Evaluation of a commercially available molybdate formulation and zinc oxide boluses in preventing hepatic copper accumulation and thus enzootic icterus in sheep

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ABSTRACT

The efficacy of a molybdate formulation and a zinc oxide bolus as prophylactic agents for enzootic icterus was evaluated in sheep. Before copper loading, liver biopsies were performed on 12 male, 6-month-old, Mutton Merino sheep to determine hepatic copper (Cu) and zinc (Zn) concentrations. The animals were restrictively randomised according to liver copper concentrations to 3 treatment groups ($n = 4$) to achieve similar mean liver copper concentrations per group. All sheep received 4 mℓ/kg of a 0.5 % aqueous solution of CuSO$_4$·5H$_2$O intraruminally 7 days per week for 10 weeks. On Day 0 the sheep in the Mo-group were injected subcutaneously with 42 mg molybdenum (Mo) contained in a commercial molybdate formulation. The animals in the Zn-group each received a zinc oxide bolus, containing 43 g zinc oxide, via a rumen cannula. Treatment was repeated on Day 42. Four animals served as untreated controls. Urinary copper excretion, plasma copper concentration, haematocrit and glutamate dehydrogenase (GLDH) activity were determined throughout the trial. The animals were sacrificed after 10 weeks and liver samples were submitted for histopathological examination. Liver and kidney copper and zinc concentrations were determined. Neither the molybdate treatment nor the zinc oxide boluses prevented hepatic copper accumulation. The urinary copper excretion, plasma copper concentration, haematocrit and GLDH activity were not significantly different ($P > 0.05$) from the controls.

Key words: chronic copper poisoning, copper, molybdate, sheep, zinc.

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INTRODUCTION

In the Karoo and southern Free State regions of South Africa a form of chronic copper poisoning, referred to as 'enzootic icterus' or 'geelsiekte', occurs in sheep under natural grazing conditions. Many of the edible plants in these areas have copper concentrations exceeding 10 ppm dry mass (DM) and few plants have molybdenum concentrations above 0.5 ppm DM[1]. Ingestion of these plants over a number of years results in gradual hepatic copper accumulation[1]. The condition is extremely rare in sheep younger than 6-tooth, while clinical cases and mortality are most prevalent in old worn-mouth animals[1]. Morbidity varies between 0.5 and 5 % of the flock and mortality of clinically affected animals is 90–100 %[1]. It is estimated that 1000–2500 sheep die annually as a result of this form of chronic copper poisoning in these regions[1,15].

Copper accumulation is a prerequisite for enzootic icterus in South Africa. A variety of stressors precipitate copper release and thus the syndrome, and all attempts to prevent the syndrome are aimed at limiting these[1]. Farmers in the enzootic icterus-endemic areas are advised to prophylactically administer ammonium- or sodium molybdate orally to all sheep in the flock to curtail mortalities during the most stressful time of the year[1], generally from August to November (G F Bath, Faculty of Veterinary Science, University of Pretoria, pers comm., 1993). Sheep are dosed orally with 5–10 mℓ of a 2 % solution of ammonium or sodium molybdate daily, every 2nd day or weekly (supplying 100–200 mg per sheep)[1]. Some farmers include molybdates in licks or in drinking water[1].

The appropriate ratio between copper and molybdenum in feed that will avert copper accumulation in sheep seems to be 10:1[1,19], but Suttle[24] reported that a 17:1 ratio also prevents copper poisoning. Excessive copper absorption can also be prevented by the inclusion of sulphur with molybdates in the ration to promote the formation of thiomolybdates in the rumen. Suttle[23] was the first to propose that the formation of cupric thiomolybdate in the rumen antagonises copper absorption. Both organic and inorganic sulphur are degraded by rumen bacteria to sulphide that interacts with molybdenum and copper to form a biologically unavailable complex containing all 3 elements[23]. Addition of 4 mg molybdenum plus 2 g sulphur per kg of commercial concentrates will limit the accumulation of copper in sheep[24]. Dietary sulphur levels of 2.9, 4 and 5.3 g/sheep/day decrease hepatic storage of copper and the 2 higher concentrations significantly reduce molybdenum concentrations in plasma[26].

