

# Assessment of Cu-Zn EDTA Parenteral Toxicity in Calves

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**Abstract** Copper (Cu) parenteral administration is used in a beef cow-calf operations to prevent or correct Cu deficiency in bovines. At present, Zinc (Zn) salts have been incorporated to complement Cu antioxidant effect. A risk of hepatotoxicity generated by overdose is a negative consequence of injectable Cu application. Cu-Zn EDTA appears as an alternative; however, data about its toxicity is unknown. The aim of this study was to assess toxicity risk of different doses of Cu-Zn EDTA in calves. Thirty two Aberdeen Angus calves of 162 ( $\pm 20$ ) kg BW were assigned to 4 groups ( $n = 8$ ), homogeneous in weight, sex, and age. Cu-Zn EDTA was administrated in doses of 0.3 mg/kg BW (group 1X); 0.6 mg/kg BW (group 2X); 0.9 mg/kg BW (group 3X) and sterile saline solution (control group-with no treatment). Clinical and blood parameters in animals were monitored during 28 days. In groups' control, 1X and 2X there were no alterations in the assessed parameters. In group 3X, one of the animals showed depression, permanent decubitus, and muscular twitching; that animal had to be killed in extremis for humanitarian reasons. Necropsy and Cu tissue concentration findings confirmed intoxication in the clinically affected animal. The rest of the animals in group 3X showed only a temporary increase in liver enzymes. The results indicate that a dose of 0.9 mg/kg BW of Cu as Cu-Zn EDTA is potentially hepatotoxic, this dose is similar to other soluble salts of parenteral administration.

**Keywords** Copper · Zinc · Liver damage · Cattle

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## Introduction

Parenteral administration of Cu injectable salts is a sanitary measure frequently used in extensive livestock systems affected by hypocuprosis [1, 2]. Due to high requirements, growing calves represent the most susceptible category to Cu deficiency, which results in less weight gain [3]. However, as a result of parenteral supplementation, cases of iatrogenic intoxication usually occur due to overdose, and this generates deaths by hepatotoxicity [4–6]. The lethal factor in iatrogenic poisoning is the transference Cu rate from the injection site to the liver, and it also depends on the milligrams per kilogram of body weight (kg BW) administrated [7]. There are salts which are slowly released minimizing the death risk, but can cause local reactions in the administration site and they are not efficient to generate Cu liver storage [8]. Both inconveniences can be solved administrating soluble salts, such as Cu edetate; however, they have a narrow margin between safety and adverse toxic effects [6]. At the same time, there were a wide range of Cu edetate doses which have generated toxicity, ranging between less than 1 and up to 4 mg/kg BW [6, 9]. This data arises specially from accidental clinical cases reports, as there is little research on toxic dose identification. At the same time, the cited communications have used Cu-Ca EDTA, and at present, there exists the possibility of using Cu-Zn EDTA as an alternative. Cu-Zn EDTA might have the advantage of providing Zn, which shares its antioxidant function with the Cu being part of Cu-Zn superoxide dismutase (Cu-Zn SOD), main antioxidant metalloenzyme at a cytoplasmatic level [10]. However, there are not any works that assess Cu-Zn EDTA toxicity. The aim of this study was to assess the effect of increasing doses of Cu-Zn EDTA in clinical, blood, liver and kidney functionality parameters in calves on growth.

## Materials and Methods

All the proceedings in this study were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Sciences of La Plata National University. Registered under the Protocol for the Use of Animals in Scientific Research (Internal identification code N° 57-3-16P).

### Animals

Thirty-two calves Aberdeen Angus of 7 to 8 months old, clinically healthy, were distributed according to sex and body weight in an ascending order and then randomly assigned to four groups ( $n = 8$ ). Group 1X (dose recommended by the manufacturer laboratory), group 2X (double dose recommended by the manufacturer laboratory), group 3X (triple dose recommended by the manufacturer laboratory) and a control group (injected with saline solution). The average weights ( $\pm$  standard deviation) in each group were as follows: group 1X, 161.5 ( $\pm 22$ ); group 2X, 168.9 ( $\pm 25$ ); group 3X, 156 ( $\pm 19$ ); and 162.4 ( $\pm 16$ ) kg BW in the control group ( $p = 0.68$ ).

