Plasma ascorbic acid, antioxidant capacity, and reactive oxygen species in healthy foals

Alessandro Migliorisi, DVM, MS; Kelsey Hart, DVM, PhD; Sarah Vaughn, MPhil; Scott Austin, DVM, MS; Brian Aldridge, BVSc, PhD; Pamela Wilkins, DVM, PhD*

1Department of Clinical Sciences, Colorado State University, Fort Collins, CO
2Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA
3Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL
*Corresponding author: Dr. Wilkins (pawilkin@illinois.edu)

https://doi.org/10.2460/ajvr.22.02.0025

OBJECTIVE
To describe ascorbic acid (AA) concentrations, plasma antioxidant capacity (PAC) and markers of oxidative stress, as measured by derivatives of reactive oxygen metabolites (dROMs), in healthy foals at birth and during the first month of life.

SAMPLES
Venous blood samples were collected from healthy Standardbred (n = 13) and Quarter Horse (n = 10) foals. Plasma AA, PAC, and dROMs were assessed at 3 to 12 hours, 3 days, and 1, 2, and 4 weeks of age.

PROCEDURES
AA was measured via high-performance liquid chromatography. PAC and dROMs were measured with a free radical analytical system. Comparisons of AA, PAC, and dROMs at different time points were assessed.

RESULTS
Mean ± standard deviation AA concentrations at 3 to 12 hours (44.7 ± 19.6 μmol/L; P ≤ .01), 1 (48.6 ± 22.5 μmol/L; P ≤ .001), and 2 weeks (41.8 ± 15.8 μmol/L; P ≤ .001) were higher than at 4 weeks of age (28.5 ± 12.7 μmol/L). Both PAC and dROMs significantly increased at different time points compared to 3 to 12 hours of age.

CLINICAL RELEVANCE
Healthy foals have higher plasma AA concentrations shortly after birth, which then gradually decrease throughout the first month of life, suggesting that AA may represent a key antioxidant in the postnatal period. The concurrent increase in PAC and dROMs suggests that dynamic development of oxidative balance occurs after birth in foals. Development of AA, PAC, and dROM reference ranges in healthy foals could be used to guide therapeutic interventions and monitor during disease states characterized by increased oxidative stress.

Ascorbic acid (AA) is a water-soluble antioxidant first isolated from adrenal glands of animals. Ascorbic acid functions as an antioxidant by serving as an electron donor in free radical reactions, resulting in the generation of an ascorbate radical. The ascorbate radical is then reduced back to AA by NADH- and NADPH-dependent reductases. Ascorbic acid also plays an important role in recycling other antioxidants, such as α-tocopherol, and many enzymatic cofactors that contain either copper or iron.

Carotenoids, and flavonoids found in the lipid phase. Ascorbic acid is a water-soluble antioxidant first isolated from adrenal glands of animals. Ascorbic acid functions as an antioxidant by serving as an electron donor in free radical reactions, resulting in the generation of an ascorbate radical. The ascorbate radical is then reduced back to AA by NADH- and NADPH-dependent reductases. Ascorbic acid also plays an important role in recycling other antioxidants, such as α-tocopherol, and many enzymatic cofactors that contain either copper or iron.

In some animal species, such as humans and guinea pigs, AA is an essential micronutrient due to lack of enzymatic pathways for endogenous hepatic synthesis, but in several domestic species, including horses, AA can be endogenously synthetized.

Plasma AA concentrations were recently described in a population of healthy neonatal foals, but sampling...
did not extend beyond 5 days of age. In the same study, Wong et al. showed significantly lower AA concentrations in septic and sick nonseptic foals compared to healthy subjects, suggesting an association between disease severity and development of hypoascorbemia. Currently, measurement of PAC is only described in healthy newborn foals. Similarly, measurement of oxidative status markers such as dROMs is described in newborn foals but has not been reported in growing foals. Further characterization of oxidative balance and AA in the healthy newborn and growing foal could provide useful information for a correct interpretation of markers of oxidative stress in critically ill foals of different ages, as well as help direct therapeutic interventions to mitigate excessive oxidative stress.

