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Case Report

Copper Toxicity in Horses: Does it Exist?

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ABSTRACT

Copper toxicity is thought to be a rare condition in horses. However, the number of cases diagnosed in Brazil is growing. This article aims to describe cases of copper toxicity involving horses from different geographic locations and discuss findings of physical examinations, differential diagnoses and potential causes. Five cases referred from 4 different properties where at least 15 other horses were affected were described. Hemolytic anemia and hemoglobinuria, presence of Heinz bodies and elevated aspartate aminotransferase and gamaglutamil transferase levels were detected in all cases. The diagnosis was based on clinical history and signs, laboratory tests results, copper level determination in feed and/or soil and histopathological findings. Two horses progressed to acute death; remaining horses responded to clinical management with or without blood transfusion, depending on disease severity. However, one of these horses, after several returns to the veterinary hospital, was euthanized due to complications. One horse was treated with ammonium tetrathiomolybdate. Two horses had several recurring episodes over the course of several months, an uncommon presentation in ruminants suffering from copper toxicity. Excess copper was associated with soil fertilization with poultry litter or treatment of previous or neighbor crops with copper-containing products. It can be concluded that copper toxicity does occur in horses and may arise from several sources and/or be associated with predisposing dietary factors. Given the growing number of cases, the condition should be included in the differential diagnosis list and proper preventive dietary and pasture fertilization measures adopted.

1. Introduction

Copper is an oligoelement found in high concentrations in the brain, liver and kidneys, binding to ceruloplasmin at the hepatic level, which transports copper from the liver to peripheral tissues. Approximately 50%of copper is excreted in the bile, while the rest is eliminated through other gastrointestinal secretions. Thus, the digestive system plays an important role in the homeostasis of this element in the animal organism [1, 2]. However, excessive intake causes copper to accumulate in tissues, particularly in the liver, with the free copper ions capable of damaging cellular components, inducing not only oxidative stress, but also damage to DNA and decreased cell proliferation. [3,4]. Sheep are the most susceptible domestic animal species to copper toxicity due to low metallothionein levels in the liver [5]. Horses are able to tolerate higher doses of dietary copper (50 mg/kg DM) compared to sheep (15 mg/kg DM), while pigs and poultry are very resistant of even higher doses (250 mg/kg and 500 mg/kg DM respectively) [6]. Ponies (Shetland and Shetland by Welsh cross ponies) are thought to be most resistant among equine breeds (791 mg/kg DM) [7]. Still, novel therapeutic and dietary copper-containing compounds and environmental contamination may increase the risk of accidental toxicity.

Effects of long-term copper intake in horses remain to be determined. Acute experimental toxicity induced by iatrogenic exposure (subcutaneous administration of copper-D-penicillamine complex) has been described [8]. Acute and cumulative copper toxicity in response to natural or induced copper sulfate intake have also

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Animal welfare/ethical statement: This study was based on clinical outcomes and did not involve experimental procedures. It was therefore exempt from ethics approval or informed consent.

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Table 1
Physical examination parameters at the time of the initial clinical evaluation and previous treatments.

	Heart Rate	Respiratory Frequency	Rectal Temperature	Capillary Fill Time	Mucosal Staining	Previous Treatment
Case 1	76 bpm	20 bpm	37.6°C	2-3″	Icteric with halo	Phenylbutazone Imidocarb dipropionate Gentamicin [a]Ornitil
Case 2	-	-	-	-	-	Imidocarb dipropionate Oxytetracycline Blood transfusion
Case 3 (mare)	76 bpm	34 mpm	38.6°C	2″	Pale and icteric	Imidocarb dipropionate [b]Pentabiótico
Case 4 (gelding)	80 bpm	40 mpm	38°C	-	Cyanotic/Icteric	-
Case 5	50 bpm	26 mpm	37.4°C	2″	Icteric	Imidocarb dipropionate Oxytetracycline

[a] Active principles: L-Ornithine aspartate; L-Arginine Hydrochloride; L-Citrulline, Acetylmethionine.
[b] Active principles: Benzylpenicillin benzathine; Benzylpenicillin procaine; Benzylpenicillin potassium; Dihydrostreptomycin base (sulfate); Streptomycin base (sulfate).

been reported [9]. Two cases of hemoglobinuria-related death have been attributed to liver damage associated with ragwort (*Senecio jacobea)* toxicity and chewing of copper-rich wood [10]. Copper-related cirrhosis of the liver has been described in a 6-year-old horse [11]. In spite of progression to hemoglobinuria and death (common findings in cumulative toxicity in sheep) in that case [11], it was not possible to determine whether copper accumulation in the liver was due to primary (genetic) or secondary causes.

This paper describes 5 clinical cases of cumulative copper toxicity in horses from 4 different geographical locations. Increased awareness of this diagnostic hypothesis and discussion of potential causes and preventive measures are expected contributions of this study.

2. Case reports

Cases reported involved privately owned horses referred for hospital management. Owners reported similar clinical manifestations in other horses in some of the properties.

2.1. Case 1

A 2-year-old Quarter Horse mare was referred with a 4-day history of apathy, anorexia, fever, dark brown urine and icterus. Table 1 shows the parameters of the physical examination at the time of the clinical consultation, as well as the previous treatment received by the patient before being referred to the veterinary hospital.

Horses at that property were fed commercial feed, mineralized salt and *Cynodon nlemfuensis* grass. Pastures had been treated with poultry litter (3 ton/ha) over the last 6 months. Two horses and 1 cow developed icterus, fever and apathy and progressed to death. The cause of death was not determined.

Physical examination revealed dehydration, apathy, yellow tinged, toxic looking mucous membranes, tachycardia, green nasal discharge, pulmonary stridor, decreased gut motility and dark brown urine (Fig. 1) unrelated to muscle damage.

Complete blood count and serum biochemistry panel revealed severe regenerative hemolytic macrocytic normochromic anemia, polychromasia, anisocytosis, presence of Howell-Jolly and Heinz bodies, kidney failure and liver damage (Table 1). Urinalysis revealed proteinuria, hemoglobinuria and hematuria. The mare tested negative for leptospirosis and blood culture was also negative (i.e., no bacterial growth).

Emergency blood transfusion (2.5 liters) and fluid therapy were carried out immediately. Nine hours later, the mare became recumbent and presented with abdominal discomfort, neurological signs and seizures, progressing to death within the next hour. Table 2 shows the clinical evolution of the animal until the moment of death. Before dying, the mare had nystagmus and convulsed.

