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SIGNALMENT

1-year-old Quarter Horse

HISTORY AND CLINICAL FINDINGS

A yearling Quarter Horse colt was presented to the University of Wisconsin Large Animal Hospital for castration, having been rescued two weeks earlier with 10 other horses from a farm where horses exhibited signs of neglect and starvation. Prior medical history was not known by the rescue organization. The colt was dewormed with pyrantel pamoate 10 days prior to admission. No other medications were given. He had been fed hay and grain after being rescued. He did not have access to pasture.

On physical examination, the colt was slightly lethargic, with poor body condition and minimal muscle mass (body score 2.5/9). His rectal temperature was normal, respiratory rate was mildly increased (20-28 breaths per minute) and heart rate was increased (60-80 beats per minute). Oral mucosa and conjunctiva were remarkably pale. Pitting edema was present in the hind fetlocks and capillary refill time was slightly prolonged. Increased cranial ventral right lung sounds and a grade 2/5 heart murmur were auscultated. An echocardiogram of the heart did not reveal an abnormality, nor did an ultrasound examination of the abdomen and chest.

LABORATORY FINDINGS

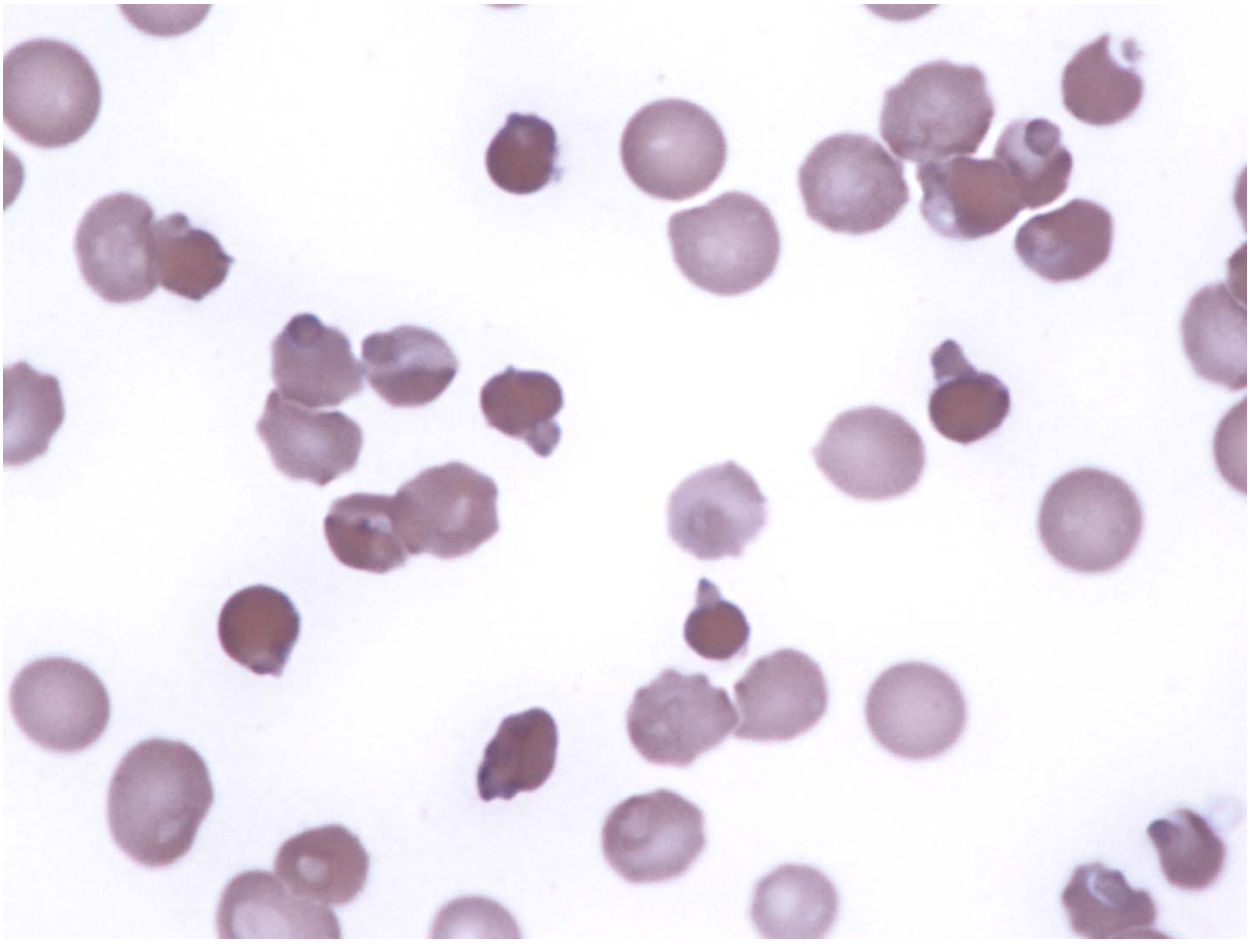
Blood was collected for a CBC (Advia 120) and clinical chemistry panel (Hitachi 912), and urine was collected for a urinalysis. CBC results are given in Table 1.

