Harper Adams University

A Thesis Submitted for the Degree of Doctor of Philosophy at

Harper Adams University

HARPER ADAMS UNIVERSITY

Effect of nano copper oxide and nano zinc oxide on mineral bioavailability, performance, and health of ruminants

Angharad Williams

BSc (Hons) Bioveterinary Science, University of Liverpool, 2014

MSc Animal Nutrition, University of Nottingham, 2015

Thesis submitted in fulfilment of the requirement of the degree of Doctor of Philosophy at Harper Adams University

Submitted: 26 May 2020

Declaration:

I declare that the work in this thesis is original and was composed by myself. None of this work has been presented in any previous application for any degree or qualification. I have acknowledged all sources of information used in this thesis by means of references.

Angharad Williams

Publications

Williams, A, Mackenzie, A.M. and Sinclair, L.A., 2018. Determination of the bioavailability of trace minerals supplied as nanoparticles in sheep and their effect on performance and health. Proceedings of the British Society of Animal Science, Volume 9, pp. 14

Williams, A, Mackenzie, A.M, Bennison, J.J and Sinclair, L.A., 2019. Determination of the bioavailability of nanoparticle copper oxide using a copper, depletion, repletion technique in growing lambs. Proceedings of the British Society of Animal Science, Volume 10, pp. 50

Williams, A, Mackenzie, A.M, Bennison, J.J and Sinclair, L.A., 2019. Effect of nanoparticle copper oxide fed alone or in combination with dietary antagonists on mineral status and performance of dairy cows. ADSA Annual Conference Program, Volume 102, pp. 279.

Acknowledgements

A big thank you to Professor Liam Sinclair and Dr Sandy Mackenzie for supervising this project. I am extremely appreciative of the opportunity to study for a PhD at Harper Adams University with your support and guidance. Thank you to Agrimin Ltd, especially Dr Jamie Bennison for funding this research and providing guidance throughout this process. Thankyou, to Dr Fabio Veronesi, for his input into trial design and statistical analysis of the studies.

I am very grateful to the farm and APAS staff for their help in conducting both of my lamb trial and dairy cow trials. Also, to undergraduate and post graduate students, Sioned Price, Connie Dale, Harry Bowers and Emma Taylor who assisted in the experimental work as part of their final year projects.

Thank you, to Dr David Price, Perkin Elmer, who's encouragement and technical help were invaluable during the method development of nanoparticle size analysis by SP-ICP-MS. I am also grateful for David's input into the written chapter for the method development.

Thank you to all of my friends and the postgraduate community at HAU who have been so supportive during the PhD process. Special thanks to Cara, Ellie, Emily and Katie for their help with my horses when I was very busy with trial work, having my appendix removed or recovering from a torn ankle ligament! A big thank you to Ellie for putting up with living with me for a year and your encouragement when everything seemed to be going wrong. Thanks to my mum, Jess and Alex for the many hours you have spent proof reading – I am very grateful.

Thanks to Joe for encouraging me to actually sit down and write my thesis and rewarding me with gin! I am very grateful for your support, encouragement, and love while I attempted to balance thesis writing and my post education career.

Finally, thank you to my family, friends and colleagues for your support during my education and career, I couldn't do it without you all.

Abstract

Ruminants are often supplemented with trace minerals to maintain health, productivity, and profitability. Trace minerals can be supplemented in an oxide form but compared to other forms available, such as sulphate, oxides have a relatively low bioavailability. For example, the bioavailability of Cu oxide is approximately 0.15 compared to Cu sulphate. Improving the bioavailability of Cu oxide could reduce the dose required to maintain animals Cu status and reduce excretion into the environment. Studies have reported an improvement in the bioavailability of trace elements when supplied as nanoparticles, and three studies were conducted to evaluate this hypothesis.

The first study, conducted in lambs, compared the bioavailability of Cu and Zn oxide (nano and conventional) with Cu and Zn sulphate. Differences in Zn status were difficult to detect because the basal ration had a higher Zn concentration than predicted therefore the supplement was only a small proportion of total Zn intake. Final liver Cu concentration indicated that nano Cu oxide may have a greater bioavailability than conventional Cu oxide, but greater statistical power was required. To increase statistical power, the second study reduced the variation in Cu status between lambs using a Cu depletion, repletion technique and the number or replicates was increased. The bioavailability of nano Cu oxide was greater than conventional as indicated by the increased ($P < 0.05$) storage of Cu in the liver and higher ($P = 0.033$) whole blood SOD activity. The third study was conducted to determine the bioavailability of nano Cu oxide in the presence of antagonists (S and Mo) in dairy cows. Liver Cu concentration was increased ($P = 0.042$) in cows fed nano Cu oxide compared to conventional Cu oxide and this effect was particularly pronounced when there were additional antagonists in the diet. These results indicate that nano Cu oxide does have a greater bioavailability than conventional Cu oxide, but further work is required to identify a relative bioavailability value, which is between 0.23 – 0.81 compared to Cu sulphate.

There are concerns over the safety of nanoparticles, but parameters measured in these three studies indicated that there was no effect on animal performance and health. Plasma GGT activity, an indicator of liver health, and haematology profile did not differ between Cu sources. This series of studies, was however, relatively short term therefore longer studies would be required to confirm this. Nanoparticles were unable to be detected in milk and blood therefore it is possible that particles are not retained in their original form in the digestive tract. Further method development work is required using SP-ICP-MS for detection of nanoparticles in biological samples.

List of Abbreviations

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AAS	Atomic absorption spectrometry
BCS	Body condition score
Cp	Ceruloplasmin
CP	Crude Protein
Cu	Copper
d	Day
DM	Dry matter
DMI	Dry matter intake
EDTA	Ethylenediaminetetraacetic acid
GGT	Gamma-glutamyl transferase
g	Gram
h	Hour
HCT	Haematocrit
Hb	Haemoglobin
ICP-MS	Inductively coupled plasma mass spectrometry
kg	Kilogram
mg	Milligram
ME	Metabolisable energy
Mo	Molybdenum
NDF	Neutral detergent fibre
P	Probability

RBC	Red blood cell
SP-ICP-MS	Single particle inductively coupled plasma mass spectrometry
Se	Selenium
SED	Standard error of the difference
S	Sulphur
SOD	Superoxide dismutase
TMR	Total mixed ration
WBC	White blood cell
Zn	Zinc

Table of Contents

List of Tables

List of Figures

1. Literature review

1.1. Introduction

Trace minerals are vital to animal health and productivity, therefore almost all cattle and sheep receive some form of trace mineral supplementation because of low concentrations in the diet or interference from antagonists (Herdt & Hoff, 2011; Kendall, et al., 2012; Spears & Weiss, 2014). Two trace elements commonly supplemented are copper (Cu) and zinc (Zn) (Herdt & Hoff, 2011). Copper is required for development of the nervous system, growth, including wool and hair, bone and connective tissue development, pigmentation of coat, iron (Fe) transportation, immunity and fertility. Zinc in ruminants is required for gene expression, appetite control, fat absorption and antioxidant defense (Suttle, 2010).

There are various methods of supplementation of trace minerals including, in feed incorporation using premixes, via water systems, free access supplements, injectables and boluses (Sinclair & Atkins, 2015). Boluses can provide a sustained daily release of trace elements for 6 – 8 months and eliminate the problem of individual intake (Kendall, et al., 2012). Boluses are particularly suited to grass based systems where animals receive little to no supplementary feed (Grace & Knowles, 2012). However, there are some drawbacks to the boluses currently available. Some of the trace elements used in boluses have a low bioavailability, for example approximately 7% of Cu released from boluses containing Cu oxide is subsequently stored in the liver (Parkins et al., 1994). Low bioavailability also results in significant excretion into the environment (Brugger & Windisch, 2015).

Improving the bioavailability and precision of trace mineral supply could reduce environmental excretion as well as decreasing the dose required to maintain the mineral status of the animal (Lopez-Alonso, 2012; Overton & Yasui, 2014; Brugger & Windisch, 2015). Studies have reported a significant improvement in the bioavailability of trace elements in ruminants when supplied as nanoparticles compared to larger particle sizes (Pelyhe & Mezes, 2013; Zhao, et al., 2014). Particles in the nano scale have at least one dimension less than 100 nm (De Jong & Borm, 2008; Auffan, et al., 2009) and supplying the mineral as nanoparticles changes the physical properties, such as surface area to volume ratio and density but the chemical properties of the mineral remain the same (Daniel & Astruc, 2004; Auffan, et al., 2009). The research on nanoparticle supplementation in ruminants is limited and most of the research

undertaken, has focussed on selenium (Se) and to a lesser extent Zn (Xun, et al., 2012; Pelyhe and Mezes, 2013) but there has been no research on Cu. This programme of studies aimed to investigate the effects of nano Cu and Zn oxide on the mineral status and performance of sheep and cattle. The overall aim was to include Cu oxide and Zn oxide with a higher bioavailability in boluses, but in order to accurately assess the bioavailability it was more appropriate to incorporate nanoparticles in feed. This method removed the variable of bolus release rate and ensured that all animals on study receive the same daily amount of Cu or Zn. Thus, the studies detailed in this thesis will be for ruminants fed nano and conventional minerals in feed. Future research will be required to include the most bioavailable mineral sources in a bolus, confirm bolus release rates and measure the effect of the bolus on mineral status, performance, and health of ruminants.