### Treatment

Groups 1X, 2X, and 3X were treated with Cu and Zn edetate solution, which provided 15 mg of Cu/mL and 50 mg of Zn/mL (Suplenut®, Biogénesis-Bagó). The animals were weighted to received a subcutaneous administration in the neck region of the following doses: group 1X, 1 mL/50 kg BW (0.3 mg of Cu and 1 mg of Zn/kg); group 2X, 2 mL/50 kg BW (0.6 of Cu and 2 mg of Zn/kg); and group 3X, 3 mL/50 kg BW (0.9 of Cu and 3 mg of Zn/kg). The control group received sterile saline solution (1 mL/50 kg BW).

### Clinical Assessments and Samplings

On treatment day (day 0) and then on days +0.25 (6 h post-treatment), +1, +2, +3, +7, +14, and +28 individual clinical assessments were carried out, including heart rate, respiratory rate, and rectal temperature. On days 0, +3, +7, +14, and +28 blood samples with and without anticoagulant were collected via jugular venipuncture. Kidney function was monitored by means of urea and creatinine concentrations, while liver function, by means of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), and total bilirubin (TB) concentration. Muscular damage was assessed by means of the activity of the creatine kinase (CK) enzymes. Hematological tests included red cell count, hemoglobin concentration (Hgb), hematocrit percentage (Hct), and hematimetric indices. The leukocyte hemogram included white blood cell count, absolute and relative leukocyte formula, and platelets count.

## Design and Statistical Analysis

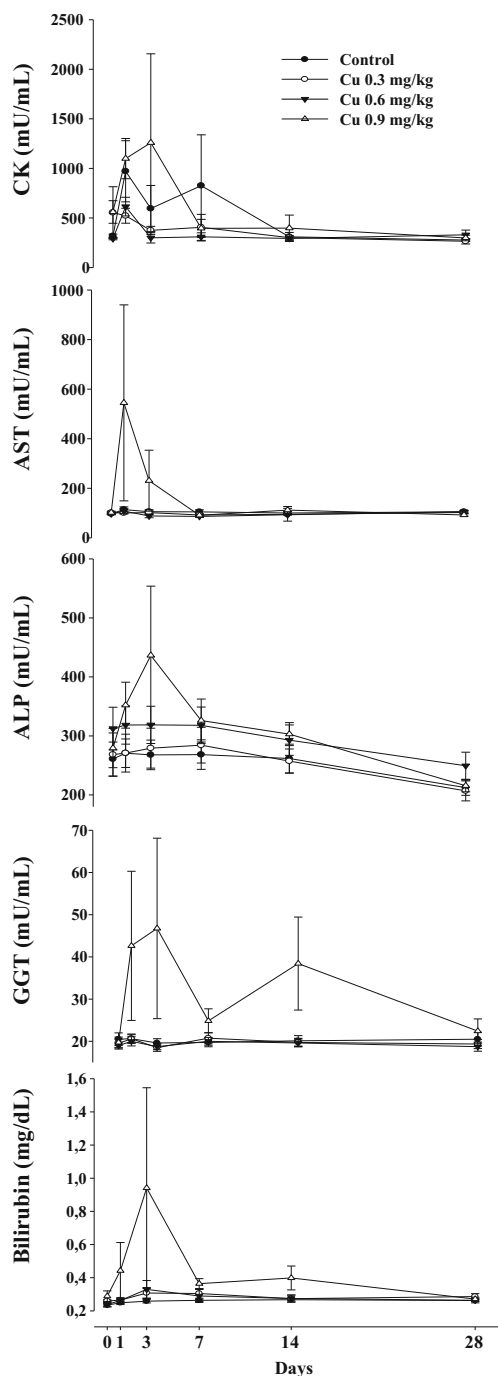
A completely random design was used, with a mixed model of measures repeated in time, where treatment, time, and interaction (time  $\times$  treatment) were fixed variables, and animals, random variables. The model used is  $Y = \mu + T + e$ , where  $Y$  is the dependent variable,  $\mu$  is the average of the population under study,  $T$  is the treatment or independent variable, while  $e$  is the error. The variables were analyzed using the statistical package SAS (9.1). The Slice option was used to detect statistical differences at each time. A  $p$  value  $< 0.05$  was considered statistically significant.