Post-mortem examination findings were as follows: icterus, serous fluid in the thoracic cavity and pericardial sac, petechiae and suffusions in epicardial and intestinal serosal surfaces, pulmonary edema, orange colored liver, greenish-brown kidneys and dark red urine. Histopathology revealed multifocal liver necrosis and severe liver fibrosis, hepatocytes filled with dark brown pigment granules, hepatic hemosiderosis (Perls stain) and dark brown pigments and vacuolization in renal tubular epithelial cells. Copper accumulation was visible in hepatocytes and renal tubular epithelial cells (rubeanic acid stain). Liver and kidney changes were consistent with cumulative copper poisoning.

There is limited information on copper levels in horses and values are thought to vary according to breed, geographical location and management. Still, liver, kidney and serum copper levels (Table 3) were above normal ranges, regardless of reference values adopted, which vary widely in literature (12.2–28.6 µmol/L or 8.8–11.9 µmol/L) [12,13,14,15].

2.2. Case 2

A 2.5 year-old male *Brasileiro de Hipismo* horse was referred with a history of anemia, apathy, fever and hemoglobinuria in the last 24 hours and similar episodes of anemia and hepatic changes over the last 18 months. The physical examination parameters on the day of admission to the veterinary hospital as well as previous therapies are listed in Table 1. Previous episodes were treated with anti-hemoparasitic agents and blood transfusion at a different hospital. The horse had tested negative for leptospirosis and *Trypanosoma evansi* and positive for *Babesia caballi*. Dietary management consisted of commercial feed, mineralized salt, coast-cross pasture and well water. At least 10 other horses living in the same property had a history of similar, non-recurrent episodes of hyperthermia and hemoglobinuria around the age of 3 years.

The horse did not make it to the hospital alive and was quickly referred for postmortem examination. Urine sediment contained

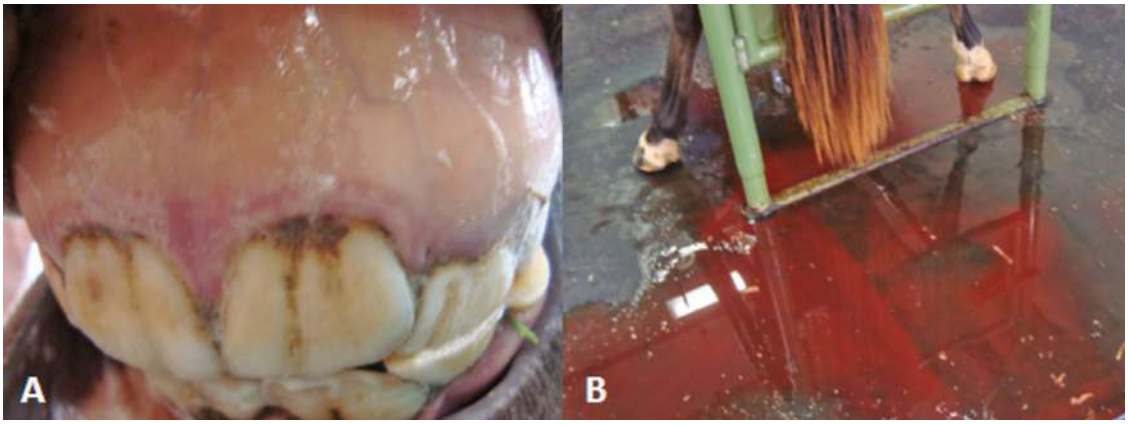

Fig 1. Case 1. (A) Oral mucosa yellow and with toxic appearance. (B) Dark brown urine.

Table 2
Clinical evolution of the mare after emergency treatment.

Hour	Heart Rate	Respiratory Frequency	Rectal Temperature	Capillary Fill Time	Mucosal Staining	Motility
[a]02:20 pm	68 bpm	28 mpm	37.6°C	3-4″	Cyanotic halo	Hypomotility
5:30 pm	60 bpm	20 mpm	37.6°C	3″	Toxemic	Hypomotility
7:00 pm	68 bpm	16 mpm	37.5°C	2″	Pale with cyanotic halo	Hypomotility
10:00 pm	DEATH					

[a] Fluid therapy performed with 16 liters of Ringer Lactate.

Table 3
Laboratory, clinical history and follow-up findings for comparative analysis of equine copper toxicity cases.

	Case 1	Case 2	Case 3 (F)	Case 4 (M)	Case 5	Ref [12,13]
Red blood cells (x10^6/⌈L)	2.1	NA	2.5	1.5	3.4	6.9-10.7
Ht (%)	13.0	NA	15.0	7.0	21.0	31-48
Hb (g/dL)	4.0	NA	4.8	5.9	7.4	11.3-17.9
AST (U/L)	1.091.5	NA	1.324.0	991.0	740.0	141-330
GGT (U/L)	108.0	NA	137.9	358.0	389.0	4-44
Hemoglobinuria	+	+	+	+	+	-
Heinz bodies	+	+	+	+	+	-
Blood Cu levels (⌈mol/L)	45.33	38.0[a]	NA	NA	31.49[b]	16-28
Liver Cu (⌈mol/g)	18.96	1.61	NA	NA	NA	0.27–0.31
Kidney Cu (⌈mol/g)	1.62	1.95	NA	NA	NA	0.36–0.47
Soil/feed Cu	NA	NA	↑	↑	NA	-
Other animals affected	Yes	Yes	Yes	Yes	No	-
Death	Yes	Yes	No	No	No	-
Recurrence	No	No	Yes	Yes	Yes	-

AST, aspartate aminotransferase; F, female; GGT, gamaglutamil transferase; M, male; NA, not assessed; Ref., references.
[a] blood sample collected after death.
[b] upon recurrence.

no red blood cells. Blood smears revealed Heinz bodies. Major gross findings were as follows: yellow tinged mucous membranes, light brown colored, firm liver, small-sized spleen and dark urine. Histopathological examination revealed bridging liver fibrosis with ductal hyperplasia, cholestasis and pigments consistent with iron and copper accumulation (Perls and rodhamine stains) in hepatocytes and Kupffer cells. Hemoglobinuric nephrosis and pigments consistent with iron and copper accumulation in kidney epithelial tubular cells were also noted. Blood, liver, and kidney samples were sent for copper level determination (Table 3).

The pasture where horses were kept was cared for by expert professionals and the rotational grazing system adopted. The owner was not informed of products used and there were no invasive plants. The pasture area was next to and level with a neighbour coffee plantation submitted to periodic treatment with copper hydroxide fungicide (691 g/kg), among other products.

