

Association between copper deficiency and DNA damage in cattle

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Cattle hypocuprosis is the second most widespread mineral deficiency affecting grazing cattle. The consequences of hypocuprosis include a failure of copper metalloenzymes, many of which form part of the antioxidant defence system. This work focuses on the association between copper (Cu) plasma concentration and DNA damage in Aberdeen Angus cattle. Two-hundred and ninety-nine heparinized blood samples from 2-year-old Aberdeen Angus cows were obtained from different farms located in the Salado River basin, Argentina. Plasma copper level analysis was carried out in whole samples, while cytogenetic analysis and single cell gel electrophoresis assay (comet assay) were carried out in 82 and 217 samples, respectively. Cytogenetic analysis showed a significant increase in the frequency of abnormal metaphases in moderate/severe hypocupremic groups (groups B and C) in relation to the normocupremic group (group A) (4.5 and 1.5 abnormal metaphases/100 cells, respectively, $P < 0.01$). The Spearman correlation test showed a negative association between cupremic values and the yield of chromosomal aberrations ($r = -0.708$, $P < 0.0001$). In the comet assay greater migration was observed in cells from the hypocupremic group, from a median of 54 in the severe hypocupremic group to 31 in the normocupremic group ($P < 0.01$). Accordingly, the Spearman correlation test showed a significant positive relationship between copper levels and cells without DNA migration and a significant negative relationship between copper levels and cells with a tiny tail ($P < 0.0001$ in both cases). The results obtained show that hypocupremia in cattle is associated with an increase in the frequency of chromosomal aberrations as well as in DNA migration as assessed by the comet assay. Whereas the comet assay could differentiate copper plasma level groups, chromosomal aberrations only detected differences between normal and hypocupremic animals. The increase of DNA damage found in hypocupremic animals could be explained by higher oxidative stress suffered by these animals.

Introduction

Cattle hypocuprosis is the second most widespread mineral deficiency affecting grazing cattle (Ramírez *et al.*, 1998). A survey conducted by the National Animal Health Monitoring Service classified 40.6% of US beef cattle as copper deficient

(Dargatz *et al.*, 1999). Similar or higher values were reported by Ramírez *et al.* (1998) in the Salado River Basin (Argentina), an area of 55 793 km² with 6.5 million head of beef cattle (Dillon, 1992). Copper deficiency has been linked to a variety of clinical signs, including pale coat, poor sheep fleece quality, anaemia, spontaneous fractures, poor capillary integrity, myocardial degeneration, hypomyelination of the spinal cord, impaired reproductive performance, decreased resistance to infectious disease, diarrhoea and generalized ill-health (Tessman *et al.*, 2001), causing severe economic losses. Copper is associated with several enzymes, either as a cofactor or as an allosteric component. Copper acts as an electron transfer intermediate in redox reactions, being an essential cofactor for oxidative and reductase enzymes (Uauy *et al.*, 1998).

The consequences of hypocuprosis include a failure of copper metalloenzymes, many of which form part of the antioxidant defence system [e.g. Cu/Zn superoxide dismutase (Cu/Zn SOD) and caeruloplasmin (Cp)]. Copper, as well as other essential trace elements, is an atypical antioxidant because it works indirectly. Copper is a catalytic cofactor for Cu/Zn SOD and Cp. Cu/Zn SOD catalyzes dismutation of the superoxide anion, producing molecular oxygen and hydrogen peroxide, with the latter product usually metabolized by glutathione peroxidase and catalase. The ferroxidase activity of Cp mediates the oxidation of ferrous ions to the ferric state, thereby preventing ferrous ion-dependent formation of hydroxyl radicals via the Fenton reaction. Thus, in enabling Cu/Zn SOD and Cp to function as described, copper can be classified as part of the antioxidant defence system of cells (Pan and Loo, 2000).

