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Acute Hemolytic Syndrome and Chronic Liver Disease in Water Buffalos (*Bubalus bubalis*)

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Abstract

Two dead and one in extremis four month-old dairy buffalo heifers were received for post mortem examination at the National Institute of Agricultural Technology (INTA) in Buenos Aires, Argentina. The carcasses were severely jaundice and haemorrhagic. The kidneys were hemorrhagic with reddish-dark urine discoloration, consistent with an acute haemolytic syndrome. High copper concentrations were detected in the kidneys and liver through atomic absorption spectrophotometry. Copper deposits were demonstrated by Timm's stain of the liver of the three heifers. An extra-intestinal pathogenic *Escherichia coli* was detected in one animal. The diagnosis was chronic copper poisoning, other chronic changes observed in the liver through HE stain and by immunohistochemistry (Ki67, Hepar-1 and keratins 7 and 20) suggested the additional action of a non identified toxic agent, which may have had aggravated the copper poisoning. The findings of this report remarks the importance of determining nutritional standard reference values for water buffalo mineral supplementation.

Keywords: Buffalo; Copper; Liver; Haemolysis; Jaundice; *Escherichia coli*

Introduction

Copper is an essential trace element that facilitates electron transfer in a number of key enzymes. However, copper redox properties are also responsible for its toxicity, since it can also produce free radicals which may oxidize cellular components [1]. Chronic copper poisoning (CCP) develops into an acute episode of intravascular haemolysis, which results from the release of excessive amounts of copper from hepatic storages into the blood. The haemolytic crisis might be precipitated by stress, hepatocellular overload or lysosomal injury [2].

Chronic copper poisoning in ruminants is related to prolonged intake of high dietary copper concentrations, often in association to pyrrolizidine alkaloids intoxication [3-6].

Although some species, such as sheep, are known to be more susceptible than cattle, little is known on the water-buffalo's susceptibility to CCP. A recent study [7] compared the susceptibility of water buffalo to CCP with cattle's CCP. These authors suggested that buffaloes had a lower hepatic copper accumulation threshold than cattle, thus being more prompt to clinical manifestation of the disease.

In Argentina, water buffaloes were first introduced at the beginning of last century although dairy farming is a recent industry, started in 1992 [8]. Since not much information is available in the local literature on buffalo husbandry and nutrition, farmers and veterinary practitioners often extrapolate it from the bovine system. This article reports an outbreak of acute haemolytic syndrome related to copper poisoning in water buffaloes after periodic copper supplementation.

Several buffalo deaths occurred in a buffalo dairy farm in north Buenos Aires province, affecting at least three heifers. The herd was composed by fourteen, three to four month-old heifers, fed predominantly on corn and hay. The animals had received two or three supplementations of a commercially available poly-mineral and poly-vitamin subcutaneous supplement, used in bovine dairy farms (Table 1), with an interval of one month between each application, though he clinical signs commenced more than one month after the last application. Further details on the animal's husbandry and nutrition were not available.

Clinical signs consisted in apathy, anorexia, diarrhoea and reddish discoloration of the urine. Non-steroid anti-inflammatory (dipirone) and a hepatic protector were used for medicating the symptomatic heifers, inducing a mild temporary recovery.

Two dead (case Nos. 1 and 2) and one in extremis (case No. 3) three to four month-old buffalo heifers were submitted to the Pathobiology Institute from the National Institute of Agricultural Technology (INTA) in Castelar, Buenos Aires province, Argentina, for diagnostic purposes. The dying animal was euthanized with an intravenous injection of sodic pentobarbital (Euthanyl, Brower, Argentina); according to the recommendations and approval of the Veterinary and Agricultural Research Centre (CICVyA) Animal Experimentation Ethics Committee, at INTA Castelar.

Tissue specimens from abomasum, small and large intestine,

Vitamins	Concentration	Minerals	Concentration
Vitamin A	2.000.000 U.I	Sodium selenium	0,013 g
Vitamin D3	2.000.000 U.I	Copper sulphate	0,8 g
Vitamin E	1.000 U.I	Zinc sulphate	0,05 g
Hydrolyzed casein.	10 ml	Excipient	100 ml

Table 1: Formula and concentration of the poly-vitamin and poly-mineral supplement used (each dose).

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liver, kidney, adrenal gland, spleen, mesenteric and mediastinal lymph-nodes, lung, heart and brain were collected for histological examination from all animals. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Tissue sections from the liver of the three heifers were stained with Timm's stain [9] together with a liver section from a normal and a copper-intoxicated bovine liver as negative and positive controls respectively. Immunohistochemistry (IHC) was performed on a tissue specimen from the liver of case No. 1, using ki-67 monoclonal antibody (Immunotec Research LTD, France) as a marker for cell proliferation as well as Hepar-1 and Keratins 7 and 20 (Dako, Denmark) for identifying cell-type.