Laboratory tests of other horses living in that property were requested. Low red blood cell count and increased aspartate aminotransferase (AST) and gamaglutamil transferase (GGT) levels were detected in 2 horses with a history of similar episodes. Those that had not suffered hemolytic crises (2 horses) were not anemic; however, increased GGT and mildly increased AST levels were detected in one and both horses respectively. Serum copper determination performed in 2 horses revealed copper levels within normal ranges. One of the horses had a serum copper value corresponding to 119 µ/dL, while in the other horse the measurement was 131 µ/dL. It is worth mentioning that the reference value used by the laboratory was 56.5–170.2 µ / dl. The measurement of serum copper was performed using Atomic Absorption Spectrophotometry. Water quality analysis revealed acceptable levels of copper (<0.008 mg/L). The measurement was performed using the Standard Methods for the Examination of Water and Wastewater (SMEWW) 3111B and D.

2.3. Cases 3 and 4

Two Lusitano horses (an 8-year-old mare and a 2.5-year-old gelding) were referred from the same property 28 days apart.

The mare was referred first with a 2-month history of apathy, weight loss and postpartum anemia. Three other mares kept in the same paddock had also developed mild anemia; therefore, mares were moved to a different paddock and the owner switched to a different brand of feed. The gelding had been in stall confinement for approximately 1 month and had a recent history of apathy and acute hemoglobinuria. The parameters related to the physical examination at the time of the clinical consultation at the veterinary hospital and the previous treatments of both animals are shown in Table 1.

Clinical examination of the mare revealed pale, yellow tinged mucous membranes, mild hyperthermia, tachycardia, tachypnea and dark urine. Laboratory tests findings were as follows: anemia, Heinz bodies, hemoglobinuria, increased bilirubin levels and markedly increased AST and GGT levels. The gelding had similar manifestations, except for normothermia, more pronounced tachycardia, anemia and hemoglobinuria, and more markedly elevated GGT levels (Table 1). Three other horses living in the same property had suffered similar, milder episodes; elevated liver enzyme levels had been also detected in asymptomatic horses.

The mare received fluid therapy, hepatoprotective agents and copper-free vitamin and mineral supplements. Physical signs and laboratory test results improved, but liver enzymes did not return to normal levels. The mare remained in hospital for 15 days and returned within 2 weeks of discharge due to recurring hemoglobinuria. The mare was kept in hospital for another month and has been healthy ever since.

The gelding was treated in a similar fashion, except for blood transfusion and ammonium tetrathiomolybdate solution (TTM, 3.4 mg/kg, IV, SID) given for 3 days (long enough for hemoglobinuria to resolve). Signs of laminitis appeared on the fourth day. A second hemolytic episode requiring blood transfusion occurred over the course of the 4-month hospitalization period.

Commercial feed analysis revealed copper levels ranging from 20.4 to 59.6 mg/kg, depending on the batch. Analysis of commercial feed revealed copper levels ranging from 20.4 to 59.6 mg/kg, depending on the batch. The method used for measurement was the AOAC Official Method 2013.06 [PNT005-AB]. Copper content investigation in soil and pastures was based on well-established methods. Copper levels were above the upper reference limit in most paddocks, with higher levels detected in lower-lying areas previously used as vineyards, where most affected horses were now kept. Excess copper was also detected in forages; estimated total copper intake exceeded dietary requirements for young and older horses.

2.4. Case 5

A 8-year-old *Mangalarga Marchador* gelding was referred with a one-month history of yellow tinged mucous membranes and fever unresponsive to antihemoparasitic therapy. The horse was fed hay, grass and well water. Grass crops were treated with poultry litter and there were veggie patches around the property. Hay analysis failed to reveal fungi or aflatoxins. The physical examination parameters on the day of admission to the veterinary hospital as well as previous therapies are listed in Table 1.

Physical examination revealed mild tachycardia, yellow tinged mucous membranes and red colored urine. Elevated AST and GGT (particularly GGT) levels (Table 1), macrocytic anemia, Heinz bodies, intravascular hemolysis, hemoglobinuria and bilirubinuria were the major laboratory findings. The horse tested negative for EIA and leptospirosis.

Transcutaneous liver biopsy revealed lobular necrotic lymphocytic and neutrophilic hepatitis with ductal hyperplasia, portal fibrosis, intracytoplasmic copper pigments in hepatocytes and histiocytes and hemosiderin pigments in Kupffer cells.

Laboratory test results from other horses living in the same property showed mildly increased AST and GGT levels in 2 animals.

Dietary copper analysis revealed the following levels: 9.01 mg/kg in hay; 21.65 mg/kg in commercial feed, 2.1 to 8.60 mg/kg in pastures (depending on location) and 976.16 mg/kg in mineralized salt. The method used to measure copper in the respective samples was MA-002 [NIOSH 7904:1994].

Copper toxicity was suspected and fluid therapy, hepatoprotective agents, vitamin and copper-free mineral supplementation started before completion of ancillary tests. Physical signs and laboratory test results improved, but liver enzyme (particularly GGT) levels did not return to normal. The horse remained in hospital for 22 days and returned within 5 months of discharge due to recurring hemoglobinuria and anemia. Physical examination and laboratory findings were similar; however, the liver parenchyma appeared hyperechoic and fibrotic on sonography. The same treatment was repeated and the horse discharged within 11 days of admission.

The condition recurred approximately 1 year later and was again successfully treated. At that time, blood copper levels were investigated (Table 3). The horse had 2 subsequent episodes within the next 2 months; liver biopsy was repeated on one occasion and revealed (Fig. 2) active lymphohistiocytic necrotic hepatitis with ductal hyperplasia, portal fibrosis and portal to centrilobular fibrosis, intracytoplasmic pigments consistent with copper (rodhamine stain) in centrilobubar hepatocytes and hemosiderin pigments in Kupffer cells. The reticulin framework was disturbed and characterized by multifocal loss of framework or ruptured and collapsed liver framework by reticulin stain. Subsequently, after one year and 3 months, the horse was taken for evaluation at the veterinary hospital, presenting hematuria. The physical parameters at the time of admission corresponded to a heart rate of 40 bpm, a respiratory rate of 20 mpm, a rectal temperature equal to 36.9°C, a capillary filling time of 3 seconds and pale mucous membranes. The animal arrived in decubitus, with the impossibility of getting up. The immediate treatment instituted was dexamethasone (1 mg/kg) and blood transfusion with whole blood. However, the next day, the horse developed neurological disorders and was euthanized. Necropsy findings indicated dark brown kidneys, brownish pericardial effusion, pale and contracted liver and diffuse jaundice in the tissues.