An unregistered molybdate formulation (containing 40 mg molybdenum/mℓ and no sulphur) is currently being sold in the Karoo to prevent enzootic icterus. It is recommended to inject 1–1.5 mℓ of the molybdenum solution subcutaneously 1–4 times a year and to especially include the month of August in the treatment regimen. Local farmers in the area are of the opinion that enzootic icterus can be prevented by the administration of this particular injectable molybdate formulation (J van Schalkwyk, Animal Health Technician, Fraserburg, pers comm., 1999).

Gooneratne et al.[8] were the first to report that the haemolytic crisis of chronic copper poisoning is prevented by intravenous administration of ammonium tetrathiomolybdate. Intravenous injection of 100 mg ammonium tetrathiomolybdate, twice per week, prevents copper accumulation in the liver of copper-loaded sheep[8]. Intravenous and subcutaneous ammonium tetrathiomolybdate prevent the development of exten-

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sive liver and kidney damage in copper-loaded sheep[8,12], and necrosis of liver cells is limited following intravenous thiomolybdate administration[16]. Intravenous tetrathiomolybdate decreases liver copper concentration, but increases the concentration in the kidney cortex, where it accumulates in lysosomes[10]. Kidney function is, nevertheless, not affected[10].

Intravenously administered thiomolybdate increases plasma copper to very high levels, but most of the copper is then protein-bound and thus insoluble in trichloro-acetic acid (TCA)[9,11]. It is surmised that the TCA-insoluble copper is not taken up by red blood cells and this inability to penetrate erythrocytes is the reason why haemolysis is not precipitated and increased urinary copper excretion occurs[9]. Other researchers reported that tetrathiomolybdate treatment had no effect on the rate and amount of copper excreted in the urine[3,14].

Chronic copper poisoning can also be prevented by increasing the dietary zinc concentration. Sheep supplemented with 220 ppm, or more effectively 420 ppm zinc, experienced a reduction in liver copper concentrations and a decrease in the severity of liver damage[5]. The addition of 150 and 340 ppm zinc to a ration containing 30 ppm copper, likewise, resulted in a significant reduction in copper accumulation in the liver of sheep[25]. Zinc sulphate, when added to the drinking water to supply a dose of 250–300 mg/sheep/day for 6 consecutive weeks, gave excellent results in preventing enzootic icterus (G Bath, Faculty of Veterinary Science, University of Pretoria, pers comm., 1993). In the Fraserburg and Carnarvon districts in the Karoo, an enzootic icterus-prevalent region, there are small springs and rivulets in the doleritic ridges where the sheep drink and the farmers are reluctant to add zinc to their water sources as it is impractical (J van Schalkwyk, Animal Health Technician, Fraserburg, pers comm., 1999).

The interaction between zinc and copper occurs in the intestinal mucosal cells[6]. When intracellular concentrations of zinc increase in these cells owing to high dietary levels, a promoter for the metallothionein gene is activated and thionein polypeptides are synthesised[6]. These polypeptides then bind copper and zinc[6]. Ultimately more copper will be bound, which decreases copper absorption by preventing copper transport across the basolateral membrane to the plasma[6].

A zinc oxide bolus has been developed in New Zealand for prevention of facial eczema[18]. The bolus is registered in New Zealand and is commercially available as the 'The Time Capsule for Facial Eczema'.

Two types of boluses are available, namely a bolus containing 43 g zinc (as zinc oxide) for lambs weighing 25–40 kg, and a bolus containing 58 g zinc (as zinc oxide) for sheep of 40–70 kg. Both boluses release zinc over a 6-week period and they are designed to release between 18 and 28 mg zinc/kg live mass per day (N Towers, AgResearch, New Zealand, pers comm., 1998).

The aim of this study was to determine the prophylactic potential of the commercially available molybdate formulation and the zinc oxide bolus, administered to sheep on a plane of copper loading, in preventing hepatic copper accumulation.

MATERIALS AND METHODS

Animals

Twelve Mutton Merino sheep (6 wethers and 6 rams), c. 6 months old and weighing approximately 30 kg, were purchased. The animals were housed at the Onderstepoort Veterinary Academic Research Unit in individual sheep pens. They were fed milled lucerne hay and a pelleted maize-based concentrate. The sheep had free access to drinking water. A water sample was collected and representative feed samples were collected every 4 weeks (n = 3) for copper, molybdenum, zinc, sulphur and iron determination by the Institute for Soil, Climate and Water (ISCW) using Inductively Coupled Plasma-Mass Spectrometer analysis (ICP-MS; VG Plasma Quad).

