Therapeutic efficacy and pharmacological safety of parenteral supplementation of different concentrations of copper in cows

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Abstract

The objective of the present study was to determine the parenteral dosage of the trace mineral copper (Cu) which re-establishes serum and hepatic levels in hypocupperhemic animals without causing intoxication in the liver or residuals in milk. The experiment was carried out in two phases; in the first phase, 20 hypocupperhemic animals were used. Those were divided into 4 groups of 5 cows each: 3 groups which received 25, 50 and 100 mg of Cu respectively and 1 control group without supplementation, but with induced copraemia 5 days before and after the treatment. The milk of the cows treated with 100 mg Cu was examined for residuals of this element for one month. In the second phase, 25 animals were selected and divided into 5 groups of 5 cows each: 1 control group and 4 groups which received 25, 50, 100 and 200 mg of Cu respectively. The impact of this Cu dosages on liver reserves of this element, on hepatic enzymes (alanine-amine-transaminase, aspartate-amine-transferase, alkaline phosphatase and glutamate dehydrogenase) and on bilirubin was analyzed. The groups treated with 50 and 100 mg Cu showed increased levels in blood serum and hepatic tissues (P<0.05) in comparison to the control group and the one treated with 25 mg, obtaining the physiological level considered normal in cows during 60 days without an effect to the level of Cu in milk or to the liver’s enzymatic activity. We conclude that treatment with this dosage results in a restoration of the Cu concentration in blood serum and hepatic tissues without toxic effects or residuals.

Keywords: Copper, trace mineral, hypocupperhemic, supplementation

Introduction

Next to phosphate deficiency, Cu deficiency or hypocuprosis is considered the second most frequent mineral deficiency in grazing cows all over the world, especially in tropical countries (McDowell & Arthington 2005). This was reported in Oceanica (Grace & Wilson 2002) Latin America (Picco et al. 2002) and Europa (Pavlata et al. 2005, Slavik et al. 2006, Enjalbert et al. 2006).

In Cuba, this is widely spread, especially in the central part of the country where high proportions of cows, heifers and seminal bulls were diagnosed with hypocupperhemia, which has negative effects on reproduction and milk production (García 2008).
Different injectable Cu drugs have been introduced to the market to prevent and cure hypocuprosis in the affected grazing zones, in which they are administered massively (Daugherty et al. 2002, Fazzio et al. 2003). Those drugs have a high efficacy; they prevent negative effects of antagonists in the digestive tract (Van Niekerk et al. 1994) and portion out adequate levels of Cu in the animals, especially in periods of high demand, such as during production and reproduction (Gee et al. 2000, Smith 2003).

Cu compounds may provoke acute intoxications since they have a relatively small safety margin, especially in calves and young animals (Goldfrank et al. 1998, EMEA 1998); on the other hand, the presence of Cu in dairy products has become a public topic recently because it can be very toxic to humans in high concentrations and it accumulates in the trophic chain (Mubbasher et al. 2003).

However, although cases of intoxication due to injectable Cu compounds are known, there are no references to controlled studies about the toxic effects and residuals in milk. Therefore, the objective of this study is to determine the parenteral Cu dosage which reinstates the serum and hepatic concentrations in hypocupperhemic animals without causing toxic effects in the liver or leaving residuals in milk.

Materials and methods

Characterization of the area of study

The experiment was carried out during the rainy season (June-July) on a dairy farm in the central southern zone of the province Villa Clara, 22° 53 N and 82° 02 W with an altitude of 90 to 100 m above the sea level. The mean annual temperature is 24.3 °C, during the period of the experiment the mean temperature was 25.9 °C. The mean annual rainfall is 1 530 mm and during the experiment the monthly rainfall was 267.4 mm. On the other hand, the relative humidity was 87.5 % according to the data collected from the provincial meteorological station.

The animals were in a rotational grazing system with a distribution of 1.5 animals ha⁻¹, 16 grazing hours daily, a composition of the main pasture of 60% Cynodon nlemfuensis, 18% Paspalum notatum (18 %) and 12 % Dichantium annulatum, with a disposability of 27 kg of dry matter per animal and a supplementation of sugarcane of 10 kg/animal/day and molasses.