1.2. Copper

1.2.1. Copper Function

Copper is required as a component of more than 300 different proteins, with a variety of functions in the body (Suttle, 2010). Some Cu dependent enzymes that are found in mammalian tissues and their function are listed in Table 1.1.

Table 1.1 Some Cu-dependent enzymes found in mammalian tissues and their functions (Suttle, 2010).

Enzyme	Functions
Ceruloplasmin	Conversion of Fe^{2+} to Fe^{3+}, Fe transport and an antioxidant
Cytochrome c oxidase	Terminal electon-transfer respiratory chain
Diamine oxidase	Oxidative deamination of diamines and their derivatives
Dopamine-β-monooxygenase	Catecholamine metabolism
Hephaestin	Export of iron from intestine
Ferroxidase II	Iron oxidation
Lysyl oxidases	Desmosine cross-linkages in connective tissues
Monoamine oxidase	Oxidative deamination of monoamines
Superoxide dismutase	Intracellular and extracellular dismutation of O_2- to H_20_2
Thiol oxidase	Disulphide bond formation
Tyrosinase	Conversion of tyrosine to melanin

As a result of the varied function of Cu in biological processes in the body, Cu deficiency can have negative effects on the animal's nervous, immune, cardiovascular and reproductive systems (Suttle, 2010). Clinical signs of Cu deficiency in ruminants include ataxia, abnormal wool and hair, depigmentation, anaemia, bone and connective tissue disorders, cardiovascular disorders, reduced immunity, diarrhoea, infertility and impaired growth rates (Suttle, 2010; Herdt & Hoff, 2011).

Lambs can present with ataxia if the ewe had a Cu deficiency during pregnancy, this condition is commonly known as "swayback" (Suttle, 2012b). The main clinical signs that are observed are spastic paralysis, especially of the hind limbs, severe un-coordination, convulsions and blindness (Gambling, et al., 2011). Swayback is a result of insufficient Cu availability at the critical stage of myelination during mid-pregnancy (Suttle, 2010). Insufficient Cu is associated with a reduction in cytochrome C oxidase (CCO) activity, which is important for lipid synthesis, thus, if there is a reduction in activity of the enzyme there is reduced myelination and pathological lesions occur in the white matter of the central nervous system (Barlow, et al., 1960; Howell, 1968; Patterson, et al., 1971). Sheep are susceptible to this condition because in mid-pregnancy there is a rapid phase of myelination in the foetal central nervous system (Suttle, 2010). After birth lambs undergo a second phase of spinal cord myelination and delayed onset ataxia can occur if there is insufficient Cu availability in late pregnancy (Patterson, et al., 1974). These are serious clinical consequences of Cu deprivation and the lesions of swayback are irreversible (Suttle, 2010). This condition does not occur in cattle because they undergo a much slower myelination of the central nervous system (Suttle, 1987).

In sheep low Cu availability can also result in abnormal wool growth whereby the wool loses its tensile strength and elastic properties and appears "stringy" (Suttle, 2010). The same appearance can be found in Cu deficient cattle where the coat has a thin and wavy appearance (Suttle & Angus, 1978). Similarly, in cattle with pigmented coats loss of coat colour, especially around the eyes, can be a sign of Cu deficiency (Mills, et al., 1976). Depigmentation is likely to be due to a reduction in the activity of tyrosinase which functions to convert tyrosine to melanin (Bonham, et al., 2002; Prohaska, 2006).

The enzyme ceruloplasmin (Cp) is an important antioxidant but also functions as part of the Fe transport chain therefore the Cu status of animals can also affect Fe status (Collins, et al., 2010). Hephaestin and ferroxidase II are also important Cu dependent enzymes that are involved in Fe absorption and metabolism, and low Cu status can therefore lead to anaemia (Prohaska, 2006). If Cu deficiency occurs in growing animals

this can cause bone disorders, osteochondrosis, osteitis fibrosa (Mills, et al., 1976), “beading” of the ribs from overgrowth of costochondral junctions, osteoporosis and incidence of spontaneous bone fractures (Cunningham, 1950; Whitelaw, et al., 1979). There are also some, anecdotal reports of connective tissue disorders in Cu deficient sheep and cattle, and this is likely to be linked to reduction in the activity of lysyl oxidases (Bonham, et al., 2002; Prohaska, 2006).

Cytochrome c oxidase is a critical enzyme in the respiratory chain, responsible for electron transfer and therefore important for energy generation in tissues (Srinivasan & Avadhani, 2012). As a result, reductions in CCO can cause a range of pathogenic consequences, mainly related to impairment and breakdown of cells (Fell, et al., 1985; Mills, et al., 1976; Jones & Suttle, 1987). A reduction in CCO activity can be due to Cu deficiency. Superoxide dismutase (SOD) is another vital enzyme that can be responsive to Cu in the diet, it’s role is in protection of tissues from free radical oxidants (Ighodaro & Akinloye, 2018). The activity of SOD converts oxygen free radicals to hydrogen peroxide as a method of disposal, but activity is reduced in animals of low Cu status (Suttle, 2010).

Diarrhoea has been reported in Cu deficient grazing cattle (Mills, et al., 1976) and sheep on high molybdenum (Mo) diets (Suttle & Field, 1968; Hogan, et al., 1971). This clinical manifestation is thought to be due to acute localised thiomolybdate induced Cu depletion of the intestinal mucosa (Suttle, 1991). Cardiovascular disorders have also been linked to Cu deficiency but most of the evidence for this is anecdotal (Suttle, 2010). Infertility, due to delayed or depressed oestrus has also been reported but there may actually be a stronger relationship between fertility and dietary Mo (Humphries, et al., 1983; Phillippo, et al., 1987). Similarly, poor growth is often reported in Cu deficient sheep (Whitelaw, et al., 1979; Wooliams, et al., 1986), and cattle (Thornton, et al., 1972a; Thornton, et al., 1972b; Whitelaw, et al., 1984) but these are mostly reported in studies where the Cu deficiency was induced by feeding diets high in Mo (Humphries, et al., 1983; Phillippo, et al., 1987; Gengelbach, et al., 1994).

1.2.2. Copper Requirement

The Cu requirement of sheep based on NRC (2007) is 3 – 7 mg/kg DM when the Mo content of the diet is less than 1 mg/kg DM (Table 1.2). The Cu requirement of dairy cattle is calculated more accurately depending on stage of production from growing heifer to lactating cow (NRC, 2001). For example, the requirement of a lactating cow

yielding 40 kg of milk per day is 16 mg/kg DM when the Mo content of the diet is less than 1 mg/kg DM and sulphur (S) is less than 2.5 g/kg DM. Dietary content of Mo, S and Fe all have an effect on Cu requirement an explanation is detailed in Section 1.2.7.

Table 1.2 Copper requirement (mg/kg DM) of sheep (NRC, 2007) and dairy cattle (NRC, 2001) depending on stage of production and when the diet contains less than 1 mg Mo/kg DM.

Animal	Cu requirement mg/kg DM
Sheep	3 – 7
Lactating cow, 40 kg milk/d	11
Late gestation cow, 270 d gestation	12 – 18
Growing heifer, 300 kg live weight, ADG* 0.7 kg	10

*Average Daily Gain

The maximum tolerable intake of Cu for sheep is 25 mg/kg DM (NRC, 1985). Cattle are more tolerant of high levels of dietary Cu than sheep with a maximum tolerable intake of 40 mg/kg DM unless dietary Mo is greatly elevated in which case it may be higher (NRC, 2001). According to EU regulation 2018/1039 on additives in animal feed the maximum permitted level for ruminating cattle is 30 mg/kg at 88% DM which equates to 34 mg/kg DM. The rule still stands that supplementation above this level can only be undertaken following a full risk assessment and written prescription by a veterinary surgeon (Bone, et al., 2011). If cattle feed is less than 20 mg Cu/kg the label must contain the warning “the level of copper in this feedingstuff may cause copper deficiencies in cattle grazing pastures with high contents of molybdenum or sulphur” (ACAF, 2010). For sheep, the maximum permitted level is 15 mg/kg at 88% DM which equates to 17 mg/kg DM. However if sheep feed exceeds 10 mg Cu/kg the label must state “the level of copper in this feedingstuff may cause poisoning in certain breeds of sheep” (ACAF, 2010). Sheep differ in their Cu requirement by breed because of changes in storage in the liver (Wiener, et al., 1978; Wooliams, et al., 1983), see Section 1.2.5.