Table 1. Hematology findings

Parameter	Colt Day 1	Colt Day 3	Reference Interval
HCT (L/L)	0.12	0.13	0.32-0.49
MCV (fL)	48	48	34-58
MCHC (g/L)	362	364	310-370
CHCM (g/L)	365	364	Not Determined
Total Protein (g/L)	86	88	60-85
Fibrinogen (g/L)	6	6	1-4
Platelets ($x10^6$/L)	300	315	100-400
Total leukocytes ($x10^6$/L)	19.1	18.1	5.0-12.5
Neutrophils ($x10^6$/L)	15.6	15.1	2.7-6.7
Lymphocytes ($x10^6$/L)	3.2	2.5	1.5-5.5
Monocytes ($x10^6$/L)	0	0.2	0-0.8
Eosinophils ($x10^6$/L)	0.2	0.2	0-0.9

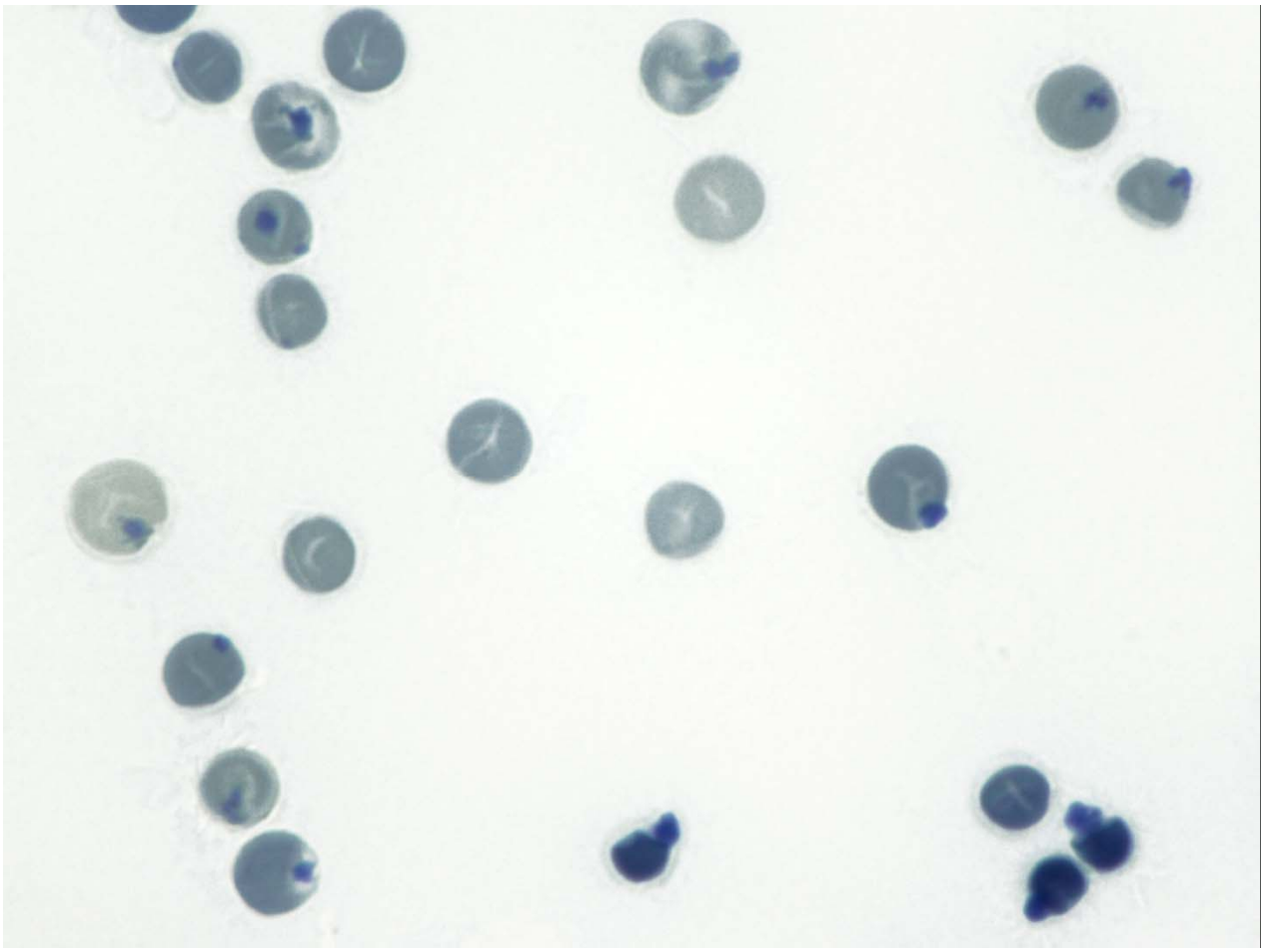
Microscopic examination of erythrocyte morphology revealed 2+ anisocytosis and 3+ Heinz bodies (Figure 1). Some erythrocytes were spherocytic with a single Heinz body protruding from the cell. Heinz bodies appeared as pale inclusions in some cells and occasional lysed erythrocytes containing Heinz bodies were seen (Figure 3A).

Figure 1. Wright-Giemsa stained blood film.



