PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(9):651-661, September 2020
DOI: 10.1590/1678-5150-PVB-6526

Original Article
Livestock Diseases

Chronic copper poisoning in beef cattle in the state of Mato Grosso, Brazil[1]

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ABSTRACT.- Martins K.P.F., Padilha V.H.T., Damasceno T.K., Souza M.A., Silva E.M.S., Ribeiro M., Pereira A.H.B. & Colodel E.M. 2020. **Chronic copper poisoning in beef cattle in the state of Mato Grosso, Brazil**. *Pesquisa Veterinária Brasileira 40(9):651-661.* Faculdade de Medicina Veterinária, Universidade Federal do Mato Grosso, Av. Fernando Corrêa da Costa 2673, Bairro Boa Esperança, Cuiabá, MT 78068-900, Brazil. E-mail: moleta@gmail.com

Copper is an essential micromineral in animal feed; however, when consumed in excess, it can cause liver necrosis, hemolytic crisis, hemoglobinuric nephrosis and death in cattle. Although uncommon in this species, copper poisoning occurs as a result of exacerbated supplementation, deficiency of antagonist microminerals, or previous liver lesions. An outbreak of chronic copper poisoning is reported in semi-confined cattle after supplementation with 50 mg/Kg of dry matter copper. The cattle showed clinical signs characterized by anorexia, motor incoordination, loss of balance, jaundice, brownish or black urine, diarrhea and death, or were found dead, 10 to 302 days after consumption. Of the 35 cattle that died, 20 underwent necropsy, whose frequent findings were jaundice, enlarged liver with evident lobular pattern, black kidneys, and urinary bladder with brownish to blackish content. Microscopically, the liver showed vacuolar degeneration and/or zonal hepatocellular centrilobular or paracentral coagulative necrosis, in addition to cholestasis, mild periacinal fibrosis, apoptotic bodies, and mild to moderate mononuclear inflammation. Degeneration and necrosis of the tubular epithelium and intratubular hemoglobin cylinders were observed in the kidneys. Copper levels in the liver and kidneys ranged from 5,901.24 to 28,373.14 µmol/kg and from 303.72 to 14,021 µmol/kg, respectively. In conclusion, copper poisoning due to excessive nutritional supplementation is an important cause of jaundice, hemoglobinuria, and death in semi-confined cattle.

INDEX TERMS: Copper poisoning, beef cattle, Mato Grosso, Brazil, semi-confinement, mineral supplementation, cattle diseases, trace elements, jaundice.

RESUMO.- [**Intoxicação crônica por cobre em bovinos de corte no Estado de Mato Grosso, Brasil.**] Cobre é um micromineral essencial, que quando em excesso induz necrose hepática, crise hemolítica, nefrose hemoglobínurica e morte em bovinos. As intoxicações, apesar de incomuns nessa espécie, ocorrem devido a suplementação exacerbada de cobre, pela deficiência de microminerais antagonistas ou secundária a lesão hepática prévia. Relata-se um surto de intoxicação crônica por cobre em bovinos semiconfinados após suplementação com 50mg/kg de cobre em matéria seca. Os bovinos manifestaram sinais clínicos caracterizados por anorexia, incoordenação motora, perda de equilíbrio, icterícia, urina acastanhada ou negra, diarreia e morte ou foram encontrados mortos, após 10 a 302 dias do início de consumo. De 35 bovinos que morreram 20 foram submetidos à necropsia sendo achada frequente icterícia, fígado aumentado e com padrão lobular evidente, rins pretos e bexiga urinária repleta de conteúdo acastanhado a enegrecida. Microscopicamente, no fígado havia degeneração vacuolar e ou necrose coagulativa hepatocelular zonal, centrolobular ou paracentral, além de degeneração vacuolar com corpúsculos de Councilman, colestase, fibrose periascinar leve, e inflamação de discreta a moderada. Nos

[1] Received on February 13, 2020.
Accepted for publication on June 23, 2020.
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rins havia degeneração e necrose do epitélio tubular assim como cilindros de hemoglobina intratubulares. Os níveis de cobre no fígado e rim foram de 5.901,24 a 28.373,14µmol/kg e 303,72 a 14.021µmol/kg respectivamente. A suplementação nutricional excessiva com cobre pode causar doença hemolítica com icterícia, hemoglobinúria e morte de bovinos mantidos em sistema de semiconfinamento.

TERMOS DE INDEXAÇÃO: Intoxicação por cobre, bovinos de corte, Mato Grosso, Brasil, semiconfinamento, suplementação mineral, doenças de bovinos, oligoelementos, icterícia.

INTRODUCTION

Copper is an essential trace element that participates in biological processes. These processes include the activity of enzymes involved in cellular respiration, inactivation of free radicals, neurotransmission, connective tissue synthesis, production of pigments, iron cellular metabolism, and hemoglobin formation (Underwood & Suttle 1999, Festa & Thiele 2011).

Because of its extensive functionality and the finding of cases of deficiency in cattle in Brazil, copper has been widely used in mineral supplements (Malafaia et al. 2014). Supplementation, added to the levels of copper present in pastures and water, should contribute to cattle reaching the daily copper requirement of 4-8 mg/kg of dry matter (DM) (Suttle 2010). After ingestion, copper is absorbed by enterocytes in the small intestine, accesses the portal circulation, binds to albumin and transcuprein, and is then taken to the liver and stored in hepatocytes (Suttle 2010). Homeostasis maintenance between the accumulation of hepatic copper and bile excretion occurs through copper-dependent metalloenzymes such as ceruloplasmin and metallothionein (Rosa & Mattioli 2002, Tapia et al. 2004). In ruminants, this metabolism is influenced by the absorption and storage capacities of individuals, which differ according to race, animal category, and antagonism with other microminerals (Blanco-Penedo et al. 2006, Cuttance et al. 2018).