3. Discussion

Copper toxicity may be acute or cumulative (chronic). Acute toxicity occurs when large amounts of copper are ingested within a short period of time. Ionic copper binds to the gastric and intestinal mucosa, leading to erosion and ulceration and ultimately to severe gastroenteritis and bleeding. Liver damage, hemolytic anemia, and nephrosis may result from absorption of copper in large quantities [16,17].

In cumulative toxicity, copper accumulates asymptomatically in the liver for long periods of time and serum biochemistry changes derived from progressive liver damage appear around the time of hemolytic episodes [18]. When liver storage capacity is exceeded (which may take a few weeks to a year to occur), copper is released into the bloodstream, leading to massive hemolysis, renal and hepatic failure. Affected animals tend to progress to death in 2–5 days [19]. Typical histopathological findings include cirrhosis of the liver or toxic hepatitis, coagulative necrosis or degenerative liver changes and nephrosis with hemoglobin casts [3]. It is noteworthy that the copper content in the liver can be calculated based

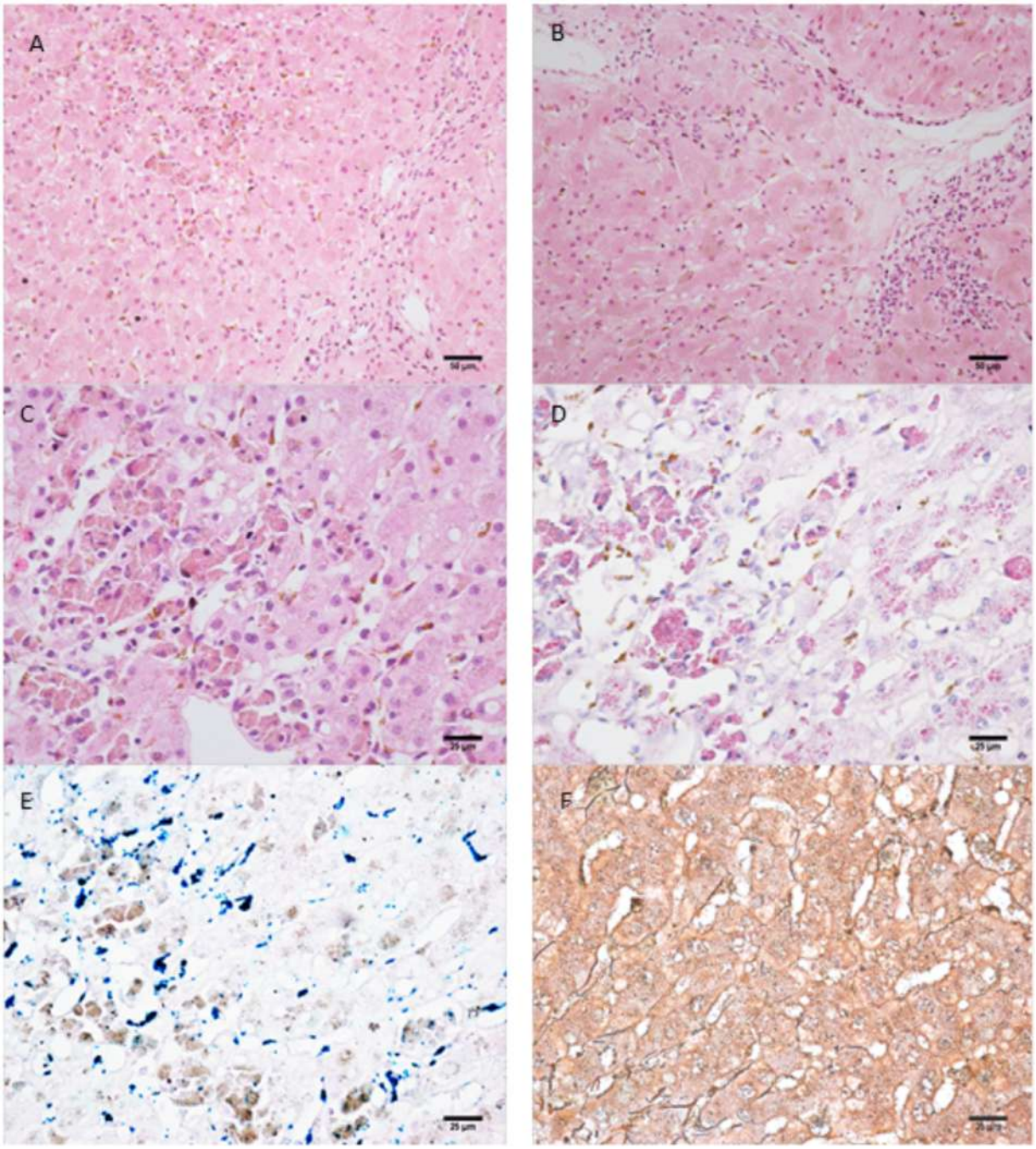

Fig 2. Case 5. Histopathological lesions of liver. (A) Lymphohistiocytic necrotic hepatitis with lobular activity, portal fibrosis and intracytoplasmic dark brown pigments in centrilobubar hepatocytes and portal macrophages, and golden pigments in Kupffer cells. Hematoxylin and eosin (HE), 200x; (B) Lymphocityc portal hepatitis with portal fibrosis and intracytoplasmic dark brown pigments in hepatocytes and portal macrophages and golden pigments in Kupffer cells. HE, 200x; (C) Active lymphohistiocytic necrotic hepatitis with intracytoplasmic dark brown pigments in hepatocytes, and golden pigments in Kupffer cells, HE, 400x; (D) Intracytoplasmic cooper pigments in red in centrilobubar hepatocytes and golden pigments in Kupffer cells, Rodhamine stain, 400x. (E) Intracytoplasmic hemosiderin pigments in blue in Kupffer cells, Perls stain, 400x; (F) collapsed, ruptured and loss of reticulin framework of liver, Reticulin stain, 400x.

on dry matter, with the possibility of such measurement being carried out post mortem [20].

Although animals' excessive intake of copper is a possible cause of intoxication, previous liver diseases can contribute to its occurrence since there is concomitant impairment of liver function in addition to accumulation of copper in the organ. Therefore, it is important to investigate conditions that can lead to liver diseases, without forgetting recent discoveries such as the new equine parvovirus, identified in 2018, responsible for causing death to a horse due to installed hepatitis [21]. Also, equine hepacivirus can also cause liver damage [22]. In the cases studied, no other concomitant cause of hepatic involvement was found. Furthermore, the histopathology of the cases presented was not consistent with the findings in the literature for viral liver diseases, as shown in the research carried out by Tomlinson et al. [23], in which lymphocytic infiltrates and necrotic hepatocytes infected with equine parvovirus were identified.

