Rumen By-Pass Copper

'Koper voorbij de Pens'

R.M.A. Goselink

LIVESTOCK RESEARCH
WAGENINGEN UR

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This research was conducted with financial support of the Dutch Dairy Board and the Dutch Product Board Animal Feed, within the Research Programme “Feed4Foodure”, research line “More with Less”, theme 5: Reduction of Copper and Zinc Losses

Dit onderzoek is uitgevoerd door Wageningen UR Livestock Research, met financiële ondersteuning van het Productschap Zuivel en het Productschap Diervoeder als onderdeel van de PPS Feed4Foodure (projectnummer BO – 31.03-005-001)

Wageningen UR Livestock Research
Wageningen, October 2015

Livestock Research Report 905

LIVESTOCK RESEARCH
WAGENINGEN UR

Goselink, R.M.A., 2014. *Rumen By-Pass Copper; "Koper voorbij de Pens".* Wageningen, Wageningen UR (University & Research centre) Livestock Research, Livestock Research Report 902.

Samenvatting NL De absorptie van koper (Cu) bij herkauwers is relatief laag vanwege de interacties tussen Cu en andere bestanddelen van het rantsoen, waardoor onoplosbare complexen gevormd worden die niet worden geabsorbeerd in de dunne darm. Dit kan mogelijk verbeterd worden door pensbestendige Cu bronnen aan te bieden, waardoor de Cu uitscheiding via de mest naar het milieu verminderd kan worden. Het doel van dit project was het evalueren van het effect van pensbestendige Cu bronnen op de Cu absorptie bij herkauwers. Dit is onderzocht met behulp van een in vitro model waarbij verschillende Cu bronnen zijn geïncubeerd om de fermentatie- en verteringsprocessen in het maagdarmkanaal te simuleren. Daarna is een proef uitgevoerd met 18 vleeskalveren waarbij twee pensbestendige Cu bronnen zijn vergeleken met kopersulfaat als controle. Het verschil in Cu absorptie tussen pensbestendige Cu bronnen en kopersulfaat was echter onvoldoende om in deze beperkte proefopzet aangetoond te kunnen worden.

Summary UK Copper (Cu) absorption in ruminants is impaired by interactions between Cu and other feed components in the rumen, forming insoluble complexes that cannot be absorbed in the small intestine. Cu absorption may be increased by using rumen-protected sources of Cu, thereby reducing Cu excretion in the environment. Goal of the present project was to evaluate the effect of rumen-protected Cu sources on Cu absorption in ruminants. This was tested in an in vitro model, incubating test tubes with different Cu sources, simulating rumen fermentation and gastrointestinal digestion. Secondly, the absorption of two rumen-protected Cu sources was tested against copper sulphate as a control in an in vivo trial with 18 veal calves. The difference in Cu absorption of rumen-protected sources relative to copper sulphate was however not large enough to be visible in the current trial set-up.

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Foreword

This study was conducted with financial support of the Dutch Dairy Board and the Dutch Product Board Animal Feed, incorporated within the research programme “Feed4Foodure”: a public-private partnership between the Dutch Ministry of Economic Affairs and a consortium of various organizations within animal feed industry and the animal production chain. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market.

The research programme comprises three main research lines: socially responsible livestock farming; nutrition, gut health and immunity; and more-with-less by efficient nutrient use. The aim of this third research line, “More with Less”, is to reduce the footprint of the Dutch livestock sector in the field of phosphate, nitrate, copper, zinc, ammonia and greenhouse gases. New nutritional models and measurement techniques will be developed to improve efficient use of nutrients in livestock farming.

The present report entitled “Rumen By-Pass Copper” was written within research line “More with Less”, theme 5 “Reduction of copper and zinc losses”. Main aim of this subproject is to gain insight in options to improve copper absorption in ruminants, preventing complexation of copper in the rumen.

We would like to thank the Dutch Dairy Board and the Dutch Product Board Animal Feed for their financial contribution, Dr. Machiel Blok and Dr. Age Jongbloed for their contribution in developing the idea of ‘rumen by-pass copper’, and prof. Lyuba Kuchkarova from Tashkent University, Uzbekistan for her scientific and practical work in both trials.

Roselinde Goselink

Summary

Copper (Cu) absorption in ruminants is impaired by interactions between Cu and other feed components in the rumen, forming insoluble complexes that cannot be absorbed in the small intestine. Cu absorption may be increased by using rumen-protected sources of Cu, thereby reducing Cu excretion in the environment. Goal of the present project was to evaluate the effect of rumen-protected Cu sources on Cu absorption in ruminants. This was tested in an in vitro model, incubating test tubes with different Cu sources, simulating rumen fermentation and gastrointestinal digestion. Secondly, the absorption of two rumen-protected Cu sources was tested against copper sulphate as a control in an in vivo trial with 18 veal calves.