The objective of this prospective study was to describe the effects of age on plasma AA, PAC, and dROMs concentration in healthy foals during the first month of life. We hypothesized that plasma AA, PAC, and dROMs concentration in healthy foals will change over the course of the first 4 weeks of life.

Materials and Methods

Animals

Animals were privately- or university-owned foals. Animals were determined to be healthy based on physical examination within 12 hours of birth. Newborn foals also had plasma IgG routinely measured and determined to be above 800 mg/dL after 12 hours via spectrophotometric analysis, glutaraldehyde coagulation test, or SNAP Foal IgG test (IDEXX Laboratories).

Foals were excluded from the final analysis if the plasma IgG concentration was less than 800 mg/dL and/or required hyperimmune plasma administration or developed, at any study time point, signs of systemic or localized disease.

Sample collection

Informed client consent was obtained for privately-owned foals and sampling protocols for university-owned foals were approved by the Institutional Animal Care and Use Committee prior to sampling (University of Illinois IACUC No. 19023; University of Georgia Animal Use protocol No. A2019 01-019-Y3-A2). Blood samples were collected from all foals during the spring of 2019 and 2020. Foals were briefly manually restrained, and blood was obtained by jugular venipuncture once within the first 12 hours of age after the foal was able to stand and nurse independently, and again at 3 days and 1, 2, and 4 weeks of age.

Blood samples were immediately placed into lithium heparin tubes, refrigerated in an ice bath, protected from light and centrifuged within 30 minutes of collection at 3,000 X g for 10 minutes at room temperature (approximately 21 °C). Plasma aliquots were obtained, transferred into amber transport tubes (ARUP Laboratories), and stored at −80 °C within 45 to 60 minutes of sampling until further analysis.

Ascorbic acid analysis

Plasma AA concentration was measured by high-performance liquid chromatography (HPLC) and electrochemical detection (ECD) following a previously described protocol to which specific modifications were applied. Modifications included use of smaller aliquots of heparinized plasma (200 µL) without a preservative; use of 3,4-dihydroxybenzylamine (DHBA) as the internal standard, which has the advantage of not being an endogenous metabolite; and the use of a high-throughput mode utilizing a 2-LC pump HPLC-ECD system with automatic alternating column regeneration, which allows a shortened turnaround time. All solutions and thawed samples were kept on ice during preparation. Chromatographic separation was achieved using a Syngery Hydro-RP column (100 X 3.0 mm, 2.5-µm particles, 100-A pores) with a Security Guard cartridge holder containing 2 C-18 4 X 3.0-mm cartridges. For the automatic alternating column regeneration method, the HPLC system was plumbed with 2 binary pumps, 2 degassers, an autosampler with a 40-µL sample loop, and a temperature-controlled column compartment with a 2-position/10-port switching valve. Calibration range was extended to 5,000 µmol/L to allow measurement of high concentrations of AA, and analytical measurement range was 5 to 5,000 µmol/L. Optimal electrochemical potentials for AA and DHBA were determined using hydrodynamic voltammetry. Optimal potentials were 200 mV for AA and 250 mV for DHBA. Since it is not practical to use different potentials for closely eluting compounds, the higher optimal potential was chosen for this method (E2 = 250 mV). Validation of HPLC methodology for AA quantification in equine plasma was internally performed in the test laboratory as per standard protocol previously described, and no interfering compounds were found in equine samples.

Plasma oxidative status

PAC was assessed by evaluating the plasma ferric reducing ability based on the analytical protocol developed by Serena et al. A plasma aliquot of 10 µL is added to a solution of ferric ions, zirconium salts, and thiocyanate-containing chromogenic reagent. In approximately 1 minute and at 37 °C, a change in color occurs, the intensity of which is detected by photometric analysis with a 505-nm wavelength. Results are expressed in Cornelli units (U Cor), where 1 U Cor = 1.4 µmol of AA/L.