Formalin-fixed kidney samples from all three heifers and a fresh liver sample from case No. 3, were obtained for determining copper concentration. The samples were dried and processed by atomic absorption spectrophotometry to determine copper concentrations as described elsewhere [10].

For bacteriological examination, tissue specimens from small and large intestine, liver, kidney, spleen and lung were aseptically collected from case No. 3 and cultured on 5 % sheep-blood agar plates at 37ºC for 48 hours both aerobically and anaerobically. The isolates were identified by standard laboratory procedures. The virulence of the *E. coli* isolates were determined by PCR to detect genes encoding F5 (*f5*), F41 (*f41*), intimin (*eae*), S (*sfa*), P (*papC*), Afa-8 (*afaEVIII*), enterotoxin (*sta*), cytolethal distending toxin III and IV (*cdtBIII*, *cdtBIV*), cytotoxic necrotizing factor 1 and 2 (*cnf1*, *cnf2*) and Shiga toxins 1 and 2 (stx_1, stx_2), according to previous reports [11,12]. Direct immunofluorescence was used for detection of *Leptospira* antigens (CDC, USA). These antibodies cross-reacted with several *Leptospira* serovars (*pomona, canicola* or *wolfii*).

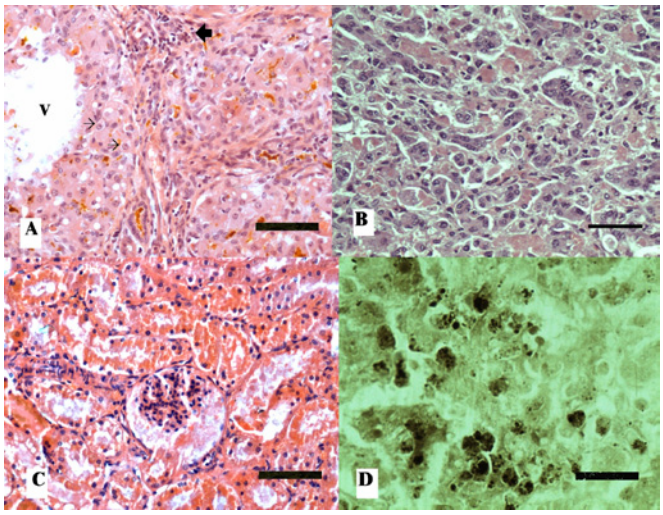

Figure 1: A) Liver section from case Nº 2 in which bile duct proliferation and fibrosis with mononuclear infiltration can be seen (large arrow). Towards the mid-zone and the hepatic vein (V), vacuolar changes and megalocytosis (small arrows) are evident. HE, bar= 50µm. B) Liver section from case Nº 1, displaying sinusoidal disarrangement, and abundant cells exhibiting neoplastic changes such as altered nucleo-cytoplasm ratio and abnormal nuclear shape and size. Degenerative cells exhibiting nuclear pyknosis can also be seen. HE, bar= 50µm. C) Kidney section from calf Nº 1 exhibiting tubulorrhexis with abundant strongly acidophilic material, resembling hemoglobin. Proteinaceous material is also evident in the capsule of the glomeruli. HE, bar= 50µm. D) Liver section from case Nº 1 in which strongly dark copper deposits can be seen in the cytoplasm of many cells. Timm's stain, bar= 25µm.

Bovine viral diarrhoea virus (BVDV) infection in two (case Nos.1 and 3) of the three animals in this study was previously reported by Craig and others (2008) [13].

All animals exhibited depression, apathy, diarrhoea and sudden death. Dark red urine discoloration was also observed during the clinical examination.

At post mortem examination, severe jaundice, predominantly in the peritoneum, aorta, cartilages and pleura was the most evident change observed. Transparent fluid in the abdominal cavity and reddish staining in the serosa of the abomasum, rumen, jejunum and gall-bladder was present in all carcasses. The livers were enlarged, firm and pale, displaying an evident acinar pattern while the gall-bladders were distended and filled with dark fluid. The gall-bladder, kidneys, spleen and lymph-nodes looked dark and congested.

In light microscopy, fibrosis, bile-duct proliferation and multi-focal mononuclear infiltrations were observed in all the livers (Figure 1A). One heifer (case No. 1) exhibited hepatocytes with cytoplasmatic vacuolation, megalocytosis and abundant cellular pleomorphism (Figure 1B). These cells were positive to Hepar-1 but not to keratins 7 or 20. The same tissue was also positive to ki-67 in approximately 15% of the hepatocytes.

Timm's s copper stain, revealed abundant copper deposits in the cytoplasm of the hepatocytes of all heifers (Figure 1D) as well as in the positive but not in the negative control livers.