Adaptation period

A 4-week adaptation period was allowed. All the animals were dewormed with levamisole (2.56 % m/v, Promisol, Sentraal Wes) and niclosamide (20 % m/v, Ex-A-Lint, Intervet SA) upon arrival and identified with numbered ear-tags. A liver biopsy was performed on all the sheep via the right 10th to 12th intercostal space[7] 1 week after arrival. Ultrasound scanning was used to demarcate the position of the liver, to avoid puncturing major blood vessels and to determine the most appropriate site for biopsy. The biopsies were performed aseptically following local anaesthesia (Lignocaine 2 %, Centaur). A 4 mm-diameter stainless steel liver biopsy needle was used. The liver samples were stored frozen until analysed. Pre- and post-surgical amoxycillin (Clamoxyl RTU, Pfizer AH) cover was administered. Seven days later small rumen cannulae were inserted into all the sheep to facilitate copper dosing. The cannulae were inserted following tranquillisation with acetylpromazine (Aceprom 2% Inject, Bayer AH) and local anaesthesia (Lignocaine 2 %, Centaur). Amoxycillin (Clamoxyl

RTU, Pfizer AH) and phenylbutazone (Phenylarthrite, Bayer AH) were administered to prevent bacterial infection and alleviate post-surgical inflammation. During the last week of adaptation the sheep were placed in metabolic crates to facilitate urine collection to establish base-line concentrations prior to copper loading. After 3 days the sheep were removed from the crates, individually stabled and copper loading commenced.

Copper loading

The sheep were weighed individually and each received 20 mg copper sulphate per kg body mass[2,22] as a 0.5 % m/v aqueous solution (4 mℓ of a 0.5 % m/v solution of $CuSO_4 \cdot 5H_2O$/kg) daily for 10 weeks. The sheep were weighed weekly and the dose adjusted accordingly. The copper was administered directly into the rumen via the rumen cannula. Copper dosing ceased after 70 consecutive days.

Treatment groups

Three randomised, parallel treatment groups (n = 4) were used. The sheep were ranked on the basis of liver copper concentration obtained from the biopsy at the start of the study, and randomly assigned to 3 experimental groups, each with similar mean liver copper concentrations. The treatment groups consisted of a molybdenum-treated group (Mo-group); a zinc-treated group (Zn-group) and an unmedicated control group. At the start of copper loading (Day 0) all the animals in the Mo-group were injected with 1.5 mℓ of the commercial molybdate formulation, purported to contain 40 mg molybdenum/mℓ, subcutaneously, and the sheep in the Zn-group received the New Zealand-registered zinc oxide bolus, containing 43 g zinc (as zinc oxide) through the rumen cannula. The molybdenum and zinc treatment were repeated after 6 weeks.

Molybdate formulation

The molybdenum concentration of the injectable formulation was also ascertained by the ISCW.

Clinical chemistry

Glutamate dehydrogenase (GLDH) activity was analysed using an automated chemical analyser (Technicon RA-XT Analyzer, Technicon Instruments Corporation) and the manufacturer's methods and reagents. The haematocrit was determined using a Cell-dyn 3700, (Abbott Diagnostic Division, Abbott Laboratories). Both parameters were measured on Days –21, –14, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70. The analyses were performed by the Clinical Pathology Labora-

tory, Faculty of Veterinary Science, University of Pretoria.

Urinary copper concentration

The sheep were placed in metabolic crates for 24 hours and the volume of urine voided per day was recorded. Urine samples (aliquots of 20 mℓ of the daily volume voided) were collected before and once a week during the copper-loading period. The urine samples were frozen immediately after collection and forwarded at the end of the trial to the analytical laboratory of the ISCW for copper analysis.

Copper and zinc concentrations

Plasma copper concentrations were monitored every 2 weeks. The liver biopsy samples were submitted for determination of copper and zinc concentrations. At the end of the experiment (Day 70) the sheep were euthanased with an overdose of pentobarbitone sodium (Eutha-Naze, Bayer AH) and liver and kidney samples (±100 g) were collected, frozen and submitted for copper and zinc analysis. The site for liver sampling was guided by the scar tissue that remained following the liver biopsy. The copper and zinc concentrations were determined by the ISCW.