The cows were milked manually twice a day at 2 a.m. and 5 p.m. The dominating soils in the area of study are loam and clay soil with Cu contents of 0.19±0.02 ppm and the pastures have Cu contents of 5.63±1.20 ppm (García 2008), which classifies as deficient in this microelement (McDowell & Arthington 2005).

Experimental procedures

The experiment was carried out in two stages, using 45 apparently healthy cows of the genotype Cuban Siboney (a crossbreed of Holstein × 3/8 Zebu) in their reproductive stages with a serum deficiency in Cu (<11.77 µmol/L), a body condition (BC) between 3 and 4 according to clinical diagnosis (Cuesta et al. 2007) and a body weight of 426.3±13.3 kg. Preceding complementary analyses were carried out to determine the levels of haemoglobin and haematocrit and to diagnose the presence of parasites in the feces, especially of trematodes like Fasciola hepatica.
In the first phase, 20 cows in their second or third lactation, with an average age of 6 to 7 years, a lactation length of 90-120 days and an average milk production of 5.2 L/cow/day were chosen. They were divided into 5 groups with 4 cows each; 3 groups received a single parenteral dosage of 25, 50 and 100 mg of Cu respectively (the Cu was applied as ethylenedinitrilotetraacetate of Cu, 25 mg/ml). Besides that, a control group without Cu supplementation was utilized, in which copraemia was induced 5 days before and after the treatment, determining the blood concentration and depletion time of Cu. After 60 days, a final analysis was carried out to determine the level of Cu at this point. A 5 day interval study to examine Cu traces in the milk was carried out for one month from the day of supplementation on the cows which were treated with 100 mg of Cu.

The second phase included 25 animals with an average age of 6 years which were to be slaughtered because of low milk production; they were divided in 5 experimental groups of 5 cows each. The effect of subcutaneous supplementation of 25, 50, 100 and 200 mg of Cu respectively (Cu as ethylenedinitrilotetraacetate of Cu, 25 mg/ml) was determined on Cu reserves and hepatic enzymes; measured against a control group without supplementation. Animals were slaughtered 5 days after the treatment to evaluate the hepatic concentration of Cu in each group, coinciding with the Cu deficiency of the phase before the supplementation. Before slaughtering, blood samples were taken to determine hepatic enzyme levels. After the Cu administration, frequent and continuous clinical examinations (once every 12 h) were carried out to determine any manifestations of intoxication; they included the general state of the animals, and the state of the skin and the mucous membranes.

Sample collection and analytic procedures

Blood samples to determine Cu levels in the serum were drawn by venipuncture of the jugular vein. 10 mL of blood were deposited in sterilized and demineralized sample tubes without anticoagulant; they were centrifuged at 1 500 rpm for 10 min and stored at a temperature of −10 °C while awaiting the analysis. For analysis of bilirubin and enzymatic activity, blood samples were collected using the same method and immediately taken to the laboratory for analysis.

Milk samples were collected in sterilized and demineralized sample tubes according to ISO 707 (1997) norms; no metallic instruments were used to collect the samples in any process of the analysis. 15 min after the animals were slaughtered, a general visceral inspection was performed to identify alterations, and liver samples were collected on the right ventral margin of the lobule (15 cm$^3$) and preserved at −10 °C prior to analysis.

The determination of Cu levels in serum, milk and hepatic tissues was carried out by spectrophotometry of atomic absorption (Miles et al. 2001), with Pye Unicam AAS (Pye Unicam Ltd., Cambridge, UK according to the manufacturer’s instructions.

