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Effects of parenteral supplementation of Cu in female cattle with different levels of cupremia

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Abstract

The experiment was carried out in two stages with the objective of determining the effect of parenteral supplementation of 50 mg Cu on the reproductive behaviour of both hypo- and normocupremic cyclic female cattle. In the first stage, 62 hypocupremic, cyclic animals were selected, 32 heifers (16 in the control group and 16 in the treatment group) and 30 cows (12 in the control group and 18 in the treatment group). In the second stage, 98 normocupremic, cyclic heifers were selected (49 in the control group and 49 in the treatment group). 50 mg of Cu were administered to the animals in the treatment groups parenterally (subcutaneously) every two months until three successive applications were completed, while the control animals were not given copper supplementation. The effects of the copper therapy on cupremia and the percentage of heat and gestations presented were determined. The relative risk of treated animals of presentating anoestrus and service repetition was evaluated by the formation of a 2×2 contingency table and the application of a χ^2 test. The levels of serum copper were compared using a t-test, while heat and gestations were compared using a proportion comparism test. The parenteral administration of 50 mg Cu in hypocupremic, cyclic heifers and cows led to significant increases of cupremia ($P<0.001$) and the percentages of heat presentations and gestations ($P<0.01$); in the normocupremic animals, there were significant increases in the values of serum copper ($P<0.0001$), heat presentations ($P<0.01$) and gestations ($P<0.001$), especially in animals with cupremia values below 14 mmol/L. The treatment reduced the relative risk of reproductive disorders. It is concluded that the parenteral administration of 50 mg Cu in female cattle with Cu blood serum level <14 mmol/L increased cupremia and the percentages of heat presentations and gestations, and reduced the relative risk of reproductive disorders.

Keywords: Cu, cupremia, heat, gestation, heifers and cows

Introduction

Copper deficiency or hypocuprosis is considered the second most frequent mineral deficiency in pasturing cattle all over the world, next to phosphorus deficiency (Wikse *et al.* 1992). In Cuba, this deficiency is widely distributed, especially in the central region of the country, where diagnoses showed high proportions of heifers, cows and bulls with hypocupremia, which affects reproduction and milk production (García *et al.* 2010a).

There are various clinical symptoms associated with hypocupremia, even though it has been suggested that reproductive problems are the principal manifestation of hypocuprosis in adult animals (Kendall *et al.* 2003, Ahola *et al.* 2005). Reports have shown a negative correlation between cupremia in the range of 9.8±1.0 to 14.0±0.9 µmo/L and reproductive indicators, as much in animals with spontaneous cupremia as with the induced form. These animals show an increased risk of presenting anoestrus and service repetition, the principal indicators of reproductive efficiency (García *et al.* 2010b).

In order to prevent and cure hypocuprosis in the affected livestock zones, different injectable form of Cu have been introduced to the market. In cows supplemented parenterally with Cu, it was observed that there was an increase in the numbers of heat presentation, an increased rate of pregnancy per service and an elevated percentage of pregnancy (Brem *et al.* 2002). Furthermore, the parenteral administration of Cu in dairy cows reduced first service-birth, gestation-birth, and birth-to-birth intervals (Black & French 2004, García *et al.* 2007).

However, despite all the results stated earlier, nothing is known about the influence of parenteral administration of Cu on reproductive behaviour in normocupremic animals, the optimum level at which adequate reproductive performance can be obtained and the critical limit of Cu supplementation for maximum results, that is, a standardized limit to which this element must be supplemented in order to obtain normal serum values in this species under tropical conditions.

The objective of this study, therefore, was to determine the effect of parenteral supplementation of 50 mg Cu on the reproductive behaviour of cyclic female cattle, under hypo- and normocupremic conditions.

Materials and methods

The experiment was conducted using animals from 3 heifer centers and 2 cattle stables of the south-central part of the province Villa Clara, Cuba. They were pastured in a restrained rotational system, with an intensity of 244.8 livestock units per ha and day ha^{-1} day^{-1}, a global load of 1.5 animals per ha, an exposure to pasture for 16 h daily. The basal feed was fodder, with dicalcium phosphate and honey as mineral supplements. The cows were milked once daily between 2.00 and 5.00; using a manual restrain method.