1.2.3. Copper Toxicity

The potential for Cu toxicity is greater than any other required trace mineral because the concentration that can cause toxicity is only four fold greater than the amount required for normal status (Goff, 2018). Sheep are more susceptible to Cu toxicity than cattle because at the same dietary Cu concentrations cattle limit Cu storage before sheep (Phillippo & Grace, 1983). Acute Cu toxicity is uncommon but has been reported

when sheep or cattle are administered with excessive therapeutic doses or ingest very large doses of Cu; for example from contaminated feed (Bidewell, et al., 2002). Chronic toxicity is more common and usually occurs when there is an acute release of Cu from the liver following a prolonged period of Cu excess in the diet (e.g. 20 mg/kg DM in sheep or 100 mg/kg DM in cattle) but this can also depend on the Mo and S content of the diet (Bidewell, et al., 2002; Herdt & Hoff, 2011). Over time Cu accumulates in the liver and eventually the capacity for storage is exceeded and hepatocyte necrosis occurs leading to release of Cu from the liver (Bidewell, et al., 2002). When Cu is not bound to proteins, as it normally is for transport and storage, the free Cu ions cause oxidation of exposed cells causing acute haemolytic crisis (Suttle, 2010).

Prior to acute haemolytic crisis there are variable clinical signs that have been reported (Bremner, 1998; Bidewell, et al., 2002). Rumen stasis or erratic rumen movements, bloat, depression, congested mucous membranes, nasal discharge, jaundice, recumbency, anorexia, ataxia, head pressing as result of hepatic encephalopathy, tachycardia, dyspnoea, photo-sensitisation, discoloured urine, pyrexia, polyuria, polydipsia and diarrhoea have all been reported as symptoms of Cu toxicity in sheep and cattle (Gummow, 1996; Bidewell, et al., 2002; Suttle, 2010).

1.2.4. Copper Absorption

The primary site of absorption for Cu is the duodenum of the small intestine, with a minority in the abomasum and colon (Turner, et al., 1987; Gooneratne, et al., 1989; Goff, 2018). If the Cu in the diet is soluble in the conditions of the rumen, for example Cu sulphate, it dissociates to Cu^{2+} ions (Goff, 2018). These ions can then interact with other substances in the rumen fluid such as thiomolybdates (Section 1.2.7), ammonium ions, volatile fatty acids and lignin (Ghodrat, et al., 2015; Goff, 2018). Depending on the substance that the Cu binds with it can form an insoluble or indigestible compound that is excreted in the faeces (McDonald, et al., 1996; Spears, 2003), a soluble compound that is transported into the abomasum (Reid & Attaelmannan, 1998) or some Cu will remain unbound (Goff, 2018). Some Cu in the diet of ruminants is not soluble in rumen fluid, for example Cu oxide, therefore unless there is interference from Cu antagonists (Section 1.2.7), Cu reaches the acidic abomasum (Spears, 2003). An acidic environment causes the Cu to dissociate to Cu^{2+} ions (Judson, et al., 1982; Goff, 2018). The proportion of Cu that dissociates is related to the time spent in the acidic conditions of the abomasum before reaching the intestine (Judson, et al., 1982).

Copper is unusual compared to other minerals in that the denatured proteins in the abomasum have an affinity for Cu^{2+} and therefore the majority of ions do not remain in solution (Bremner, 1970; Ward & Spears, 1993). The proteins and Cu^{2+} form an insoluble complex (Ivan, et al., 1986). In the duodenum the pH of 7 to 7.3 causes a proportion of the Cu to be released as the protein changes conformation (Bremner, 1970; Ward & Spears, 1993). The Cu ions that remain unbound and those that had interacted with volatile fatty acids and organic compounds then reach the intestine where they can be absorbed (Goff, 2018)

In order for Cu to be absorbed in the intestines it must cross the single layer of epithelial cells joined together by proteins to form tight junctions that line both the small and large intestine. The surface of the epithelial cells in contact with the lumen of the gut is referred to as the apical membrane and its surface area is increased by microvilli known as the brush border to increase absorption (Goff, 2018). The surface of the apical membrane is covered in glycocalyx, a mixture of mucus and glycoproteins (Johansson, et al., 2011). A water layer adheres to the glycocalyx by surface tension. The surface of the epithelial cells in contact with the lamina propria is known as the basolateral membrane. The lamina propria is made up of loose connective tissue with extracellular fluids and an extensive vascular and lymphatic network (Goff, 2018).

The major pathway for Cu absorption across the epithelial cells of the intestine is transcellular transport (Figure 1.1; Van de Berghe & Klomp, 2009; Goff, 2018). The Cu^{2+} ions move from the digestive fluids through the unstirred water layer and glycolayx and reach the apical membrane. At the apical membrane brush border Cu metalloreductases reduce Cu^{2+} to Cu^{+} (Goff, 2018). It is thought that Cu can only move across the apical membrane into enterocytes in the Cu^{+} form, primarily using a specific high affinity Cu^{+} transport protein, CRT1 (Hashimoto & Kambe, 2015). The divalent metal transporter 1 protein, DMT1 (also transports Fe, Zn and manganese (Mn)) can also be used to move Cu^{+} into enterocytes (Arredondo, et al., 2003; Lutsenko, et al., 2007). More recently, as a result of gene knockout experiments in mice, a third non-CTR1 mechanism for movement of Cu across the apical membrane has been suggested as an anion transport system (Zimnicka, et al., 2011).

Once inside the enterocyte the Cu is collected by chaperone proteins that direct it to different sites in the cell (Goff, 2018). Chaperone proteins deliver Cu for inclusion in many of the enzymes discussed in Section 1.2.1 such as CCO, hephaestin and SOD (Markossian & Kurganov, 2003). If the animal has inadequate Cu stores then the

majority is collected by a specific chaperone protein, Atox1 (Lutsenko, et al., 2007). The protein Atox1 transports Cu to the golgi apparatus where it is transferred to another protein, ATP7A (Lutsenko, et al., 2008). A complex of ATP7A and six Cu^{+} ions is formed and incorporated in a Golgi transport vesicle and translocated to the basolateral membrane where the Cu^{+} is converted back to Cu^{2+} by Cu oxidase and released into extracellular fluids in the interstitial space by exocytosis using energy from ATP (Lutsenko, et al., 2007; Lutsenko, et al., 2008; Van de Berghe & Klomp, 2009). Energy is required to pump Cu^{+} across the concentration gradient from the low Cu concentration inside the cell compared to the extracellular fluid (Goff, 2018). From the extracellular fluids the Cu^{2+} ions diffuse into plasma where the majority are bound to albumin and histidine and transported to the liver (Hashimoto & Kambe, 2015).

If dietary intake of Cu is high then large amounts of Cu^{+} enter the cell because the process of Cu entering the enterocyte via the apical membrane from the freely ionised soluble Cu pool is approximately 40 to 60% efficient (Goff, 2018). The high concentration of Cu in the cytoplasm triggers ATP7A to become more active and release Cu^{+} at a higher rate into the bloodstream in order to reduce the cytoplasm content (Goff, 2018). If the animal has an adequate store of Cu the enterocytes also start to produce metallothionein in large amounts (Van de Berghe & Klomp, 2009). When Cu^{+} ions cross the apical membrane of the enterocyte the majority are bound to metallothionein opposed to Atox1 (Goff, 2018). Some of the Cu^{+} bound to metallothionein is given up to Atox1 but the majority remains bound and when the enterocyte dies it gets sloughed off so that Cu bound to the metallothionein in the dead cell is excreted in faeces (Van de Berghe & Klomp, 2009). The brush border Cu metalloreductase and CTR1 are also down regulated when Cu stores are adequate (Kaplan & Lutsenko, 2009; Van de Berghe & Klomp, 2009).

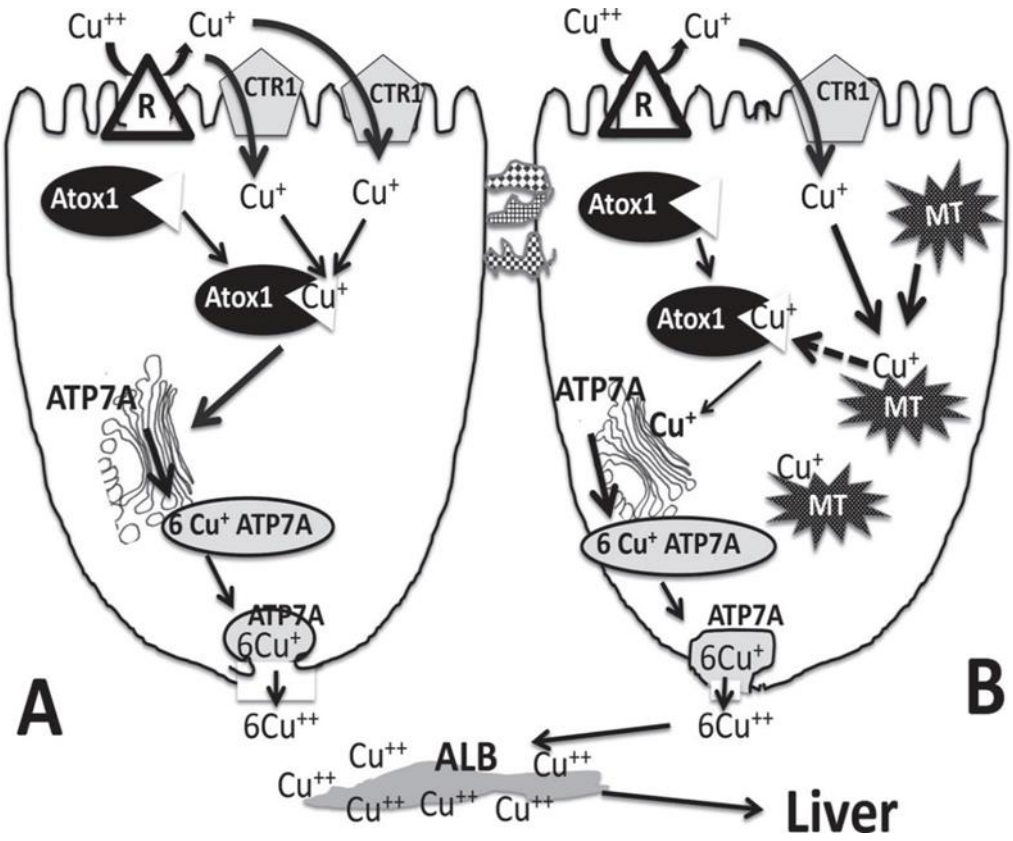

Figure 1.1 Copper absorption via transcellular transport through the enterocyte in the intestine. Firstly, brush border reductase converts Cu^{2+} to Cu^{+}.