The determination of Total Bilirubin (TB), Direct Bilirubin (DB), Indirect Bilirubin (IB) and the enzymes alanine-amine-transaminase (ALAT), aspartate-amine-transferase (ASAT), alkaline phosphatase (ALP) and glutamate dehydrogenase was effected by kinetic methods via UV, optimizing IFCC; using an Airone 200 (Crony Instruments SRL, Rome, Italy) according to the manufacturer’s instructions. For the control of reproducibility, the Elitrol-I, lot R60148 model manufactured by Eltech was employed.
Statistical analysis

The data were analysed in detail, using the statistical package Statgraphics 5 (Statpoint Technologies, Warrenton, VA, USA). Descriptive variables were calculated for all variables; for the different stages in which the dynamic of the dosage of Cu was evaluated in the blood, for the concentrations of microelements and the level of hepatic enzymes, a variance analysis of simple classification (ANOVA) was carried out. Previously, the base trials were done applying the LSD test for mean comparison. A regression analysis and simple lineal correlation was carried out on the dynamic of copraemia, the Cu dosage applied, as well as serum and hepatic Cu concentrations.

Results and discussion

First phase

The dynamic of Cu in the serum of the animals in this study (Figure 1) demonstrated hypocupperhemic values in the control groups throughout the experiment. In the animals treated with 25 mg of Cu, the cupperhemia reached a maximum 5 day after treatment although it was still lower than 11.77 µmol/L, a value which is below the physiological parameters and indicates hypocupperhemia (Álvarez 2001, McDowell & Arthington 2005). There were no statistical differences in serum Cu concentrations between this group and the control group which indicates that the dosage is insufficient to restore the required serum Cu levels.

The serum Cu concentration increased in the groups treated with 50 and 100 mg of this microelement, reaching the required physiological levels of the bovine species as suggested by the authors mentioned earlier. The animals reached a maximum cupperhemia peak 5 days post treatment, with no statistical differences between these two dosages, but significantly higher ($P<0.05$) throughout the treatment than the levels of animals treated with 25 mg of Cu and the control group.

![Graph](image.png)

Average values with different letters within the series at the same day indicate significant differences ($P<0.05$) for LSD. DL: deficiency Limit

Figure 1

Dynamic of Cu levels in the serum of animals treated with different parenteral dosages of Cu.
After the maximum peak, the cupperhemia stabilizes and begins descending after day 15. From this moment, there is a highly significant decline ($P<0.0001$) of serum Cu levels in the animals treated with dosages of 50 ($r=-0.975$) and 100 mg ($r=-0.952$). A similar situation was found in the group treated with 25 mg of Cu, although the decline was less significant ($P<0.01$) and ($r=-0.511$). There was no significant decline in serum Cu values in the control group during the experiment (Figure 2).

The Cu serum levels were found to be within the physiological parameters of the bovine species 55 days after 50 or 100 mg of this element were administered to the animals. However, as a consequence of the decrease, after 60 days cupperhemias of 10.54, 10.67, 11.63 and 11.66 µmol/L were found in the groups treated with 0, 25, 50 and 100 mg respectively. The average decline of cupperhemia from one timepoint to the next was 0.17 µmol/L and 0.24 µmol/L in the animals treated with 50 and 100 mg respectively. These results demonstrate on the one hand the efficiency of the treatment applied to increase the blood Cu concentrations, and on the other hand, the fact that after 60 days the values were back to being below the lower limits of the physiological parameters for this species. Nevertheless, to establish the exact timepoint at which to repeat the treatment, it is important to conduct more prolonged studies.

A positive and significant correlation ($P<0.05$) was reported between the Cu dosages injected and the blood serum Cu concentrations on the 5th day post treatment ($r=0.90; R^2=0.81$), coinciding with the cupperhemia peak in the animals on day 15 ($r=0.89; R^2=0.80$), the moment in which the coppper level starts to decline. This demonstrates that the administration of an increased Cu dosage led to a significant increment of this microelement in the blood.

The results obtained in this experiment correspond with the ones obtained in previous studies in which the administration of 125 mg of Cu in cows and a repeat of this treatment after three months increased ($P<0.01$) Cu concentrations in the blood serum (Balbuena et al.).
1999 and Castelli et al. 2001). In Cuba, parenteral administration of Cu in gestating cows also incremented \((P<0.001)\) the Cu concentration in blood serum (García et al. 2007).