The investigation was carried out in two stages; in the first stage, 62 animals were selected and diagnosed as hypocupremic and cyclic; amongst these were 32 heifers (16 in the control and 16 in the treatment group) and 30 cows (12 in the control and 18 in the treatment group). In the second stage, 98 heifers were selected and diagnosed as normocupremic and cyclic (49 in the control and 49 in the treatment group), which were then stratified into two distinct levels (lower and higher than 14 mmol/L), based on the upper limit of correlation between cupremia and reproductive indicators as shown in previous experiments (García *et al.* 2010 b). Animals presenting serum Cu values below 11.77 mmol/L were considered hypocupremic (Álvarez 2001).

Both stages were conducted during the rainy season (June-November), using female cattle with body conditions between 3 and 4 points on a scale limit of 5 (Rodenburg 2004) in all cases. The ages of the heifers ranged from 21 to 24 months and average live weights were between 300 and 310 kg. The cows were aged between 6 and 8 years. There was a voluntary waiting period of 60 days for inseminating the cows again after gestation (Cuban technical norm).

In both experimental stages, all treated animals were injected parenterally (subcutaneously) with 50 mg Cu (Cu as ethylenediaminetetraacetate of Cu), every two months until three applications were completed, while the control animals were not administered any treatment. The effects of the Cu therapy were then investigated based on the concentration of serum Cu and the percentage of heat and gestations presented.

A relative risk analysis was conducted on the appearance of the following reproductive disorders: anoestrus, service repetition and total reproductive problems (the sum of the two) in cows and heifers treated with the Cu complex, both hypo- and normocupremics, totalling 132 female animals.

Animal/ha load was determined according to Hernández *et al.* (1997). The reproductive indicators were determined as described in the procedure of Brito (2001). Blood samples were taken by venopuncture of the jugular vein into previously sterilized and demineralized test tubes without anticoagulant, gently depositing 10 ml of blood. The samples were later centrifuged at 3 500 rpm for 10 min in order to obtain blood serum, and later stored at –10 °C until the analyses were carried out.

Cu determination was done using atomic absorption spectrophotometry (Miranda *et al.* 2000), in an SP-9 equipment (Pye Unicam Ltd., Cambridge, UK) used according to the manufacturer's instructions. The quality control during the study was realized using certified reference material (Pig Kidney CRM 186, BCR Reference Materials), in which Cu values of 11.21±0.64 µmol/L (mean±SD with a 95 % confidence interval) were determined in five analysed samples. The certified material contained 10.97±0.68 µmol/L, showing concordance with the obtained values.

Gynecological examinations were carried out on all animals in order to determine possible pathologies of the reproductive apparatus and to identify cyclic animals (those that presented a *corpus luteum* or evidence of follicle development). Gestations were confirmed employing the method as described by Holy (1987), through rectal palpation; vaginal examinations were carried out using a sterilized speculum. Diagnoses were carried out at the beginning of the experiment, coinciding with the initial application of Cu, at 60 and 120 days, coinciding with the second administration and 30 days after the last application of the treatment. In this experiment, animals that needed more than one artificial insemination before becoming pregnant were considered candidates for repeated service.

Heat detection was carried out between 6.00-10.00 and 14.00-18.00 by an experienced herdsman and assisted by a bull heat detector (bull with deviated penis) using a 1:25 bull\cow ratio (Pedroso & Roller 2004). Artificial insemination was conducted by a trained expert with a 50-55 % efficiency in the last four years of his career, using the deep cervix method, and the frozen tablet form of proven fertile bull sperm (stored at –196 °C in liquid nitrogen and defrozen before the procedure).

