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Subcellular distribution of hepatic copper in beef cattle receiving high copper supplementation

Marta López-Alonso[a], Paloma Carbajales[b], Marta Miranda[b,*], Victor Pereira[a]

[a] Department of Animal Pathology, Faculty of Veterinary Medicine, Universidade de Santiago de Compostela, 27002 Lugo, Spain
[b] Department of Anatomy, Animal Production and Clinical Veterinary Sciences, Faculty of Veterinary Medicine, Universidade de Santiago de Compostela, 27002 Lugo, Spain

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ABSTRACT

Previous studies of intensively reared cattle in NW Spain have reported significantly higher copper (Cu) accumulation in the liver in Holstein-Friesian (HF) animals than in Galician Blonde (GB) or GBxHF crosses when receiving a diet supplemented at the maximum Cu concentrations allowed in the EU legislation (35 mg/kg). The present study aimed to evaluate whether this difference is due to the pattern of subcellular accumulation of Cu in the liver. For this purpose, liver samples from 10 GB, 9 HF and 10 GBxHF young bulls were analysed to determine the content of metallothionein (MT) and Cu and zinc (Zn) (in the liver (Cu-liver and Zn-liver) and bound to metallothionein (Cu-MT and Zn-MT)). The Cu distribution within the main subcellular compartments (nuclei, large granule, microsomes and cytosol) was also determined. Even though HF animals showed significantly higher ($P < 0.05$) Cu concentrations in the liver (161 ± 10 mg/kg wet weight) compared with GB (132 ± 8 mg/kg), no breed-related differences were observed for any of the parameters considered in this study. Overall, the pattern of hepatic subcellular accumulation was similar to that previously described in cattle: (i) MT concentrations were lower than in other animal species but strongly related to hepatic Zn; (ii) a low proportion of Cu (6.61%) was bound to MT but this was strongly and negatively related to the Cu:Zn ratio in the liver cell; and (iii) the highest proportion of Cu (57.3%) was found in the large granule (lysosome containing) fraction. All these results indicate a low capacity of cattle to excrete Cu by the bile resulting in a high Cu accumulation in the liver cell.

1. Introduction

Trace element supplementation has been used routinely in the animal feed industry without full consideration of the background concentrations in the feed materials. This has led to the provision of amounts of trace elements greatly exceeding the physiological requirements of the animals [1]. However, in recent years the philosophy of trace element supplementation has changed substantially. In addition to considering the efficacy of trace element additives in relation to animal health and performance, their safety for consumers (via animal-source food) and maximum levels in the environment must be clearly addressed [2].

The problems associated with excessive trace element supplementation are exemplified by considering copper (Cu) requirements in animals, particularly in ruminants. Cu deficiency is very frequent in ruminants worldwide [3] and many animals benefit from Cu being included in their diet. However, if the amounts of Cu supplied exceed the physiological needs, large amounts of Cu may accumulate in the liver, potentially leading to chronic Cu toxicity [4]. This has potential consequences for the consumer, and maximum regulatory limits (MRL) have been proposed in order to address this problem [5].

Previous studies on intensively reared beef cattle in NW Spain [6] have shown that young bulls of Holstein-Friesian (HF) breed accumulate more Cu in the liver than these of the Galician Blonde (GB) breed or the crosses of GB and HF (GBxHF). Moreover, in a substantial proportion of the HF young bulls (42%), the hepatic Cu concentrations are above safe-adequate levels [6]. This may have consequences for animal health as oxidative damage has been described [7] and may pose a risk to the consumer. Our findings are consistent with other recent findings across Europe showing that subclinical chronic Cu toxicity in cattle is more frequent than generally assumed [8]. This poses a problem in animal nutrition.

Sheep are particularly susceptible to chronic Cu poisoning, and hepatic Cu metabolism has therefore been widely studied in this species [9,10]. The susceptibility is thought to be related to the fact that the sheep liver cannot accumulate large amounts of metallothionein (MT)-

* Corresponding author.
E-mail address: marta.miranda@usc.es (M. Miranda).

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bound Cu (Cu-MT). In mammals, Cu absorbed in the intestine is generally transported to the liver, where it is utilized in normal hepatocyte metabolism, stored as Cu-MT or, if present in excess, excreted in bile [11]. MT seems to play a major role in the excretion of Cu in the bile, both by a direct route through the hepatocyte cytoplasm or, more importantly, by the hepatolysosomal route, in which Cu-MT is sequestered by lysosomes for excretion in the bile [12]. If there is a large influx of Cu into the liver, the capacity of the MT to bind Cu and of the lysosomes to remove Cu from the cytosol may be exceeded. The Cu is then accumulated at a higher rate in other organelles (mainly in the nucleus) or, when particularly high concentrations of Cu are stored, it may remain in the cytosol as free Cu ions; in both cases Cu is responsible for important alterations in liver structure and function [11,13].

Sheep breeds display large differences in Cu metabolism, and some breeds have even been identified as resistant or tolerant to Cu [3]. These differences seem to be related to the capacity of the animal to excrete Cu in the bile [14]. Although some breed-related differences in Cu requirements have been described in cattle (Simmental and Charolaise appear to have higher Cu requirements than Aberdeen Angus [15]) and attributed to differences in Cu biliary excretion, studies of subcellular hepatic Cu metabolism in cattle are scarce.

The aim of this study was to determine whether the pattern of subcellular hepatic accumulation of Cu explains the higher hepatic Cu accumulation observed in HF than in GB young bulls in Galicia (NW Spain). For this purpose, we evaluated the role of MT in binding Cu and analysed the subcellular distribution of Cu in different liver compartments in HF, GB and GBxHF crosses.