The in vitro model was not successful in finding differences in Cu absorption after the different incubation steps, as the Cu concentration in the supernatant was below the detection limit for all Cu sources. This may be related to the sensitivity and conditions of the in vitro model itself. These results do not necessarily imply a similar behaviour and absorbability of these Cu sources in vivo.

In the in vivo trial, three groups of six veal calves each were used to test two potential rumen-protected Cu sources in a 4-week supplementation period against a $CuSO_4$ control supplementation. Liver Cu content was determined as an indicator of Cu absorption surplus. At the end of the trial, no significant differences were found in the liver Cu content. The increase in Cu absorption of the rumen-protected sources relative to $CuSO_4$ may have been insufficient to show differences in this small trial (trial design aimed at finding a difference of at least twice the absorption percentage of $CuSO_4$ of 4%). Also, the formation of insoluble complexes in the lower intestine cannot be excluded. A larger study with adult ruminants at less extreme depletion and repletion diets is needed to determine the true bioavailability of rumen-protected Cu sources.

1 Introduction

1.1 Copper in animal physiology

Copper (Cu) is an important trace element in human and animal physiology. It is part of many enzymes, involved in the formation of blood cells as a constituent of haemoglobin, and important in the composition of connective tissue, skin and hair. Cu is mainly absorbed in the small intestine, after which it is attached to albumin for transport to the liver. In the liver Cu can be stored or bound to carrier protein ceruloplasmin for further distribution in the body, e.g. secretion in milk. Excretion of surplus Cu takes place through faeces (COMV, 2005).

In ruminants, sulphides are formed in the rumen from sulphur containing substances in the ration. These sulphides can easily precipitate with Cu, making Cu unavailable for absorption. In combination with high molybdenum levels in the ration, Cu absorption is further reduced, by precipitation of insoluble thiomolybdate complexes (Jongbloed et al., 2004a).

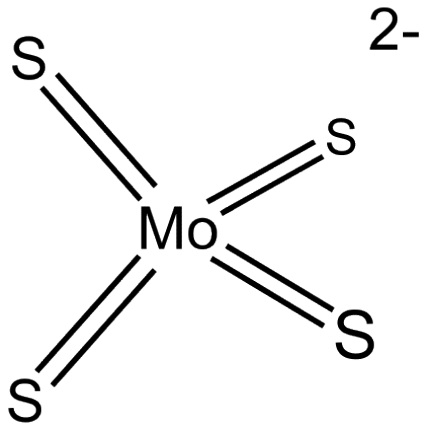

Figure 1.1 *Tetrathiomolybdate.*

By these precipitation reactions in the rumen, the absorption of dietary Cu in adult ruminants is only 2-10% (Jongbloed et al., 2004a). The remaining unabsorbed Cu complexes will be excreted with the faeces; thus contributing to Cu accumulation in the environment.

1.2 Copper in the environment

Copper is not only an essential element to living organisms; it is also a heavy metal with detrimental consequences for the environment when present in high concentrations. It can damage protein, lipids and DNA, resulting in an exotoxicological impact on soil microorganisms, plants and aquatic life. Accumulation in the environment should therefore be prevented where possible.
The contribution of agriculture in the Netherlands to total Cu pollution in surface water is 12%, and the contribution to total Cu accumulation in soils is even higher with 60% (Emissieregistratie, 2012). The largest part of this soil accumulation of Cu originates from animal manure (see Table 1.1).

Table 1.1
Accumulation of copper on Dutch agricultural soils (x1,000 kg).

	1980	1990	2000	2005	2009
Total input	**1,360**	**970**	**780**	**515**	**465**
of which animal manure	1,050	750	700	435	405
fertilizer	150	120	50	40	25
deposition	80	50	20	20	20
other	80	50	10	20	15
Total output (crops)	**140**	**130**	**100**	**95**	**100**
Net accumulation	**1,220**	**840**	**680**	**420**	**365**

Source: CBS Statline, 2013.

For (adult) ruminants, only 2-10% of dietary Cu is absorbed, leaving 90-98% of dietary Cu unabsorbed in the faeces. Thus, if Cu absorption could be increased, the Cu concentration in animal diets and Cu excretion in faeces can be reduced, preventing further accumulation of Cu in the environment.