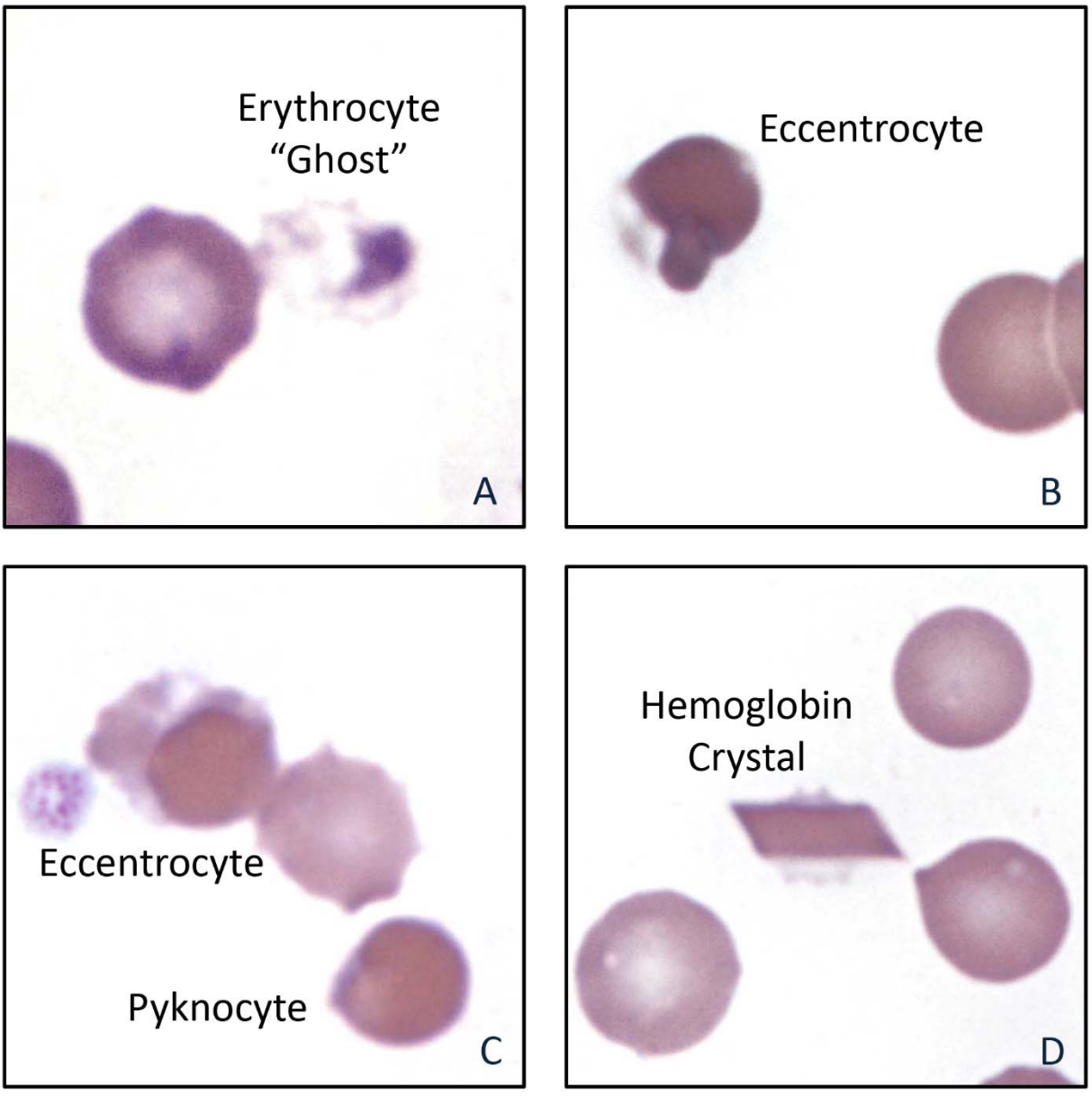
A reticulocyte stain revealed additional Heinz bodies not easily identified by the Wright-Giemsa stain (Figure 2). Spherocytic erythrocytes containing Heinz bodies stained deeply basophilic in the reticulocyte stain (Figure 2, bottom right)

Figure 2. Reticulocyte stained blood film



Eccentrocytes were uncommon, and when present, they also contained a single Heinz body (Figure 3B) or loose aggregates of precipitated hemoglobin (Figure 3C). Some erythrocytes appeared as pyknocytes (Figure 3C). Rare hemoglobin crystals were seen by visual scanning blood films (Figure 3D).

Figure 3. Wright-Giemsa stained blood films



All serum chemistry analytes were within reference intervals except bilirubin (103 μmol/L, reference interval 7-56 μmol/L), total protein (85 g/L, reference interval 52-82 g/L), albumin (27 g/L, reference interval 28-38 g/L), and total globulin (58 g/L, reference interval 21-38 g/L). Serum protein electrophoresis revealed a mild increase in beta-2 globulins.

Results from the urinalysis (Table 2) were considered normal except for being positive for bilirubin. Methemoglobin was 8% of total hemoglobin when measured spectrophotometrically. A Coggins test for equine infectious anemia was negative.