Histopathology

After euthanasia, liver samples were collected and preserved in 10 % buffered formalin for histological examination. The tissues were routinely processed and stained with haematoxylin and eosin (HE). The number of neutrophil clusters around apoptotic bodies in 10 adjacent fields under ×20 magnification were counted.

Data analysis

The data of each variable were captured. The data were statistically evaluated using the computer software programme Sigma Stat (Jandel Scientific). One-way analysis of variance (ANOVA) and Kruskal-Wallis one-way ANOVA on Ranks were performed and statistical significance was set at $P < 0.05$. The Tukey test was used for all pairwise comparisons of the mean responses to the various treatment groups.

RESULTS

Neither the subcutaneous administration of 1.5 mℓ of the molybdate formulation nor the intraruminal zinc oxide bolus prevented hepatic copper accumulation in sheep (Fig. 1). In all 3 treatment groups the mean liver copper concentrations following 10 weeks of copper dosing were significantly higher ($P < 0.05$) than before
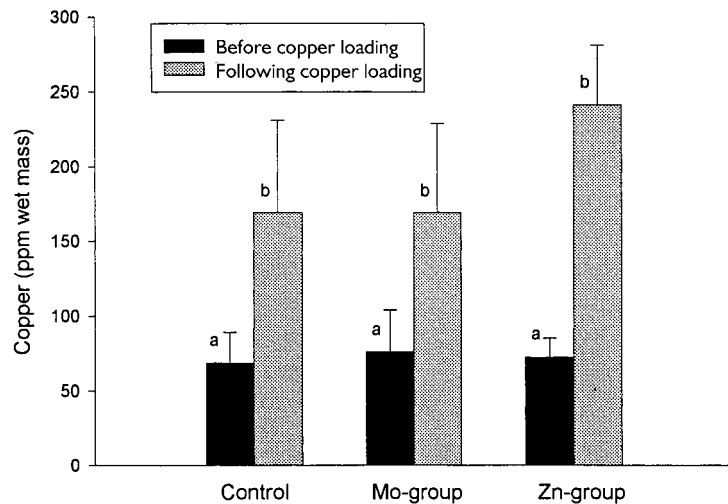

Fig. 1: **Mean liver copper concentration before and after copper loading in the 3 treatment groups. Dissimilar letters indicate significant differences** ($P < 0.05$)**.**
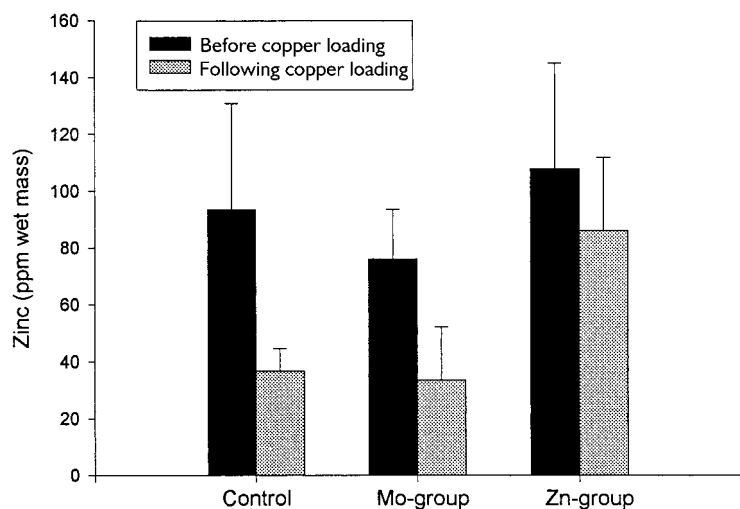

Fig. 2: **Mean liver zinc concentration before and after copper loading in the 3 treatment groups.**

dosing began. The mean hepatic copper concentrations in the control group and the Mo-group were similar, namely 169.23 (±61.64) and 169.27 (±59.53) ppm wet mass (WM), respectively. On the other hand, the liver zinc concentrations decreased compared to the concentrations obtained at the start of the experiment in all the groups. No significant difference occurred between the pre- and post-treatment concentrations within groups (Fig. 2). The total number of apoptotic bodies counted in the liver section in the field examined was 6 in the control group, 32 in the Mo-group and 4 in the Zn-group.