Several reports have shown the impact of copper deficiency on the antioxidant defence system and oxidative damage to cellular components. The activity of Cu/Zn SOD, catalase and glutathione peroxidase is decreased in animals with copper deficiency (Strain, 1994). Lower Cu/Zn SOD activity with an increase in superoxide anions, lipoperoxides and oxidation of proteins was reported in rats, mice and chickens (Prohaska, 1990; Sukalsky *et al.*, 1997; Cockel and Belonje, 2002; Hawk *et al.*, 2003). Decreased Cu/Zn SOD and cytochrome c oxidase (CCO) activity and increased carbonylated proteins were reported in HL60 cells deprived of copper (Percival, 1998; Johnson and Thomas, 1999). Cattle hypocuprosis was associated with a decrease in Cu/Zn SOD, Cp and CCO activity and with an increase in lipid peroxidation (Xin *et al.*, 1991; Arthington *et al.*, 1996; Ward and Spears, 1997; Gengelbach and Spears, 1998; Cerone *et al.*, 2000a,b). Collectively, these studies indicate that copper deficiency weakens the antioxidant defence systems.

The association between Cu deficiency and DNA damage was first proposed by Webster *et al.* (1996), who observed an increase in nuclear enzymes associated with DNA repair in rats feeding low Cu diets. Later, Pan and Loo (2000) showed an increase in DNA damage measured by the comet assay in

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Jurkat T lymphocytes cultured with the copper chelator 2,3,2-tetraamine after exposure to hydrogen peroxide. They concluded that normal copper levels are necessary to maintain the structural integrity of DNA during oxidative stress. However, Cu deficiency in Jurkat T lymphocytes itself did not have genotoxic effects. Recently, clastogenic and the genotoxic effects of hypocuprosis in a limited number of Aberdeen Angus cows was described (Abba *et al.*, 2000; Picco *et al.*, 2001).

The aim of this work was to confirm the association between Cu plasma concentration and DNA damage evaluated by cytogenetic analysis and the comet assay in a representative number of animals. In addition, the relationship between DNA damage and individual Cu status was also evaluated.

Materials and methods

Two-hundred and ninety-nine heparinized blood samples were obtained from Aberdeen Angus cows. Samples were collected in Vacutainer[®] tubes (Franklin Lakes, NJ) from different farms located in the Salado River basin (Buenos Aires Province, Argentina). Plasma Cu level analysis was carried out for all the samples. Chromosomal aberrations were analysed in samples from 82 animals and the comet assay was performed in 127 other blood samples.

Plasma Cu analysis

For plasma copper determination blood samples were centrifuged and the plasma was treated with 10% (w/v) trichloroacetic acid, separating the supernatant for Cu analysis. Copper concentration was analysed by flame atomic absorption spectrophotometry (double beam atomic absorption spectrophotometer GBC 902) using internal quality control (Piper and Higgins, 1967). Plasma copper level was classified according to Suttle (1983) as normocupremia (>60 µg Cu/dl plasma), moderate hypocupremia (30–59 µg/dl) and severe hypocupremia (<30 µg/dl).

Cytogenetic analysis

Blood samples were cultured for 54 h in Ham's F12 culture medium, supplemented with pokeweed mitogen (Gibco BRL, Gaithersburg, MD) at the concentration recommended by the supplier, penicillin (60 IU) and streptomycin (50 µg/ml). Forty-five minutes before harvesting, colchicine (1 µg/ml) was added to the medium. After centrifugation, lymphocytes were resuspended in 5 ml of 0.075 M KCl and incubated at 37°C for 30 min. Fixation was carried out with methanol/acetic acid (3:1) at room temperature. Chromosomal preparations were made by dropping the cell suspension onto slides, and these were subsequently stained with 5% Giemsa solution. Chromosomes were counted and scored for aberrations blind on coded slides. A total of 200 metaphases per animal were scored to check the incidence of structural chromosome aberrations.