Table 2. Urinalysis

TEST	RESULT	TEST	RESULTS
Color	Amber	pH	8.5
Appearance	Cloudy	Protein/SSA	Neg
SG	1.020	Glucose	Neg
Sediment: many calcium carbonate crystals		Ketones	Neg
		Bilirubin	Pos
		Heme	Neg

QUESTIONS:

What would be included in your differential diagnostic list?

What additional history would you inquire about?

What additional tests would you request?

ADDITIONAL FINDINGS:

Heinz bodies form following oxidative damage to hemoglobin, resulting in its denaturation and precipitation. The humane society staff and referring veterinarian were questioned about possible sources of environmental and drug toxicities. No potential dietary sources, such as red maple leaves, onions, or garlic were identified and no drugs other than pyrantel pamoate were given. Heavy metals (principally copper and zinc) in the environment were considered possible toxicants, but they were not increased in serum (copper and zinc) or whole blood (lead) when measured using atomic absorption (Table 3).

Table 3. Heavy metal assays

Analyte	Colt	Reference Values
Copper (mmol/L)	19	13-31
Zinc (mmol/L)	13	9-26
Lead (mmol/L)	<0.2	0.2-1.2

The colt was released on vitamin E therapy with instructions that the referring veterinarian should monitor the anemia. Because the colt was young and no oxidant drug or environmental causes for Heinz body formation were identified, EDTA anticoagulated blood samples from Days 3 and 81 was sent by overnight courier to the University of Florida for erythrocyte biochemical analysis for a possible hereditary defect. Erythrocyte biochemical finding are shown in Table 4.[1] Hemoglobin electrophoresis by agarose gel electrophoresis did not reveal an abnormal hemoglobin.

Table 4. Erythrocyte biochemical assays from a colt with Heinz body hemolytic anemia

Parameter	Day 3	Day 81	Day 122	Reference Values
PCV (L/L)	0.13	0.19	0.24	0.31-0.44
Heinz bodies (%)	65	50	22	0
Methemoglobin (%)	13.0	12.6	5	0-1.6
GSH (mmol/L)	1.2	1.5	1.6	1.8-3.0
G6PD (U/g Hb)	28	-	-	13-26
6PGD (U/g Hb)	5.8	-	-	1.5-4.3
GR without FAD (U/g Hb)	4.4	-	-	0.8-5.6
GR with FAD (U/g Hb)	4.8	-	-	1.2-6.6
Cb5R (U/g Hb)	5.6	6.5	-	6.5-19.2
GPx (U/g Hb)	-	140	-	142-265

Reference values are minimum and maximum values for 28 to 38 healthy horses. PCV = packed cell volume, GSH = reduced glutathione, Hb = hemoglobin, G6PD = glucose-6-phosphate dehydrogenase, 6PGD = 6-phosphogluconate dehydrogenase;

GR = glutathione reductase, FAD = flavin adenine dinucleotide, Cb5R = cytochrome-b5 reductase, GPx = glutathione peroxidase.

The colt's physical condition improved somewhat following release, and his PCV was 0.24 L/L on Day 61 when readmitted for castration. Sternal core and aspirate bone marrow biopsies were done while the horse was under anesthesia for surgery. The bone marrow aspirate was judged to be hypercellular, but the core was judged to be hypocellular with slight myelofibrosis. Megakaryocyte numbers were adequate. The M:E ratio was markedly reduced. Unidentified basophilic blast cells (<5% of all nucleated cells) were seen in the aspirate smear. These cells resembled lymphoblasts, or possibly myeloblasts; however, increased blast cells were not appreciated in the core biopsy. Plasma cells were present in normal numbers. Hemosiderin was recognized in the aspirate, but not the core biopsy.

The colt's appetite, attitude, and appearance were further improved when reexamined at the University of Wisconsin on Day 81, although mucous membranes remained pale pink and the heart murmur was still present. Results from the CBC at that time (Table 5) were similar to values from the initial examination. Serum clinical chemistry analytes were within normal limits except for increased bilirubin (89 μmol/L, reference interval 7-56 μmol/L) and slightly increased phosphorus (2.1 μmol/L, reference interval 0.6-1.9 μmol/L).