The mean kidney copper concentrations in the Mo-group was 6.89 (± 4.3; median 6.13), in the Zn-group 10.54 (±6.19; median 10,22) and in the control group 5.23 (±1.31; median 5.31) ppm WM, which were not significantly different. Median kidney zinc concentration of the animals in the group receiving additional zinc was rather high at 78.25 ppm WM, but was only significantly different

from the Mo-group (median 17.6 ppm WM) and not the control group (median 23.1 ppm WM).

The mean plasma copper concentration of the Mo-group was only slightly higher when compared to the control and the Zn-group (Fig. 3). Urinary copper excretion increased following copper loading, but no noticeable difference between the various treatment groups was evident (Fig. 4). The mean packed cell volume determined in all the groups fluctuated within the normal range of 22–44 % (Fig. 5). After 4 weeks of copper dosing the haematocrit of the control group showed a substantial decrease, but this was attributed to an individual animal with a haematocrit of only 12.4 %. If this result was excluded then no difference within and between groups was evident. On D 35 this animal's haematocrit had again increased to 24.3 %.

The GLDH activities varied substantially within and between the groups (Fig. 6). Although the mean GLDH activity in the Mo-group tended to be higher,
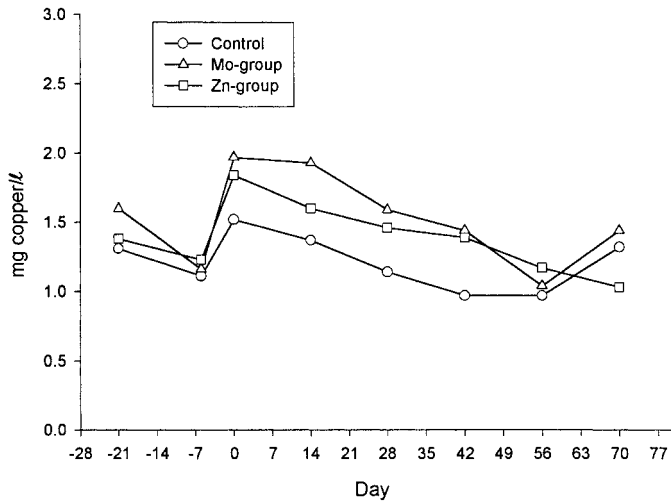
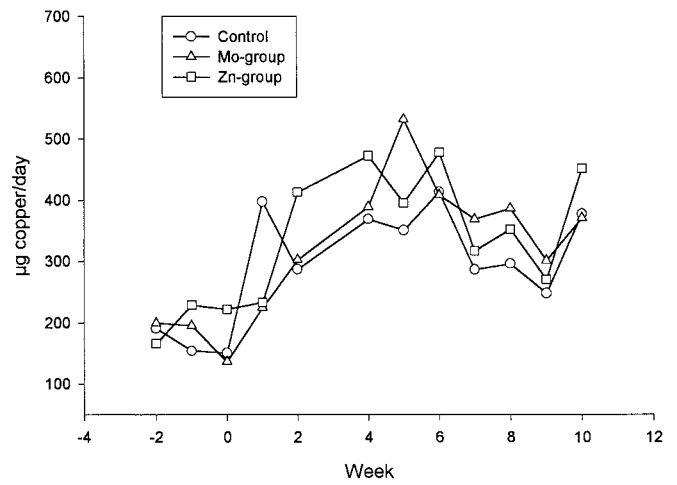Fig. 3: **Mean plasma copper concentration before and after copper loading in the 3 treatment groups.**

Fig. 4: **Mean urinary copper excretion before and after copper loading in the 3 treatment groups.**
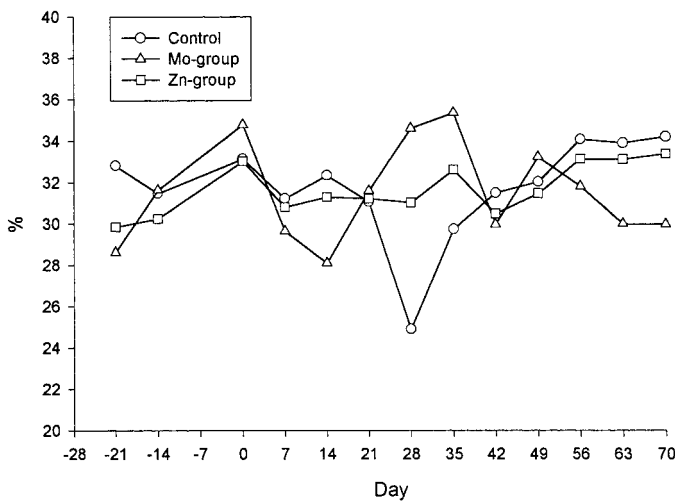