In general, cattle tolerate up to 5 g of dietary copper sulfate per day - a value considered to be higher than the daily needs (Tokarnia et al. 2010). However, when the storage capacity of the hepatocytes is exceeded, the free micromineral becomes toxic because of its potential oxidative effect (García-Vaquero et al. 2012, López-Alonso et al. 2017), and it causes hepatocellular degeneration and necrosis, hemolysis, and hemoglobinuric nephrosis (Cullen & Stalker 2016). The induction of these lesions is classified as acute when a large amount of copper is exposed in a short period, and this form is associated with cupric parenteral supplementation in calves (Mylrea & Byrne 1974, Sullivan et al. 1991, Hamar et al. 1997). In contrast, the chronic form is related to constant excessive mineral supplementation (López-Alonso et al. 2000, Hunter et al. 2013, Newcomer et al. 2016). After a subclinical period (weeks to months), cattle present acute manifestations that range from characteristic clinical signs, such as hemoglobinuria, jaundice, mild diarrhea and oliguria, to nonspecific signs such as apathy, dehydration, and weight loss (Minervino et al. 2009, Johnston et al. 2014).

Despite the intense livestock activity in Brazil (IBGE 2017), analysis of surveys addressing the causes of bovine mortality in different regions of the country from 1964 to 2014 showed no reports of deaths due to excessive copper supplementation in cattle (Lucena et al. 2010, Souza et al. 2015, Rondelli et al. 2017), except for one description by Tokarnia et al. (2000). Conversely, spontaneous cases of copper poisoning have been reported in Europe and Oceania in intensive dairy and beef cattle breeding, which suggests that confined cattle that receive nutritional supplementation with copper are part of a category susceptible to poisoning (López-Alonso et al. 2000, Bidewell et al. 2012, Hunter et al. 2013, Johnston et al. 2014, Kendall et al. 2015). This study aimed to report an outbreak of chronic poisoning caused by excessive copper supplementation in semi-confined cattle in the state of Mato Grosso, Brazil.

MATERIALS AND METHODS

Epidemiological investigation and clinical examination. This study was carried out on a farm that raises beef cattle under semi-confinement management located in the municipality of Rondonópolis, state of Mato Grosso, Brazil. Information was obtained through interviews with the owner, veterinarian and employees of the property and during visits made by technicians from the Veterinary Pathology Laboratory of Federal University of Mato Grosso (LPV-UFMT), Cuiabá Campus, who carried out surveys on the herd and housing areas. All paddocks in the property were inspected, and a clinical inspection of the herd was also performed.

Biochemical and molecular analysis. For biochemical analyses, blood samples of ten bovines were collected through venipuncture. The samples were placed in sterile tubes without EDTA. Biochemical tests were performed using a Wiener Lab CM-200 automatic biochemistry and turbidimetry analyzer.

Three blood samples from different cattle with clinical signs were also collected into a Vacuette® 6 ml tube without additive for serum copper measurement. The samples were analyzed at the Hermes Pardini Laboratory (Belo Horizonte/MG) by flame atomic absorption spectrophotometry (in-house method).

Blood smears from 20 cattle and spleen and liver impression smears from three cattle were made during necropsy and evaluated, after rapid panoptic staining, to investigate hemiparasites (Bain 2016).

Polymerase chain reaction (PCR) was used to detect *Ehrlichia* spp. and *Babesia* spp.. The tests were carried out at the Virology and Rickettsiosis Laboratory of UFMT (LVR-UFMT), Cuiabá campus, using a BIO-RAD T100™ thermocycler, and the images of the gels were captured and processed in a photo-documentation system (ChemiDoc XRS+, Bio-Rad) (Almeida et al. 2012).

Necropsy and histopathological examination. Twenty cattle were submitted to necropsy, nine of which showed clinical signs and died spontaneously, two were euthanized, and nine were found dead. Morphological changes were noted and photographic records were taken. Fragments of the organs of these animals were collected, fixed in 10% formalin, identified in sequence from B1 to B20, and sent to the LPV-UFMT (Table 1).

Seventeen samples from cattle submitted to necropsy by the property's veterinarian are identified in sequence from B1 to B7 and B11 to B20. Samples B1 to B7 were received between May 10, 2018 and May 23, 2018. Samples B11 to B20 were from cattle that died between May 27, 2018 and July 5, 2018. Three cattle, B8, B9 and B10, presenting clinical alterations, were collected between May 24 and 26, 2018 and submitted to necropsy by the LPV_UFMT team. Fragments of the organs received were cleaved, routinely processed, embedded in paraffin, and stained with hematoxylin and eosin (HE). Sections of the liver from a bovine were subjected to the Von Kossa histological technique (calcium). Liver and kidney fragments from all 20 cattle were subjected to histochemical staining with rubeanic acid for copper detection.

Analysis of feed and pasture, chemical analysis of soil, and evaluation of levels of copper (Cu), molybdenum (Mo) and sulfur (S) in offal. Pasture and soil samples were collected in two paddocks where animal deaths occurred. These were identified and sent to "Agroanálise Laboratórios Integrados" (Cuiabá/MT) to determine copper and sulfur levels by atomic absorption spectrophotometry.

Liver and kidney fragments, conserved in 10% buffered formalin solution, were sent to the Institute of Biosciences, Ecology Center of the "Federal University of Rio Grande do Sul (UFRGS) for sulfur, copper and molybdenum determination. These samples were analyzed by Atomic Absorption/ Air-Acetylene Flame spectrophotometry and microwave-assisted nitric acid digestion (EPA 3052).

RESULTS

Epidemiological investigation and clinical signs

The property contains 700 ha of pasture formed with *Brachiaria brizantha*, Ipyporã cultivar, and *Panicum maximum*, Mombaça and Massai cultivars, divided into 40 paddocks. The deaths occurred in seven different paddocks, and four of them were fertilized with monobasic phosphate, ammonium sulfate, potassium chloride, and limestone in December 2017. In January 2018, the farm hosted 3218 cattle under semi-confinement management, with daily consumption estimated at 1.56% of the diet's live weight with a high roughage to concentrate ratio. Concentrate consumption was estimated at 8.3 kg of DM/head/day for animals with average weight of 530 kg, with observed weight gain of 1.5-1.8 kg/animal/day.