## Results

In the clinic inspection during the 6 first hours post-treatment, clinical signs were observed only in group 3X, in which 5 of the 8 animals showed head and neck laterality movements, salivation, eye discharge, and intermittent sternal decubitus. On day +2 the animals were alert, with normal coordination, except for one animal from group 3X which showed depression and intermittent decubitus. The same animal, on day +3, showed marked depression, lateral decubitus, and muscular twitching on thoracic and pelvic limbs. After the clinical assessment the animal was euthanized for humanitarian reasons. The necropsy showed accentuated liver lobular pattern (nutmeg liver) widespread distributed in the parenchyma. On the gallbladder serosa petechial and ecchymotic hemorrhages were observed. The pericardium presented petechial and ecchymotic hemorrhages. In the rest of the organs and systems, no injuries were observed. The liver microscopic observation showed marked necrosis of peri-acinar distribution (centrilobular) with loss of the structure and hepatic cords disposition. In the injury area, hepatocytes with degeneration and marked nuclear pyknosis and karyorrhexis, were observed. The Cu concentration in the liver was within the normal range (360 ppm-MS) however, Cu concentration in the kidney was high (41.5 ppm-MS).

The registered clinical parameters, heart rate, respiratory rate, and rectal temperature evidenced no significant differences between groups ( $p$ : 0.93; 0.52, and 0.82 respectively) and no interactions between groups and sampling ( $p$ : 0.89; 0.52, and 0.99 respectively) during this study. The mean values for the three parameters remained within normal range during the whole study.

Clinical enzymology evidenced the existence of liver injury in group 3X, but with a significant individual variability. This situation, together with the sacrifice of the most affected animal on day +3, increased data dispersion and no statistical differences were observed between groups for ALP ( $p = 0.27$ ), AST ( $p = 0.13$ ), GGT ( $p = 0.09$ ), and TB ( $p = 0.30$ ). However, ALP and AST mean values increased over normal range in group 3X (Fig. 1), and showed more sensitivity to identify the



**Fig. 1** Average values of creatine kinase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, and total bilirubin in the different groups. As from day +7, group 3X had one animal less ( $n = 7$ ) since an affected animal was euthanized for humanitarian reasons

existence of liver injury (Table 1). In the animal which was euthanized ALP, AST, GGT, and TB concentration increased over the range considered as normal, and the same occurred with CK activity, probably as a result of permanent decubitus (Table 2).

**Table 1** Fraction of animals over the range of normal parameters of liver functionality in group 3X (0.9 mg Cu/kg BW)

Survey period	ALP (>320 mU/mL)	AST (>132 mU/mL)	GGT (>48 mU/mL)	TB (>1.0 mg/100 mL)
Day 0 <sup>a</sup>	3/8	0/8	0/8	0/8
Day +1	6/8	5/8	1/8	1/8
Day +3	6/8	3/8	1/8	1/8
Day +7	5/7	0/7	0/7	0/7
Day +14	2/7	2/7	2/7	0/7
Day +28	0/7	0/7	0/7	0/7

ALP serum alkaline phosphatase, AST aspartate aminotransferase, GGT gamma glutamyl transpeptidase, TB total bilirubin

<sup>a</sup> prior treatment

All blood and kidney (urea and creatinine) parameters, remained within range considered as normal for the age and species under survey, and without variations attributable to the treatment during the study.

### Discussion

The assessment of clinical, blood, and clinical enzymology parameters show that the doses of 0.3 and 0.6 mg/kg BW of Cu as Cu-Zn EDTA—groups 1X and 2X, respectively—do not generate variations in clinical, blood, liver, kidney, or muscular function parameters in calves. Toxicity was evident only in group 3X, which received 0.9 mg Cu/kg BW. Clinical and serological changes were variable in this group. In this sense, only 5 out of 8 animals showed clinical signs during the first hours post-treatment and four of them were able to recover on day +1. On the other side, animals in group 3X showed an increase in liver enzymes levels, but with different behavior and severe value dispersion, specially the animal which was euthanized on day +3. The different susceptibility of animals