A) A Cu transporter 1 protein (CTR1) facilitates diffusion of the Cu^{+} across the apical membrane. The Cu^{+} becomes bound to a Cu chaperone protein (Atox1). Atox1 shuttles the Cu^{+} to the Golgi apparatus, where it is transferred to a Cu transport protein (ATP7) capable of holding 6 Cu^{+} ions that is within the membrane of a Golgi transport vesicle. The ATP7A vesicle carries the 6 Cu^{+} safely across the cell. The transport vesicle membrane melds with the basolateral membrane releasing the Cu^{+} into the extracellular fluid by exocytosis. The ATP7A transport vesicle membrane contains a Cu oxidase, which oxidises the Cu^{+} to Cu^{2+} as it is released to the interstitial space. The Cu^{2+} ions become bound to albumin (ALB) for transport.

If the body has adequate Cu stores then pathway B commences.

B) The enterocytes begin to produce metallothionein (MT) in large amounts. The Cu^{+} ions entering the cell are now more likely to be bound to MT than to the Atox1 chaperone. Metallothionein can give up Cu^{+} to the Atox1, but only very slowly, and much of the metallothionein-bound Cu may be trapped in the enterocyte when it dies and is sloughed off and is excreted with the faeces. High Cu status also reduces the amount of CTR1 in the apical membrane (Goff, 2018).

Paracellular transport can absorb a small proportion of Cu across the intestinal wall (Goff, 2018). This is likely to be minimal because there is a positive electrical potential difference across the tight junction which will cause resistance to absorption of cations (Field, 2003). This resistance can be overcome if there is a large concentration gradient of Cu between the gut lumen and the interstitial space between enterocytes. For this to occur the Cu must not be bound to any organic molecules, such as proteins, and must be in solution. In this case the force created by the concentration gradient can be large enough to push the Cu across the tight junction, into the interstitial space, and then into the capillary in a process known as paracellular absorption (Goff, 2018). Paracellular absorption is a non-saturable process that is completely dependent on concentration gradient, mineral size and charge on the ion (Nellans, 1991; Goff, 2018).

Solvent drag could be responsible for greater movement of Cu than paracellular transport, particularly because the acidic environment in the abomasum promotes movement of Cu ions into solution (Judson, et al., 1982). Solvent drag moves dissolved minerals across tight junctions with the bulk flow of water (Nellans, 1991; Goff, 2018).

1.2.5. Copper Transport and Storage

Copper bound to proteins in the plasma is transported to the liver and stored rapidly (Weiss & Linder, 1985). In the hepatocytes Cu^{2+} is converted to Cu^{+} by Cu reductase and CTR1 is used to move it across the cell membranes (Kim, et al., 2008; Van de Berghe & Klomp, 2009). Similarly to the intestine Cu is moved around the hepatocyte by chaperone proteins which direct it to enzyme pathways such as CCO and SOD (Goff, 2018). The Cu in the liver maintains the Cu concentration in the bloodstream within a very tight range using homeostatic mechanisms (Herdt & Hoff, 2011). If there is excess Cu in the diet it is stored in the liver and when dietary intake is insufficient to meet physiological needs Cu is mobilised from the liver, therefore, concentration of Cu in the blood will not be reduced until liver Cu is less than 40 mg/kg (Claypool, et al., 1975).

As a result of homeostatic mechanisms controlling blood Cu concentration, the liver can store large amounts of Cu (Suttle, 2010). In the liver Cu is present in mitochondria, microsomes, nuclei, lysosomes and liver parenchymal cells (soluble Cu fraction) (Gooneratne, et al., 1989). To store Cu in the liver it is bound to metallochaperones and metallothionein (Goff, 2018). Studies have shown that Cu storage in the liver is dependent on breed in sheep (Wiener, et al., 1978; Gooneratne, et al., 1989). For

example, Wooliams et al. (1983) reported that Welsh Mountain sheep store more Cu in their liver than Scottish Blackface fed the same concentration of Cu in the diet (Figure 1.2).

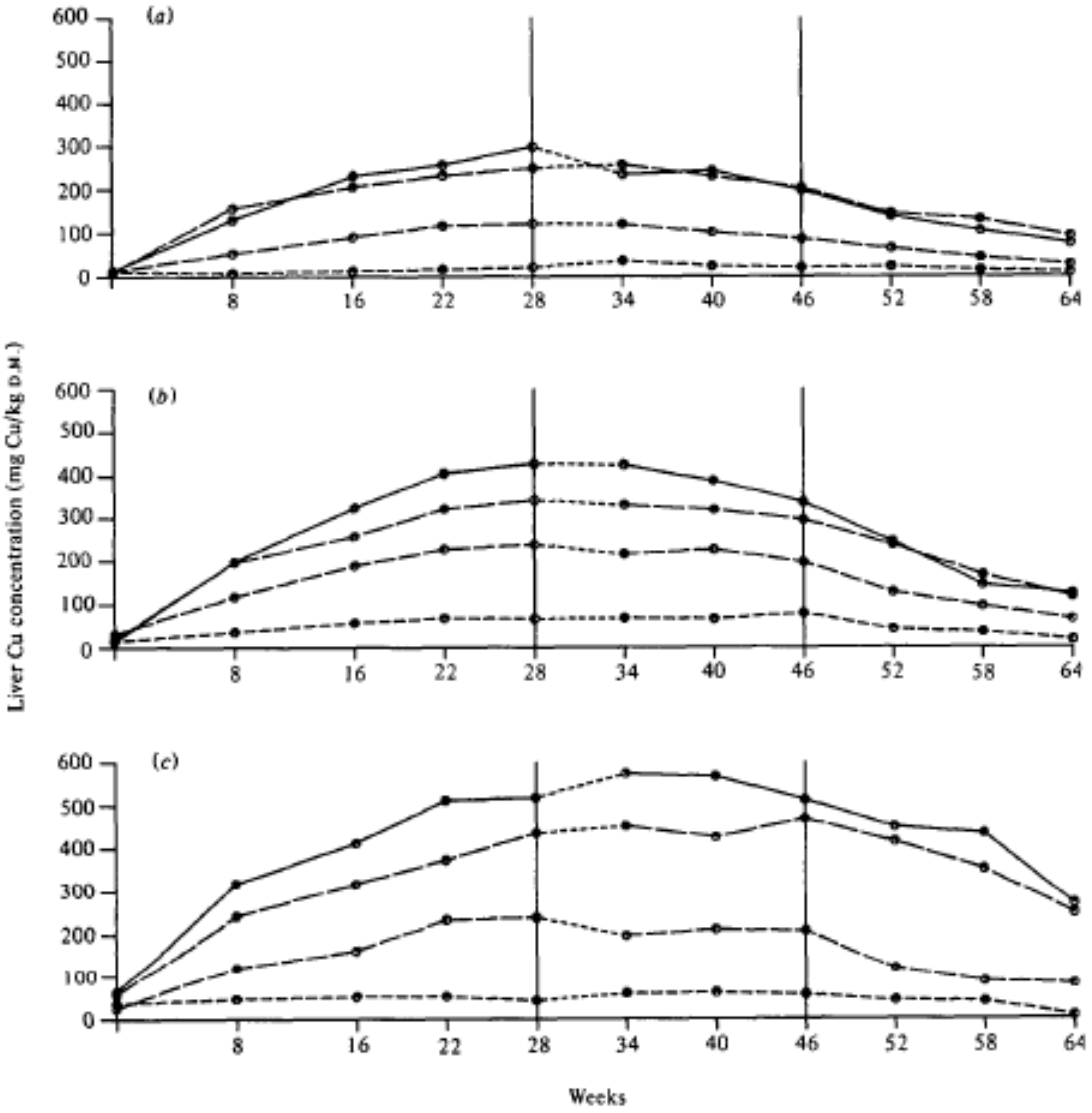

Figure 1.2 Concentration of Cu in the liver (mg/kg DM) of Scottish Blackface (a), Scottish Blackface x Welsh Mountain (b) and Welsh Mountain sheep fed 4 - - - -, 9 -- -- --, 17 --- --- --- or 29 -------- mg Cu/kg DM (Wooliams, et al., 1983). Period is indicated by the vertical lines on the figure.