Comet assay

For the comet assay blood samples were stored in the dark at 4°C for no more than 30 min. The comet assay was performed according to Singh *et al.* (1988), with some minor modifications. Aliquots of 15 µl of whole blood were mixed with 75 µl of 0.5% low melting point agarose (Gibco BRL, NY), seeded on a slide coated with 0.5% normal melting point agarose (Promega) and cooled until solidification. Two slides per animal were made. After this, the cells were lysed in detergent solution [100 mM EDTA (Gibco BRL, NY), 2.5 M NaCl (Gibco BRL, NY), 10 mM Tris (USBiological, MA), 1% Triton X-100 (Sigma, St Louis, MO) and 10% dimethyl sulfoxide] for at least 1 h and stored until electrophoresis. Before electrophoresis, the slides were equilibrated in alkaline electrophoresis solution [1 mM EDTA (Gibco BRL, NY), 300 mM NaOH (Carlo Erba, Milano, Italy), pH > 13] for 20 min. Electrophoresis was carried

out for 30 min at 25 V and 300 mA (1.25 V/cm). Afterwards, slides were neutralized by washing three times with Tris buffer (pH 7.5) for 5 min each time and distilled water. Slides were stained with 1/1000 SYBR Green I (Molecular Probes, Eugene, OR) solution (Ward and Marples, 2000) and analysis was carried out using an Olympus BX 40 microscope provided with a 100 W high pressure mercury lamp (USHIO USH 102 D). Images were captured with a Sony CCD camera and saved using Image Pro Plus[®] software. The comet assay was carried out blind on coded slides. A total of 100 cells per animal were scored. DNA damage was classified according to Kobayashi *et al.* (1995), with some minor modifications, as type 1 (comets without a tail), type 2 (comets with a tiny tail), type 3 (comets with a dim tail), type 4 (cells with a clear tail), and type 5 (cells with a clear decrease in the diameter of the head and a clear tail).

Statistical analysis

The incidence of chromosomal aberrations and DNA damage assessed by comet assay were compared with different plasma Cu level groups by means of the medians test and the χ^2 for contrast test as suggested by Duez *et al.* (2003). The relationships between plasma Cu levels and chromosomal aberration yields as well as between plasma Cu levels and DNA damage were determined by the Spearman correlation test. The data were analysed using the SPSS (Release 11) statistical package.

Results

Samples were divided into three different groups taking into account plasma Cu levels as suggested by Suttle (1983): group A, normocupremic animals (60–120 µg/dl); group B, moderate hypocupremic animals (30–59 µg/dl); group C, severe hypocupremic animals (<30 µg/dl).

The comet assay was performed for 64 animals belonging to group A, 81 cows belonging to group B and 154 animals belonging to group C. Average plasma Cu levels were 73.1 ± 1.3 (SEM), 39.2 ± 2.03 and 18.3 ± 0.44 for groups A, B and C, respectively.

Cytogenetic analysis

In the cytogenetic analysis of 82 blood samples achromatic lesions and monochromatid and isochromatid breaks were found (Table I). Statistical analysis showed a significant increase in the frequency of abnormal metaphases in the moderate/severe hypocupremia group in relation to the normocupremic group (4.5 versus 1.5) ($P < 0.01$). When the frequency of different chromosomal aberrations was compared, significant differences were found in achromatic lesions and monochromatid breaks between the normocupremic and moderate/severe hypocupremic groups ($P < 0.01$). No significant differences were found between the two hypocupremic groups in chromosomal aberration incidence ($P > 0.05$). The Spearman correlation test showed a negative association between cupremic values and chromosomal aberrations incidences ($P < 0.0001$) (Table III).

