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Control of hepatic copper retention in Texel ram lambs by dietary supplementation with copper antagonists followed by a copper depletion regimen

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ABSTRACT

Two groups of six Texel ram lambs were individually fed a commercial feed concentrate containing, on average, 24 mg Cu and 400 mg Fe kg/dry matter (DM), with (Group A) or without (Group O) three copper-antagonists being: Mo 2, S 3000 and Zn 250 mg/kg, for 96 d to assess their combined effect on hepatic Cu retention (HCR). Concentrate feed allowances increased initially from 1 to 2 kg/d, with 0.1–0.2 kg/d chopped hay, but losses of one lamb from each group led to the restriction of concentrate to 1.4 kg/d with an increase in hay allowance to 0.3 kg/d after 26 d. Lambs were weighed on d 0, 26, 61 and 96 before liver biopsy samples were obtained for Cu analysis. Increases in total liver Cu content during the three consecutive intervals between biopsies were estimated on the assumption that dry livers weighed 5.15 g/kg live weight. Plasma samples were collected weekly for the analysis of Cu concentration and γ-glutamyl transpeptidase (EC 2.3.2.2; GGT) activity. HCR as a fraction of Cu intake (FHCR) was lower in Group A than O (0.056 *versus* 0.097 ± 0.0152; P<0.01) and tended to decrease with time (P<0.1) but final liver Cu concentrations reached values associated with chronic Cu toxicity of 1069 ± 123.4 and 760 ± 63.2 mg Cu/kg DM in Groups O and A, respectively. Plasma GGT increased more in Group O than in Group A (time × group interaction, P<0.05) to final values of 118 ± 16.4 and 64 ± 10.2 IU/l, respectively, which were both above a normal limit of 44 IU/l. After 96 d, sheep were group fed 1 kg/d of a common depletion regimen based on whole barley grain coated with 3000 mg S/kg and containing 3–5 mg Cu/kg DM. After 22 wks of Cu depletion, all but one lamb had GGT values within a normal range. Liver Cu accretion was reduced by feeding the antagonists, but insufficiently to avoid mild hepato-toxicity, which was resolved upon feeding a grain-based diet low in available Cu.

1. Introduction

Breeds of sheep vary widely in the efficiency with which they absorb Cu and those that absorb Cu well, such as the North Ronaldsay, are relatively susceptible to chronic Cu poisoning (CCP; Wiener et al., 1978). Hepatic copper retention (HCR) reflects absorptive efficiency and is higher in the Texel than in many other breeds (Woolliams et al., 1982; Van der Berg et al., 1983; Suttle et al., 2002). Dietary supplements of the Cu antagonists Fe (Rosa et al., 1986), Zn (Bremner et al., 1976) and

Abbreviations: AAS, atomic absorption spectrometry; AST, aspartate transaminase; CCP, chronic Cu poisoning; DM, dry matter; GDH, glutamate dehydrogenase; GGT, γ-glutamyl transpeptidase; FHCR, fractional hepatic Cu retention; HCR, hepatic Cu retention; LW, live weight; mpl, maximum permitted level.

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Table 1
Composition of Cu depletion regimen fed for 22 wks following the formal experiment.

Ingredient	kg/100 kg	Ingredient	g/100 kg
Whole barley grain	90.8	Vitamin A + D3[a]	0.44
Urea	1.8	Vitamin E[a]	11.0
Calcium sulphate	1.4	$FeSO_4 \cdot 7H_2O$	19.2
Sodium bicarbonate	1.8	$MnSO_4 \cdot H_2O$	6.0
Potassium chloride[c]	0.46	Potassium iodide	0.13
Sugar	1.28	Cobalt chloride	0.04
Water[b]	2.36		

[a] Vitamin premixes provided 3000, 1000 and 25 IU/kg of vitamins A, D and E respectively.
[b] Water was used to dissolve the sugar and coat the barley before adding other constituents.
[c] Potassium chloride rather than sodium chloride was used because the diet contained another source of supplementary Na and would otherwise have been low in K. Note that Se was not added to avoid risks of overdose with Se when reformulated under farm conditions.