Figure 1.2 shows the liver concentration results from a study by Wooliams et al. (1983) which was conducted to investigate the accumulation and depletion of Cu in different breeds of sheep fed diets differing in their Cu content, 4, 9, 17 and 29 mg Cu/kg DM over 28 weeks, followed by an 18 week period when all sheep were fed 4 mg Cu/kg DM, and finally an 18 week period when all sheep were fed 4 mg Cu/kg DM and 4 mg Mo/kg DM was added to the diet. The study periods are indicated by the vertical lines

on Figure 1.2. During the first period there was a dose response on liver Cu concentration related to Cu content of the diet and there was a greater Cu accumulation in the liver of Welsh Mountains compared to Scottish Blackface with the cross breed in the middle (Wooliams, et al., 1983). Similarly, it has been reported that Scottish Blackface x Texel lambs retain twice as much Cu in their liver as pure-bred Scottish Blackface lambs when fed the same dietary Cu concentration (Wooliams, et al., 1982). In a Cu repletion study it was also reported that based on plasma Cu concentrations Scottish Blackface exhibited a low rate of Cu repletion compared to Welsh Mountain lambs and cross breeds were in the middle (Wiener, et al., 1978). There are fewer reports of breed differences for Cu absorption and retention for cattle (Gooneratne, et al., 1989) but there is growing evidence (Suttle, 2010). Jersey cows have been reported to have a higher Cu requirement than Holsteins cows (Du, et al., 1996) and Simmental higher than Aberdeen Angus (Mullis, et al., 2003; Stahlhut, et al., 2005). Similarly, to the sheep studies of Wooliams et al. (1982 and 1983), Friesian calves have been reported to have higher liver Cu concentration than Galician Blond calves and cross bred calves were intermediary (Miranda, et al., 2006).

Wooliams et al. (1983) reported that in period one of their study all breeds retained proportionally less Cu in their livers as Cu concentration in the diet increased. The Scottish Blackface (Section A on Figure 1.2) were extreme in that they did not retain any more Cu from diets containing 29 mg/kg DM than they did from diets containing 17 mg/kg DM (Wooliams, et al., 1983). Balemi et al. (2010) also reported that when supplementing non-lactating dairy cows with different forms of Cu, that initial liver Cu concentration affects the rate of accumulation. If the initial concentration was less than 1100 µmol/kg the average increase was 4.1 µmol/kg whereas rates were more variable and for some cows negative, when initial concentrations were greater (Balemi, et al., 2010). Similarly, Suttle (2013) reported that changes in liver Cu concentration depend upon initial concentration. The reason for initial liver Cu concentration affecting the change in concentration is reported differently between researchers but it is likely to be a combination of increased endogenous losses (Wooliams, et al., 1983; Grace, et al., 1988; Suttle, 2013) and changes to Cu absorption efficiency (Wiener, et al., 1978).

During the second study period of Wooliams et al. (1983) liver Cu concentration decreased for all breeds (Figure 1.2). The greatest decrease was reported for sheep with the highest liver Cu concentrations, but at similar concentrations the decrease was greater in Scottish Blackface compared to Welsh Mountain. In the final phase liver Cu concentration also decreased because of the addition of antagonists to the diet, but

there was no effect of breed (Wooliams, et al., 1983). Decreases in liver Cu concentration indicated that in period two and three of the study the sheep had to mobilise Cu from the liver in order to maintain plasma Cu concentration (Herdt & Hoff, 2011). Many studies have also reported reductions in liver Cu concentration in sheep and cattle fed diets containing high concentrations of Cu absorption antagonists (Ward, et al., 1996; Ward & Spears, 1997; Sinclair, et al., 2013).

1.2.6. Copper Excretion

Copper is absorbed in small proportions from the diet therefore the majority of ingested Cu moves through the gastrointestinal tract and is excreted in the faeces (Goff, 2018). Active excretion of Cu also occurs via bile in the faeces but the ability of ruminants to control this excretion pathway is limited (Soli & Rambaek, 1978). A small amount of Cu is excreted in urine (Gooneratne, et al., 1989) and is largely unaffected by dietary Cu concentration, although can be increased in sheep if dietary Mo concentrations are high (Smith, et al., 1968). As a result of the limited ability to actively excrete Cu in bile or urine, and their lack of a control mechanism for Cu absorption, ruminants have an increased susceptibility to Cu toxicity compared with monogastrics (Herdt & Hoff, 2011). Although, cattle limit storage of Cu in the liver at lower Cu concentrations than sheep by increasing biliary secretion and are therefore less susceptible to toxicity (Phillippo & Grace, 1983). One variable that does increase Cu excretion in bile, in both cattle and sheep, is high dietary Mo and S concentrations because the Cu is excreted as part of insoluble thiomolybdates molecules (Gooneratne, et al., 1994). Genetic differences in Cu absorption have not been reported in cattle, but differences in excretion have. For example, the Aberdeen Angus breed has been reported to have a higher requirement for Cu than the Simmental breed because they excrete significantly less in the faeces (Gooneratne, et al., 1987; Gooneratne, et al., 1988).

1.2.7. Copper Responsive Disorders

1.2.7.1. Primary

A primary Cu deficiency is a result of the diet containing insufficient Cu resulting in the animal having to mobilise Cu from its liver to maintain blood concentrations until eventually the liver is depleted of its Cu stores (Herdt & Hoff, 2011). When blood Cu concentration falls the animal can no longer maintain all the activity of the Cu

containing enzymes and the signs of Cu deficiency as a result of inadequate function discussed in Section 1.2.1 occur (Suttle, 2010; Herdt & Hoff, 2011).

1.2.7.2. Secondary

Secondary Cu responsive disorders are more common than primary and occur as a result of Cu antagonists in the diet, and not as a direct result of the concentration of Cu in the diet (Suttle, 2010; Herdt & Hoff, 2011). There are three main antagonists to Cu absorption; Mo, S and Fe and they can reduce Cu absorption individually or in combination (Suttle, 2010). Zinc can also reduce Cu absorption (Taylor, et al., 1991 ; Goff, 2018) and this is discussed in Section 1.4.

In the rumen sources of S are reduced to sulphides, which react with Mo to form thiomolybdate compounds (Figure 1.3; Gould & Kendall, 2011). The formation of thiomolybdates is a reversible reaction that is temperature and pH dependent, and driven by the availability of molybdate and sulphide (Price, et al., 1987; Gould & Kendall, 2011). Thiomolybdates are formed in a stepwise dehydration reaction from mono-thiomolybdate to tetra-thiomolybdate whereby one of the O on mono-thiomolydate is replaced by S from hydrogen sulphide and the co-product is water (Gould & Kendall, 2011).

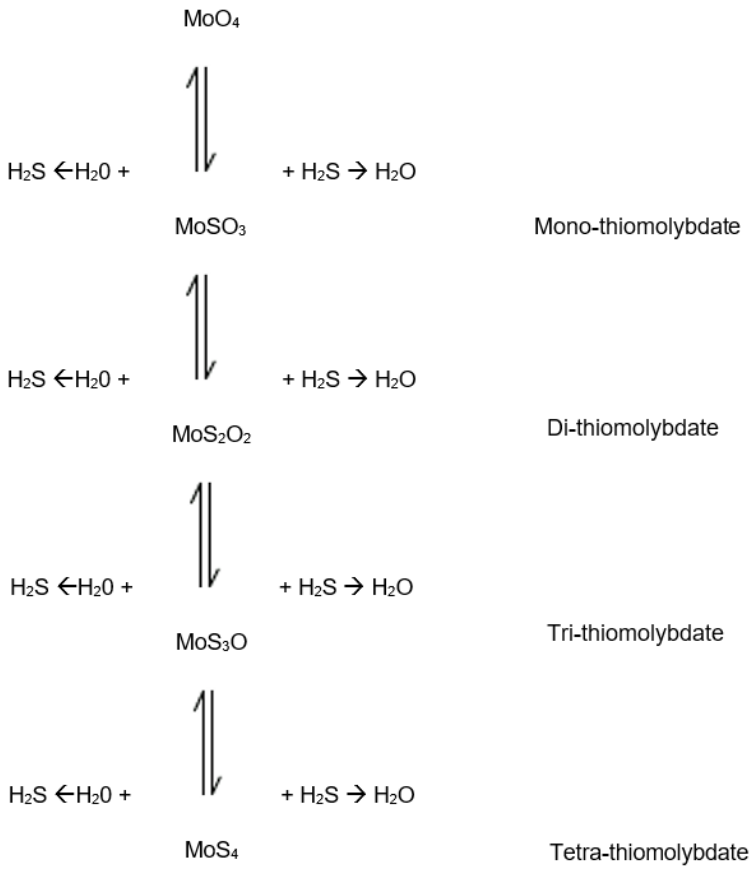

Figure 1.3 Formation of thiomoybdates in the rumen adapted from Gould & Kendall (2011).