Comet assay

Table II shows plasma Cu levels and DNA damage measured by the comet assay in each group. The increase in DNA

Table I. Plasma Cu levels (mean \pm SEM) and chromosomal aberrations incidence per 100 cells [median (25–75 percentile)]

Group	Plasma Cu (µg/dl)	Cells scored	Abnormal metaphases ^a	Achromatic lesions	Monochromatid breaks	Isochromatid breaks
A (n = 35)	75.6 \pm 11.5	7000	1.5 ^b (1–2.5)	2.5 ^b (1.5–4)	1.5 ^b (1–2)	0.5 (0–1)
B (n = 14)	39.6 \pm 9.8	2800	4.25 ^c (2.75–5)	4.75 ^c (2.9–5.6)	4 ^c (1.5–6)	1.25 (0.5–3)
C (n = 33)	19.2 \pm 5	6600	4.5 ^c (3.5–6.5)	8 ^c (4.5–10.5)	4 ^c (2.5–6)	1.5 (0.75–2.75)

Group A, cupremia >60 µg/dl; Group B, cupremia between 30 and 60 µg/dl; Group C, cupremia <30 µg/dl.

^aMetaphases with at least one chromosomal aberration. Metaphases showing only gaps were not scored as abnormal.

^{b,c}Different letters indicate significantly different ($P < 0.01$).

Table II. Plasma Cu levels (mean \pm SEM) and DNA damage measured by the comet assay [median (25–75 percentile)]

Group	Plasma Cu ($\mu\text{g}/\text{dl}$)	Type 1	Type 2	Type 3	Type 4	Type 5
A ($n = 29$)	70.1 \pm 7.9	70 ^a (63–74.5)	24 ^a (16–27.5)	6 (4–8)	1 (0–3.5)	1 (0–2)
B ($n = 67$)	39.2 \pm 8.2	54 ^b (46–64)	38 ^b (26–45)	6 (4–8)	2 (0–4)	0 (0–2)
C ($n = 121$)	18.1 \pm 5.6	47 ^c (42–54)	44 ^c (38–48)	6 (3.5–10)	2 (0–3.5)	0 (0–2)

Group A, cupremia $>60 \mu\text{g}/\text{dl}$; Group B, cupremia between 30 and 60 $\mu\text{g}/\text{dl}$; Group C, cupremia $<30 \mu\text{g}/\text{dl}$.

Type 1, cells without a tail; type 2, cells with a tiny tail; type 3, cells with a dim tail; type 4, cells with a clear tail; type 5, cells with a clear tail and a clear decrease in the diameter of the head.

^{a,b,c}Different letters indicate significantly different ($P < 0.01$).

Table III. Spearman correlation coefficient between cupremia values and genotoxic effect measures

Genotoxic effect	r_s^a
Chromosomal aberrations	
Abnormal metaphases	–0.708
Achromatic lesions	–0.617
Monochromatid breaks	–0.589
Isochromatid breaks	–0.428
Comet assay	
Type 1	0.553
Type 2	–0.538

^aSignificant statistical differences at $P < 0.0001$.

damage was mostly evidenced by a decrease in comets of type 1 (cells without damage), from a median of 70 in the normocupremic group to 47 in the severe hypocupremic group ($P < 0.01$), and a concomitant increase in comets of type 2 (cells with a tiny tail), from a median of 24 in the normocupremic group to 44 in the severe hypocupremic group ($P < 0.01$). No significant differences were observed between cupremic groups in relation to comets of types 3–5 ($P > 0.3$). Table III shows significant relationships between plasma Cu levels and comets of types 1 and 2 ($P < 0.0001$).

Discussion

The results obtained show that hypocupremia in cattle is associated with an increase in the frequency of chromosomal aberrations as well as in the percentage of cells with DNA migration as assessed by the comet assay. These data corroborate the clastogenic and genotoxic effects of copper deficiency in cattle. According to Suttle's classification, while moderate hypocupremia reflects liver incapacity to maintain a physiological cupremia level, severe hypocupremia represents a dysfunctional stage in which clinical alterations can be observed. In this sense, the comet assay could differentiate each plasma Cu level group. On the other hand, chromosomal aberrations detected statistically significant differences between the normal and moderate/severe hypocupremia groups. However, correlation coefficient values obtained for comparison of abnormal metaphases and cupremia were higher than those obtained for the comet assay and cupremia. This finding could reflect specific differences between the comet assay and the chromosomal aberration test.