The oxidant ability in the plasma was assessed by measuring the dROMs with the d-ROMLab test (Innovatics Laboratories Inc). The d-ROMLab test is a method of analysis that provides an indirect estimate of organic hydroperoxides (ROOH) present in a sample. Organic hydroperoxides are the main contributor to oxidant ability, but other reactive species, such as organic chloramines, may also influence the test results. The methodology is based on iron (or copper) oxidation via Fenton’s reaction. The change in color that occurs by adding the oxidizable chromogen substrate N,N-diethyl-paraphenylenediamine
is then measured photometrically. Results are expressed as chemical equivalence in “Carratelli units” (U Carr), where 1 U Carr is equivalent to 0.08 mg of H₂O₂ (hydrogen peroxide)/100 mL.

PAC and dROMs testing was performed using the Free Radical Analytical System FRAS5 (Innovatics Laboratories Inc) according to the manufacturer’s directions after validation for the use of FRAS5 on equine samples was performed. Briefly, dROMs measurement was validated by spiking aliquots of a single blood sample with various concentrations of H₂O₂ to provide known concentrations of reactive oxygen metabolites. The samples demonstrated a dose-dependent increase in dROMs. To validate the PAC assay for use in horses, aliquots of the same blood sample were spiked with different concentrations of AA. Samples demonstrated a dose-dependent response until appearing to reach saturation at 12.5 mM (S. Vaughn, MPhil, College of Veterinary Medicine, University of Georgia, 2021, unpublished data).

Statistical analysis

Data were analyzed using a commercially available software program (GraphPad Software). Normality of data was assessed using the Shapiro-Wilk test. Continuous data are presented as mean ± standard deviation if normally distributed and median and interquartile range (IQR, 25th to 75th) if not normally distributed. The ROUT method was used to identify outliers with Q set at 1%. Outliers were excluded from final analysis. A mixed-model 1-way ANOVA was used to assess the effect of age on AA, PAC, and dROMs at different time collection points. Tukey’s multiple comparison test was used for post hoc analysis. Because the data were not normally distributed, Spearman’s correlation analysis was used to investigate the association between AA concentration and PAC, and between AA and dROMs. Significance was accepted at *P < .05.*

Results

Animals

A total of 23 healthy foals were sampled for the study. Breeds represented were Standardbred (n = 13) and Quarter horse (10). The first postbirth blood sample was collected between 3 and 12 hours of age, as some foalings occurred unobserved overnight and the foals were sampled first thing the following morning. For AA analysis, a total of 22 foal samples were available at 3 to 12 hours and at 3 days and 1 and 2 weeks of age, and 20 samples were available at 4 weeks of age. For the PAC analysis, 23 foal samples were available at 3 to 12 hours and at 3 days and 1 week of age, 21 at 2 weeks of age, and 20 at 4 weeks of age. For the dROMS analysis, 21 foal samples were available at 3 to 12 hours and at 2 weeks of age, 22 at 3 days and 1 week of age, and 20 at 4 weeks of age. This discrepancy in sample numbers at various time points was due to horse movement related to breeding and mare-and-foal selling operations, exclusion of foals that were administered hyperimmune plasma, analytical technical issues, and accidental thawing of some samples during shipment for analysis. The Standardbred foal population was housed in the midwest United States, while the Quarter horse foal population was housed in the southeast United States.

Effect of age on AA concentration

There was a significant effect of age on plasma AA concentrations (Figure 1; Table 1). Specifically, AA concentrations were higher 3 to 12 hours after birth and at 1 week of age and then steadily and significantly decreased at 2 and 4 weeks of age. Significant differences in plasma AA concentration were found between 3 and 12 hours and 4 weeks of age (P ≤ .01), between weeks 1 and 4 (P ≤ .001), and between weeks 2 and 4 (P ≤ .001). There was no difference at any time point between Standardbred and Quarter horse plasma AA concentrations, and no influence of geographical location on plasma AA concentrations at any time point (P > .2).