In December 2017, some cattle from different paddocks presented neurological clinical signs and died. The presumed diagnosis, without morphological confirmation, was polioencephalomalacia. After this event, the formulation of the feed used on the property was changed in order to antagonize sulfur absorption and prevent copper deficiency. At the beginning of January 2018, copper was added to the feed mix, with an increase from 20 to 50 mg/kg of DM. The levels of S, Mo, and total iron (Fe) were not significantly changed.

From January 10, 2018 to July 5, 2018, 35 Nelore cattle, with predominance of males aged 18-36 months, died on this farm. However, 15 deaths occurred before April 20, when detailed clinical inspections or collections for complementary exams of these cattle began. In January 2018, herders found six (6/15) dead cattle during paddock inspection. Two of these cattle were examined by collaborators, who noticed marked jaundice, increased gallbladder volume, and bladders with brownish to blackish content. Subsequently, the veterinarian at the property reported that nine deaths (9/15) occurred in April 2018 in different paddocks. One of the animals found dead was submitted to necropsy, which showed serous and yellowish fat (jaundice). Samples were not collected for complementary exams.

From April 20 to July 5, 2018, monitoring showed 20 deaths in the property, and the first collection for further exams occurred on May 9, 2018. Nine cattle (9/20) were found dead without any clinical changes. In the other (11/20), clinical signs were noted, ranging from apathy, reluctance to move, and distancing from the herd. Motor incoordination, loss of balance, visual impairment, diarrhea, or dry stools were also observed. Four cattle (4/11) showed jaundice, and in one animal (1/4) presented blackened urine. In May, treatment for bovine parasitic sadness (Terramicina LA®, Ganaseg®, Borgal®, and Mercepton®) was started in three cattle, and no clinical response was observed.

Table 1. Identification of samples and gross characteristics of cattle with chronic copper poisoning in the municipality of Rondonópolis/MT from April to July 2018

Bovine	Age (months)	Sex	Breed	Feed intake time (days) 50 mg copper/kg/DM	Major gross changes				
					Jaundice	Liver volume	Disclosure of the lobular pattern	Urine color	Kidney color
B1	24	M	Nelore	282	++	+	N	AC	E
B2	24	M	Nelore	198	+++	+	**	AC	-
B3	18	M	Nelore	99	+	NA	+++	AC	N
B4	24	M	Nelore	205	-	-	-	-	-
B5	24	M	Nelore	201	+++	++	N	AC	E
B6	24	M	Nelore	212	++	++	+	AC	E
B7	18	M	Nelore	106	+	NA	+++	N	P
B8	24	M	Nelore	302	+++	++	-	AC	E
B9	24	M	Nelore	216	NA	NA	N	N	P
B10	36	M	Nelore	290	+++	++	+	AC	E
B11	24	M	Nelore	110	+++	++	+	AC	E
B12	24	M	Nelore	117	NA	NA	-	-	-
B13	24	M	Nelore	232	++	NA	A	AC	E
B14	36	F	Crossbred	297	NA	NA	N	-	P
B15	24	M	Nelore	211	+++	+++	+	AC	E
B16	24	M	Nelore	208	+++	+++	++	AC	E
B17	18	M	Nelore	76	NA	++	+++	N	N
B18	24	M	Nelore	100	+	+	++	N	P
B19	36	M	Crossbred	297	+++	++	+++	CC	E
B20	24	M	Nelore	211	+++	+++	++	CC	E

M = Male, F = female, NA = no alterations, - no information; Jaundice/enlarged liver/evident lobular pattern/distended gallbladder: A = autolysis, N = normal, + mild, ++ moderate, +++ severe; Urine color: N = normal (citrus yellow), AC = brownish, CC = black (Coca-cola color); Kidney color: N = normal, P = pale, E = blackish (metallic black).

In addition, in May, given the suspicion of chronic copper poisoning, it was recommended that this micromineral be suppressed in the feed. Cu level was adjusted from 50 to 20 mg/kg of DM, and later in that month the level was reduced to 11 mg/kg of DM, keeping the S and total Fe values at 0.2 and 17 mg/kg, respectively.

Necropsy findings

The main gross changes found at necropsy are shown in Table 1. In one case (1/20), no information on gross changes was obtained because collection was carried out by the farm employees. In cases which these findings were described, gross changes were similar, with variation in intensity and/or presence of jaundice (15/19), enlarged and brownish liver (13/19) (Fig.1A), and evidence of lobular pattern (11/16) (Fig.1B). In four cases, observations were related to the gallbladders, which were all distended, with liquid to pasty to lumpy content ranging from green to brown.

Gross changes were described for kidneys and urinary bladder content in 17 cases. The kidneys were predominantly black (11/17) (Fig.1C), pale (Fig.1D) (4/17), or had a normal color (2/17). Urine color was brownish (11/17), blackish (2/17), or normal (citrus yellow) (4/17).

In the thoracic cavity of B19, petechiae and ecchymosis were observed in the parietal pleura. B20 showed multifocal, irregular, brownish, 3 cm diameter areas in the myocardium. In B10, the mucosa of the omasum and small intestine had multifocal ulcers, whereas ulcers in the abomasum and rectum were red.