Little is known about the increase in DNA damage during copper deficiency in mammalian cells. Ide *et al.* (1994) showed an increase in DNA damage in pancreatic cells of rats maintained on a copper-deficient diet for 7 weeks. Indirect evidence of DNA damage was observed by Webster *et al.* (1996), who

found an increase in nuclear enzymes associated with DNA repair in rats fed a low copper diet. In Cu-deficient rat embryos a tendency to higher 8-hydroxy-2-deoxyguanosine concentrations was observed by Hawk *et al.* (2003). On the other hand, Pan and Loo (2000), using Jurkat T lymphocytes deprived of copper by culture with a high-affinity copper chelator, found a higher susceptibility of copper-deficient cells to suffer DNA damage after exposure to hydrogen peroxide. However, copper deficiency itself did not induce DNA damage. In contrast, our results show a clear relationship between copper deficiency and the yield of DNA damage. The reason is not clear, but is probably related to the fact that whereas the results of Pan and Loo (2000) were obtained in an *in vitro* experimental model, our study was performed *in vivo* in cattle suffering long-term (many months) copper deficiency. In addition, copper deficiency was provoked indirectly by a copper chelator in the experiment of Pan and Loo (2000) while we observed the effect of a natural direct decrease in copper at the physiological level.

The increase in DNA damage found in hypocupremic cattle could be explained by higher oxidative stress suffered by these animals. The impact of copper deficiency on the antioxidant system and oxidative damage of cellular components has been reported in several species and tissues, as well as in cultured cells. This suggestion of induction of oxidative stress is based on two lines of evidence, suggesting extracellular and intracellular pathways leading to genotoxic effect. First, CP is the main cupremic determinant and appears to be one of the enzymes most sensitive to copper deficiency (Bingley and Anderson, 1972; Blakley and Hamilton, 1985). In this sense, it is well known that low CP levels are related to an increased susceptibility to infections and tissue injuries. It has been suggested that CP in plasma acts as an extracellular scavenger of free radicals and may thus protect the cells against reactive oxygen species released from neutrophils and macrophages (Weiss, 1989; Saenko *et al.*, 1994). On the other hand, the ferroxidase activity of Cp mediates oxidation of ferrous ions to the ferric state, thereby preventing ferrous ion-dependent formation of hydroxyl radicals via the Fenton reaction. It is also possible that there is an increase in ferrous ions when the activity of Cp is lower (Miyajima *et al.*, 2003). Simultaneously, the DNA molecule in nuclear chromatin, having an abundance of phosphate anions and oxygen donor groups, is an ideal partner to bind metal cations such as Fe. The chromatin proteins can also bind metals (Kasprzak, 2002). Therefore, nuclear components can be damaged by hydroxyl radicals generated by the presence of O_2^- and H_2O_2 (Kasprzak, 2002). Second, a significant decrease in one of the main antioxidant enzymes, Cu/Zn SOD, has been found, along with a decrease in the catalytic activity of the respiratory chain enzyme CCO in animals suffering from hypocuprosis. Thus, partial oxygen reduction

leading to an increase in free radicals and insufficient antioxidant activity of Cu/Zn-SOD could increase oxidative stress. Furthermore, several enzymes with antioxidant activity which do not require copper as a cofactor, such as catalase and glutathione peroxidase, are known to be negatively influenced by copper deficiency, increasing free radicals generated in the cells (Strain, 1994).

Therefore, due to the relation between Cu deficiency and impairment of the antioxidant system, genotoxic effects as a consequence of an increase in oxidative stress could be suggested.

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Received on May 15, 2004; revised on September 1, 2004;
accepted on September 7, 2004