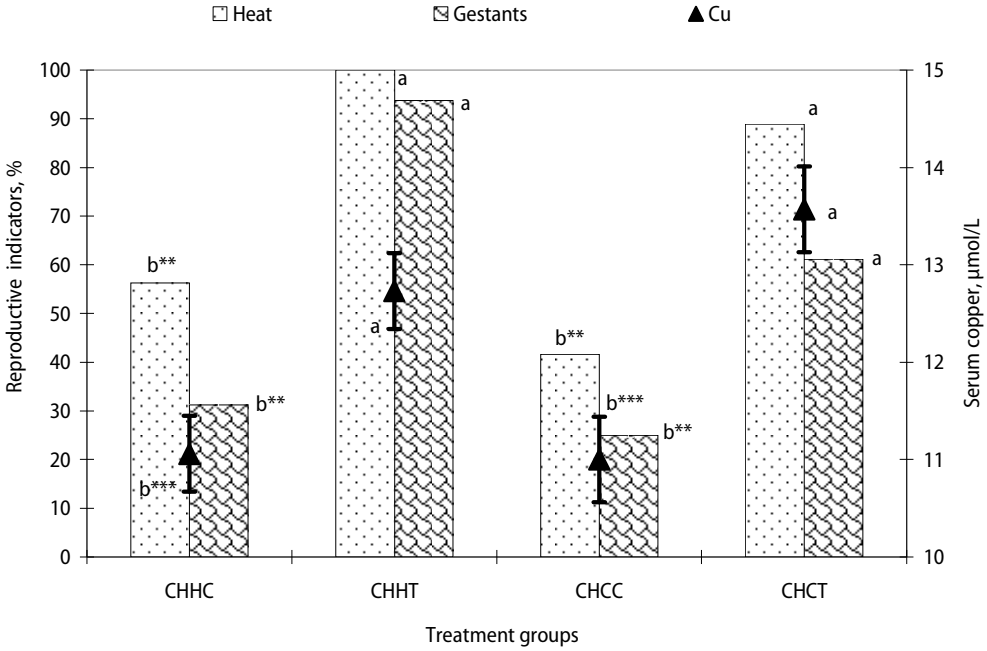
Relative risk of hypocupremic and normocupremic animals to present anoestrus, service repetition and total reproductive disorders was determined using a 2×2 contingency table (Thrusfield 1995) and the application of a χ^2 test, using the statistical packages Epidat 3.1 (PAHO, Washington, DC, USA) and Win Episcope 2.0 (Thrusfield *et al.* 2001). A cohort type prospective study was carried out with a 95 % confidence interval (CI) and a bilateral hypothesis.

For general statistical processing, Statgraphics 5.0 (Statpoint Technologies, Inc., Warrenton, VA, USA) version was employed, while for the comparism of treatment and serum Cu levels,

a t-test was used. In cases where heat and gestations were presented, an analysis was carried out using proportional comparism.

Results

The application of 50 mg Cu every two months in cyclic hypocupremic animals (Figure 1), increased the levels of serum Cu ($P<0.001$) significantly in the treated groups when compared to the control groups, both in cows and heifers. In both categories, the supplemented animals showed a significant increase in the percentages of heat presentations and gestations ($P<0.01$) when compared to the control groups.



CHHC: Cyclic hypocupremic heifers control (n=16), CHHT: Cyclic hypocupremic heifers treated (n=16), CHCC: Cyclic hypocupremic cows control (n=12), CHCT: Cyclic hypocupremic cows treated (n=18). Different letters indicate significant differences between groups, ***$P<0.001$, **$P<0.01$

Figure 1
Effect of parenteral administration of three doses of 50 mg Cu in cyclic hypocupremic female cattle on cupremia and reproductive behaviour

There was no significant difference between heifers and cows in the levels of Cu in blood serum and the percentages of heat and gestation presentation. Neither was there any significant difference between both categories of animals used in the control groups, which demonstrated a lower reproductive potential.

Cu supplementation in normocupremic animals is efficient, as demonstrated by a significant increase in the values of Cu blood serum ($P<0.0001$), heat presentation ($P<0.01$)