Histopathological results

Nineteen of the 20 liver and kidney samples presented morphological changes, and one was excluded from the evaluation due to marked *postmortem* changes. The main histopathological alterations observed in all livers (Table 2) were zonal coagulative necrosis (15/19) and/or hepatocellular vacuolar degeneration (14/19). Centrilobular necrosis associated with congestion and hemorrhage (Fig.2A) was seen in 13 cases (13/15), two (2/13) of which occasionally had a paracentral pattern, and four (4/13) extended to the mediozonal region. Necrosis was massive in two cases (2/15). Individual hepatocyte necrosis was present in 12 cases (12/19)

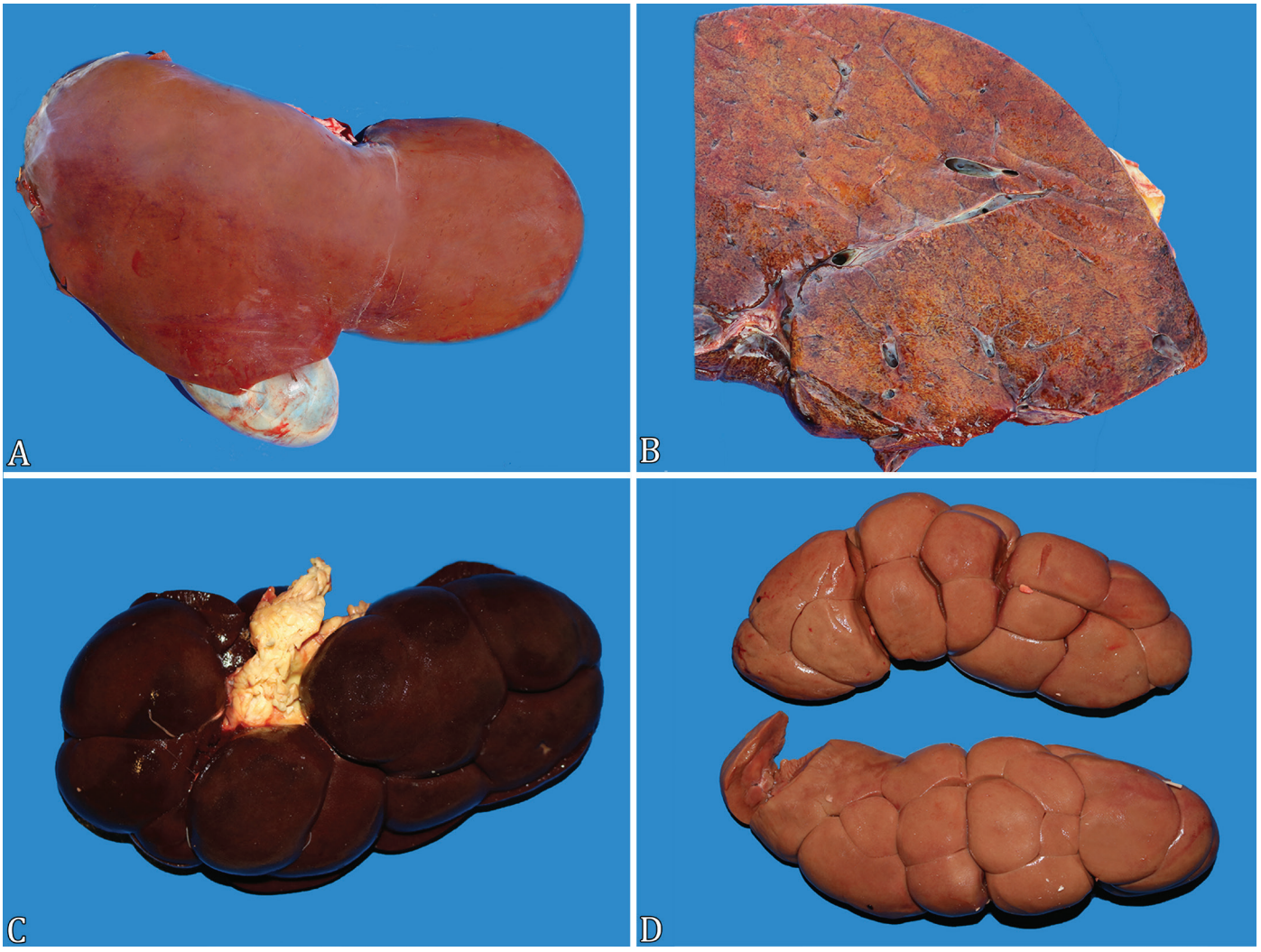

Fig.1. Gross findings in liver and kidney of cattle chronically poisoned by copper. (**A**) Liver with swelling and brownish capsular surface (13,171 µmol/kg of Cu) (B18). (**B**) On the cut surface, an evident lobular pattern (28,373 µmol/kg of Cu) is observed (B19). (**C**) Diffuse and moderately blackened kidney (13,391 µmol/kg of Cu) (B16). (**D**) Kidney with diffuse and moderate pallor (8,639 µmol/kg of Cu) (B17).

and were associated with degenerative changes and in areas adjacent to zonal necrosis (7/12), or were noted in cases where hepatocellular degeneration predominated (4/12), or only associated with the limits of areas where there was zonal necrosis (1/12).

Hepatocellular degeneration (14/19), predominantly periacinal (8/14), was classified as macrovesicular (7/8) or microvesicular (1/8). Less frequently, massive (Fig. 2B) (4/14) and centrilobular (2/14) degeneration was also observed. Proliferation of the periportal fibrous connective tissue was found in 11 samples, seven considered discrete (7/11) and four moderate (4/11). Cholestasis was present in 12 cases, six of which were mild (6/12), three moderate (3/12), and three severe (3/12). Councilman's bodies were observed in nine cases. Finally, a mild inflammatory infiltrate, adjacent to necrosis, was observed in 15 cases, 13 (13/15) neutrophilic and two (2/15) mixed.

The main renal changes (Table 3) observed in 20 cattle submitted to necropsy were coagulative necrosis and vacuolar degeneration of the tubular epithelium (Fig.2C). Tubular coagulative necrosis prevailed in nine cases (9/19), tubular degeneration was found in three cases (3/19), and they occurred with the same intensity in seven cases (7/19). Hemoglobin cylinders in the tubular lumen were verified in 14 cases (Fig.2D) (14/19). Mild to moderate glomerulonephritis was identified in nine animals. Mild to moderate interstitial inflammation was found in 11 kidneys, and interstitial fibrosis was present in four samples, three moderate and one mild.

Findings in other organs included mild vacuolization of the white matter of the brain in B2, occasional coagulative necrosis of cardiomyocytes in the myocardium in B20, and multifocal necrotizing omasitis and enterocolitis in B10.