Figure 1—Box-and-whisker plots for plasma ascorbic acid (AA) concentrations in a population of healthy foals over the first month of life. Box lower and upper limits indicate 25th and 75th interquartile range, respectively. Whiskers indicate minimum and maximum values. Mean is indicated by “+”. Median is indicated by horizontal line across box plot. *Significant differences were found between 3 and 12 hours and 4 weeks of age, between 1 and 4 weeks of age, and between 2 and 4 weeks of age.
There was a consistent and significant increase in dROMs during the first month of life in foals (Figure 3; Table 1). Significant differences in dROMs were found between the day of birth and 1 (P = .01), 2 (P ≤ .001), and 4 weeks of age (P ≤ .001), between 3 and 12 hours and 2 weeks of age, and between 3 days and 4 weeks of age. U Cor = Cornelli units. See Figure 1 for key.

**Table 1**—Ascorbic acid (AA), plasma antioxidant capacity (PAC), and derivatives of reactive oxygen metabolites (dROMs) in healthy foals at different time points.

<table>
<thead>
<tr>
<th></th>
<th>3–12 hours</th>
<th>3 days</th>
<th>1 week</th>
<th>2 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA µmol/L</td>
<td>44.7 ± 19.6</td>
<td>38.7 ± 19.6</td>
<td>48.6 ± 22.5</td>
<td>41.8 ± 15.8</td>
<td>28.5 ± 12.7</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>PAC U Cor</td>
<td>2,901 ± 576.2</td>
<td>3,003 ± 655.9</td>
<td>3,474 ± 750.8</td>
<td>3,667 ± 924.7</td>
<td>3,277 (2,967–3,813)</td>
</tr>
<tr>
<td>n</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>dROMs U Carr</td>
<td>52.4 ± 16.1</td>
<td>63.4 ± 29.2</td>
<td>77.5 ± 29.9</td>
<td>102.5 (90.2–126.8)</td>
<td>161 (128–185)</td>
</tr>
<tr>
<td>n</td>
<td>21</td>
<td>22</td>
<td>22</td>
<td>21</td>
<td>20</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (SD) or as median and 25th–75th interquartile range (IQR). U Carr = Carratelli units. U Cor = Cornelli units.

**Figure 2**—Box-and-whisker plots for plasma antioxidant capacity (PAC) in a population of healthy foals over the first month of life. Significant differences were found between 3 and 12 hours and 1 week of age, between 3 and 12 hours and 2 weeks of age, and between 3 days and 2 weeks of age. U Cor = Cornelli units. See Figure 1 for key.

**Figure 3**—Box-and-whisker plots for dROMs concentration in a population of healthy foals over the first month of life. Significant increases from baseline (3–12 hours) were found at 1, 2, and 4 weeks of age. Carr U = Carratelli units. See Figure 1 for key.

There was a consistent and significant increase in dROMs during the first month of life in foals (Figure 3; Table 1). Significant differences in dROMs were found between the day of birth and 1 (P = .01), 2 (P ≤ .001), and 4 weeks of age (P ≤ .001), between 3 and 12 hours and 2 weeks of age, and between 3 days and 4 weeks of age (P ≤ .001).

**Associations between plasma AA concentration and oxidative stress**

A weak but significant negative correlation was found between AA and dROMs (P = .002; Spearman ρ = −0.3). There was no significant correlation between AA and PAC (P = .77; Spearman ρ = −0.02).