Fig. 5: **Mean haematocrit determined in the 3 treatment groups throughout the trial.**
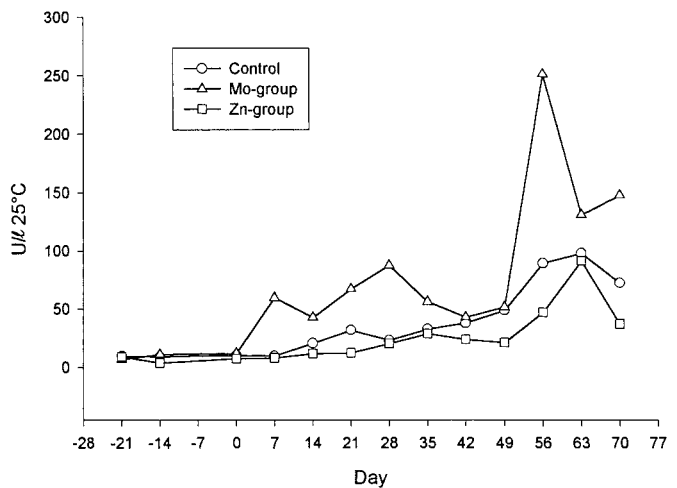

Fig. 6: **Mean GLDH activity determined throughout the trial in the 3 treatment groups.**

only 2 animals in this group contributed to the higher activity measured. On D 70, when the sheep were sacrificed, GLDH activity in these 2 sheep measured 305 and 266 U/l, which coincided with the highest number of apoptotic bodies (17 and 14, respectively) counted in the specified histological field examined.

Analysis of the molybdate formulation revealed only 27.8 mg molybdenum/mℓ, instead of 40 mg/ml. The subcutaneous administration of the molybdate formulation was painful and caused transient discomfort to the animals, but no long-term tissue reaction was observed.

The results of the feed analysis are pre-

sented in Table 1. Molybdenum analysis of the feed was only performed twice. Water analysis revealed the following concentrations: 40.4 µg/ℓ copper; <0.001 mg/ℓ iron; 19.26 µg/ℓ molybdenum and 5.86 mg/ℓ sulphur-sulphate (S-SO$_4$).

DISCUSSION

In this study, 2 subcutaneous treatments of the molybdate formulation did not prevent copper accumulation in the liver of sheep receiving additional copper. A possible explanation for inefficacy could be the absence of sulphur/sulphates in the formulation. The formation of thiomolybdates in the rumen is necessary to form complexes with copper and reduce copper absorption from the intestines[17]. Thiomolybdates that are absorbed will interact with tissue copper and increase copper elimination[17].

Insufficient dosage could be another reason for the lack of efficacy, as the dose was either too small and the dosing interval too long, or both. In this study only

Table 1: **Feed analysis performed during the trial.**

Sample	Cu (mg/kg)	Fe (mg/kg)	Mo (µg/kg)	S (%)	Zn (mg/kg)
Milled lucerne					
A	8.21	346.3	1.61	0.25	18.31
B	6.04	516.7	<0.001	0.26	20.22
C	9.4	1270.3	–	0.31	12.3
Mean	7.88	711.1	–	0.27	16.94
SD	1.7	491.7	–	0.03	4.13
Sheep pellets					
A	39.32	664.9	1.28	0.25	126.34
B	38.62	665.8	<0.001	0.23	130.36
C	43.3	807.5	–	0.25	79.5
Mean	40.41	712.73	–	0.24	112.07
SD	2.52	82.07	–	0.01	28.27

42 mg molybdenum (1.5 mℓ of the formulation which contained 28 mg molybdenum/mℓ) was administered subcutaneously at a 6-week interval. This dose exceeded the manufacturers' recommendation of 1–1.5 mℓ subcutaneously 1–4 times a year. Intravenous administration of 50 mg tetrathiomolybdate, twice weekly for 11 weeks, prevents chronic copper poisoning in copper-loaded sheep[16]. Gooneratne et al.[8] also concluded that intravenous injection of 100 mg ammonium tetrathiomolybdate (approximately 37 mg molybdenum), twice weekly, averts copper accumulation in the liver of copper-loaded sheep. In the Karoo, dosing a molybdate solution daily, every 2nd day, or weekly, is sometimes recommended (G F Bath, Faculty of Veterinary Science, University of Pretoria, pers comm., 1993), and a number of farmers include molybdates in licks or in drinking water[1], resulting in a more continuous intake.