Formation of tetra-thiomolybdates increases as pH decreases, and are therefore likely to become more predominant as the starch levels in diets increase and therefore rumen pH decreases (Gould & Kendall, 2011). The reactions are also responsive to the rate at which thiomolybdates are removed from the rumen. Removal from the rumen can be a result of absorption through the rumen wall, movement down the digestive tract to the abomasum, or formation of insoluble Cu thiomolybdates in the rumen which are excreted (Gould & Kendall, 2011). Association of the thiomolybdates with either the solid or liquid phase of the rumen digesta can also have an important role (Price, et al., 1987; Gould & Kendall, 2011). When thiomolybdates are associated with the solid phase (bacteria, protozoa and undigested feed particles), usually tri and tetra complexes, they have a greater stability, whereas when they are associated with the liquid phase they are readily hydrolysed to mono and di-thiomolybdates (Price, et al., 1987; Spears, 2003).

Thiomolybdates readily bind with Cu to form insoluble complexes and prevent Cu from being absorbed (Gould & Kendall, 2011). If Cu in the diet is not absorbed then stores in

the liver will be depleted and the animal will start to reach a state of Cu deficiency (Herdt & Hoff, 2011). Several functions in the body rely on adequate Cu and functioning enzymes therefore the animal will start to exhibit the clinical signs reported in Section 1.2.1 (Suttle, 2010). Tetra-thiomolybdates have a greater affinity for Cu than tri-thiomolybdates, which have a greater affinity than di-thiomolybdates, therefore conditions in the rumen which elicit an effect on the reversible thiomolybdates reactions can have an effect on Cu antagonism and therefore Cu status of the animal (Suttle & Field, 1983). The association of thiomolybdates with either the liquid or solid phase of the rumen digesta can also have an effect. In the rumen Cu is generally associated with the solid phase, thus formation of insoluble Cu-thiomolybdates is more likely if the thiomolybdates are associated with this phase (Price & Chesters, 1985). Additionally, if the thiomolybdate is associated with the solid phase the Cu, it is not released from the complex even under acidic conditions (Allen & Gawthorne, 1987).

If there is insufficient Cu in the rumen then a proportion of the thiomolybdates that are formed may be absorbed into the bloodstream where they will readily bind with any Cu containing substances and potentially inhibit enzyme activity (Suttle, 2010; Gould & Kendall, 2011). Chidambaram (1984) showed that tetra-thiomolybdates can inhibit Cp, CCO, SOD, ascorbate oxidase and tyrosinase activity. Thus, secondary Cu deficiency clinical signs can also be elicited by absorption of thiomolybdates into the bloodstream (Suttle, 2010).

Suttle & Price (1976) produced an equation to predict Cu absorption in the presence of S and Mo in the diet:

$$\text{Log (CuAbsorbability)} = -1.153 - 0.076(S) - 0.013(S \times Mo)$$

This equation, has been criticised for its simplicity because the effect of sulphur and molybdenum varies depending on the source of Cu (Suttle, 2010). There is also a ceiling of 5 mg/kg DM, above which increases in dietary Mo do not elicit a greater inhibitory effect on Cu absorption (Suttle, 2010). The equation also does not account for the third mineral which is a major antagonist to Cu absorption, Fe.

The mechanism by which Fe reduces Cu availability has less supporting evidence than Mo and S and the formation of thiomolydbdates (Gould & Kendall, 2011). One theory is that Fe and sulphide react to form Fe-S, and then Cu displaces Fe to form Cu-S which is not absorbed by the animal (Suttle, 1974). An alternative theory is that in the rumen Fe can react with sulphide and Cu to produce an Fe-Cu-S complex which is not absorbed by the animal (Suttle & Peter, 1985). There is uncertainty about the

mechanism by which Fe reduces Cu availability, partly because studies have reported varying results on Cu storage and metabolism when adding Fe to the diet of ruminants (Campbell, et al., 1974; Rosa, et al., 1986; Sinclair & Mackenzie., 2013).

Recently, Sinclair & Mackenzie (2013) reported that supplementation of Fe reduced liver Cu concentration in dairy cows. Similarly, supplementation of Fe decreased plasma and liver Cu concentration in post-weaning calves but there were no clinical signs of Cu deficiency (Bremner, et al., 1987) and supplementation of Fe, at a rate of 30 mg/kg LW, reduced liver Cu concentration from 179 mg/kg DM to 7 mg/kg DM and reduced plasma Cu concentration and Cp activity in growing cattle (Campbell, et al., 1974). In contrast Rosa et al. (1986) reported that there was no effect of adding 1 g Fe/kg to the diet on serum, liver or spleen Cu concentration in sheep. The sheep in this study were fed a diet low in S, and potentially there was insufficient S present in the rumen to form an Fe-S complex, which could bind with Cu and make it unavailable for absorption. This is in agreement with Suttle (1974) who stated that the Cu-Fe interaction is in part dependent on S.

The effect of S and Mo on Cu metabolism can also be affected by other components of the diet (Sinclair, et al., 2017). For example, Sinclair et al. (2017) reported that added dietary sulphur and molybdenum had a greater influence on liver Cu concentration when dairy cows were fed a grass silage rather than maize silage based diet. The Zn content of the diet in relation to its antagonist effect on Cu must also be considered (Goff, 2018). Zinc can interfere with Cu absorption via upregulation of synthesis of metallothionein or by competition for the DMT1 transporter (Taylor, et al., 1991 ; Lutsenko, et al., 2007). These mechanisms are discussed in detail in Section 1.4.

1.2.8. Copper supplementation

Adequate Cu supply is vital for animal health and performance (Herdt & Hoff, 2011). Feed sources can be variable in their Cu content but more importantly absorption of Cu from the feed stuff can also differ (Suttle, 2010). The reasons for differences in absorption are poorly understood but estimates of the absorption of Cu from common feed sources for Scottish Blackface sheep are shown in Table 1.3.

Table 1.3 Estimates of the absorption of Cu (ACu %) in feed sources of Mo concentration less than 2 mg/kg DM in Scottish Blackface ewes (Suttle, 2010).

	ACu (Mean ± SD)	Number of estimates
Grazed herbage (July)	2.5 ± 1.09	7
Grazed herbage (September/October)	1.4 ± 0.86	6
Silage	4.9 ± 3.2	7
Hay	7.3 ± 1.8	5
Root brassicas	6.7 ± 0.9	2
Cereals	9.1 ± 0.97	3

If cattle or sheep are fed feed sources low in Cu they are often supplemented in order to prevent the negative problems associated with Cu deprivation (Herdt & Hoff, 2011) detailed in Section 1.2.1. More commonly ruminants require Cu supplementation because of high levels of minerals that inhibit absorption, mainly Mo and S (Suttle, 1991; Gould & Kendall, 2011) as detailed in Section 1.2.7. If supplementation is required there are many different sources of Cu available which vary in their bioavailability (Baker & Ammerman, 1995a).

1.2.9. Bioavailability of Cu sources for ruminants

Mineral bioavailability has been defined as “The efficiency with which a natural or manufactured source of an element delivers the element to storage or supplies it to metabolically active tissue” (Wapnir, 1998). In order to asses bioavailability the following factors should be considered; the level of mineral in relation to physiological requirement, an assessment of which tissues should be analysed to examine bioavailability of the mineral, the range of linear response between dose and chosen assessment criteria and the results of a slope ratio analysis to determine relative bioavailability (Wapnir, 1998).

1.2.9.1. Bioavailability of Cu sources

The relative bioavailability of supplemental Cu sources compared to Cu sulphate for cattle and sheep has been collated by Baker and Ammerman (1995a; Table 1.4). The bioavailability data was based on several different methods of measuring Cu bioavailability; liver Cu concentrations, plasma Cu concentrations, Cp, CCO and SOD activity. The standard Cu source to which other Cu sources are commonly compared is Cu sulphate (Baker & Ammerman, 1995a). Copper lysine and cuprous acetate have a

similar bioavailability to Cu sulphate whereas Cu EDTA has a marginally lower bioavailability and cupric oxide and cupric sulfide have a considerably lower bioavailability. In cattle, the bioavailability of Cu sulphate is over six times greater than Cu oxide.

Table 1.4 Relative bioavailability[a] of supplemental Cu sources for cattle and sheep (Baker and Ammerman, 1995a).

	Species	
Source	Cattle	Sheep
Cu sulphate	1	1.00
Cu EDTA	0.95	1.20
Cu lysine	1	-
Cu proteinate	-	1.30
Cupric chloride	1.15	1.15
Cu oxide	0.15	-
Cu sulphide	0.25	-
Cu acetate	1.00	-

[a]Average values rounded to the nearest "0.05" and expressed relative to response obtained with cupric sulphate. Terminology for source is that of the author.