Mo plus S (Suttle, 1977) have each reduced liver Cu levels in breeds less susceptible to CCP. Zinc (Van der Schee et al., 1983) and Mo (Crosby et al., 2004) reduced HCR in the Texel or Texel cross but levels used (*i.e.*, 479 mg Zn and 4 or 8 mg/kg DM, respectively) all exceeded European Community maximum permitted levels (mpl). Mixtures of antagonists are routinely added to compound feeds for sheep in Europe to reduce the risk of CCP in vulnerable breeds, but composition and inclusion rate have varied and the additivity of protective effects has not been demonstrated.

The experiment now reported examined the ability of a blend of Mo, S and Zn at mpl to restrict HCR by Texel lambs, fed a pelleted commercial concentrate high in Cu from normal feed ingredients. A simple Cu depletion regimen, based on barley grain which is normally relatively low in Cu, was formulated and fed for 22 wks after the formal experiment to reverse any hepatotoxicity that may have accrued during the experiment.

2. Materials and methods

2.1. Animals, diets and treatments

Twelve Texel ram lambs, 49.6 ± 2.29 SEM kg live weight (LW), were individually penned, bedded on straw, and allocated within pairs to one of the two groups according to plasma Cu concentration, which was 11.8–24.3 μmol/l, after ranking on the basis of initial liver biopsy Cu concentrations of 272–706 mg/kg dry matter (DM). A fattening lamb concentrate was blended to commercial specification (BOCM Pauls, Ipswich, UK), with distiller grains included to provide a high Cu concentration. Prior to pelleting, the blend was divided into two batches, A and O, one of which (A) was supplemented with antagonists (Rowett Research Services, Aberdeen, UK) being: Mo (2 mg/kg as molybdate), S (3000 mg/kg as the powdered element) and Zn (250 mg/kg as zinc sulphate). Concentrate was initially fed to appetite, commencing with 1 kg plus 0.1 kg chopped grass hay (composition not ascertained) fed in two equal portions at 9.00 and 16.00 h, in a 96 d experiment. Feed refusals were recorded but rarely occurred. Live weights were recorded on the day of each liver biopsy prior to feeding. At the end of the formal experiment, the diet was gradually changed over 7 d to 1 kg/d of a Cu depletion ration of whole barley grain coated with urea, vitamins and minerals, based on the drought-feeding regimen of Bogdanovic (1983), with a single macro-mineral antagonist, S, added as 3.6 g gypsum ($CaSO_4 \cdot 2H_2O$)/kg DM (Table 1). Lambs were group fed this depletion diet, which contained 3–5 mg Cu/kg DM and was fed for 22 wks.

2.2. Sampling and analytical techniques

Blood samples were collected at weekly intervals and prior to liver biopsies on 0, 26, 61 and 96 d, into heparinised, evacuated tubes (Becton Dickinson, Oxford, UK), centrifuged at 1000 × *g* for 10 min at 18–20 °C. Plasma was retrieved and stored at 4 °C prior to batch analysis within 2 d of sampling. Standard sera were used to determine and, if necessary, correct for systematic error. Plasma was analysed for Cu concentration by flame atomic absorption spectrometry (AAS), using a Philips PU9200X spectrophotometer fitted with a 'split-tube' burner with samples diluted 1:5 in n-butanol at 60 g/l. Activity of γ-glutamyl transferase (GGT) was chosen as the only indicator of liver damage for the formal experiment but, at the end of the depletion period, glutamate dehydogenase (GDH) and aspartate transaminase (AST) activities were also measured to ensure that any liver damage had resolved. All enzyme activities were measured at 30 °C using assay kits (Boehringer Mannheim UK, Lewes, Sussex). Liver biopsy samples were obtained by aspiration biopsy under general anaesthesia and assayed for Cu by AAS using methods and standardisation of Suttle et al. (2002). The experiment was conducted under UK Home Office Licence with the approval of the local ethical standards committee.