2. Material and methods

2.1. Liver samples

In the present study, liver samples originated from 10 GB, 9 HF and 10 GBxHF young bulls of a previous investigation of liver Cu status after feeding a high Cu-supplemented diet were used [17]. Briefly, the bulls were grown from age 12–36 weeks (body weight range ca. 130–390 kg) in a commercial feedlot and were fed a concentrate diet containing a standard mineral supplement (Co (0.5), Fe (32), I (0.5), Mn (40), Se (0.1) and Zn (32) mg/kg of concentrate) with the maximum level of Cu supplementation allowed by the EU legistation (35 mg Cu as copper sulphate/kg feed) [16]. For further details about the animals and diets, see Miranda et al. [17]. Samples were taken from the caudate lobe of the livers immediately after the animals were slaughtered at age 36 weeks. The samples were frozen in liquid nitrogen and stored at −80 °C before being processed.

2.2. Subcellular fractionation

About 1 g of liver was homogenized in 6 vols of 0.25 M sucrose (pH 8, 4 °C) with a Potter–Elvehjem homogenizer. The material was fractionated as described by Corbett et al. [18]. Briefly, the homogenate was centrifuged (Beckham centrifuge, model J2–21) at 600*g* for 10 min to separate the nuclear pellet (nuclei, plasma membranes, unbroken cells), and the resulting supernatant was centrifuged at 8500 g for 12 min to separate the large-granule pellet (mitochondria and lysosomes). The supernatant obtained in this step was centrifuged (Beckman ultracentrifuge, model L7–80, 70 Ti rotor) at 105,000*g* for 60 min to separate the microsomal pellet (endoplasmatic reticulum, Golgi apparatus, ribosomes) from the cytosol (including Cu-MT, other soluble proteins and free ions). Enzyme assays were conducted to confirm the effectiveness of the fractionation procedure, as also described by Corbett et al. [18].

Table 1
Liver content of metallothionein (MT) and copper and zinc content in the liver (Cu-liver and Zn-liver) and bound to metallothionein (Cu-MT and Zn-MT) in Holstein Friesian (HF), Galician Blonde (GB) and their crosses (GBxHF). Data are expressed in arithmetic mean ± SEM. Different superscript letters indicate statistical differences between groups ($p < 0.05$).

	HF (n = 9)	GB (n = 10)	GBxHF (n = 10)	P
MT (mg/kg WW)	177 ± 48	114 ± 10	132 ± 8	0.222
Cu-liver (mg/kg WW)	161 ± 10[a]	132 ± 8[b]	154 ± 6[ab]	0.028
Cu-MT (mg/kg WW)	12.53 ± 3.91	6.62 ± 0.88	8.62 ± 1.14	0.201
%Cu-MT*	7.51 ± 1.90	5.09 ± 0.64	5.65 ± 0.72	0.336
Zn-liver (mg/kg WW)	44.9 ± 5.5	38.7 ± 1.1	40.5 ± 1.4	0.370
Zn-MT (mg/kg WW)	4.47 ± 1.01	4.21 ± 0.88	3.79 ± 0.46	0.878
%Zn-MT*	9.50 ± 1.40	11.02 ± 2.45	9.24 ± 0.95	0.734

WW: wet weight.
* %Cu-MT and% Zn-MT: expressed as percentage of the Cu or the Zn content in the liver.

2.3. MT assays

MT was determined by a modification of the silver (Ag) saturation method [19]. Briefly, aliquots of between 0.1 and 0.5 mL of liver cytosol were first adjusted to a sample volume of 2.4 mL with 0.5 M glycine pH 8.5. The samples were mixed with 1 mL of AgNO3 solution (20 µg Ag/mL glycine buffer). Complete saturation of the samples was ensured by using various aliquots of the sample. Excess Ag was removed and precipitated by addition of 0.2 mL of 2% haemoglobin solution (in buffer). The solution was then heat treated in a water bath (about 100 °C for 1 min) and centrifuged at 1000*g* for 5 min at room temperature. These steps were repeated two more times. The final supernatant fraction was analysed to determine the amount of Ag by ICP-OES, and the MT concentrations were calculated assuming a molar ratio of Ag(I)/MT of 17 [19].

2.4. Metal content in liver, bound to MT and other subcellular fractions

To determine Cu and Zn concentrations in the liver, 0.5 g of tissue was digested with concentrated nitric acid (Suprapur, Merck) and hydrogen peroxide by microwave-assisted digestion.

The fractions present in the liver supernatant after heat treatment were assumed to be the Cu and Zn bound to MT (Cu-MT, Zn-MT) [20]. The supernatant was obtained by first heating (72 °C for 5 min) and then chilling (in an ice-cold bath) one mL of liver cytosol; the heat-denatured proteins were removed by centrifugation at 1600*g* for 5 min, and the final supernatant was analysed by inductively coupled plasma atomic emission spectrometry (ICP-OES, Perkin–Elmer Optima 4300 DV, Perkin Elmer Instruments, Norwalk, CT USA).

Subsamples (0.5–1 mL) of liver homogenate and of subcellular fractions were digested in 1 mL of 69% concentrated nitric acid and 0.2 mL of 33% w/v hydrogen peroxide. Digested samples were transferred to polypropylene sample tubes and diluted to 5 mL with Milli-Q ultrapure water. Metal concentrations in the digest were determined by ICP-OES.

2.5. Analytical quality control

Analytical quality control was applied throughout the study. Two blanks and an in-house reference material (−80° C cattle liver homogenate) were processed with each batch of 6 samples to detect any background contamination and to monitor the between-batch consistency of analysis. A certified reference material (SRM: Standard Reference Material® 1577c Bovine Liver; National Institute of Standards & Technology) was also analysed with each batch of samples to determine the analytical recovery. Blank absorbance values were