Table 5. Hematology findings from a colt with Heinz body hemolytic anemia

Parameter	Day 81	Day 122	Day 155	Day 289	Reference Interval
HCT (L/L)	0.20	0.24	0.32	0.26	0.32-0.49
MCV (fL)	45	44	42	42	34-58
MCHC (g/L)	397	373	335	373	310-370
Total Protein (g/L)	73	76	ND	ND	60-85
Fibrinogen (g/L)	3	5	ND	ND	1-4
Platelets ($x10^6$/L)	423	302	161	278	100-400
Total leukocytes ($x10^6$/L)	15.1	12.4	13.1	11.3	5.0-12.5
Neutrophils ($x10^6$/L)	12.9	7.4	7.5	6.2	2.7-6.7
Lymphocytes ($x10^6$/L)	1.8	4.4	4.8	4.2	1.5-5.5
Monocytes ($x10^6$/L)	0.3	0.4	0.5	0.5	0-0.8
Eosinophils ($x10^6$/L)	0	0.2	0.3	0.4	0-0.9
Metarubricytes ($x10^6$/L)	0.2	0	0	0	0

Although his attitude and body condition improved with a healthy diet, the horse failed to thrive and was never deemed adoptable. Additional CBCs were performed by the University of Florida Veterinary Clinical Pathology Laboratory (Day 122) and IDEXX Laboratories (Days 155 and 289) as shown in Table 5. Heinz bodies were recognized during microscopic blood film reviews (including reticulocyte stain) at the University of Florida and by an IDEXX veterinary clinical pathologist on day 155. The blood film on day 289 was not reviewed by a veterinary clinical pathologist. The colt remained housed

at the humane society until he died one year after the initial examination. A necropsy was not done.

DISCUSSION

Erythrocytes carry oxygen bound to hemoglobin, making them highly prone to oxidative injury. The primary source for endogenous reactive oxygen species in healthy human erythrocytes is reported to be the autoxidation of oxyhemoglobin, which generates methemoglobin and superoxide free radicals,[2-4] Erythrocytes also have membranes rich in polyunsaturated fatty acids and the heme group of hemoglobin can initiate a wide array of free radical reactions. Additionally, other metabolic reactions in the body generate oxidants that may damage circulating erythrocytes.[5]

Proteomic studies in mice have revealed many cysteine-containing proteins that are sensitive to oxidation. These oxidant-sensitive proteins include ones related to the maintenance of erythrocyte lifespan (e.g., cytoskeletal proteins), metabolic enzymes, stress-induced proteins, and transport proteins.[6] Erythrocyte oxidative injuries that are easily recognized in diagnostic laboratories include methemoglobin, Heinz bodies, and eccentrocytes. Methemoglobin develops when ferrous iron (+2) in heme is oxidized to the ferric (+3) state. Methemoglobin cannot bind oxygen; however, it can be reduced back to the ferrous, oxygen-binding state within erythrocytes using NADH (generated by anaerobic glycolysis) and a cytochrome b5 reductase (methemoglobin reductase) enzyme. Heinz bodies develop when the sulfhydryl groups in globin chains of hemoglobin are oxidized and resultant denatured molecules precipitate into large inclusions that can be identified microscopically in stained blood films. Eccentrocytes form when erythrocyte membranes bind tightly together following oxidative injury. As a result of adjacent membrane adhesion, the remaining part of the cell becomes spherical, with hemoglobin located eccentrically.[7] The presence of methemoglobinemia and Heinz bodies within erythrocytes of this horse, plus small numbers of eccentrocytes, indicated that the anemia resulted from oxidative damage to erythrocytes.