The dynamic of the average Cu concentrations in blood serum and in milk of the cows treated with 100 mg of Cu (Figure 3) demonstrates that although the copperhemia values augmented significantly, Cu concentration did not increase in milk; those were found to be within the normal parameters for this species, 2.8-3.2 µmol/L, as stated by (Grace & Wilson 2002); this complies with the standard which states 8.55 µmol/L as the maximum tolerable limit specified by other authors (Muntean et al. 2004, Rodríguez et al. 2005).

These are the first records in Cuba about Cu content in cow’s milk, which constitutes an important nationwide contribution.

This study did not show a correlation between the Cu concentrations in blood and milk \((r=0.035)\), which is consistent with a previous study in which no correlation between Cu in blood serum and in cow’s colostrum could be detected (Pavlata et al. 2004).

The intoxication after parenteral treatment takes a hyperacute course, with the possibility of the appearance of clinical symptoms (Fazzio et al. 2003); however the single subcutaneous administration of of Cu did not result in any clinical symptoms of toxicity in animals 4 days after treatment. Over the observation period, frequent and continuous clinical examinations were performed, which were confirmed by the macroscopic necropsy findings in which the typical lesions (Jubb & Kennedy 2007) of this intoxication were absent.

**Second phase**

The hepatic Cu concentration (Figure 4) does not demonstrate statistical differences between the control group and the group treated with 25 mg of Cu, indicating an application of that 25 mg of Cu is insufficient to restore the required levels in the liver. Significant differences \((P<0.05)\) were found between the values of these groups and the ones attained in the other groups (50 and 100 mg of Cu respectively), in which the Cu levels in the hepatic tissue could be increased up to normal parameters of the bovine species (Radostits et al. 2000).

The hepatic levels of Cu rose to 365.15 ppm in the groups treated with 100 mg, which is within the established parameters, and less than 600 ppm, an indicator of intoxication...
A higher synthesis of ceruloplasmine (Cp) can be observed due to the increase in hepatic Cu depots following the application of the same, which helps maintain the required concentration by transporting the excess part from the hepatic cells to other tissues (López-Alonso et al. 2005).

Those results coincide with some studies in which the parenteral Cu application increased hepatic copper reserves in the treated animals which maintained high copper levels even after 90 days post treatment (Quiroga et al. 1995).

In the present experiment, there was a positive correlation \((P < 0.05)\) between the injected Cu dosage and the hepatic concentration \((r = 0.95; R^2 = 0.90)\) which indicates that an increased dosage of the administered Cu led to a significant augmentation in its concentration in the hepatic tissue.

The comparison of the results in the studied groups treated with different Cu dosages (Table 1) did not reveal significant statistical differences between them for the enzymes ASAT, ALAT, GLDH and ALP. All of these were found to be within the physiological parameters for the bovine species (Kaneko et al. 2002), and to be similar to the values reported for bovine races kept in tropical conditions (Villa et al. 1999, Lago et al. 2004, Sánchez et al. 2004, Campos et al. 2007).

Table 1

<table>
<thead>
<tr>
<th>Dosage</th>
<th>n</th>
<th>ALAT, IU/L</th>
<th>ASAT, IU/L</th>
<th>GLDH, IU/L</th>
<th>ALP, IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg</td>
<td>5</td>
<td>38.92±2.36</td>
<td>69.34±7.10</td>
<td>4.07±0.17</td>
<td>143.66±13.19</td>
</tr>
<tr>
<td>25 mg</td>
<td>5</td>
<td>39.18±0.78</td>
<td>65.06±2.45</td>
<td>4.07±0.04</td>
<td>138.62±7.33</td>
</tr>
<tr>
<td>50 mg</td>
<td>5</td>
<td>39.94±2.72</td>
<td>77.31±7.78</td>
<td>4.13±0.12</td>
<td>150.22±14.26</td>
</tr>
<tr>
<td>100 mg</td>
<td>5</td>
<td>38.16±1.79</td>
<td>73.23±3.38</td>
<td>4.14±0.07</td>
<td>142.73±15.26</td>
</tr>
<tr>
<td>200 mg</td>
<td>5</td>
<td>38.4±1.84</td>
<td>73.75±2.35</td>
<td>4.13±0.07</td>
<td>145.09±6.15</td>
</tr>
</tbody>
</table>