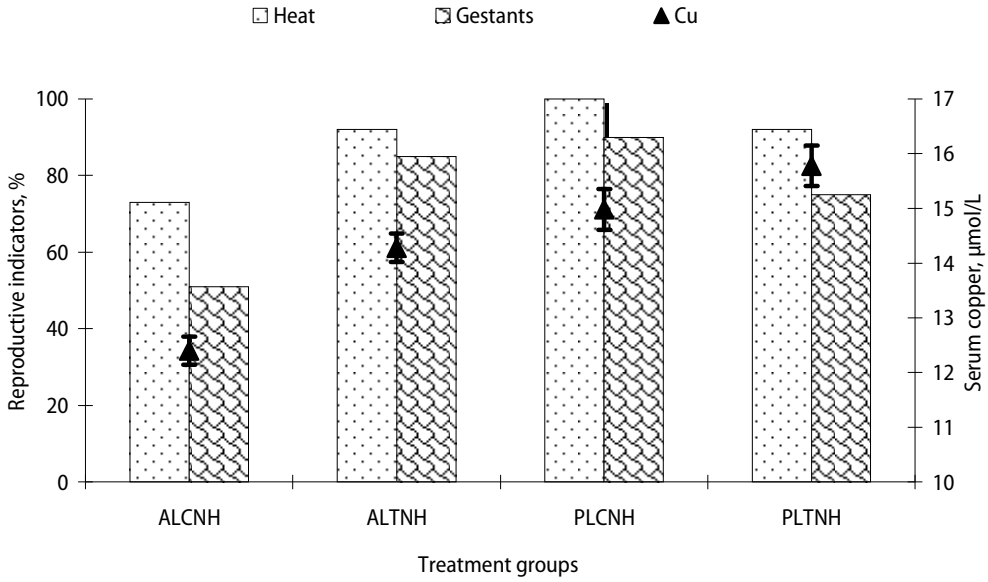
and the proportion of pregnancy ($P<0.001$) in the treated animals in comparison to the control groups (Table 1).

Table 1
Cupremia, heat presentation and gestations in cyclic normocupremic heifers treated parenterally with three doses of 50 mg Cu

Indicators	Treated group	Control group	SEM ±
Experimental Animals, n	49	49	-
Copper, µmol/L	14.58[a]	12.79[b]	0.23***
Heat presentation, %	94[a]	78[b]	-*
Gestations, %	86[a]	57[b]	-**

Mean values with different superscripts in the same row are significantly different, *$P<0.01$, **$P<0.001$, ***$P<0.0001$

The stratification of cyclic normocupremic heifers into two distinct levels (lower and higher than 14 mmol/L) clearly demonstrated that in animals with normal concentrations of Cu or values <14 mmol/L, the supplementation of this element increased serum Cu ($P<0.0001$), heat presentation ($P<0.01$) and percentage of pregnancy ($P<0.001$) compared to those in the control groups. Even though, animals with Cu concentration above 14 mmol/L showed an increase in cupremia ($P<0.001$), the reproductive variables did not show any statistically significant difference between the treated animals and the control groups (Figure 2).



ALCNH: Average level of Cu in control cyclic normocupremic heifers (11.77-14mmol/L) (n=37), ALTNH: Average level of Cu in treated cyclic normocupremic heifers (11.77-14mmol/L) (n=39), PLCNH: Peak level of Cu in control cyclic normocupremic heifers (>14mmol/L) (n=12), PLTNH: Peak level of Cu in treated cyclic normocupremic heifers (>14mmol/L) (n=10). Different letters indicate significant differences between groups, ***$P<0.001$, ****$P<0.0001$.

Figure 2
Effect of parenteral administration of three doses of 50 mg Cu in cyclic normocupremic heifers, stratified in two levels of cupremia, on reproductive behaviour and cupremia

The study of relative risk in heifers and cows exposed to parenteral treatment with Cu (Figure 3), demonstrated that Cu has a positive effect on reproductive disorders, as evidenced by the decrease in disorder presentation in both categories of animals. An independent analysis showed values for anestrus in heifers of 0.09 ($P<0.0001$), and a 95 % CI of 0.03-0.29, for service repetition of 0.80 (95 % CI of 0.50-1.28) and for the sum of both of 0.47 ($P<0.001$), with a 95 % CI of 0.31-0.69.

In treated cows, the risk index for anestrus was 0.34 ($P<0.01$), with a 95 % CI of 0.13-0.84, for service repetition it was 0.92 (95 % CI of 0.48-1.77) and for the sum of both it amounted to 0.62 ($P<0.05$), with a 95 % CI of 0.36-0.97, an indication for a lesser risk for heifers.