2.3. Calculations

Calculation of fractional hepatic Cu retention rates (FHCR) allows adjustment for unplanned differences in Cu intake while providing a measure of the efficiency of Cu absorption (ARC, 1980) and were calculated as: firstly, liver sizes were estimated from a relationship with LW recorded previously in Texel lambs at 5.15 g liver DM/kg LW; Suttle et al., 2002): secondly, liver

Table 2
Analysed or estimated (e) concentrations of Cu and four mineral antagonists in two commercial concentrates fed to two groups of Texel lambs either supplemented (Group A) or not supplemented (Group O) with three antagonists to reduce risk of chronic Cu poisoning.

	Mineral concentration (mg/kg DM)				
	Cu	Fe	Mo	S	Zn
Group O	25.6	395	0.3 (e)[a]	2000 (e)	181
Group A	22.5	405	2.5 (e)	5400 (e)	452

[a] Estimated values were based on previous analyses of similar unsupplemented concentrates and accurate additions of the intended Mo and S supplements.

Cu contents were calculated by multiplying estimated liver dry weights by Cu concentrations in the biopsy samples; thirdly, individual daily rates of change in liver Cu content were calculated for the three intervals between successive biopsies and expressed as a fraction of Cu intakes in the two groups.

2.4. Statistical methods

Sequential values were subjected to repeated measures (mixed model), two-way analysis of variance (GraphPad Software Inc., La Jolla, CA, USA). Liver Cu concentrations were logarithmically transformed to give homogenous variances. All means are given with their SEM. and some final means were compared by Students' *t*-test.

3. Results

3.1. Concentrate composition and food intake

The analysed or estimated concentrations of antagonists in samples of the two diets are in Table 2. The higher Cu concentration in the diet for Group O than A is noteworthy.

Individual lamb intakes of concentrate and hay rose to maxima of 2 kg and 0.2 kg/d, respectively, within 2 wks and one lamb from each group died from the nutritional disorders bloat and urinary calculi. No data from these two lambs was used. From d 27, all lambs were restricted to 1.4 kg/d (1.23 kg DM/d) of concentrate, their average intake up to that time, with 0.3 kg/d of chopped hay. Subsequent feed refusals were negligible.

3.2. Live weight and liver size

Mean LW at each biopsy are in Table 3. All lambs gained weight during the experiment and gain was negatively correlated with initial weight ($P<0.001$), unaffected by dietary treatment and averaged 177 g/d. Liver dry weights increased from 255 to 343 g during the experiment.

3.3. Liver Cu accretion

Liver Cu concentrations throughout the experiment are in Fig. 1 and were initially similar for the two groups but close to the upper limit of normality at 400 mg/kg DM (Suttle, 2010). Mean liver Cu concentration rose more rapidly in Group O than in Group A during the first 61 d. Thereafter, values continued to increase in Group O, albeit at a slower rate than previously, but decreased slightly in Group A, resulting in a group × time interaction ($P<0.01$) after log transformation. Increases in liver Cu concentration over the whole experiment were 341 ± 64.6 and 733 ± 122.4 mg Cu/kg DM in Groups A and O, respectively, and differed ($P<0.05$). The livers of Group A lambs contained more Cu than those of Group O by the end of the experiment (Table 3). Estimates of mean FHCR throughout the experiment are in Fig. 2 and were always lower in Group A than in Group O ($P<0.01$). The FHCR tended to be lowest ($P<0.1$) between days 61 and 96 but there was no interaction between group and time.

Table 3
Live weights (LW), liver dry matter (DM) and total liver Cu contents in two groups of Texel rams given a predominantly concentrate diet high in Cu with (Group A) or without (Group O) a mixture of three copper antagonists for 96 d.

Day	0		26		61		96		SEM
Group	A	O	A	O	A	O	A	O	
LW (kg)	49.1	50.0	58.1	60.1	60.0	60.3	66.5	66.5	0.82
Liver DM[a] (g)	253	257	297	310	309	310	342	344	4.2
Liver Cu (mg)	109	87	152	170	238	275	258a	364b	14.0

Different letters (a, b) denotes a difference between groups (*i.e.*, $P<0.05$) within day.

[a] Estimated on the assumption that liver DM was 5.15 g/kg LW (Suttle et al., 2002).