Erythrocyte oxidative injury results from either the presence of excessive amounts of oxidants or in a decreased ability of erythrocytes to protect themselves against low levels of oxidants produced naturally in the body. Heinz body hemolytic anemia has been reported in horses consuming wilted red maple (*Acer rubrum*) leaves,[8] onions,[9] and garlic.[10] Leaves from other maple species also have the potential to cause erythrocyte oxidative injury.[11] Methemoglobinemia and hemolytic anemia with eccentrocytosis has been reported in horses ingesting *Pistacia* species leaves, but neither the presence nor absence of Heinz bodies was reported.[12,13] None of these plants or *Brassica* species, reported to cause Heinz body hemolytic anemia in ruminants, were available for consumption by this horse.[14] Phenothiazine anthelmintics can cause Heinz body hemolytic anemia in horses but this type of anthelmintic was not given to this horse.[15] Copper toxicity causes Heinz body hemolytic anemia in sheep and goats,[16,17] and a single report of Heinz body hemolytic anemia in a horse with hepatic

copper overload has been reported,[18] but plasma copper was normal in this patient. Zinc toxicity causes Heinz body hemolytic anemia in dogs,[19,20] but zinc concentration in plasma was normal in this horse.

Erythrocytes lack the capacity for protein synthesis that could replace damaged proteins; consequently, they have an extensive antioxidant defense system to mitigate the continuous exposure to oxidative stress. The possibility of an intrinsic erythrocyte defect was considered in this colt because an external oxidant could not be identified.

Superoxide (O_2^-) is produced in vivo during autoxidation of hemoglobin, adrenaline, and certain flavins; neutrophil and monocyte oxidant bursts; enzymatic reactions such as xanthine oxidase; mitochondrial reactions; and other metabolic processes.[21] Superoxide is a relatively unreactive free radical that rapidly dismutates to hydrogen peroxide (H_2O_2) even without the superoxide dismutase enzyme. In the presence of a metal ion such as iron or copper, O_2^- and H_2O_2 interact to form the highly reactive hydroxyl radical ($OH^{\cdot}$).[21] Hydrogen peroxide is degraded by catalase, (GPx) and peroxiredoxin (Prx). The latter two enzymes depend on glutathione/glutathione reductase and thioredoxin/thioredoxin reductase systems, respectively, and both of these enzyme systems derive reducing equivalents (electrons) from NADPH that is generated by the pentose phosphate pathway. NADPH is also important in maintaining catalase in a functional form. Defects in the pentose phosphate pathway can render erythrocytes susceptible to endogenous and exogenous oxidant injury. Glucose-6-phosphate dehydrogenase (G6PD) is the rate controlling enzyme in this pathway, and a persistent hemolytic anemia with eccentrocytosis has been described in an American saddlebred colt with <1% of normal G6PD activity.[22] Erythrocyte G6PD activity was normal or slightly increased in the colt.

Reduced glutathione (GSH) is a tripeptide containing a highly reactive sulfhydryl group that may act nonenzymatically as a free radical acceptor to counteract oxidant damage. GSH also functions as an electron donor in various reductive enzyme reactions including GPx, phospholipid hydroperoxide glutathione peroxidase, glutathione S-transferase, and glutaredoxin. Following oxidation, glutathione forms a disulfide (GSSG) that can be reduced back to GSH by the flavin adenine dinucleotide (FAD)-dependent glutathione reductase (GR) enzyme, using NADPH as the source of electrons. Decreased erythrocyte GSH in the colt is consistent with increased oxidation or decreased reduction of GSSG back to GSH. Horses with erythrocyte FAD deficiency have markedly reduced GR activity, decreased GSH concentration, and prominent eccentrocytosis.[23,24] These FAD deficient horses also have methemoglobinemia because the cytochrome b5 reductase enzyme also requires FAD as a cofactor. This colt was not FAD deficient because he had normal GR activity without the addition of FAD to the GR assay. GSH binds to various oxidized thiols, including those present in hemoglobin and Prx,[5,25] which might account for the reduced GSH concentration in the colt. These glutathionylation reactions can be reversed by glutaredoxin and GSH.[25] Alternatively, GSSG may have been transported out of erythrocytes.[5]