**Table 2** Liver enzymes activity, muscular enzyme, and total bilirubin concentration in animal with Cu iatrogenic poisoning

	ALP <sup>1</sup>	AST <sup>2</sup>	GGT <sup>3</sup>	TB <sup>4</sup>	CK <sup>5</sup>
Units	mU/mL	mU/mL	mU/mL	mg/dL	mIU/mL
Normal range	68–320	78–132	5–48	0.01–1.0	44–228
Day 0 <sup>a</sup>	139	85	19	0.25	362
Day +1	576	3311	165	1.62	1608
Day +3 <sup>b</sup>	1231	1089	195	5.17	7530

ALP serum alkaline phosphatase, AST aspartate aminotransferase, GGT gamma glutamyl transpeptidase, TB total bilirubin, CK creatine kinase

<sup>a</sup> Prior treatment

<sup>b</sup> Sample collected prior the animal was euthanized for humanitarian reasons

from the same group has been demonstrated in chronic poisoning cases, where intoxicated animals accumulate four times more Cu than the others in the same group [11]. In the acute Cu poisoning the cause of different susceptibility is unknown, and the key factor will be the Cu transfer rate from the injection site to the liver [7]. This rate is maximum in salts such as edetate which enable to minimise local reactions and to optimise liver Cu storage. It also has lower therapeutic margin [12]. The toxic dose of 0.9 mg/kg BW agrees with doses mentioned in accidental cases [6], but it is lower to other trial that administrated up to 4 mg/kg BW to provoke hemolytic crisis but no death [9]. Possibly, the same factors which generate individual susceptibility in each study also generate differences between studies. One of these factors could be the liver metallothionein (MT) concentration, enzyme in charge of capturing Cu which comes from the injection site [13]. Curiously, Zn induces the synthesis of MT and this coincides with chronic poisoning cases in which the animals that accumulated higher content of Cu also had the higher concentrations of Zn [11, 14].

ALP, AST, GGT, and TB determinations showed liver disease in group 3X, with different sensitivity, though. Raise in ALP can be unspecific in calves due to the fact that it is a ubiquitous enzyme [15] and it shows low correlation with liver Cu concentration in cases of chronic poisoning. AST activity was a sensitive indicator, with mean values which increased faster than those of ALP enzymes. In chronic poisoning cases, AST activity revealed a good correlation with Cu liver concentration in cattle [16]. However, AST is present in muscles as well, for this reason it must be evaluated together with CK [15]. GGT is a good indicator of severe liver damage, including bile ducts, contrary to AST which mainly indicates hepatocellular damage [12]. Its behavior as damage indicator was relatively sensitive, but very specific since it increased only in the animal which was euthanized. TB concentration showed the same behavior.

Hemolysis in bovines is an expected consequence in chronic Cu poisoning cases [11]. However, in acute Cu-Ca EDTA poisoning, the reports are variable since in some cases, death without previous hemolysis are generated [4, 6] while in others severe hemolytic crisis are informed [9]. During the present study, no hemolysis was evidenced, not even in the animal which then was euthanized. In the same way, there are discrepancies in the Cu concentrations in the tissues which allow intoxication diagnosis. High Cu concentration in the liver is characteristic of a chronic poisoning [17]. In acute cases, even in fatal cases, there is no liver Cu recharge, so liver Cu levels can be found within the normal range or even below it [6, 18]. Renal Cu concentrations have been proposed as an alternative to diagnosis in acute cases. In previous trial [19] evaluated Cu concentration in renal cortex in endemic area of hypocuprosis, and they established a limit of 19.9 ppm (MS), over which they diagnosed acute poisoning. In a subsequent trial [18] the authors used the mentioned range and they were able to ratify its value as a Cu over dose indicator in

93% of confirmed cases. Other authors propose values as from 40 ppm MS [17, 20, 21]. In this study, the animal which was euthanized had a kidney Cu concentration (41.5 ppm MS) higher than those proposed.

## Conclusions

Cu supplementation as Cu-Zn EDTA was safe in doses of 0.3 and 0.6 mg Cu/kg BW in calves, however, in doses of 0.9 mg/kg BW clinical signs were observed, as well as an increase in liver damage indicators.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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