The rubeanic acid technique was performed on all 20 samples. A positive, green to blackish, mild to moderate, intracytoplasmic marking in hepatocytes was observed in 17 samples and in the epithelium or renal tubular lumen in eight samples.

Cu, S and Mo in liver and kidney and serum Cu

Copper levels in the liver and kidneys ranged from 5,901.24 to 28,373.14 µmol/kg and from 303.72 to 14,021 µmol/kg, respectively. Of the three animals analyzed, S levels ranged from 52.55 to 217.97 µmol/kg, whereas Mo levels varied between 0.019476 and 0.45284 µmol/kg. In blood serum, Cu levels ranged from 116 to 350mcg/dL (Table 4).

Biochemical and molecular analyses

The serum activities of liver enzymes that indicate injuries, such as *Glutamic-Oxaloacetic Transaminase* (GOT) and Glutamic-Pyruvic Transaminase (GPT), were above the reference values (Table 5) in seven animals (7/7) and only GPT in six (6/7). Creatinine was measured in seven animals, with six (6/7) values overlapping the reference. In the 20 blood smears, there were no morphological structures compatible with *Babesia* spp. or *Anaplasma* spp. Serum samples from animals B10, B18, and B19 were negative for *Ehrlichia* spp. and *Babesia* spp.

Pasture and soil analyses for Cu, Mo, and S levels

Mean copper values in the pastures varied between 6.7 (*Panicum maximum* var. Mombaça) and 6.5 mg/kg (*Brachiaria brizantha*). Sulfur values averaged 1.2 mg/kg in both pastures (*Panicum maximum* var. Mombaça and *Brachiraria brizantha*). In the soil, the mean levels of Cu and S were 0.25 and 29.1 mg/dm^3, respectively.

Table 2. Histopathological characteristics of cattle livers with chronic copper poisoning

Bovine	Centrilobular necrosis	Massive necrosis	Individual necrosis	Centrilobular degeneration	Periacinal degeneration	Massive degeneration	Fibrosis	Cholestasis	Councilman body	Inflammation
B1	+++	-	-	-	++ Ma	-	-	-	+	+ N
B2	+++*	-	-	-	+Ma	-	+	+	-	+N
B3	+++	-	-		+Ma	-	-	-	-	-
B4	-	-	+	+Mi	-	-	-	-	-	-
B5	-	-	++	-	-	+++Ma	++	+	+	-
B6	++**	-	++	-	-	+++ Ma		+++	-	+N
B7	+++	-	-	-	-	-	++	-	-	+N
B8	++**	-	-	-	-	-	+	+	-	+N
B9	-	+++	-	-	-	-	-	++	-	++N
B10	++*	-	+	-	-	+++Ma	+	+++	+	+N
B11	++**	-	+	-	-	-	+	+	-	+N
B12	+++	-	+	-	+ Ma	-	-	-	+	+N
B13	A	A	A	A	A	A	A	A	A	A
B14	+++**	-	+	-	++Ma	-	+	+	-	++M
B15	-	-	+++	-	-	+++Ma	++	++	++	+N
B16	+++	-	+	-	++Ma	-	++	+++	+	+M
B17	-	+++	-	-	-	-	-	-	-	++M
B18	+++	-	+	-	++Mi	-	+	+	+	+N
B19	-	-	+	++Mi	-	-	-	-	+	-
B20	++	-	+	-	+ Ma	-	+	++	+	+N

* Centrilobular to paracentral necrosis, ** centrilobular to mediozonal necrosis; Ma macrovesicular, Mi microvesicular; N neutrophilic, M mixed; A = autolysis; + mild, ++ moderate, +++ severe.