Columns with different letters indicate significant differences \((P < 0.05)\) for LSD, SE: standard error.
The blood serum levels of the enzymes ASAT and ALAP in this experiment are a lot higher than the ones reported by various Cuban authors in the 1980s. These differences could be a result of the breed and feeding systems; some of the studies were carried out in Holstein cows (Margolles et al. 1987, Zaldivar et al. 1989) and others in zebu cows (Colomé et al. 1989), whereby in both cases the animals were fed high quantities of concentrated feeds. In a study to evaluate hepatic function in 7 breeds specialized in milk production under tropical conditions, it was demonstrated that Holstein cows presented lower levels \( P<0.05 \) of these enzymes (Campos et al. 2007).

On the other hand, the mentioned authors realized their experiments using calorimetric methods and all proceedings were performed manually, whereas in this study a modern method was applied (a kinetic UV method optimized by IFCC) and the proceedings were performed exclusively computerized, which definitely could have influenced the results.

The bilirubin level is another important indicator of hepatic function (Table 2); it was found within the physiological parameters for the bovine species (Kaneko et al. 2002) without any significant statistical differences between the animals treated with different Cu dosages. Similar results have been reported in dairy cows within the tropics (Campos et al. 2007).

<table>
<thead>
<tr>
<th>Dosage</th>
<th>TB, μmol/L</th>
<th>DB, μmol/L</th>
<th>IB, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg</td>
<td>10.40±1.19</td>
<td>4.56±0.35</td>
<td>5.84±1.04</td>
</tr>
<tr>
<td>25 mg</td>
<td>10.42±0.42</td>
<td>4.63±0.31</td>
<td>5.76±0.63</td>
</tr>
<tr>
<td>50 mg</td>
<td>10.41±0.39</td>
<td>4.80±0.30</td>
<td>5.45±0.49</td>
</tr>
<tr>
<td>100 mg</td>
<td>10.88±0.60</td>
<td>4.36±0.21</td>
<td>6.53±0.60</td>
</tr>
<tr>
<td>200 mg</td>
<td>10.34±0.27</td>
<td>4.74±0.28</td>
<td>5.60±0.36</td>
</tr>
</tbody>
</table>

The results of the determination of the hepatic enzymes and bilirubin permit the conclusion that the Cu concentrations applied do not provoke alterations in liver function, and that the administration of this product does not have acute effects on this organ. The liver is vulnerable to acute intoxication by Cu, approximately within 24 h of the intake, since three quarters of the available Cu is incorporated into it. This rapid incorporation provokes toxicity symptoms characterized by hepatic damage, including severe hepatic necrosis, because of enzymatic alterations (Atkinson et al. 2004).

Acute Cu intoxication in sheep after parenteral administration of the Cu compound is associated with the increment of ALP, TB and DB and the Total and Direct Bilirubin (Uzal et al. 1992, Robles et al. 1993); while ASAT and ALAT are augmented in sheep fed with poultry-bedding bed containing high levels of Cu, causing some marked hepatic alterations (Cantón Castillo et al. 1994, Vivas 2002).

The prolonged consumption of the mentioned diet (7 months) with high Cu contents incremented the levels of ASAT, ALAT and the bilirubin above the normal level for the species, which provoked slight to moderate hepatic necrosis (Pérez 2004).

In conclusion, the parenteral Cu dosages of 50 and 100 mg are effective in restoring the Cu concentration in blood serum and in hepatic tissue for 60 days in the treated animals up to the reference level for the bovine species without causing toxic effects or leaving residuals.
These results may have important positive repercussions for the production and reproduction in dairy farming.

References


Garcia-Diaz et al.: Efficacy and pharmacological security of parenteral supplementation of copper in cows


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