1.3 Scope and objective

In non-ruminating calves little fermentation of feed takes places in the rumen and relative Cu absorption is about 10-fold higher (around 70%) than absorption in adult ruminants (COMV, 2005). This suggests that the diet composition and the digestive process in adult ruminating cattle is responsible for the relatively low Cu absorption. The main contribution of Cu in dairy cattle rations is through concentrates. Grass silage contains around 8 mg Cu per kg DM and maize silage around 4 mg Cu/kg DM; standard concentrates contain on average 28 mg Cu/kg DM and high protein concentrates even 35 mg Cu/kg DM (COMV, 2005). By using rumen-protected sources of Cu as additives in concentrate instead of the regularly used Cu salts, the Cu absorption may be increased in adult ruminants in the direction of the high absorption level of non-ruminating calves. This may reduce Cu excretion in the environment up to 20-50% expressed on the total dairy ration, depending on the relative contribution of concentrate over forage, and will thereby help to improve the sustainability of the dairy industry.

Goal of the present project was to evaluate options to improve Cu utilization in dairy cattle with rumen-protected Cu sources and to estimate the potential effect of these options regarding the reduction of Cu excretion in the environment. In part 1, an in vitro methods was used to determine solubility after various incubation steps simulating rumen fermentation and gastrointestinal digestion. In part 2, the absorption of two rumen-protected Cu sources was tested against copper sulphate as a control in an in vivo trial.

2 In vitro experiment

2.1 Introduction

Different Cu sources, organic or inorganic, will have varying biochemical properties regarding their degradability by rumen flora or endogenous enzymes and solubility at different pH. If Cu solubilizes in the rumen, rumen fermentation may result in the formation of insoluble Cu complexes with sulphides and molybdenum. These complexes cannot be absorbed along the gastrointestinal tract, leaving Cu unavailable to the ruminant and excreted with faeces. High levels of free Cu in the rumen are also undesirable, as these are toxic to rumen microorganisms, in vivo as well as in in vitro. At 0.5-1 mg Cu/l the cellulase activity was already decreased (Martinez & Church, 1970; Hubbert et al., 1958) and at 21 mg Cu/l in vitro gas production was inhibited by 50% (Forsberg, 1978).

2.1.1 Copper sources

Cupric sulfate (**$CuSO_4$**) is a commonly used inorganic Cu form in ruminant feeds. It is relatively well soluble in the rumen, and will serve as a negative control (not rumen-protected Cu source) in the current trial. Cupric oxide (**CuO**) is quite insoluble along the whole gastrointestinal tract, resulting in a very low absorption in ruminants (Spears, 2003). Therefore CuO does not seem to have beneficial characteristics to reduce Cu excretion. Tribasic copper chloride (**Cu_2OH_3Cl**) is an inorganic Cu form with a specific, pH dependent solubility. At the high pH of the rumen the solubility of Cu from Cu_2OH_3Cl is low, while at the low pH of the abomasum, Cu release is increased. In diets low in Mo the absorption of Cu from Cu_2OH_3Cl was comparable to CuSO4, but at diets high in Mo and S, Cu absorption from Cu_2OH_3Cl supplementation reached 196% of the absorption from $CuSO_4$ (Spears et al., 2004).

Organic (chelated) Cu sources, such as **Cu proteinate** with Cu bound to amino acids have shown to have a higher bioavailability than $CuSO_4$ (Kincaid et al., 1986; Ward et al., 1996; Hansen et al., 2008) and may pass the rumen without releasing Cu.

Specific encapsulation techniques have been developed to allow nutrients to pass the rumen microflora, to be released postruminally (in the abomasum or small intestine) for absorption. Encapsulation of nutrients can be reached by a denatured protein layer, e.g. a whey protein emulsion protecting unsaturated fatty acids against biohydrogenation (Van Vuuren et al., 2010). **Fat encapsulation** is a technique used to protect various feed additives such as essential amino acids (Papas et al., 1984), unsaturated fatty acids or (semi-)vitamins. Fat protected globules may differ in absorption depending on their diameter; intestinal absorption may be higher with decreasing sphere diameter (Li et al., 2012).

2.1.2 Objective

To screen different Cu sources for their potential use as a rumen-protected Cu source, five organic and inorganic Cu supplements were compared with $CuSO_4$ for their solubility along the gastrointestinal tract, simulated by in vitro techniques.

2.2 Materials and Methods

2.2.1 Tested materials

The following Cu sources have been included in the in vitro study:

- $CuSO_4$ (control; $CuSO_4 \cdot 5H_2O$)
- Tribasic cupric chloride (Cu_2OH_3Cl)
- Cu proteinate
- $CuSO_4 \cdot 5H_2O$ encapsulated in fat for rumen-protection, *large* globules
- $CuSO_4 \cdot 5H_2O$ encapsulated in fat for rumen-protection, *small* globules

For each source, three 50-ml centrifuge tubes were prepared for in vitro incubations, by adding 60µg of Cu to each tube. To dose the small amount of Cu needed per test tube, 600 µg Cu of each source was first diluted in 2000 mg (dry) glucose powder and mixed overnight. Each test tube received 200 mg of glucose + 60 µg Cu.