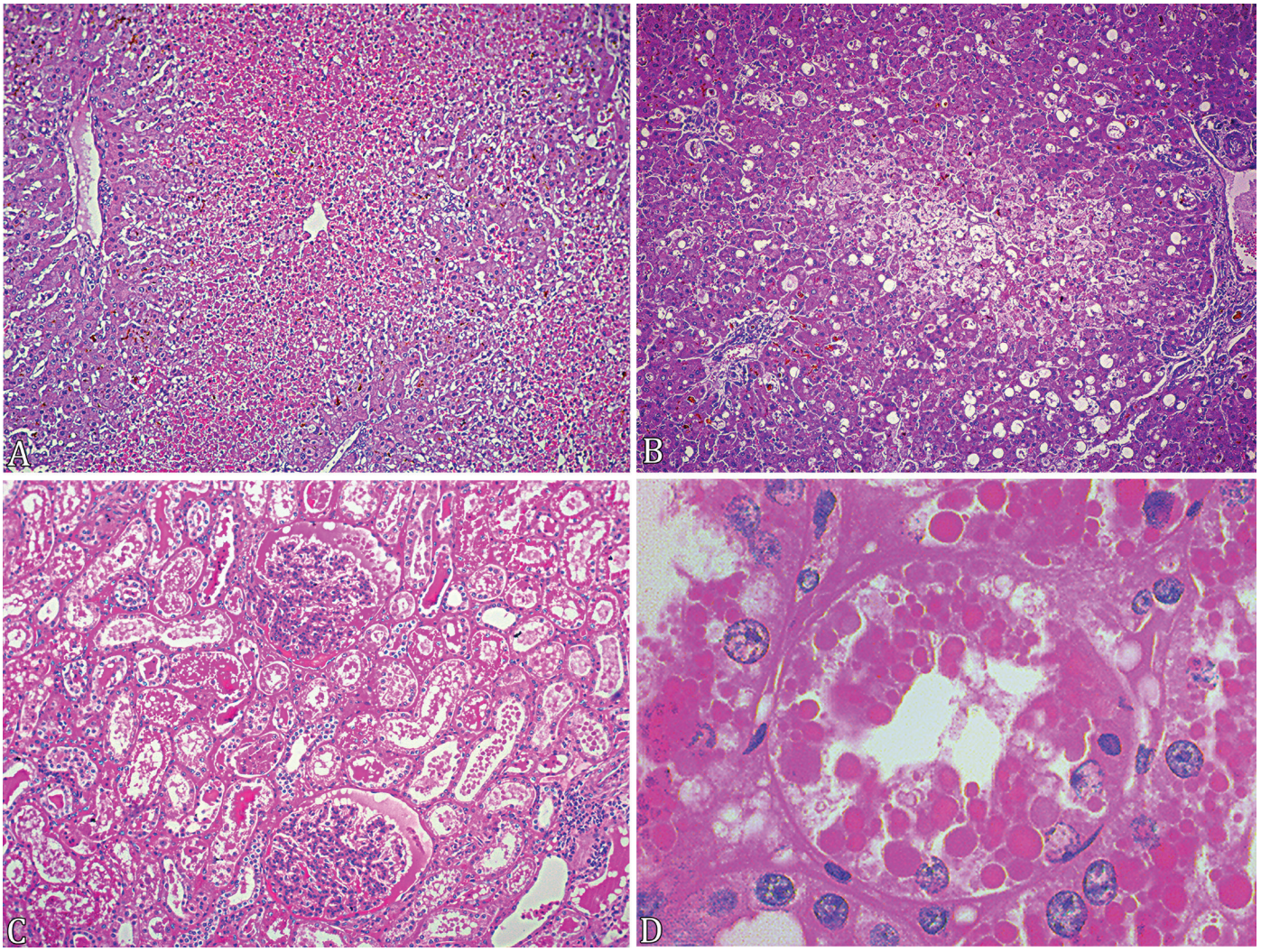

Fig.2. Histopathological findings of liver and kidney of cattle chronically poisoned by copper. (**A**) Centrilobular coagulative hepatocellular necrosis associated with congestion and hemorrhage. HE, obj.10x. (**B**) Hepatocellular, macrovesicular, massive degeneration associated with centrilobular necrosis and cholestasis. HE, obj.10x. (**C**) Kidney with multifocal areas of tubular epithelial degeneration and necrosis. In the tubular light and the Bowman space, there is amorphous and strongly eosinophilic material compatible with hemoglobin. HE, obj.10x. (**D**) There is hyaline globular material (hemoglobin) in greater magnitude in addition to cytoplasmolysis, karyorrhexis and nuclear pycnosis. HE, obj.100x.

DISCUSSION

The epidemiological, clinical and pathological aspects and the serum, hepatic and renal Cu values found in the described outbreak are consistent with chronic copper poisoning in cattle associated with excessive mineral supplementation. Copper poisoning was first investigated in Australian cattle in the 1940s (Dick & Bull 1945). From that period to the present day, cases have been reported in several countries. They are often associated with parenteral or nutritional supplementation in dairy cattle herds under intensive nutritional management (Galey et al. 1991, Bradley 1993, Steffen et al. 1997, Bidewell et al. 2012, Johnston et al. 2014). In contrast to the aforementioned description, cases of spontaneous Cu poisoning are rare in Brazil, with only one report of deaths in beef cattle after feeding with chicken litter (Tokarnia et al. 2000), and there are no reports on natural occurrence of Cu poisoning in beef cattle due to excessive supplementation.

In the present case, Cu supplementation per kg of DM was increased from 20 to 50mg/kg. Considering the last value and the daily consumption per bovine of approximately 8.3 kg of concentrate, each animal ingested an average of 415 mg Cu/day, which represents a surplus of 352 mg of Cu when considering the required value of 63.28 mg for maintenance and weight gain of male Zebu cattle (Silva et al. 2016). It is also verified that the value of 50 mg Cu/kg DM represents 20 mg Cu/kg DM in excess to the maximum limit established as safe by the European Union (EU) Commission Implementing Regulation in 2018. The EU regulation made this determination after observing that regular diets with excessive values caused poisoning (Bidewell et al. 2012, Kendall et al. 2015, Sinclair & Atkins 2015). It is believed that the gradual change of the Brazilian livestock breeding system, specifically in the state of Mato Grosso, to confinement or semi-confinement systems (IMEA 2019) allows cases of poisoning to occur due to the use of exaggerated amounts of Cu in nutrition.

Table 3. Histopathological characteristics of cattle kidneys with chronic copper poisoning

Bovine	Tubular necrosis	Tubular degeneration	Hemoglobin cylinder	Glomerulonephritis	Fibrosis	Inflammation
B1	+++	-	++	++ P	-	+ NS
B2	++	+++	+++	+MP	-	+NS
B3	++	+++	+++	++ MP	-	-
B4	-	-	-	-	++	-
B5	++	++	++	+P	-	+ M
B6	++	+	+	-	-	-
B7	++	-	-	-	-	-
B8	+++	+	++	+P	-	++NS
B9	++	+	-	++MP	++	-
B10	+	+++	+++	++P	++	-
B11	++	++	+++	+P	-	-
B12	++	+	+	++MP	-	-
B13	A	A	++	A	-	+NS
B14	++	+	++	-	-	+NS
B15	++	++	+++	-	-	+NS
B16	++	+	+++	-	-	+NS
B17	+	+	-	-	-	-
B18	+	+	-	-	-	+NS
B19	-	+	-	-	-	+NS
B20	+++	++	+++	-	+	+M

P Proliferative, MP membranoproliferative, NS nonsuppurative, M mixed; A = autolysis.

Table 4. Levels of copper, sulfur, molybdenum in the liver, kidney and serum of cattle with chronic copper poisoning

Bovine	Liver			Kidney	Blood serum
	Copper (µmol/kg)	Molybdenum (µmol/kg)	Sulfur (µmol/kg)	Copper (µmol/kg)	Copper (mcg/dL)
B2	10.606	-	-	-	-
B3	5.901	-		1.410	-
B5	10.417	-	-	-	-
B6	20.552	-		634	116
B7	18.805	-		14.021	-
B8	8.293	-	-	511	-
B9	9.536	-	-	6.168	-
B10	7.836	-		321	192
B16	13.391	0,044	217,97	303	-
B17	8.639	-	-	-	-
B18	13.171	-	-	7.427	-
B19	28.373	0,019	52,55	428	350
B20	9.992	0,045	176,38	6.341	350
Reference values	->8.000 µmol/kg*	0,191-0,383***	900-3.000****	600-800 µmol/kg **	19 -38 mcg/dl**

* Kendall et al. (2015), ** Suttle (2010), *** Underwood (1977), **** Dias et al. (2013); - unrealized.