**Discussion**

In this study we demonstrated higher plasma AA concentrations in healthy foals for the first week of life, followed by a gradual decrease over the first month of life that occurred concurrently with a gradual increase in plasma reactive oxygen metabolites. These findings support a significant impact of age on the measured parameters. Possible explanations include age-related variation in oxidative stress and dietary and metabolic changes as the foal transitions from intrauterine to extrauterine life. Our results for AA concentrations in healthy foals within 12 hours of birth are similar to what was previously reported. The AA concentrations at 3 days are instead lower than what reported by Wong et al.²
a finding potentially related to the lower sample size in that study. Interestingly, in our foal population AA at all time points was significantly higher than AA concentration in a group of adult healthy mares sampled within 12 hours of parturition (PA Wilkins, DVM, PhD, College of Veterinary Medicine University of Illinois, unpublished data, 2021), as well as than concentrations reported in a group of healthy adult horses in one study. This latter finding could indicate a major antioxidant role for AA in younger animals compared to adult horses.

A possible explanation for the higher plasma AA concentration in foals during the first week of life may relate to AA intake via colostrum ingestion. Despite being unable to synthesize adequate amounts of AA, newborn calves were found to have a similar pattern of increased AA concentration shortly after birth followed by a gradual decrease in the postnatal period, and colostrum was demonstrated to be a major source of AA for calves in the postnatal period. AA concentration in the colostrum of the mares was also found to be significantly higher than normal milk. All foal samples in this study occurred after colostrum ingestion, so it is possible that equine colostrum could similarly represent an important source of AA for the newborn foal.

Based on what observed in other species, another possible explanation for the higher plasma AA concentration in newborn foals compared to older foals and horses could be peripartum increased synthesis and/or release of stored AA in preparation for the transition to the oxygen-rich extrauterine environment. Intrauterine equine fetal development occurs in a relatively hypoxic environment. Following birth, the newborn is exposed to a markedly higher environmental partial pressure of oxygen, leading to increased oxygen saturation and peripheral tissue oxygenation but also potentiation of the formation of ROS. In human infants, rabbits, and rodents, the activity and availability of some antioxidants increase near the time of birth in preparation for this substantial periparturient increase in ROS production.

Endogenous synthesis of AA occurs in the liver, and a variety of compounds can upregulate its hepatic production, including catecholamines and glucagon. Following hepatic synthesis, AA in many species is actively stored in adrenal gland, and release is stimulated by acute stress in rats and exogenous ACTH administration in humans. Currently, there are no data on the AA tissue distribution in foals or adult horses. Nonetheless, it is known that in the equine fetus the maturation of the foal’s endocrine function toward late gestation leads to significant weight gain of adrenal glands, so it is also possible that the lower levels of these hormones observed in the days following birth are associated with the progressive reduction in circulating AA concentration we observed. This reduction in AA concentrations as foals age might be offset by the concurrent development of other antioxidant pathways, but further study is needed to investigate this theory.

PAC in our foal population was higher than what has been previously reported, a finding potentially related to timing of sample collection. All foals in our study were sampled after colostrum ingestion, which was shown to be a significant source of some nonenzymatic antioxidants. Albumin, uric acid, AA, tocopherols, retinol, bilirubin, glutathione, and metal-binding proteins, such as ceruloplasmin, are considered the main components of nonenzymatic PAC, which is quantified by the assay used in this study. Several of these specific antioxidant components have been investigated in equine medicine. At 12 hours from birth, foals were found to have an increased concentration of unconjugated bilirubin compared to 3 days and 1 week of age, while ceruloplasmin concentration was extremely low during the first week of life compared to 4 weeks of age. Additionally, plasma alpha-tocopherol and beta-carotene concentrations were lower in neonatal Przewalski foals than adult horses, and Finnhorse foals had lower α-tocopherol and retinol concentrations than their dams. Lastly, uric acid concentrations were lower in Thoroughbred and Arabian foals at birth than at 4 weeks of age. In our population, this potential for age-related maturation of other antioxidant mechanisms is supported by the finding that, despite a decrease in plasma AA concentration, the foal’s PAC gradually increased during the neonatal period. The explanation for the increased PAC is unlikely to be driven by ingestion of mare’s milk, as this was found to contain lower concentrations of AA and retinols, and similar amounts of tocopherols, compared to colostrum. Instead, the observed
increase in PAC could be explained, at least in part, by increased dietary intake of tocopherols and retinols as the foal’s diet transitions from milk to feed and forage as they mature. However, dietary tocopherols and retinols in the various diets that foals in this study had access to at the different locations during the study were not quantified. This makes it difficult to determine the dietary contribution for various antioxidants for each foal in this population, which is a limitation of this work.