2.2.2 In vitro incubation

To simulate the full gastrointestinal pathway of Cu from ingestion to absorption an in vitro incubation protocol was used with three successive phases: 1) rumen incubation; 2) abomasal incubation; and 3) intestinal incubation; comparable to the procedure used by Ward and Spears (1993).

Rumen incubation

Rumen fermentation was simulated by a 4 h rumen incubation. First, rumen fluid was collected from 3 dry cows, immediately mixed in a pre-warmed thermos and transported to the laboratory. In the lab, rumen fluid was strained through a cheesecloth and mixed with a carbonate phosphate buffer at a 1:1 ratio.

The buffer solution was prepared similar to the composition of ruminant saliva, as described by Baumgardt et al. (1962) with some modifications. Buffer composition per litre was 9.80 g $NaHCO_3$, 4.65 g $Na_2HPO_4 \cdot 2H_2O$, 0.57 g NaCl, 0.47 g KCl, 0.04 g $CaCl_2$ and 0.12 g $MgSO_4 \cdot 7H_2O$. To reach anaerobic circumstances, the buffer was bubbled with CO_2 for 30 min at 39°C and a reducing agent (67 ml H_2O with 107 mg NaOH and 220 mg $Na_2S \cdot 3H_2O$) was added, 5 minutes before mixing the buffer with the strained rumen fluid.

Each 50-ml centrifuge tube with Cu and glucose (as an energy substrate for rumen microbial growth and metabolism) was supplemented with 0.75 ml of a Mo and S solution (containing 13.33 µg of Mo as Na_2MoO_4 and 10.0 mg of S as Na_2SO_4 per ml), to stimulate thiomolybdate formation. Next, 30 ml of buffered rumen fluid (pH = 6.7, 15 ml rumen fluid with 15 ml buffer mixture) was added to each tube. Tubes were bubbled with CO_2 to create anaerobic circumstances, closed with screw-caps and stored at 38°C for 4h. The tubes were swirled every hour and after 1h, the screw-cap was slightly opened to release the gases produced during incubation.

Abomasal incubation

After 4h, 1 tube of the 3 replicates per Cu source was put on ice to stop microbial activity. The other 2 tubes were used in the abomasum incubation phase. In each tube 4.7 ml of pepsin (Sigma Aldrich P7000) solubilized in 1 M HCl was added, to lower the pH to approximately 2.5. Tubes were swirled and stored at 38°C for 1.5h.

Intestinal incubation

After 1.5h, 1 tube of the 2 remaining replicates per Cu source was put on ice to stop enzymatic activity. The other tube was used in the intestinal incubation phase. In this tube 6.5 ml of 1 M $NaHCO_3$ was added to restore the pH to approximately 6.6, and 0.2 ml of 20% pancreatin solution (Sigma-Aldrich P3292) was added to simulate duodenal digestion. The tubes were swirled and stored at 38°C for 2h and then put on ice.

All tubes were centrifuged for 30 min at 21,000 g after which 1.5 ml of supernatant was collected for (soluble) Cu analysis.

2.2.3 Analysis

Copper analysis
Rumen fluid samples were acidified to pH<2 with HNO_3 after which Cu concentration was measured by inductively coupled plasma atomic emission spectroscopy (ICP) analysis with lowest detection limit 0.5 mg/l.

Amylase activity
Adding 60 µg of Cu to 30 ml of buffered rumen fluid results in a Cu concentration of 2 mg/l, which may slightly inhibit microbial fermentation (Martinez & Church, 1970; Hubbert et al., 1958). Therefore a quick scan for amylase activity was performed as a screening method to test for microbial activity (Hristov et al., 1999; Engvall, 1980). Amylase activity was determined according to Ugolev (1969). In short, 1 ml of rumen fluid was taken from each of the tubes at the end of rumen incubation (n=3 per Cu source) and added to a tube with soluble wheat starch. After incubation at 37°C for 1h, iodine reagent was added and starch disappearance was measured by spectrophotometry (at 620 nm).

2.3 Results and Discussion

2.3.1 Amylase activity

Amylase activity is sensitive to toxic effects of Cu, but activity was not severely reduced by adding 60µg of Cu to buffered rumen fluid to reach a concentration of 2 µg/ml. Addition of $CuSO_4$ salt or the fat protected $CuSO_4$ in small globules resulted in a small but significant reduction of enzyme activity or amylase producing microorganisms; amylase activity was however still 49-53 g/l per h (Table 2.3). The addition of just Mo and S to rumen fluid or either of the Cu sources Cu proteinate or fat protected Cu in large globules did not significantly affect amylase activity.
In a study by Ward and Spears (1993), microbial cellulase activity was reduced significantly from 39% to 33% when adding 4 µg Cu/ml. At levels of 10-15 µg Cu/ml (100 µg Cu lysine or an equal amount of Cu as $CuSO_4$), cellulase activity decreased from 33% to only 3-9% proving high Cu levels to be toxic to rumen microorganisms in vitro.