Clinical and laboratory findings described in this study are typical of cumulative copper toxicity. Even asymptomatic animals liv-

ing in contact with affected horses had liver enzyme changes suggestive of chronic toxicity, as reported in ruminants.

Copper toxicity was suspected in these horses due to liver damage combined with Heinz body-mediated intravascular hemolysis, which is seldom observed in other clinical conditions. Several diseases may cause liver damage in horses and some may be associated with elevated GGT levels, but concurrent intravascular hemolysis is uncommon [13].

It is interesting to observe that cases 1 and 5 presented hepatic hemosiderosis. Copper causes direct damage to the renal proximal tubule in cases of intoxication, but intravascular hemolysis plays an important role in the pathogenesis of renal failure. When considering the occurrence of hemolysis, resulting from excessive destruction of red blood cells, it is occurred hemosiderin formation and accumulation in the tissue, including the liver [24].

In adult horses, hemolysis may be caused by toxic changes (red maple, onions, phenothiazines, methylene blue), immunological disorders (primary or secondary immune-mediated response to bacterial or viral infections, adverse drug reactions and neoplastic diseases), infectious (piroplasmosis, granulocytic ehrlichiosis), iatrogenic (hypotonic fluid therapy, DMSO>10%) and other (disseminated intravascular coagulation, familial methemoglobinemia) conditions [13]. However, few conditions are able to induce Heinz body formation [13], the most common in horses being poisonous plant, onion or garlic intake, phenotiazine toxicity and DMSO administration. These hypotheses have all been ruled out in cases reported.

Copper toxicity diagnosis was based on history of excess dietary/environmental copper and/or copper level determination in blood, kidney and liver samples collected from affected animals.

An important limitation of our study is that we did not analyze animal hair for the presence of copper, as the presence of the element in the hair indicates long-term copper intake. If the copper content in the hair was low, it is likely that there is another cause, not just intoxication, for the accumulation of copper in the liver. On the other hand, if the copper content in the hair was high, this result would be a strong argument to justify the diagnosis of copper poisoning, along with the other findings reported. To emphasize this statement, it is known that metals can accumulate both inside and outside the hair. In mammals, hair is mainly composed of keratin, which contains ample amounts of sulfhydryl that can bind various metals [25]. Another important limitation to be highlighted is that not all necessary data was collected in all cases, since the animals were brought to the hospital for care, and it was not always possible to have access to samples and other information by owner's choice."

Horses are thought to be less susceptible to copper toxicity [6,7]. However, studies investigating elevated copper levels in this species are scarce.

Pasture treatment with poultry litter is a major cause of copper toxicity in sheep [26]. Poultry diets are rich in phosphorus and may contain arsenic, copper and zinc, which are used as growth promoters and excreted in feces and urine. Therefore, poultry litter is a rich source of these minerals [27]. Also, poultry litter may be treated with copper-based fungicides [3]. Copper levels in poultry litter may range from 25 to 1003 mg/kg (473 mg/kg on average) [26]. Copper-rich poultry litter was thought to be the cause of soil and pasture contamination in cases 1 and 5. Poultry litter feeding ban in Brazil led to increased use of poultry manure as soil fertilizer in an effort to reduce fertilization costs and eliminate poultry farming waste. Horses are highly tolerant of copper; still pasture fertilization with poultry litter should be done with extreme caution due to potential toxicity.

On the other hand, according to Kuziemska et al. [28], fertilizers such as poultry manure (not the poultry litter) may not only be a valuable source of plant nutrients, but may also reduce the phytotoxicity of heavy metals, including copper. These researchers investigated the effect of increasing doses of copper applications in combination with various organic fertilizers (cattle and poultry manure, and mushroom substrate) on Cocksfoot Grass yield, as well as copper absorption by them. As a result, all organic fertilizers were able to reduce the toxic effect of copper, especially cattle manure. Therefore, the use of such fertilizers could be an alternative to the use of poultry litter, contributing to lower the levels of copper in the pasture and, consequently, excessive intake of it by animals.

Pastures contaminated with copper fungicides have been associated with copper toxicity in ruminants [3]. One must therefore be aware of crops requiring wide use of copper-based products in the vicinity of animal herds, even horse herds. In Brazil, as provided by the Ministry of Agriculture, Livestock and Supply, several copper fungicides are marketed, containing different molecules, dosages and granulometry [29]. As indicated by the Fungicide Resistance Action Committee (FRAC), despite the diversity of existing formulations, copper fungicides must form a uniform and long-lasting film on the cultivated content, without accumulating in it [30].

As an example, to demonstrate the importance of precautions when using copper fungicides, frequent applications of such formulations increased the soil surface copper content in some places in Serra Gaúcha (Brazil) exceeding the critical soil content of 0.40 mg/L [31] and the maximum adsorption capacity of soils, which can poison plants and water sources [32,33]. This accumulation has also been observed in other countries, such as France, where the long-term application of cupric fungicides to control downy mildew (*Plasmopara viticola*) resulted in an increase in copper concentration in the order of 100–1500 mg/kg [34]. Based on such evidence, the appropriate concentration of cupric fungicide must be used according to the goal that is desired with the planting in question, so that excess copper does not occur later in the soil and in the cultivated content, avoiding, thus, a possible poisoning of animals that have access to the cultivation site.

In cases 2, 3 and 4, toxicity occurred in response to long-term intake of copper derived from soil contaminated with products used in crops grown in the same property (coffee crops treated with copper fungicide, case 2; soil and pasture contamination from previous grape farming, cases 3 and 4). Copper-based products are widely used in coffee plantations and vineyards. Regular monitoring of nutrient levels (including copper) in soil and pasture is recommended for deficiency and toxicity prevention purposes.

Only 2 confirmed cases of naturally occurring copper toxicity have been reported in horses: copper sulphate intake [9] and liver damage induced by pyrrolizidine alkaloids found in *Senecio jacobea* and chewing of copper-contaminated wood [10].

Potential copper toxicity associated with ingestion of pyrrolizidine alkaloid-rich plants has been reported in sheep. These alkaloids promote hepatocyte damage and copper toxicity – or rather phytogenic copper toxicity [3]. Horses in this study had no signs of pre-existing liver damage and pyrrolizidine alkaloid containing plants were not present in their diet or environment, nor were histopathological findings consistent with this type of toxicity. Still, potential associations support the need to investigate primary liver damage as a predisposing factor to copper toxicity.