Thus, while further study is needed to comprehensively and concurrently assess the availability of the spectrum of antioxidants in foals as they age, it seems possible that AA might represent a key antioxidant in the immediate postnatal period in the foal, which may then gradually become less vital as other antioxidant systems and pathways mature. This theory is supported by the significant but weak correlation between AA concentration and circulating reactive oxygen metabolites (dROMs) that we noted.

Only one other study has quantified dROMs in foals. Sgorbini et al. documented more than 2-fold higher dROM concentrations in presuckle samples collected from foals immediately after delivery than we observed in foals between 3 and 12 hours of age, but older foals were not sampled in that study. It is likely that differences in the timing of blood collection from the foal explain these disparate results, with the postfoaling, presuckle samples possibly reflecting parturition-associated stress. Another possible explanation for this discrepancy might relate to the different dROMs analytic equipment used in the 2 studies.

Interestingly, circulating dROMs rose steadily during the first 4 weeks of life in foals in this study, despite the slight but gradual increase in PAC that we observed. This has several possible explanations. It seems likely that the aforementioned maturing antioxidant pathways may not yet be fully capable of coping with ongoing ROS production. Additionally, inflammation is a well-known cause of increased oxidative stress. Several inflammatory cytokines have been linked to an increased production of ROS, including interferon-γ (INF-γ), interleukin-β (IL-β), tumor necrosis factor-α (TNF-α), and tumor growth factor-β (TGF-β). IFN-γ and TGF-β are necessary to stimulate the appropriate maturation of the adaptive immunity system and have been shown to markedly increase throughout the first 4 weeks of life in foals.

Further study is needed to determine if these cytokines or maturation of other immune and inflammatory processes in the neonatal foal directly stimulate ROS production.

It is also possible that this increase in circulating ROS is appropriate and physiologically important. When present in low amounts, ROS are involved in physiological functions such as cell signaling, regulation of growth, proliferation, and apoptosis. Perhaps the steady increase in dROMs observed in our healthy foal population is a necessary condition for physiologic postnatal tissue development. Lastly, dROM concentrations in healthy horses are also reportedly higher than in other species, so the increase in dROMs we observed in foals from birth to 4 weeks of age may just reflect a trend toward higher species-specific range.

This study has several additional limitations. Ascorbic acid was the only nonenzymatic antioxidant measured in this study, and a more comprehensive analysis of the components of PAC could have provided more complete information on antioxidant and AA kinetics in the first month of life in horses. Also, unpredictable technical issues prevented the analysis of all samples collected. Lastly, several plasma samples accidentally thawed during shipment, which also contributed to inconsistent numbers of available foal samples at different time points.

These data demonstrate that healthy foals have higher plasma concentrations of AA during the first week of life, which then gradually decline through, at least, the first 4 weeks of age. Plasma pro-oxidant markers, as measured by dROMs, increased during the same time period. However, despite this increase in circulating ROS, PAC also increased during the neonatal period, likely demonstrating development and interplay of other antioxidant mechanisms. AA thus may represent a key nonenzymatic antioxidant during the neonatal period in the foal.

Further studies are needed to determine if oxidative stress is increased in foals with suboptimal AA plasma concentrations.

Acknowledgments

This work was supported by the Morris Animal Foundation (D19EQ-025) and by the College of Veterinary Medicine at the University of Illinois. The authors declare there were no conflicts of interest. The authors thank Georgia Macy, Meg Lemons, Shyla Giancola, Jessie Cathcart, and Chelsea McClellan. The data that support the findings of this study are available from the corresponding author upon request.

References