Table 5. Serum activity of liver and kidney enzymes of cattle with chronic copper poisoning

Bovine	GGT (UI/L)	GOT (U/L)	GPT (U/L)	Creatinine (mg/dL)
B3	30.09	-	-	2.2
B6	-	633	86	-
B14	-	3166	1190	2.9
B16	-	2100	2422	26.5
B17	-	5550	1520	44.3
B18	-	182	20	1.6
B19	-	1489	40	3.2
B20	-	1150	150	6
Reference values*	≥26	45 to 110	7 to 36	0.6 to 1.8

GGT = Gamma-glutamyl transferase, GOT = glutamic-oxaloacetic transaminase, GPT = glutamic-pyruvic transaminase; * Minervino et al. (2009).

In the outbreak described in this study, the reports state that the change in feed formulation, with an increase in Cu supplementation, was made after the alleged occurrence of polioencephalomalacia assigned to S excess, and assuming that S excess could cause negative effects related to the formation of thiomolybdates (TM) in the rumen environment (Suttle 1974, Dick et al. 1975). Absorbed TM have an affinity for enzymes and other organic components that contain Cu, causing decreased enzyme activity which, consequently, can induce clinical signs of Cu deficiency. In ruminants, the signs of Cu deficiency are nonspecific or subclinical, and include a reduction in weight gain, decreased food consumption or inefficient feed conversion, altered hair pigmentation, and negative impacts on reproduction (Gould & Kendall 2011). In this occurrence, the effects related to the formation of cupro-thiomolybdates were not evaluated, and only the toxic consequences of Cu hepatocellular accumulation were observed. The antagonism that other trace elements other than S, such as Mo, Fe and zinc (Zn), have on the absorption and use of Cu is an essential factor to be considered when planning supplementation (López-Alonso et al. 2017, Sinclair et al. 2017, Skalny et al. 2019). However, the values of these microminerals were not established before changes in the Cu content were introduced in the feed.

Other less common causes of Cu poisoning in cattle include ingestion of pastures with normal Cu content and low in antagonistic microminerals (phytogenic poisoning) (López-Alonso et al. 2000) or previous liver damage (hepatogenic poisoning) (Newcomer et al. 2016). Phytogenic and hepatogenic poisoning events were ruled out due to the finding of an excessive exogenous Cu source and the absence of clinical and pathological findings that indicated previous liver damage.

The variation in the clinical course of poisoning was evident when the time of ingestion of the Cu-rich diet, the subsequent manifestation of clinical signs, or eventual death of the cattle were observed. This wide variation can be justified by the distinct individual susceptibility, which is influenced by sex, breed, and age (Littledick et al. 1995, Miranda et al. 2010, López-Alonso et al. 2017). Considering the reports of injuries made by the property's employees, it is believed that death of cattle compatible with Cu poisoning occurred 10 days after changing the feed formulation to 50 mg of copper/kg DM. Previous supplementation (20 mg copper/kg DM) possibly contributed to the hepatocellular Cu deposit and clinical manifestation of hemolysis in a shorter period after the Cu increase in the diet. However, the values of visceral Cu deposits were not measured before the onset of mortality.

Regarding the age of the bovines at the time of visceral Cu accumulation measurements, the three youngest individuals (aged 18 months) to manifest the disease were those that ingested the feed for the shortest time (76-106 days). Speed of hepatic Cu accumulation in younger animals is favored by the smaller Cu deposit in the muscles during a period of lower growth rate or when the animals are very young and receive Cu during lactation (Littledick et al. 1995, Miranda et al. 2010, López-Alonso et al. 2017). Greater breed susceptibility is observed in Holstein compared with Jersey and Angus and with Simental (Gooneratne et al. 1994, Littledick et al. 1995, Miranda et al. 2010). However, there are no studies addressing susceptibility in Nelore to establish comparison.

Of the 20 animals evaluated, nine were found dead without observation of clinical symptoms. This finding can be assigned to the type of management adopted on the farm, high stocking, and once daily inspection of herds, on average, which are facts that allow animals with shorter disease evolution to go unnoticed. This type of occurrence, added to the variation in clinical signs, demonstrate the need for *post-mortem* examinations and liver Cu measurements for diagnostic completion (Cullen & Stalker 2016). In 11 cattle, apathy, reluctance to move, and distancing from the herd were continuously observed. Although unspecific, these findings have been commonly described in cattle poisoned by copper (Galey et al. 1991, Tremblay & Baird 1991, Bradley 1993, Bidewell et al. 2012, Suttle et al. 2013). Blackened urine related to hemoglobinuria was clinically visualized in one bovine, and jaundice was observed in four cattle. These clinical signs, which are considered classic in Cu poisoning (Tokarnia et al. 2000, Minervino et al. 2009), are not always described in this disease in cattle (Galey et al. 1991, Minervino et al. 2009, Bidewell et al. 2012, Suttle et al. 2013). Minervino et al. (2009), when inducing chronic copper poisoning, obtained in most cattle, in addition to nonspecific clinical signs, a clinical condition considered atypical, such as diarrhea or dry stools, ruminal atony, and oliguria. Changes in the central nervous system, such as motor incoordination, difficulty in moving and visual impairment (Perrin et al. 1990, Bradley 1993), which were also reported in the present outbreak, have already been associated with a condition known as hepatic encephalopathy (Howell et al. 1974, Bidewell et al. 2002).