Clinical progression in cases studied differed from descriptions in ruminants. Sheep may make a full recovery if properly treated or even in rare, untreated cases, in spite of the highly lethal nature of copper toxicity and the high risk of hemolysis- and/or kidney failure-related death in this species. Surviving horses in this case series took around 2 weeks to recover from liver damage and had one or more recurring hemolytic episodes. Similar data were extracted from the clinical history of horses living in contact with affected patients. Recurrence is seldom reported in ruminants [35], except in cases of repeated excess copper intake. It should also be

considered that once the diagnosis of copper poisoning is made, changes are expected in animal management, reducing the probability of recurrent poison with excess copper ingested. This procedure was carried out with the surviving animals and with the horses that had contact with places with excess copper in our study. Dietary changes were made, and the animals began to graze in other places, as pertinent to each case.

Three phases of cumulative toxicity have been described in ruminants: pre-hemolytic, hemolytic and posthemolytic. The pre-hemolytic phase may last anywhere from one month to 2 years and is characterized by asymptomatic copper accumulation in the liver, in the ten days preceding the hemolytic crisis. Once liver storage capacity is exceeded, copper becomes free in hepatocytes, leading to hepatic necrosis and a surge of copper release into the bloodstream [16,17,36]. Liver enzyme changes had been detected long before hemolytic episodes in several patients in this series, including those that did not progress to the hemolytic phase.

A recent study conducted with rats to investigate the effects of high copper concentrations in the cecum and rectum, demonstrated the occurrence of lesions and interference in microbiota homeostasis [37]. When considering the importance of proper functioning of the gastrointestinal tract for the horse to have a healthy life, the information that excess copper at the intestinal level can contribute to the deterioration of the clinical condition of intoxicated animals should not be discarded.

Clinical features of copper toxicity in horses are thought to be similar to those described in young ruminants prior to rumen development. Different from adult patients, liver damage may precede severe anemia and related hemolytic episodes by several weeks in these animals, with impaired growth and potential chronic toxicity if dietary copper levels are not adjusted. Icterus and a small-sized, fibrotic liver may be found at slaughter [38]. Cirrhosis of the liver has been described in ruminants; however, toxic hepatitis is the prevailing finding [3]. In horses, chronic liver damage (as evidenced by sonographic and histopathologic findings in case 5) may translate into increased risks of recurrent copper-induced hemolytic episodes.

Given the rare nature of the disease in horses, no treatment protocols have been established to date. The effectiveness of drugs such as penicillamine, zinc, trientine, and tetrathiomolybdate has been reported in other species [39], but not in horses. Cases described in literature were treated in a similar fashion to those in this series (i.e., fluid therapy for kidney failure prevention and control, hepatoprotective agents and blood transfusion in cases of severe anemia). Excess dietary copper levels were also adjusted. Dietary adjustments should be considered, given the high copper content in many commercial feeds and supplements and the limited availability of copper-free iron supplements in the domestic market.

Therapeutic use of TTM in horses suspected of copper toxicity has not been described. The drug was used in case 4 with due owner consent in an attempt to control the extremely severe disease, at doses extrapolated from ruminants. However, patient survival could not be attributed to the effect of TTM alone. Also, that horse was the only one to develop laminitis. Whether laminitis was disease-related or TTM-induced remains to be determined.

One may wonder why equine copper toxicity is becoming increasingly common in Brazil. This case series describes 4 outbreaks involving several animals, some of which progressed to death. If copper toxicity has always been a problem in horses, why are case descriptions scarce? Lack of proper diagnosis? The multifactorial nature of the disease, as shown in cases 3 and 4, in which environmental contamination was involved, probably has a role to play. Copper levels in soil may vary throughout the year; still, excessive amounts of copper were detected in pastures in those cases. Also, affected horses were fed commercial feed with borderline or excess copper, considering the tolerance value of 50 mg/kg DM for the equine species [7]."

Minimum copper content in feed labels is 30 mg/kg. However, batches containing almost twice as much copper are the norm in the equine feed industry. NRC recommendations correspond to 80 mg of copper per 400 kg of body weight or 10 mg/kg in feed (NRC 2007 and 1989 respectively). Given the high tolerance of copper of horses compared to other species, only minimum amounts are accounted for in feed manufacturing, with many good brands often exceeding that limit. Equine mineral supplements follow the same minimum level rule. Copper intake levels by individual horses are therefore unknown. Cases described in this series suggest excess copper may in fact lead to toxicity and death, a remote hypothesis to date. Attention was not paid to the observation of the amount of zinc present in the feed available for horses. However, zinc can interfere with copper absorption by animals, as explained later in this discussion.

Determining a reliable upper limit of tolerability for copper is difficult due to the physiological regulation of copper reservoirs that prevent pro-oxidative states in periods of deficiency or excess. Increased uptake of enteric copper occurs when liver storage is low, while increases in the synthesis of metallothionein, a copper-binding protein that limits enteric uptake or distribution, along with bile excretion, occur when liver stocks are high. Although these self-regulatory adjustments keep the copper content in hepatocytes within safe metabolic limits, the genetic mutations that influence these mechanisms can modify or limit the appropriate physiological response. This fact can be particularly evident when food intake is close to the upper limit of tolerability [40].

Still in relation to the animals' diet, a study conducted by Minervino et al. [41] investigated whether zinc supplementation may be an option as a preventive measure to the accumulation of copper in the liver of sheep. It was concluded that 300 mg/kg of zinc in dry matter is useful in preventing excessive hepatic storage of copper. In addition, the accumulation in the liver was lower in animals that received zinc supplementation. The synthesis of metallothionein (low molecular weight proteins) is regulated by the amount of zinc ingested. When large amounts of this element are ingested, the greater amount of metallothionein is integrated, more complex metallothionein-copper forms which are subsequently excreted. Therefore, an excessive intake of zinc reduces the absorption of copper, leading to an increase in its excretion [42].

Regarding the factors that interfere with copper absorption and its interaction with other elements, in addition to its relationship with zinc, it should be considered that iron is known to be present in greater amounts in autumn forages, when the absorption of copper by animals is reduced [43]. Excessive iron intake by horses can result in haemochromatosis (accumulation of iron in hepatocytes), causing chronic hepatitis. This event, however, is rare in adult horses, requiring the ingestion of extremely high levels of iron or the ingestion of this mineral for long periods [44]. Based on this statement, it can be assumed that even if copper consumption by animals is reduced at certain times of the year, due to the large amount of iron present in forages, it is unlikely that iron intake will cause significant liver changes enough to cause intoxication. Therefore, attention should be paid to the levels of copper in the soil used for grazing and possible pre-existing hepatic alterations presented by the animals. Also, in relation to the interaction of copper with iron, in human medicine there is evidence that high levels of the first can reduce the latter absorption, causing deleterious hematological effects, such as anemia [45].