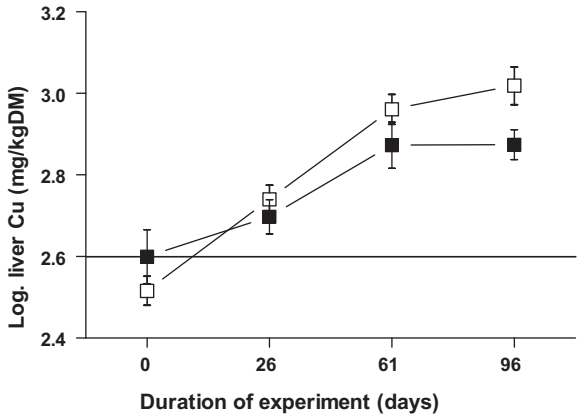

Fig. 1. Mean liver Cu concentrations in biopsy samples from two groups of Texel ram lambs fed predominantly with concentrates relatively high in Cu (22.5–25.6 mg Cu/kg DM) with (solid symbol) or without (open symbol) a blend of Cu antagonists for 96 d. Error bars denote the standard errors of means and the gridline is the upper limit of normality.

3.4. Gamma glutamyl transpeptidase activity

The GGT activities throughout the experiment are in Fig. 3 and were initially similar in the two groups, and close to the upper limit of the normal range routinely used by the Scottish Veterinary Investigation Service for samples submitted to the same laboratory at 44 IU/l (Suttle, 2010). Unsupplemented lambs showed gradual increases in GGT that gathered pace towards the end of the experiment, whereas increases were less pronounced in Group A at all stages, resulting in a time × group interaction ($P<0.01$). Divergence between groups reached significance after 12 wks of the experiment. An abrupt increase in GGT from 69 to 103 IU/l occurred in one lamb from Group A between d 61 and 70 (*i.e.*, after the third liver biopsy) and its values remained high until the end of the experiment.

3.5. Plasma Cu

There were no differences between plasma Cu concentrations in Groups O and A at any time, and no correlations between plasma Cu and either GGT or liver Cu (data not shown). However, plasma Cu was always less variable in Group A than in Group O, in which two lambs were occasionally hypercupraemic in the middle of the experiment (*e.g.*, plasma Cu 25.4 and 26.5 μmol/l on d 49). Plasma Cu concentration on d 96 was 15.7 ± 0.92 and 13.9 ± 0.50 μmol/l in Groups O and A, respectively.

3.6. Efficacy of Cu-depletion regimen

Lambs gained LW when fed the barley grain inclusive diet, with initial and final LW ranges of 59–75 and 70–93 kg, respectively. After 22 wks of Cu depletion, plasma GGT activities had decreased in all lambs and mean plasma GGT was

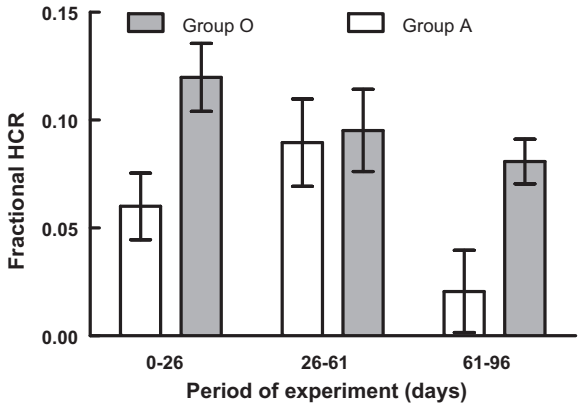

Fig. 2. Estimates of the mean daily hepatic retention of Cu, expressed as a fraction of Cu intake (FHCR, mg/mg) in whole livers of Texel ram lambs fed predominantly with concentrates relatively high in Cu with (open symbol) or without (solid symbol) a blend of Cu antagonists. Results are presented for three intervals between consecutive liver biopsies and assume that dry livers weighed 5.15 g/kg DM (Suttle et al., 2002): bars denote standard errors of means.