Table 2.1
α-amylase activity (g/l per h) after rumen incubation with different Cu sources.

	average	st dev
Rumen fluid with		
no Cu, Mo or S added	61.9	8.5
no Cu added; only Mo and S	68.4	4.8
$CuSO_4$ (control), Mo and S	49.4*	6.6
Cu proteinate, Mo and S	58.0	13.2
Fat protected, large globules, Mo and S	64.0	11.7
Fat protected, small globules, Mo and S	52.5*	5.4

* significant difference (P<0.05)
Cu_2OH_3Cl addition has not been tested for amylase activity

2.3.2 Cu concentration

None of the fluid samples after rumen incubation or after intestinal digestion had a soluble Cu concentration above the detection limit (<0.5 mg/l). Other samples (after abomasal incubation) have not been analysed after these results.
Even though solubility of each of the Cu sources tested must have been different, as shown in a pH dependent solubility trial with Cu_2OH_3Cl and $CuSO_4$ in water (Spears et al., 2004), no variation in Cu concentration in the fluid phase (supernatant) could be detected. Allen and Gawthorne (1987) determined Cu in rumen fluid supernatant after centrifugation at 25,000g for 30 min, but the concentrations were severely reduced by 59% in presence of tetrathiomolybdates (5 mg Mo/kg DM). Ward and Spears (1993) used $CuSO_4$ and added Mo + S to stimulate thiomolybdate formation. They were able to find a reduction in supernatant Cu content from 0.10 to 0.08 mg/l after incubation steps comparable to our study, but with lower Mo and S concentration (10 mg Mo and 7.5 g S per kg DM). Presumably thiomolybdate concentration in our study samples was relatively high and able to

sequester all Cu released. Because complete samples passed through the different incubation phases (abomasum, intestine), thiomolybdates produced in the rumen may have been able to sequester the Cu ions released in later phases as well. In vivo, the thiomolybdates produced in the rumen may bind other minerals passing with rumen contractions, or may even be absorbed before passing to the abomasum (Mason et al., 1988). Otherwise, unknown factors present in the rumen fluid (e.g. Mo, S, F, organic material, amino acids (Spears et al., 2004)) or rumen bacteria (Allen and Gawthorne, 1987) may have been able to bind the Cu added to the samples, reducing supernatant Cu concentration.

2.4 Conclusions

Determining the differences in solubility of various Cu sources along the gastrointestinal tract by a simple in vitro incubation method has not been successful since the Cu concentration in supernatant rumen fluid was below the detection limit for all sources. Nevertheless, Cu solubility may have been different between sources as judged by small but significant differences in amylase activity which may have been caused by Cu toxicity to rumen microorganisms.
The absence of differences in soluble Cu concentrations may be related to the sensitivity and conditions of the in vitro methods applied here. Hence, these results do not necessarily imply a similar behaviour and absorbability of Cu among these sources in vivo. Furthermore, the in vivo results may be different, due to the dynamic processes of rumen passage and absorption which cannot be fully simulated in vitro.

3 In vivo experiment

3.1 Introduction

Especially in diets with high Mo and S levels, free Cu in the rumen will be bound to thiomolybdate complexes and become insoluble. Different Cu sources, organic or inorganic, have varying biochemical properties regarding their degradability by rumen flora and endogenous enzymes and solubility at different pH. Cu sources may differ in their ability to pass the rumen and prevent Cu binding to thiomolybdate. Bioavailability of Cu sources can be tested by measuring the rate of Cu accumulation in the liver after a period of feeding different Cu supplements as described in sheep by Ledoux et al. (1995).

3.1.1 Objective

The objective of this study was to compare the relative in vivo Cu absorption from two Cu supplements with physicochemical characteristics that may increase rumen-by pass of Cu as compared to $CuSO_4$.