There are conflicting accounts of the capability of zinc to prevent copper accumulation in the liver of sheep. Supplementation of more than 150 ppm zinc to a ration containing 30 ppm copper resulted in a significant reduction in hepatic copper accumulation[25]. Significant decreases in the liver copper concentration of sheep fed rations containing 28 ppm copper and additional zinc at 220 or 420 ppm have been recorded after 8 and 12 weeks, but no significant difference was noted after 24 weeks when the experiment was terminated[5].

In the current experiment, the administration of zinc oxide boluses did not prevent intestinal copper absorption and subsequent storage in the liver of sheep when copper loaded. This is in agreement with the findings of Saylor and Leach[20], who reported that supplementation of the diet of sheep containing up to 48 ppm copper with 543 ppm zinc for 60 days did not reduce the liver copper concentration. Increases in the copper content of the diet resulted in significantly higher liver copper concentrations, and they concluded that supplementation of zinc to the diet had no effect on the hepatic concentration of either copper or zinc[20]. A possible explanation for the inability of zinc to prevent copper accumulation is the tendency of zinc to also induce hepatic metallothionein synthesis[4,21]. Metallothioneins are proteins that are capable of binding heavy metals such as copper and zinc, and function as a cellular-protective detoxification mechanism[4]. Copper has a greater affinity for the available metallothionein binding sites, and competes with and displaces zinc from the protein, although zinc induces

metallothionein synthesis[4,20]. The high daily dose of copper probably also overwhelmed the intestinal metallothioneins' protective effect. These explanations clarify the increased liver copper concentration and decreased hepatic zinc concentration detected in the respective pre- and post treatment concentrations of the Zn-group in the present study (Figs 1, 2).

Although exceptional variation in GLDH activity occurred within and between groups throughout the trial, a rising trend was noticeable following copper loading (Fig. 6). GLDH activity is a good indicator of hepatic necrosis in sheep[13], and the greater number of apoptotic bodies in 2 of the sheep in the Mo-group suggested more extensive liver damage in this group compared to the other groups. Whereas parenteral tetrathiomolybdate prevents extensive liver damage in sheep receiving additional copper[8,16], no such protective effect was noticed in the sheep in this study receiving the molybdate treatment.

As expected, urinary copper excretion increased in all 3 groups following copper loading. There are conflicting reports in the literature regarding the ability of tetrathiomolybdate to increase copper excretion in the urine[3,9,14], but in this experiment the molybdate therapy did not significantly increase cupruresis in relation to the other groups (Fig. 4). Contrary to previous reports indicating increased copper concentrations in the kidney cortex[10], the molybdate treatment in the current study did not increase kidney copper concentration substantially, although analysis was performed using both the renal cortex and medulla.

The plasma copper concentration and the haematocrit fluctuated within the normal range and no haemolytic crisis was detected during the trial. The reason for the low packed-cell volume in 1 of the control sheep is unknown. After only 28 days of copper loading, it is unlikely to have been due to haemolysis.

In spite of a large discrepancy in molybdenum concentration in successive feed samples, it is concluded that the molybdenum, sulphur/sulphates, zinc and iron concentrations in the feed and water probably did not have any preventative effect on hepatic copper accumulation, as the liver copper concentrations increased significantly in all groups.

The purported prophylactic potential of the molybdate formulation could not be confirmed and, if effective, the reason is still obscure and cannot be ascribed to reduced hepatic copper accumulation or increased urinary copper excretion. A change in the formulation to contain tetrathiomolybdate should be consid-

ered, as tetrathiomolybdate is known to prevent hepatic copper accumulation[8]. However, the possibility should be entertained that simple molybdate salts may also form copper-molybdenum complexes in the liver, which are stable or protein-bound (as in the case of thiomolybdates) and will prevent release of copper from the liver during stressful incidents.

Although the zinc oxide boluses increased hepatic copper accumulation, the copper is most probably tightly bound, as part of a cellular protective mechanism, to metallothionein[4], and might not be released under stress.

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