Increase in serum enzymes that indicate damage to liver or kidney function was constant in the animals evaluated in this study. The increase in transaminases such as GOT and GPT occurs mainly at the stage when hepatocellular necrosis has already been established (Minervino et al. 2009). Serum creatinine is an important marker of renal metabolism (Thrall 2014) and its elevation in these cattle is related to decreased glomerular filtration (Perrone et al. 1992) and hypoxia caused by the intravascular hemolytic crisis (Bozynski et al. 2009, Newman 2013).

Although serum Cu measurement has been considered insensitive for detection of hepatic Cu accumulation (López-Alonso et al. 2006), it can assist with diagnosing poisoning in animals that show clinical signs of the disease (Johnston et al. 2014). In the cattle assessed in this study, values higher the reference were observed in all four sera analyzed (Underwood & Suttle 1999), and in two (2/4) of these, the serum Cu level was at the upper detection limit of 350 mcg/dL.

The gross findings, characterized by jaundice and blackened urinary bladder content, are consistent with chronic copper poisoning and, in this case, were the most reported *postmortem* observations. Jaundice was the most frequent observation, and it was related to hepatocellular necrosis and hemolysis (Cullen & Stalker 2016). The visualization of blackened kidneys in 11 cases showed intravascular hemolysis, even if not clinically verified. Hemolysis occurs as a result of the direct damage that CU does to red blood cells by inducing oxidative stress in the erythrocyte membrane through inhibition of the enzyme glucose-6-phosphate (Fairbanks 1967).

Increased liver volume was a common finding related to excessive hepatocellular Cu deposit (Cullen & Stalker 2016). Associated with the event of oxidative stress, it causes

degenerative and necrotic changes (García-Vaquero et al. 2012), contributing to hepatocellular swelling and increased liver volume (Morgan et al. 2014).

Histopathological evaluation showed predominance of zonal hepatocellular necrosis, with centrilobular necrosis as the most frequent. Similar lesions have already been described in other Cu poisoning events in cattle (Minervino et al. 2009). They are related to the massive release of Cu and to the acute clinical phase of disease, when intravascular hemolysis occurs (Valli 2007). Hepatocellular degeneration has been associated with the pre-hemolytic phase of disease (Gooneratne et al. 1980). It is related to the gradual accumulation of Cu in the cytoplasm and nuclei of the hepatocytes (López-Alonso et al. 2017). It can trigger lipid peroxidation of the cell membrane, water accumulation, electrolytes, lipids and formation of autophagic vesicles capable of providing a vacuolar aspect to the cytoplasm (Gooneratne et al. 1980, García-Vaquero et al. 2012, Wang et al. 2014). In those livers, in which hepatocellular degeneration was the most important finding, individual hepatocyte necrosis occurred more frequently. These findings have already been described in other reports of Cu poisoning, but associated with the subclinical phase (Hunter et al. 2013) or the chronic phase of the disease in the absence of a hemolytic crisis (Newcomer et al. 2016). This association was not observed in the present report, since even in the only case (B19) where individual degeneration and necrosis were mild, jaundice and blackened kidneys were evident.

Tubular necrosis was the most frequently observed microscopic renal alteration. It was associated with mild intensity combined with damage to the renal tubular epithelium, caused by the oxidative potential of copper (Zhao et al. 2019). Changes are potentiated by tissue hypoxia (Newman 2013) that occurs during the hemolytic crisis and at the time when excess hemoglobin is present in the renal tubules (Gooneratne et al. 1986).

The histochemical staining of copper, from mild to moderate, did not occur only in liver samples with marked autolysis and putrefaction, but also in two cases where necrosis was massive. Intensity of copper staining in the hepatocyte cytoplasm is experimentally evident until the fifth week of excessive supplementation and decreases as poisoning progresses (Fuentealba et al. 1987). Renal copper staining in these cattle occurred in fewer cases and demonstrated less usefulness in histochemical diagnosis than liver staining (Evering et al. 1990). The smallest evidence of kidney Cu granules occurs after tubular necrosis that exfoliates cells overloaded with copper to the lumen, which may not be more evident mainly after the beginning of tubular regeneration (Haywood et al. 1985).

Regarding hepatic Cu levels, Suttle (2010) argues that, given the numerous factors that can individually influence the rate of Cu absorption, use and storage, a safety range should be used with acceptable values from 6,400 to 16,000 µmol of Cu/kg DM. According to Grace & Knowles (2015), interpretation of the term "adequate" has generated the idea that a more significant Cu nutritional supply than the necessary can provide greater productive and reproductive gains. In this expectation, excessive supplementation is recommended. However, as in this case, there are reports of deaths associated with chronic copper poisoning, in which the hepatic deposit oscillates between these reference values (Johnston et al. 2014, Morgan et al. 2014). This information reinforces that Cu rates in the liver identified as adequate may be present in cattle at risk for developing chronic poisoning. Therefore, the Animal and Plant Health Agency (APHA) of the United Kingdom established that values >8,000 µmol/g of Cu DM should be considered toxic, even in the absence of clinical signs (Kendall et al. 2015).

Levels between 600 and 800 µmol of Cu/kg DM are considered acceptable in the bovine kidney (Suttle 2010). In this case, chemical analyses of 10 samples of renal tissue revealed high values in six cases and within the normal range in four cases. Typically, urinary copper excretion is low (López-Alonso et al. 2017). Therefore, the interpretation of Cu evaluation results in this tissue should consider the dynamics between intensity of liver damage (Zischka et al. 2011) and the existing amount of the Cu carrier protein CTR1 (Han et al. 2009) in each animal (Gray et al. 2012).

CONCLUSIONS

Nutritional Cu supplementation for cattle with values from 20 to 50mg/kg of DM is potentially toxic. It can cause hepatocellular accumulation of this micromineral and, consequently, risk of degeneration and hepatocellular necrosis, hemolytic crisis, and nephrosis.

Copper poisoning should be considered a differential diagnosis mainly in confined or semi-confined cattle with jaundice and hemoglobinuria.

Conflict of interest statement.- There are no conflicts of interest.

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