3.2 Materials and Methods

3.2.1 Animals, housing and feeding

To study intestinal absorbability of two Cu sources which were expected to differ in rumen solubility and degree of rumen by-pass as intact product, 18 ruminating bull calves of approximately 4 months of age were blocked in groups of 3 calves with comparable live weight (average: 116 ± 11.8 kg). Calves from each group were randomly divided over three treatment groups: control (CON), rumen by-pass Cu #1 (RBC1) and rumen by-pass Cu #2 (RBC2).
Calves were kept in an indoor veal calf facility with wooden slatted floors. They were kept in three groups of 6 calves each and feed intake was registered per (treatment) group. Their diets were formulated to meet or exceed all standard requirements (NRC, 2001; CVB, 2011). Grass silage was fed ad libitum, and each morning calves received 0.1 kg DM of maize silage and 2 kg concentrate per calf. Ingredients and composition of the basal diet is shown in Table 3.1 and Appendix 1.

Table 3.1
Diet composition.

	Grass silage	Maize silage	Concentrate
	Ad libitum	*0.1 kg DM / calf*	*2 kg / calf*
Dry matter (g/kg)	564	333	900
VEVI* (/kg DM)	924	1066	1054
NE_{meat} (MJ/kg DM)	6.38	7.36	7.27
DVE (g/kg DM)	59	51	104
OEB (g/kg DM)	-14	-34	31
Cu (mg/kg DM)	4.2	3.4	8.1
Mo (mg/kg DM)	1.7	0.5	3.5
S (g/kg DM)	1.7	1.0	2.7

*Voedereenheid Vleesvee Intensief, net energy for meat production according to CVB (2011)

3.2.2 Treatments

All animals were fed with a low Cu diet with grass silage, maize silage and concentrate during a 6-week adaptation and depletion period, with high Mo and S concentration in the concentrate (Table 3.1). Thereafter a 4-week treatment period started with the same diet, but additional Cu

supplementation mixed with the maize silage and fed individually at the feeding gate. Each group received a different mixture aiming at a gross intake of 130 mg Cu/calf/day:

- Group CON (control) received a conventional copper source: $CuSO_4$.
- Group RBC1 (rumen by-pass copper 1) received Cu_2OH_3Cl
- Group RBC2 (rumen by-pass copper 2) received fat protected $CuSO_4$ (small globules).

All Cu sources were mixed with the 0.1 kg DM maize silage per calf and fed once daily.

3.2.3 Sampling and data collection

Feeding

Feed composition and feeding value were estimated by NIRS analysis and Cu content by inductively coupled plasma atomic emission spectroscopy (ICP). Roughage intake was measured per treatment group, by weighing the amount fed and the amount of feed refusals after 24 hours.

Liver

The most reliable determination of Cu status is by analysing Cu concentration in liver tissue (COMV, 2005), as surplus Cu is stored in the liver. Liver biopsies were taken at the beginning of the trial in week -6, and at the end of the trial in week 4. Biopsies were performed by percutaneous aspiration technique. In short, calves were lightly sedated (Sedamun 20 mg/ml) and fixed in a standing position. The position of the liver was located by percussion, the area was shaved, cleaned with water and a detergent and disinfected; after that, biopsy location was locally anaesthetised (Licocaine 2%). A stab incision (1 cm) was made and a biopsy needle (Mengini 170 mm × 1.8 mm) was inserted and rotated to obtain approximately 1 g of liver tissue. Then, the needle was drawn back with the outer opening of the needle closed and liver tissue was collected in sterile vials.
Liver biopsies were digested by perchloric acid, after which Cu concentration was measured by ICP analysis with a lowest detection limit of 1 mg/kg.

3.2.4 Calculations

The net Cu requirements for maintenance and growth were estimated according to COMV (2005):

- Maintenance: 7.1 µg/kg body weight
- Growth: 0.5 mg/kg growth (aiming at 1 kg growth per day)

Average gross Cu intake was calculated as a mean per group, based on daily feed intake and Cu concentration of different feed stuffs.

3.3 Results and Discussion

3.3.1 General health

Some calves showed signs of a bronchopneumonia in the first weeks of the trial but recovered well after treatment with antibiotics (animals 8154 and 3988, both RBC2).
One calf (animal 0736, CON) suffered from a chronic pneumonia and was treated with antibiotics but without sufficient response. At the end of week -3 this calf was removed from the trial.

3.3.2 Live weight

Average live weight increased from 121 kg in the first week of the depletion period (wk -6) to 161 kg in the first week of the treatment period (wk 1) and 180 kg at the end of the trial (wk 4; Table 3.2, Figure 3.1). Dietary treatment did not affect growth performance (P=0.413). Daily growth in 10 weeks was on average 0.84 kg/d, somewhat lower than expected based on the average growth of veal calves of 1 kg per day (CVB, 2011).

Table 3.2

Average live weight in kg per treatment group (standard deviation between brackets).