Multifocal haemorrhages were observed at different sites of the brains and hearts of all animals. The spleens exhibited haemorrhages and hyaline deposits together with necrosis and intravascular thrombus. These changes were also observed in the lymph-nodes together with polymorphonuclear and macrophage infiltration and sinusal hyperplasia. The gall-bladders displayed exfoliation of the epithelium and mononuclear infiltration and haemorrhages in the lamina propria. In the kidneys, hyaline intra-cytoplasmatic droplet-change and necrosis was evident in the tubular epithelium. Cylinders, cellular debris and eosinophilic material accumulated in the tubules and often in Bowman's capsule were seen together with hemorrhagic foci and greenish-brown pigment (Figure 1C). In the ileum and colon, exfoliated epithelial cells, some being strongly acidophilic, sometimes forming pseudo-membranes were observed in the lumen. Both, ileum and colon exhibited polymorphonuclear infiltration and haemorrhages in the lamina propria. In the ileum, both lymphoid tissues and villi were atrophic.

Copper concentration was 198 ppm in the liver sample examined (case No. 3) (normal 25-100 ppm wet basis for cattle) and 66, 47 and 77 ppm in the kidneys of case No.1,2 and 3 respectively (normal 4-6 ppm wet basis for cattle) [14].

Escherichia coli was isolated from the lung, the muscle, and the intestinal content of case No. 2. The genes *iucD*, encoding the siderophore aerobactin, and *f17A*, encoding the structural subunit from the fimbria F17, respectively, were identified in the *E. coli* strains. All the *E. coli* strains were negative for the genes *f5*, *f41*, *eae*, *sfa*, *papC*, *afaEVIII*, *sta*, *cdtBIII*, *cdtBIV*, *cnf1*, *cnf2*, stx_1 and stx_2, The production of a haemolysin, not corresponding to the enterohemolysin encoded by the gen *ehxA*, characteristic of enterohemorrhagic *E. coli*, was observed in the *E. coli* strains *iucD* and *f17A* positives. *Clostridium* sp. and *Salmonella* sp. were not isolated. Immunofluorescence staining was negative for *Leptospira* spp. antigens in all the samples analyzed.

The mortality in the herd affected young heifers of approximately

three to four month-old, exhibiting similar clinical signs among them. The severe jaundice and the generalized changes, such as the haemorrhages, the severe nephrosis with the reddish-dark urine discoloration, are consistent with an acute haemolytic syndrome.

Considering the high levels of copper detected in the liver and kidneys, it is likely that chronic copper intoxication was responsible for the haemolytic syndrome [15]. Since the heifers had been given a poly-vitamin and poly-mineral subcutaneous supplementation containing copper, it is likely that this was the source of the intoxication.

Copper is metabolized in the liver [2] but, since these heifers had a compromised liver, copper metabolism was certainly altered and any stressing factor –such as diarrhoea- could have triggered the haemolytic episode.

Other changes observed in the livers, such as bile duct proliferation, fibrosis, megalocytosis and cellular pleomorphism highlights a chronic, sub-clinical condition, in which malignant cell transformation was present. The fact that IHC for Hepar-1, but not for keratins 7 or 20 were positive, indicates that these neoplastic changes were, most likely, of a hepatocellular origin. Chronic changes like these, are often related to toxic agents from plants or fungi, among which, pyrrolizidine alkaloids and aflatoxins are the most common ones [16,17]. These substances are known to induce hepatocellular carcinoma in animals and humans [1,18]. It is also known that these toxins inhibit cell proliferation and, occasionally, induce apoptosis, thus aggravating CCP [1]. Unfortunately, no hay was available to determine the presence of any toxic plant or fungi.

In Argentina, there is no data on standard-reference water buffalo's tissue copper levels available in the literature. In the present study, copper levels had to be compared to bovine [14,15] as well as to African buffalo (*Syncerus caffer*) [19].

Water buffalos are susceptible to *Leptospira* spp., producing a haemolytic syndrome. This microorganism has been isolated from buffaloes in Buenos Aires province [20]. However, no *Leptospira* antigens were detected in the specimens analyzed in this study. On the other hand, *E. coli* strains encoding virulence genes related to an extra-intestinal pathotype were isolated from different organs from the animal to which bacteriological analysis was performed (case No. 3), while BVDV was present in the intestine of the other two heifers, as had been previously reported [13]. Therefore, the intestinal manifestations and pathological changes observed in the gut were probably associated with the action of these two pathogens. Infections with BVDV are known to induce immunosuppression in cattle [21]; this might have favoured *E. coli* invasion, with posterior septicaemia.

In many developing countries, buffalo milk accounts for almost 50% of drinking milk, whereas in other countries such as Italy and Argentina, most of this milk is used for mozzarella cheese production [22,23]. Aflatoxins and pyrrolizidine alkaloids are both known to cause hepatocelular carcinoma in animals and humans, and active metabolites of these substances can be detected in milk [1,18,24,25]. In this study, the changes observed in the liver of the heifers suggests that these toxins might have been present, thus highlighting a possible threat for human health.

Since the information available on buffalo husbandry and health care in Argentina is scant, the findings of this report, also remarks the importance of determining nutritional standard reference values for water buffalo mineral supplementation.

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