Leudoux et al. (1995) reported similar bioavailability values for inorganic Cu sources in sheep as those reported for cattle and sheep by Baker and Ammerman (1995a) but used the bioavailability of Cu chloride as the reference instead of Cu sulphate (Table 1.5). Similarly, to Baker and Ammerman (1995a), Cu sulphate had the highest bioavailability value which was four times greater than Cu oxide in sheep when based on liver Cu concentration. In contrast to Baker and Ammerman (1995a), Ledoux et al. (1995) reported a lower bioavailability for Cu acetate and Cu chloride compared to Cu sulphate.

Table 1.5 Relative bioavailability of inorganic Cu sources for sheep expressed relative to Cu chloride (Ledoux, et al., 1995).

Source	Relative bioavailability compared to Cu chloride
Cu chloride	1.00
Cu acetate	0.93
Cu sulphate	1.42
Cupric carbonate	1.21
Cu oxide	0.35

1.2.9.2. Inorganic vs Organic Cu sources

Ward et al. (1993) supplemented growing steers with 5 mg Cu/kg DM in the form of Cu sulphate or Cu lysine and reported that the bioavailability was similar when based on plasma Cu concentrations. Rabiansky et al. (1999) conducted a depletion, repletion study in heifers over 211 days. In the repletion phase heifers were supplemented with either 8 or 16 mg Cu/kg in the form of Cu sulphate or Cu lysine, but there was no effect of Cu source on liver and plasma Cu concentrations (Rabiansky, et al., 1999). In contrast, Pott et al. (1994) reported that Cu supplemented to lambs at 11 mg/kg DM, in the form of Cu lysine, had a lower bioavailability of 0.68 relative to Cu sulphate based on liver Cu concentrations collected via liver biopsy. An opposite result has been reported for Cu glycinate which was reported to be a more bioavailable source than Cu sulphate when fed to steers on high S and Mo diets (Hansen, et al., 2008).

Bioavailability of Cu proteinate compared with Cu sulphate has also been investigated in a number of studies. Kincaid et al. (1986) fed Holstein calves (age >12 weeks old) pellets containing 19 mg/kg of Cu as either Cu proteinate or Cu sulphate and 0.6 mg/kg of Mo with a*d libitum* hay which contained 1 mg/kg of Cu and 5 mg/kg of Mo. After 12 weeks plasma Cu concentration was higher for calves supplemented with Cu proteinate than those receiving Cu sulphate (13.7 µmol/L and 11.8 µmol/L respectively) indicating an increased bioavailability (Kincaid, et al., 1986). Similarly in the presence of 5 mg Mo/kg DM Ward et al. (1996) reported that Cu proteinate had a higher bioavailability than Cu sulphate when they were both supplemented at 5 mg/kg DM based on plasma Cu concentration and liver Cu concentration (Ward, et al., 1996). Marques et al. (2016) also reported that late gestating beef cows had higher liver Cu concentrations when supplemented with organic complexed Cu (AAC; Availa 4; Zinpro Corporation, Eden Prairie, MN) compared to Cu sulphate.

In a second experiment by Ward et al. (1996) they investigated depletion and repletion of Cu without adding Mo to the diet. In the absence of additional Mo the diet contained 3 mg Mo/kg DM and 7.3 mg Cu/kg DM and Cu proteinate and Cu sulphate were supplemented at 50 mg of Cu per day equivalent to a supplementary rate of 7.6 – 10.1 mg/kg DM depending on the size of the animal. Bioavailability of Cu proteinate and Cu sulphate was the same based on plasma Cu concentration and liver Cu concentration (Ward, et al., 1996). Similarly, in the presence of a diet containing 10 mg Mo/kg DM, Wittenberg et al. (1990) reported a similar bioavailability between Cu sulphate and Cu proteinate. Sinclair et al. (2013) also reported that plasma and liver Cu concentration

were similar between cows fed Cu sulphate and Bioplex Cu (an organic Cu source, Alltech Inc., Nicholasville, KY) with or without additional S and Mo in the diet.

Studies have reported differing bioavailability values, but Cu oxide consistently has a lower value than Cu sulphate and organic Cu sources (Baker & Ammerman, 1995a; Ledoux, et al., 1995). It is clear that experimental conditions can affect results, along with animal genetics, ratio of test element to basal diet element and interaction with other trace elements in the diet (Baker & Ammerman, 1995a). The most common technique for measuring absorption of Cu in ruminants is using a Cu depletion and repletion technique (Suttle, 1974; Suttle, 1978) this allows for more accurate prediction of bioavailability values when comparing supplements but may overestimate bioavailability of all supplements when they are fed to animals of normal Cu status. A second common technique is to evaluate the bioavailability of Cu when high levels of antagonists such as Mo and S are fed (Sinclair, et al., 2013).

1.2.10. Determining Cu Status

There are a number of variables that can be used to indicate Cu status in ruminants including concentrations of Cu in the blood, enzyme activity, and storage of Cu in organs (Herdt & Hoff, 2011). Assessment of Cu in the blood is a commonly used method but it has many limitations due to the nature of Cu metabolism in the body (Herdt & Hoff, 2011). Firstly, the liver accumulates Cu and releases it into the bloodstream to maintain plasma Cu within a narrow range between 9.4 and 20.0 µmol/L (McDowell, 1992). If excess Cu accumulates in the liver, then a stressful event can cause the liver to release Cu suddenly into the bloodstream causing haemolysis (Johnston, et al., 2014). It is only when this has occurred that plasma Cu concentration will indicate Cu toxicity (Herdt & Hoff, 2011). Similarly, plasma Cu concentration will only indicate deficiency when the concentration of Cu in the liver has declined to a level where the liver cannot release anymore Cu into the bloodstream (Claypool, et al., 1975).

The relationship between liver and blood Cu concentrations is curvilinear, therefore in most situations plasma Cu concentration is not indicative of Cu storage or dietary Cu intake, at liver concentrations greater than 30 – 50 mg/kg DM there is little correlation between the two (Herdt & Hoff, 2011). Presence of high levels of Cu antagonists in the diet can cause formation of thiomolybdates (Gould & Kendall, 2011). Thiomolybdates can be absorbed and enter the bloodstream where they can bind with Cu in the

bloodstream, rendering the Cu functionally biologically unavailable (Gould & Kendall, 2011). When assessing Cu status, plasma concentration would appear adequate despite the animal having functional Cu deficiency (Suttle, 1991). There are also physiological factors that can affect plasma Cu concentration (Etzel, et al., 1982; Small, et al., 1997). For example, Small et al. (1997) reported higher plasma Cu concentration at time of oestrus compared to 21 d after service in heifers. Similarly, in beef cows, plasma Cu concentration was reduced on the day of calving (Small, 1997). Concentrations can also be increased with infection (Etzel, et al., 1982), and therefore health should be considered in plasma Cu concentration analysis.

Approximately 80% of the Cu in the blood is present as the protein Cp (Herdt & Hoff, 2011). Ceruloplasmin can therefore be measured as an indicator of Cu status but similarly to plasma Cu concentration is under homeostatic control and is only of limited use (Suttle, 2010). Interpretation of Cp can be more challenging because it responds as an acute phase protein and is therefore upregulated as part of the general inflammatory response (Herdt & Hoff, 2011). Assessment of SOD, a Cu-dependent enzyme, can also indicate Cu status (Xin, et al., 1991), but it is not a sensitive measure because reduction of SOD activity takes longer than the reduction in plasma Cu and Cp concentration when dietary Cu is insufficient (Andrewartha & Caple, 1980; Ward & Spears, 1997; Gengelbach & Spears, 1998).

Measurement of Cu in the liver is a better indicator of the animal's Cu status because ruminants readily store Cu in the liver (Suttle, 2010). Therefore, it reflects the long term availability of dietary Cu to the animal including the effect that any Cu antagonists (Section 1.2.7) in the diet may have exhibited (Herdt & Hoff, 2011). Liver Cu concentration is therefore a more reliable indicator that can be used to assess whether the animal requires Cu supplementation (Herdt & Hoff, 2011). Liver Cu concentration can be analysed by taking sections of the whole liver at slaughter or by liver biopsy (Hogan, et al., 1971; Sinclair, et al., 2013). Liver biopsies are taken with a 14-G Tru-Cut needle through the intercostal space (Davies & Jebbet, 1981). Only a small section, between 50 and 75 mg of tissue, is required for an accurate estimate of Cu concentration (Hogan, et al.,1971). The normal range for concentration of Cu in the liver is 19 – 508 mg/kg DM (Livesey, et al., 2002). However, a recent survey that reported the liver Cu concentration of 510 cull cows at a single abattoir suggested that dairy breeds had higher concentrations than beef and 40% were over the reference range (Kendall, et al., 2015).

1.3. Zinc

1.3.1. Zinc function

Zinc functions as a component of several enzymes as well as having structural and regulatory functions in the body (Suttle, 2010). Cousins et al. (2006) reported that Zn is required for almost all signaling and metabolic pathways in the body. As Zn functions widely in the body the most commonly reported deficiencies in ruminants are those that become limiting to health and production including gene expression, appetite control, fat absorption and antioxidant defence (Suttle, 2010; Herdt & Hoff, 2011).