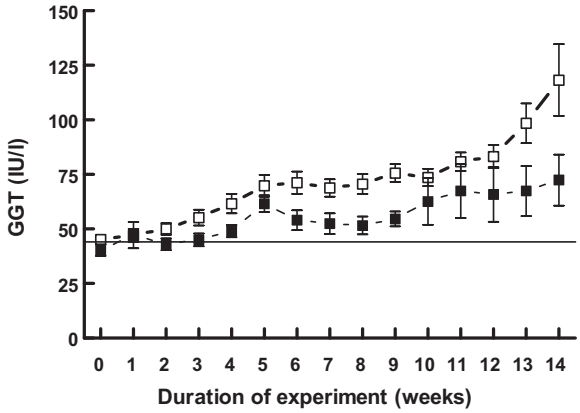

Fig. 3. Mean γ-glutamyl transpeptidase (GGT) activity (IU/l) in plasma samples collected weekly from two groups of Texel ram lambs fed predominantly with concentrates relatively high in Cu with (solid symbol) or without (open symbol) a blend of Cu antagonists for 96 d. Bars denote standard errors of means and the gridline is the upper limit of normality.

35 ± 3.3 and 29 ± 1.1 IU/l in Groups O and A, respectively, with only one unsupplemented (O) lamb having a slightly abnormal value of 47 IU/l. All AST activities, and all but one GDH activity, were within normal ranges (Suttle, 2010). The individual lamb with marginally raised GDH at 34 IU/l had normal GGT activity. Plasma Cu concentration after depletion was 13.2 ± 0.71 and 13.9 ± 1.04 μmol/l in Groups O and A, respectively, and similar to values when depletion began.

4. Discussion

4.1. Estimated effect of antagonists on Cu absorption

Fractional HCR approximates to efficiency of Cu absorption in the early linear phase of Cu accretion (Woolliams et al., 1983) and the best estimate of the effect of mixed antagonists on maximal efficiency of Cu absorption probably comes in the first 26 d when FHCR halved from 0.12 to 0.06 (Fig. 2). Two assumptions were made in deriving these values: firstly, that the dietary Cu concentrations in Table 2 accurately represent the relative amounts of Cu consumed by the two groups; secondly, that hay made little or no contribution to hepatic Cu retention. If the difference in dietary Cu was due to feed sampling error rather than mixing error, as Cu was not added as specific mineral supplements, the difference in FHCR between groups will have been underestimated. The contribution of Cu from hay was the same for both groups and unlikely to have exceeded needs for maintenance and growth. The high FHCR of 0.12 for Group O is similar to that recorded previously for pure-bred Texels (Suttle et al., 2002) but much higher than those recorded in eight other breeds or crosses fed complete diets low in antagonists (*i.e.*, 0.042–0.086; Woolliams et al., 1982, 1983). The reduction in Cu absorption is equivalent to halving the Cu concentration in a diet without antagonists from 24 to 12 mg/kg DM, yet liver Cu concentrations reached high levels in Group A rams. The mpl of 17 mg Cu/kg DM in European sheep feeds is probably too high for Texels.

4.2. Changes in hepatic Cu retention with time

The decrease in FHCR between d 62 and d 96 (Fig. 2) is typical of sheep which have acquired high liver Cu concentrations and has been partially attributed to increased losses via biliary secretion (Woolliams et al., 1983). However, the reduction in FHCR with time was proportionately higher in the group (A) with the lower liver Cu concentration and was reflected in an ever-widening gap in liver Cu concentration between groups (Fig. 1). Similar contrasts in patterns of hepatic Cu accretion have been reported between breeds that absorb Cu with different efficiencies (Welsh Mountain > Scottish Blackface; Woolliams et al., 1983; Texel > Suffolk, Suttle et al., 2002) and may have a common explanation. Since Cu absorption occurs distal to the site of biliary Cu secretion, both dietary and genetic factors which influence Cu absorption have an opportunity to affect reabsorption of secreted biliary Cu. If true, the protective role of antagonists will be enhanced at the time when it is most needed, when hepatic Cu storage capacity becomes saturated.