Just as important as the interaction of copper with other metals and what factors interferes with its absorption, especially in cases of suspected intoxication by the element, it should not be forgotten that the concentration of copper in the soil is significantly higher when compared to other metals. This can be pos-

sibly because copper ions form soluble compounds when interacting with ligands present in the soil solution, such as organic acids exuded by plants [46]. When soils pH is lower than 5, copper becomes more available since it is found in its ionic form. As the pH increases, these ions become insoluble in oxides and hydroxides forms, consequently decreasing their availability [47]. Therefore, it is possible that there are places with low levels of copper. If the analysis of the soil indeed indicates small amounts of this element, and there are cases of horses suspected of copper poisoning, it would be plausible to search for it in the feed given to the animal, as well as how fertilization is carried out of the soil and, of course, investigate the history of previous liver diseases.

The horses in this series were supposedly fed large amounts of copper derived from pastures, ration and mineralized salt. This is an assumption because we do not have all the dosages of the amount of copper ingested by each animal. However, in each case of this study a source of excess copper and/or an increased dosage of the element was found, and, in addition, the manifestations were all consistent with copper intoxication and with no further diagnosis, regardless any previous hepatic factor predisposing. From the standpoint of copper alone, horses fed copper-rich feed and/or pasture should not be given copper-rich salt. In contrast to previous nutritional deficiencies, it appears that we are heading towards a nutrient overload (and not just from an energy standpoint, as the current obesity trend in horses suggests). If horses' diets are not re-evaluated, copper toxicity may no longer be a rare condition, regardless of pre-existing liver damage, if there is a heavy copper consumption present. Furthermore, as our set of reports had different breeds of horses, the risk of toxicity may be related to the genetics of the animals, which can contribute to greater or lesser tolerance to high concentrations of the element.

4. Conclusion

Copper toxicity may occur in horses and can be associated with excess copper derived from various sources and/or predisposing dietary factors, regardless of whether or not pre-existing liver disorders are detected. Given the unusual nature of the condition in horses, little is known about the progression of the disease in this species and diagnostic and treatment protocols are insufficient. Veterinarians and owners must have a vast knowledgeable about animal diets and pasture fertilization strategies to prevent copper toxicity. Copper toxicity may occur in horses and may be associated with excess copper derived from several sources and/or with predisposing dietary factors.

Authorship

Carla Bargi Belli, Wilson Roberto Fernandes, Henrique Macedo Neuenschwander and Raquel Yvonne Arantes Baccarin were responsible for the clinical management of patients and idealized this study. Aline de Matos Curvelo de Barros was also responsible for the clinical management of 2 animals in the study and contributed to the writing of the manuscript. Luciana Neves Torres, Lilian Rose Marques de Sá and Paulo C. Maiorka performed postmortem and histopathological exams. Maria Claudia Araripe Sucupira was responsible for the determination of copper levels. This manuscript was prepared by Carla Bargi Belli; the final version was approved by all contributing authors.

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References

[1] Thompson LJ. Copper. In: Gupta RC, editor. Veterinary toxicology-basic and clinical principles. Elsevier Academic Press; 2007. p. 427–30.
[2] Collins JF, Klevay LM. Copper. Adv Nutr 2011;2:520–2.
[3] Tokarnia CH, Peixoto PV, Barbosa JD, Brito MF, Döbereiner J. Deficiências Minerais em Animais de Produção. Rio de Janeiro: Helianthus 2010.
[4] Tapiero H, Townsend DM, Tew KD. Trace elements in human physiology and pathology. Copper. Biomed Pharmacother 2003;57:386–98.
[5] Weigel RA, Ortolani EL, Sucupira MCA. Oxidative metabolism in copper poisoning sheep treated with tetrathiomolybdate and/or antioxidative vitamins. Braz J Vet Res Anim Sci 2010;47:421–8.
[6] Bozynski CC, Evans TJ, Kim DY, Johnson GC, Hughes-Hanks JM, Mitchell WJ, et al. Copper toxicosis with hemolysis and hemoglobinuric nephrosis in three adult Boer goats. J Vet Diagn Invest 2009;21:395–400.
[7] Smith JD, Jordan R, Nelson ML. Tolerance of ponies to high levels of dietary copper. J Anim Sci 1975;41:1645–9.
[8] Auer DE, Seawright AA. A suspected case of acute copper toxicity in a horse. Aust Vet J 1989;66:191–2.
[9] Bauer M. Copper sulphate poisoning in horses. Veterinarski Arhiv 1975;45:257–67.
[10] Dewes HF, Lowe MD. Haemolytic crisis associated with ragwort poisoning and rail chewing in two thoroughbred fillies. New Zealand Vet J 1985;33:160–89.
[11] Ankringa N, Wijnberg ID, Boerma S, Ijzer J. Copper-associated hepatic cirrhosis in a Friesian horse. Tijdschrift voor Diergeneeskunde 2012;137:310–14.
[12] Cymbaluk NF, Christensen DA. Copper, zinc and manganese concentrations in equine liver, kidney and plasma. Can Vet J 1986;27:206–10.
[13] Sprayberry KA, Robinson NE. Robinson's current therapy in equine medicine. 7th ed. St Louis: Elsevier; 2015.
[14] Shawaf T, Almathen F, Meligy A, El-Deeb W, Al-Bulushi S. Biochemical analysis of some serum trace elements in donkeys and horses in Eastern region of Kingdom of Saudi Arabia. Veterinary World 2017;10:1269–74.
[15] Silva MAML, Porto Filho RM, Rosas Filho AC, Maciel CC, Doretto JS, Silva HL. Determinação das concentrações de cobre e zinco séricos de eqüinos da região de Espírito Santo do Pinhal. Bol Med Vet 2007;3:24–34.
[16] Duncan A. Copper. In Practice 1997;19(9):503.
[17] Ferreira MC., Antonelli AC, Ortolani EL. Intoxicação por cobre, selênio, zinco e cloreto de sódio. In: Spinosa HS, Górniak SL, Palermo-Neto J, editors. Toxicologia Aplicada à Medicina Veterinária, Manole; 2008, p. 666-678.
[18] Ortolani EL, Machado CH, Sucupira MC. Assessment of some clinical and laboratory variables for early diagnosis of cumulative copper poisoning in sheep. Vet Hum Toxicol 2003;45:289–93.
[19] Maiorka PC, Massoco CO, De Almeida SD, Gorniak SL, Dagli ML. Copper toxicosis in sheep: a case report. Vet HumToxicol 1998;40:99–100.
[20] Johnston H, Beasley L, MacPherson N. Copper toxicity in a New Zealand dairy herd. Ir Vet J 2014;67:1–6.
[21] Diver TJ, Tennant BC, Kumar A, McDonough S, Cullen J, Bhuva N, et al. New parvovirus associated with serum hepatitis in horses after inoculation of common biological product. Emerg Infect Dis 2018;24:303–10.
[22] Date T, Sugiyama M, Lkhagvasuren D, Wakita T, Oyunsuren T, Mizokami M. Prevalence of equine hepacivirus infection in Mongolia. Virus Res 2020;282:197940.
[23] Tomlinson JE, Jager M, Struzyna A, Laverack M, Fortier LA, Dubovi E, et al. Tropism, pathology, and transmission of equine parvovirus-hepatitis. Emerg Microbes Infect 2020;9:651–63.
[24] Miraj SS;, Rao M. Clinical toxicology of copper. In: Bagchi D, Bagchi M, editors. Metal Toxicol Handbook. Boca Raton: CRC Press; 2020. cap.13, 19p.
[25] Khazaee M, Hamidian AH, Shabani AA, Ashrafi S, Mirjalili SAA, Esmaeilzadeh E. Accumulation of heavy metals and as in liver, hair, femur, and lung of Persian jird (Meriones persicus) in Darreh Zereshk copper mine. Iran. Environ Sci Pollut Res 2016;23:3860–70.
[26] Christodoulopoulos G, Roubies N. Diagnosis and treatment of copper poisoning caused by accidental feeding on poultry litter in a sheep flock. Aust Vet J 2007;85:451–3.
[27] Toor GS, Haggard BE. Phosphorus and trace metal dynamics in soils amended with poultry litter and granulates. Soil Use Manage 2009;25:409–18.
[28] Kuziemska B, Trębicka J, Wysokinski A, Jaremko D. Supplementation of organic amendments improve yield and adaptability by reducing the toxic effect of copper in cocksfoot grass (dactylis glomerata L. Cv Amera). Agronomy 2021;11:1–11.
[29] Ministério da Agricultura, Pecuária e Abastecimento – MAPA. Consulta de produtos formulados. Coordenação Geral de Agrotóxicos e Afins/DFIA/DAS. 2003. Available in: https://agrofit.agricultura.gov.br/agrofit_cons/!ap_produto_form_detalhe_cons?p_id_produto_formulado_tecnico=9299&p_tipo_janela=NEW Last accessed: 28/06/2021
[30] Fungicide Resistance Action Committee – FRAC. Fungicides sorted by model of action (including FRAC code numbering). 2017. Available in: < http://www.frac.info/docs/defaultsource/publications/frac-code-list/frac-code-list-2017-final.pdf?sfvrsn=fab94a9a_2 >Last accessed: 28/06/2021