Week	CON	RBC1	RBC2
-6	125 (14)	120 (5)	123 (13)
-5	133 (16)	127 (8)	127 (16)
-4	142 (18)	135 (7)	135 (15)
-3	148 (18)	140 (8)	142 (17)
-2	158 (21)	150 (8)	147 (16)
-1	161 (19)	152 (8)	153 (17)
1	169 (20)	161 (10)	158 (20)
2	172 (19)	161 (9)	160 (20)
3	180 (19)	170 (9)	170 (21)
4	191 (21)	177 (10)	177 (22)

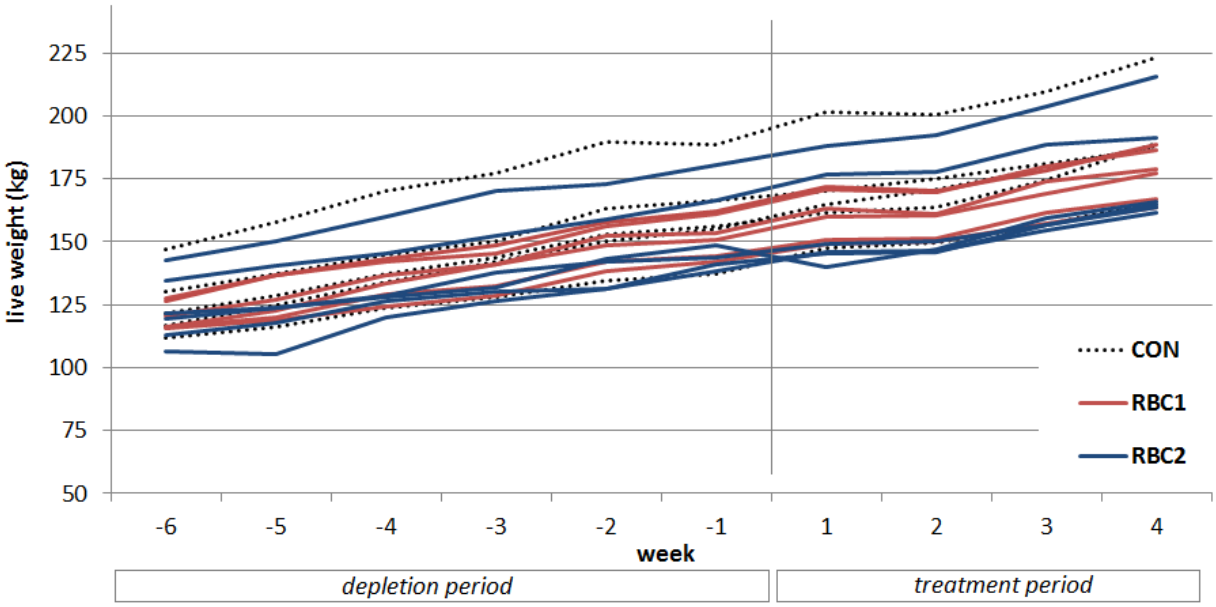

Figure 3.1 *Live weight of individual calves during the trial as assigned to one of three treatment groups: CON (black broken lines), RBC1 (red lines) and RBC2 (blue lines).*

3.3.3 Feed intake

Feed intake increased during the trial from 3.2 kg DM/d at the start to 3.8 kg DM/d at the end of the trial. Intake of maize silage and concentrates was always complete (0.1 kg DM maize silage and 2 kg concentrates per calf per day). Grass silage intake was *ad libitum*, and increased during the trial. Grass silage intake was higher in group CON (on average 0.2 kg DM per calf per day) than in other groups ($P<0.001$). The higher grass intake may be related to the numerically larger calves (on average 5.4 kg (3%) higher live weight in the treatment period).

During the first 6 weeks of the depletion period, average gross Cu intake increased from 20.9 to 23.5 mg Cu per calf per day. Average Mo concentration of the ration was 2.6 mg/kg DM and average S concentration 2.2 g/kg DM. According to the calculation of Jongbloed et al. (2004a), average Cu absorption rate from rations with comparable levels of Mo and S is estimated to be 4.0%, resulting in an average net Cu absorption of 0.88 mg/d. Requirements are estimated as 1.42 mg/d, hence net Cu intake was approximately 0.54 mg/d below Cu requirements as intended in the depletion period. During the 4 (repletion) weeks of the treatment period, gross Cu intake averaged 153 mg Cu per calf per day.

3.3.4 Liver Cu concentration

The liver Cu concentration at the beginning (wk -6) and end of the trial (wk 4) is shown for each of the 18 calves in Appendix 2, averaged per treatment group in Table 3.3. Variation in liver Cu concentration between calves was high. The amount of blood congestion or connective tissue in the liver biopsy may have influenced the results from the Cu analysis.

Table 3.3

Liver Cu concentration (in mg/kg DM) as analysed at the start (wk -6) and end (wk 4) of the trial, averaged per treatment group.