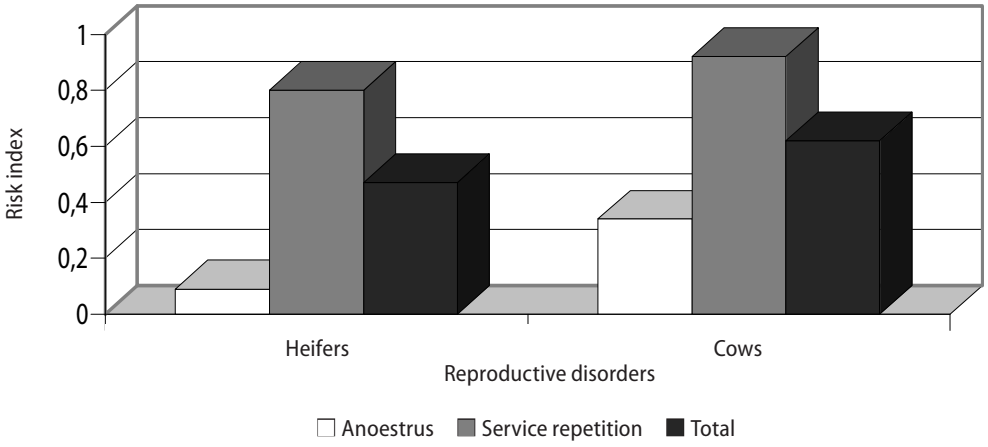


Figure 3
Relative risk of reproductive disorders in cows and heifers supplemented parenterally with 50 mg Cu

Discussion

The results obtained in this experiment demonstrate that there is an association between cupremia and reproductive behaviour in female cattle and that the parenteral application of Cu is a practical and effective method of supplementation, which coincides with earlier related studies (Mackenzie *et al.* 2001, Black & French 2004, García *et al.* 2005).

The positive effect of Cu supplementation up to 14 mmol/L indicates that this constitutes an optimum level of cupremia for reproductive efficiency in the bovine species, at least under Cuban conditions, and a standardized limit for obtaining a profitable response with Cu supplementation. This aspect is of great importance, because it serves as a starting point in the study of this topic, especially when reconsidering the normal level of this element in the bovine species under the prevailing conditions in the country mentioned earlier.

The results of the relative risk analysis demonstrated for the first time the real magnitude of the risk index for these reproductive disorders in supplemented animals, and a clear reflection of the protective effect of the parenteral supplementation of Cu against the same, thereby making this work a substantial contribution to the subject matter.

Various studies have demonstrated the non-existence of a correlation between the levels of Cu in blood serum and in hepatic tissue (reserve organ), because animals with cupremia

values considered normal, have been diagnosed with a high proportion of global or general organic hypocuprosis (Quiroz *et al.* 2003). Supplementation helps restoring the needed reserves in the viscera, from where they are mobilized to the tissues and organs to normalize physiologic processes related to it. Once these levels and the utilization capacity of the cells have been reached, the surplus Cu is then stored in the reserve organs, especially in the liver. This situation explains the fact that supplementation above 14 mmol/L is not effective on reproductive behaviour because of the limitations imposed by other competing dietary factors such as energy, proteins, vitamins and other minerals (Landau *et al.* 2000).

It has been established that Cu is related to synthesis and secretion of the hypophysiary hormones, modulating the capacity for the liberation of the luteinizing hormone (LH), suggesting that sub-fertility and other reproductive alterations are due to low levels or inhibition of the gonadotrophic hormones, which provokes lack of ovulation, poor development of the *corpus luteum* and insufficient production of P_4 (Corbellini 1998).

In the same vein, reports have shown a low amplitude of LH ($P<0.05$) in bovine livestock with Cu deficiency when compared to normocupremic animals (Xin *et al.* 1993). The administration of gonadotrophic releasing hormone (GnRH) in rats with normal levels of Cu increased the liberation of LH and follicle stimulating hormone (FSH) from the hypophysis, when compared to hypocupremic animals, probably due to Cu stimulating the GnRH receptors in the hypophysis (Kochman *et al.* 1997).

