higher than on consecutive days. This could imply that hospitalization (i.e. therapy) is decreasing oxidative stress in sick foals. However, healthy non-hospitalized foals have a similar TBARS level at day 1, which is contradicting this statement. We could therefore argue that the higher levels of TBARS on day 1 is caused by the age of the foal. However, França de Souza (2021) already established that TBARS is stable from the age of 12 hours to 168 hours. A second non-significant trend could be noted when TBARS was related to length of hospitalization. Here it appears that at hospital admission, (surviving) foals with a higher TBARS value stay in the hospital for a longer time than foals with a lower TBARS value on day 1. This implicates a prognostic value of TBARS concentration in terms of length of hospitalization.

In conclusion, we found no differences in TBARS concentrations between septic, sick non-septic and healthy foals, or in surviving versus non-surviving foals. Possible correlations with sample day and length of hospitalization are found. Additional studies using other TBARS assay kits or evaluating other oxidative stress parameters such as antioxidants are warranted to assess oxidative stress critically ill neonatal foals.

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