Group	Week -6		Week 4	
	Liver Cu (mg/kg DM)	Total liver Cu* (mg)	Liver Cu (mg/kg DM)	Total liver Cu* (mg)
CON	184	461	114	435
RBC1	172	412	100	347
RBC2	203	493	138	484

*Calculated liver mass by 2% of body weight

When comparing the results of RBC1 and RBC2 in week 4 with the control supplementation, the potentially rumen by-pass Cu sources RBC1 and RBC2 did not seem to be much more bioavailable than $CuSO_4$. The repletion of Cu storage in the liver by adding 130 mg Cu/d from RBC1 or RBC2 did not show to be more effective than $CuSO_4$ (Table 3.3).

The lower than expected levels of liver Cu for RBC1 and RBC2 at the end of the trial may have been caused by a low rate of rumen protection under the trial circumstances. Also other interactions with nutritional factors in the lower intestine may have reduced postruminal Cu availability.
A small difference in bioavailability may have been present however, but small differences could not be detected in this pilot study with 18 calves. It was designed to find at least a double increase of absorption, from an estimated 4% absorption of Cu from $CuSO_4$ to ≥8% absorption for the rumen-protected sources.

Another factor that may have influenced the result is a difference in Cu metabolism under circumstances of a negative Cu balance. An experimental design using a depletion and repletion diet helps to increase the effect of treatment on liver Cu concentration (Ward et al., 1996). In case of severe Cu deficiency however, Cu absorption will be actively increased at the intestinal transporter level, which may influence the results. In a study of Spears et al. (2004) for example, Cu_2OH_3Cl had a higher bioavailability relative to $CuSO_4$ with high dietary Mo and S but this result could not be reproduced in a repletion study with Cu deficient animals.

3.4 Conclusions

The supply of Cu from three different sources ($CuSO_4$, Cu_2OH_3Cl and fat protected $CuSO_4$) during a 4-week supplementation period after a 6-week depletion diet did not result in significant differences in the liver Cu content. These results do not confirm differences in bioavailability of Cu from different sources as hypothesised in this study. This may be caused because the increase in bioavailability of the rumen-protected sources relative to $CuSO_4$ control supplementation was not large enough to be found under the test circumstances with a depletion-repletion protocol (i.e. absorption did not increase more than twice the $CuSO_4$ absorption). Otherwise, the formation of insoluble complexes with rumen-produced thiomolbydates in the lower intestine cannot be excluded. A larger study with adult ruminants at less extreme depletion and repletion diets may be needed to determine the true bioavailability under practical circumstances.

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Appendix 1 Feed composition

	Grass silage	Maize silage	Concentrate
Dry matter (g/kg)	564	333	880
Feed composition (g/kg DM)			
RAS	74	41	79
RE	108	69	180
RC	277	190	100
RVET	30	34	43
Sugar	153	-	85
Starch	-	366	182
Feeding value (g/kg DM)			
VEVI (/kg DM)	924	1066	1054
VEM (/kg DM)	896	1010	985
DVE	59	51	104
OEB	-14	-34	31
Minerals (/kg DM)			
Cu (mg)	4.2	3.4	8.1
Mo (mg)	1.7	0.5	3.5
S (g)	1.7	1.0	2.7
Na (g)	1.2	<0.1	3.8
K (g)	29.6	10.9	12.8
Mg (g)	1.7	1.0	4.7
Ca (g)	4.0	1.2	9.1
Fe (mg)	144	62	232

Appendix 2 Liver Cu concentration

Liver Cu concentration (in mg/kg DM) as analysed at the start (wk -6) and end (wk 4) of the trial, and calculated total liver Cu storage in mg per calf.

Group	Calf	Week -6		Week 4	
		Liver Cu (mg/kg DM)	Total liver Cu* (mg)	Liver Cu (mg/kg DM)	Total liver Cu* (mg)
CON	**382**	137	307	108	360
CON	**642**	225	547	103	389
CON	**736**	241	427	n.a.	n.a.
CON	**4738**	178	463	129	482
CON	**5603**	170	500	120	536
CON	**9946**	210	489	108	406
RBC1	**639**	111	258	71	254
RBC1	**1847**	129	329	36	134
RBC1	**2305**	237	547	142	466
RBC1	**7075**	160	405	71	268
RBC1	**8053**	239	554	161	538
RBC1	**8769**	157	378	119	422
RBC2	**3988**	225	547	213	707
RBC2	**5633**	201	480	160	525
RBC2	**7221**	177	476	131	502
RBC2	**7229**	164	371	81	262
RBC2	**8154**	270	575	145	479
RBC2	**9986**	179	510	99	427

*Calculated liver mass by 2% of body weight

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Livestock Research Report 905

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