4.3. Antagonism from Zn

The specific contribution of Zn in lowering FHCR in Group A cannot be ascertained from this study but merits discussion. Antagonists such as Zn which interact with Cu beyond the rumen may add to the effects of Mo and S, which interact with Cu in the rumen (Suttle, 2010). It is by inducing metallothionein in the intestinal mucosa that Zn is believed to inhibit Cu absorption (Bremner et al., 1976). Such an effect would probably affect reabsorption of biliary Cu and thus increase in relative importance as biliary Cu secretion increases. Van der Schee et al. (1983) found that supplementary Zn reduced HCR in the

Texel, but not in the Friesian milksheep. The Texel had a higher liver Cu status which may have provided more scope for Zn to inhibit recycling of biliary Cu. Crosby et al. (2004) found that Mo and S reduced HCR less in Texel lambs reared on galvanised floors than in those bedded on straw, raising doubts about the additivity of antagonist effects. The effect of reducing the mpl from 250 to 150 mg Zn/kg (European Communities, 2002) on the CCP protection afforded to vulnerable breeds such as the Texel should be investigated.

4.4. Relationship between plasma GGT and liver Cu concentration

The experiment was not designed to provide a complete biochemical assessment of possible liver damage and would have been strengthened by the inclusion of a panel of biochemical markers such as that used at the end of the depletion phase. However, the increases in GGT activities were probably due largely to Cu-induced hepatotoxicity for three reasons: liver Cu had risen to concentrations associated with Cu toxicity; addition of Cu antagonists had lessened the increase in plasma GGT and subsequent Cu depletion was accompanied by a reduction in plasma GGT. The exceptional individual in Group A referred to earlier, had a low liver Cu concentration of 618 mg/kg DM at d 96. Isolated, abrupt post-biopsy increases in GGT have been encountered in other experiments and may be attributable to accidental damage to the biliary tract during biopsy. Ortolani et al. (2003) found that the relationship between liver Cu concentration and GGT was stronger than that with AST in sheep dosed orally with $CuSO_4$. It has long been known that liver enzyme activities rise gradually during the pre-haemolytic phase of CCP (*e.g.*, Lewis et al., 1997). The rapid increases in GGT between d 66 and 96 in Group O (Fig. 3) suggests that pre-haemolytic Cu poisoning was developing to a point where the biliary tract became damaged. Thus, hepatotoxicity developed with liver Cu concentrations in the range recently proposed as 'marginal' for CCP (*i.e.*, 400–1000 mg/kg DM; Suttle, 2010).

4.5. Depleting liver Cu stores

That addition of antagonists to the feed concentrate could not prevent increases in liver Cu concentration and plasma GGT adds to the importance of complementary reductions in dietary concentrations of available Cu in controlling an outbreak of CCP. However, associated changes in dietary composition may present short-term problems before depletion has been achieved by precipitating further haemolytic crises in a flock, but problems are probably minimised when whole grain of relatively high energy content is fed. Barley grain invariably has a relatively low Cu content, but oat or wheat grains could also be used. My formulation used a single macro-mineral antagonist, S, but not the micro-nutrient Mo because small, accurate additions of Mo are hard to achieve under practical conditions. Elemental S was used to raise S intakes in the experiment because it is easier to pellet than gypsum. Depletion of Texel lambs is not easy to achieve without antagonists since lamb requirements may be <4.3 mg Cu/kg DM (Suttle, 2010) and might be better accomplished by addition of 150 mg Zn/kg, particularly in the light of my results. However, use of such rations should be discontinued as soon as sensitive indicators of liver damage, such as GDH, indicate that hepatotoxicity has been eliminated.

5. Conclusion

Addition of the Cu antagonists Mo, S and Zn to a feed high in a fourth, Fe, halved accretion of Cu in livers of Texel ram lambs, thereby allowing them to attain a steady state with dietary Cu 32% above the current maximum permitted level (mpl) in the European Union (EC, 2002). The concentrate contained 60% more Zn than the current mpl of 150 mg/kg (EC, 2002) and that threshold may be too low to substantially reduce risk of Cu-induced hepatoxicity in vulnerable sheep breeds such as the Texel. Hepatotoxicity was safely alleviated by feeding a diet based on whole barley grain coated with sulphate, but relatively low in Cu (*i.e.*, 3–5 mg/kg DM).

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