Numerous investigations have suggested that increased oxidative stress in hypocupremic animals is associated with reduction of the antioxidant hormone Cu/Zn superoxide dismutase (SOD), of which Cu is an indispensable catalytic cofactor (Pan & Loo 2000). Also, the reduction in the activity of cytochrome c oxidase (CCO), which is involved in the correct reduction of O_2 in the respiratory chain, is accompanied by an increase in the univalent reduction of O_2, leading to a consequent increase in the intracellular concentration of superoxide anions (O_2^-). Even if CCO is not an antioxidant enzyme, the alteration of its function clearly has a pro-oxidant effect (Guetens *et al.* 2002).

For the activity of SOD to decrease significantly, there must be a severe hypocupremia (below 5 µmol/L) for a period no less than 1 month, while a decrease in the activity of ceruloplasmin (CP) is proportional to a decrease in cupremia (Picco *et al.* 2004). Taking cognizance of all these factors, it is a logical conclusion that CP plays an important role in oxidative stress.

The activity of CP is considered antioxidant because it permits the oxidation of iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+})state, which makes it impossible to utilize Fe^{2+} to produce hydroxyl radicals (^-OH) through the Fenton reaction. It has also been reported that CP has the capacity to fix Cu (6 atoms of Cu/molecule), thereby impeding the free atoms from acting as catalysts during oxidative damage, and to inhibit lipid peroxidation of membranes stimulated by Cu and Fe ions and soft barrier on H_2O_2, O_2^- and ^-OH (Miyajima *et al.* 2002).
The free radicals, such as superoxide anions (O_2^-), are produced in the *corpus luteum* during the synthesis of progesterone (P_4), and further transformed in the luteal cells to H_2O_2, which is luteolytic and an inhibitor of cholesterol transport to the mitochondria, where it is converted into pregnenolone, which leads to a decrease in the formation of P_4 (Michaluk *et al.* 2006)

In the same manner, copper deficiency generates oxidative stress, especially at the ovarian level, affecting its steroidogenic tissues with a consequent decrease in the synthesis

of estrogen and progesterone, causing damage in the oocytes, hindering embryonic development and increasing immature embryo mortality, leading to negative repercussions on reproductive indicators (Jozwik *et al.* 1999, De Matos & Furnus 2000)

CCO represents the terminal enzyme in the respiratory chain and catalyzes the transfer of 4 electrons to O_2 for the formation of two molecules of water and adenosine triphosphate, and participating in the partial obtainment of energy (Gebhard *et al.* 2001). This way, the body bondition score and energy metabolism of hypocupremic animals are affected, both of which are reported to show positive and significant correlations to cupremia (García *et al.* 2005). Furthermore, animals with hypocupremia do not maintain an adequate body bondition score and suffer a significant extension of the period between first birth and first appearance of heat. In addition, they liberate limited quantities of LH after an exogenous stimulation with GnRH, when compared to cows that are capable of maintaining their body condition (Pedroso *et al.* 2003).

During hypocuprosis, the activity of the enzyme lysyl oxidase (LOX), which is Cu dependent, suffers a considerable reduction, thereby causing a severe perturbation in the growth rate of pre-ovarian follicles, leading to reduction in the secretion of ovarian estradiol, a lower peak of estradiol in the pre-ovarian follicle, which is an endocrine sign that responsible for estrus induction, which in turn leads to perturbations of estrus and ovulation respectively (Kendall *et al.* 2003)

During Cu deficiency, the activity of the enzyme 15'15' dioxygenase is reduced; it is Cu dependent and responsible for the rupture of the central double bond of the carbon 15 of the carotene molecule, an indispensable factor in the formation of vitamin A (Izaguirre & Shimada 2001). Sharma *et al.* (2003) have reported a correlation of vitamin A with the Cu serum status of the animals with a marked significant deficiency of vitamin A in the Cu-deficient animals. Hypovitaminosis A is responsible for damages in the hypophysis and can cause ovarian sclerosis, atresia and degeneration of the ovarian follicles, reduced growth of the *corpus luteum*, reduction of P_4 level during the heat cycle, delay in heat appearance, induction of silent heat, increasing numbers of ovary cysts, reduction of the pregnancy index and premature embryo mortality.

It is concluded, that the parenteral administration of 50 mg Cu in female cattle with Cu blood serum levels <14 mmol/L increased cupremia and the percentages of heat presentation and gestation, and reduced the relative risk of producing reproductive disorders.

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