[31] Comissão de química e fertilidade do solo – RS/SC. Manual de adubação e de calagem para os estados do Rio Grande do Sul e Santa Catarina. Sociedade Brasileira de Ciência do Solo, Núcleo Regional Sul. Porto Alegre,2016.
[32] Casali CA, Moterle DF, Rheinheimer DS, Brunetto G, Corcini ALM, Kaminski J, et al. Copper forms and desorption in soils under grapevine in the Serra Gaúcha of Rio Grande do Sul. Rev Bras Ciênc Solo 2008;32:1479–87.
[33] Mirlean N, Roisenberg A, Chies JO. Metal contamination of vineyard soils in wet subtropics (Southern Brazil). Environ Pollut 2007;149:10–17.
[34] Flores-Veles LM, Ducaroir J, Jaunet A, Roberto M. Study of the distribution of copper in an acid sandy vineyard soil by three different methods. Eur J Soil Sci 1996;47:523–32.
[35] Augusto CFB, Morgado AA, Souza FN, Nunes GR, Weigel RA, Mori CS, et al. Recidiva de crise hemolítica em ovinos tratados com tetratiomolibdato e/ou vitaminas antioxidantes, após indução experimental. In: Proceedings 34th Congresso Brasileiro de Medicina Veterinária, Santos, Brazil; 2007.
[36] Sullivan JM, Janovitz EB, Robinson FR. Copper toxicosis in veal calves. J Vet Diagn Invest 1991;3:161–4.
[37] Cheng S, Mao H, Ruan Y, Wu C, Xu Z, Hu G, et al. Copper changes intestinal microbiota of the cecum and rectum in female mice by 16S rRNA gene sequencing. Biol Trace Elem Res 2020;193:445–55.
[38] McDowell LR. Minerals in animal and human nutrition. 2nd ed. Amsterdam: Elsevier; 2003.
[39] Hoffmann G. Copper–associated liver disease. Vet Clin North Am.: Small Anim Pract 2009;39:489–511.
[40] Center SA, Richter KP, Twedt DC, Wakshalag JJ, Watson P, Webster RL. Is it time to reconsider current guidelines for copper content in commercial dog foods? JAVMA 2021;258:357–64.
[41] Minervino AHH, López-Alonso M, Júnior BRA, Rodrigues FAML, Araújo CASC, Sousa RS, et al. Dietary zinc supplementation to prevent chronic copper poisoning in sheep. Animals 2018;8:227.
[42] Dolcourt BA, Paxton JH, Bora KM, Aaron CK, et al. Pennies for your thoughts: A case series of pancytopenia due to zinc-induced copper deficiency in the same patient. Clin Pract Cases Emerg Med 2019;3:34.
[43] Hill GM;, Shannon MC. Copper and zinc nutritional issues for agricultural animal production. Biol Trace Elem Res 2019;188:148–59.
[44] Theelen MJP, Beukers M, Grinwis GCM, van Oldruitenborgh-Oosterbaan MMS. Chronic iron overload causing haemochromatosis and hepatopathy in 21 horses and one donkey. Equine Vet Journal 2019;51:303–9.
[45] Angelova Asenova S, Nedkova V, Koleva-Kolarova R. Copper in the human organism. Trakia J Sci 2011;9:88–98.
[46] Meurer EJ. Fundamentos de Química do Solo. 3ed. Porto Alegre: Gênesis; 2006.
[47] Abreu C.A, Lopes AS, Santos G. Micronutrientes. In: Novais RF, editor. Fertilidade do solo. Viçosa: Sociedade Brasileira de Ciência do Solo; 2007. p. 645-736.