Selenium acts as an antioxidant when incorporated as selenocysteine at the active site of a wide range of selenoproteins, including GPx, phospholipid hydroperoxide glutathione peroxidase, and thioredoxin reductase in erythrocytes.[26] GPx deficiency is not associated with a hemolytic anemia in humans.[27] However, Heinz body hemolytic anemia has been reported in selenium-deficient cattle grazing on St. Augustine grass. [28] There is a direct correlation between whole blood selenium and GPx activity in erythrocytes. The near normal GPx activity in this colt rules out selenium deficiency as a major contributing factor to the oxidant damage present in erythrocytes from the colt.

Catalase is an enzyme that can catalyze the conversion of H_2O_2 to water and O_2 without using energy. Humans with acatalasemia and dogs with low erythrocyte catalase activity do not develop hemolytic anemia,[27,29] and the erythrocyte catalase activity is this patient was near the reference values.

Peroxiredoxins are a ubiquitous family of enzymes that reduce hydrogen peroxide, organic hydroperoxides, lipid hydroperoxides, protein hydroperoxides, and peroxynitrite.[4] Isoform composition varies by tissue. Peroxiredoxin 2 (Prx2) is the third most abundant protein in the cytoplasm of erythrocytes after hemoglobin (98% of the total protein) and carbonic anhydrase. Much lower quantities of peroxiredoxin 1 and 6 also occur in mammalian erythrocytes.[30] Prx2 appears to be more important in protecting erythrocytes against H_2O_2 generated during hemoglobin autoxidation and normal metabolic reactions than GPx or catalase.[30] Prx2 functions primarily as a noncatalytic scavenger of hydrogen peroxide; however, oxidized Prx2 is slowly regenerated using reduced thioredoxin, and oxidized thioredoxin is reduced by NADPH using thioredoxin reductase. [31,32] Prx2 is essential for preventing hemolytic anemia from oxidative stress by maintaining hemoglobin stability.[33] Prx2 knockout mice have Heinz body hemolytic anemia and increased methemoglobin.[4,31] An assay for Prx2, developed for human erythrocytes, was unsuccessful when attempted using erythrocytes from this colt and a normal equine control.

Peroxiredoxin 1 (Prx1) knockout mice were reported to develop severe Heinz body hemolytic anemia and malignant cancers including lymphoma, sarcomas, and carcinomas in mice.[34] However, later studies by other investigators found no hematologic abnormalities in Prx1 knockout mice.[35,36]

Superoxide dismutase 1 (SOD1) knockout mice had increased Heinz bodies in erythrocytes, but did not exhibit additional evidence of oxidative stress as judged from the lack of significant changes in the levels of methemoglobin, erythrocyte reduced glutathione, plasma protein thiol and carbonyl groups, and thiobarbituric-acid reactive substances in the plasma.[4,37] Superoxide dismutase was not assayed in the colt.

Vitamin E is lipid soluble and acts as a free radical scavenger in the membrane.[5] Hemolytic anemia secondary to vitamin E deficiency has been reported in monkeys and premature human infants, but Heinz body formation has not been recognized.[38,39]

Many inherited hemoglobin defects have been reported in humans that result in unstable hemoglobins and Heinz body associated hemolytic anemia.[40] We did not identify an abnormal hemoglobin by agarose gel electrophoresis, but this possibility has not been ruled out.

Based on the persistence of Heinz bodies, methemoglobinemia, low erythrocyte reduced glutathione, and anemia for months in the absence of an identifiable external oxidant, this horse most likely had a defect in the defense of erythrocytes against endogenously generated oxidants. Prx2 and SOD1 deficiencies are two possibilities that were not assayed. The more severe anemia with higher Heinz body numbers and higher methemoglobin content when initially examined, may reflect added oxidative stress associated with inflammation that appeared to be present at that time.

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