Zinc plays a critical role in DNA synthesis and protein metabolism and therefore foetal growth can be affected by Zn deprivation (Hurley, 1981). Gene expression defects that can disturb the development of the foetus are reported to be mostly derived from the effects of Zn deprivation (Chester, 1992). Feed intake, including the amount and pattern can also be affected by Zn status (Suttle, 2010). The appetite regulating hormones, cholecystokinin, cytokine hormone and leptin are upregulated when there is Zn deprivation (Cousins, et al., 2003; Kwun, et al., 2007). Leptin acts as a satiety signal therefore increased expression reduces feed intake (Heisler & Lam, 2017). Following this, changes in the concentration of neurotransmitters in the brain have also been reported, but it is not known if this is a direct result of Zn status or appetite reduction (Kwun, et al., 2007). Fat absorption is affected by Zn status because of the effect on formation of chylomicrons (Noh & Koo, 2001). Zinc dependent phospholipase A2 is secreted by the pancreas and hydrolyses phosphatidylcholine which forms chylomicrons that are important for the absorption of fat micelles (Kim, et al., 1998; Noh & Koo, 2001). The role of Zn as an antioxidant defence is also vital to animal health (Suttle, 2010). Zinc is involved in the enzyme CuZnSOD which protects cells from superoxide free radicals and Zn also offers protection from iron induced lipid peroxidation, and induces metallothionein which scavenges free radicals and protects against oxidant stress (Zago & Oteiza, 2001; Beattie & Trayhurn, 2002).

1.3.2. Zinc Requirement

The Zn requirement of sheep recommended by NRC (2007) is 18 - 35 mg/kg DM, with a maximum tolerable intake of 750 mg/kg DM (NRC, 1985; Table 1.6). The Zn requirement of cattle depends on physiological state, for example a lactating cow producing 40 kg milk per day requires 63 mg Zn/kg DM whereas a cow at 270 d of

gestation requires 23 mg/kg DM. The maximum tolerable intake for cattle is suggested to be 300 to 1000 mg/kg DM. When feeding high levels of Zn the effect on absorption and metabolism of Cu must be considered (NRC, 2001).

Table 1.6 Zinc requirement (mg/kg DM) of sheep (NRC, 2007) and dairy cattle in different physiological states (NRC, 2001).

Animal	Zn requirement mg/kg DM
Sheep	18 - 35
Lactating cow, 40 kg milk/d	63
Late gestation cow, 270 d gestation	23
Growing heifer, 300 kg live weight, ADG 0.7 kg	33

1.3.3. Zinc Toxicity

Compared to minerals, such as Cu discussed earlier, ruminants have a higher tolerance to increased dietary Zn intake (Suttle, 2010). Zinc toxicity was observed in cattle fed 900 mg Zn/kg diet (Ott, et al., 1966a). Similarly, depressed feed consumption was observed in lambs fed 1500 mg/kg diet, while 1000 mg/kg diet reduced gains and feed efficiency. Further clinical signs were not observed but prolonged consumption of high Zn levels caused death (Ott, et al., 1966b). In contrast, diets providing more than 1000 mg Zn/kg DM are routinely fed to cattle in New Zealand to prevent facial eczema without signs of Zn toxicity (NRC, 2001). Most of the research on Zn toxicity is outdated (Ott, et al., 1966a; Ott, et al., 1966b; Smith, et al., 1979) therefore further research may be required to identify the value and the factors that affect it. Method of administration is reported to be important for induction of Zn toxicity (Smith, 1977a; Smith, et al., 1979). For example, Smith et al. (1979) reported that the toxicity of Zn sulphate was greater when administered with a drenching gun compared to intraruminal intubation. This is thought to be a result of stimulation of the reticular groove reflex causing the drenches to travel directly to the abomasum (Smith, et al., 1977b; Smith, et al., 1979).

If clinical manifestations of Zn toxicity are observed they include inappetence, loss of condition, diarrhea, weakness and jaundice (Allen, et al., 1983). These clinical manifestations are reported to be a result of pathological changes in organs of the body, primarily the kidney, abomasum and pancreas. Lesions in the liver, rumen, small intestine and adrenal gland have also been reported (Allen, et al., 1983). High concentrations of Zn in liver and plasma of cattle are reported to be 300 – 600 µg/g and 2 – 5 µg/ml respectively. Toxic concentrations are reported to be greater than 1000 µg/g and 3 – 15 µg/ml respectively (Kincaid, 2000).

1.3.4. Zinc absorption

Zinc is primarily absorbed by transcellular transport in the small intestine (Figure 1.4; Goff, 2018) with small amounts in the colon (Gopalasmy, et al., 2015). Intestinal Zn is absorbed as Zn^{2+}, across the apical membrane, into the cell using ZIP4, a Zn transporting protein (Mao, et al., 2007). Similarly to Cu, Zn can also move across the apical membrane using the DMT1 protein transporter but this is a minor pathway (Goff, 2018). Inside the cell Zn is transported by ZnT transporter proteins down one of two routes, and either chaperoned to sites within the cell for use by the cell or moved to the basolateral membrane and exported into the interstitial space (Goff, 2018). There are many ZnT transporters but 2,4 and 7 are primarily responsible for moving Zn to the basolateral membrane (Cousins, 2010). From there, ZnT1 moves Zn through the basolateral membrane via transport vesicles which release it into the bloodstream where it binds to albumin or transferrin (Evans & Winter, 1975). The use of transport proteins in the transcellular transport of Zn means that it is an active saturable process with a maximum absorption efficiency of 0.75 (Suttle, 2010; Herdt & Hoff, 2011).

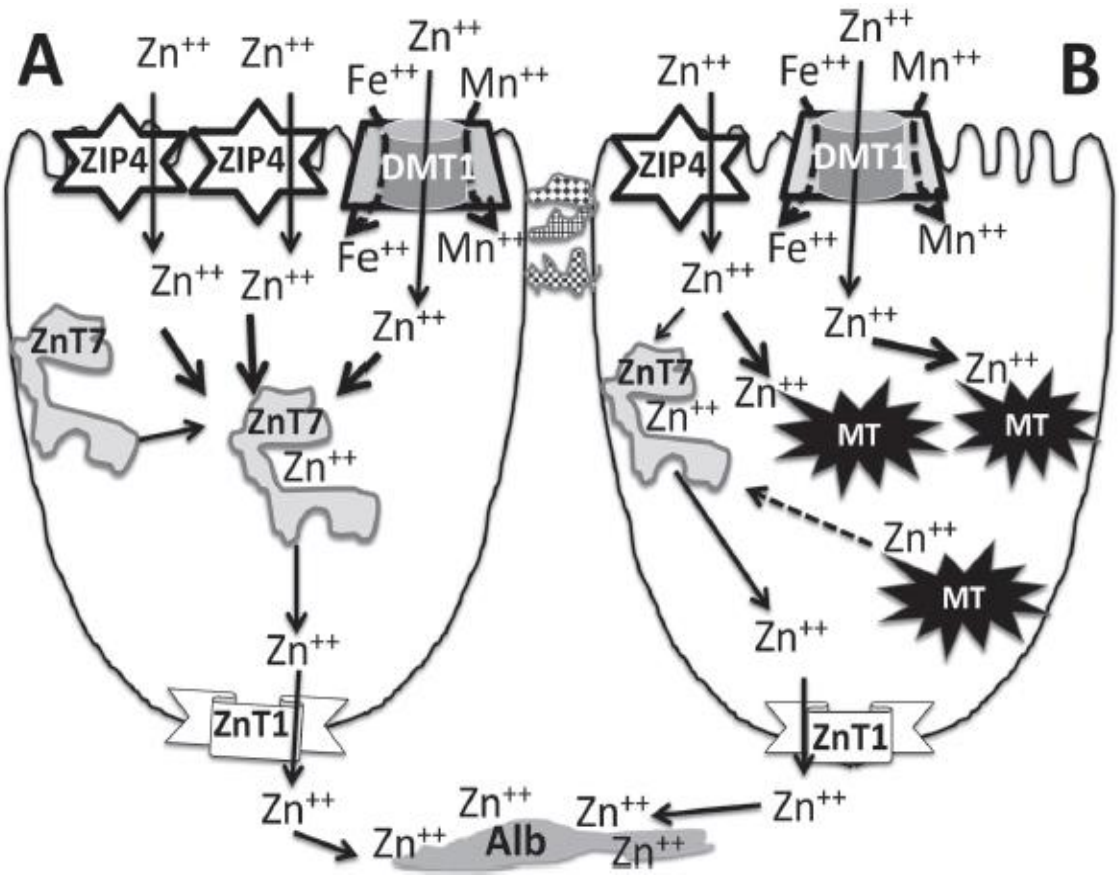

Figure 1.4 Zinc absorption via transcellular transport through enterocytes.
If Zn is required in the body then pathway A commences:
A. It is moved across the apical membrane by ZIP4 or via DMT1. Inside the enterocyte Zn chaperone proteins carry Zn to the basolateral membrane. From there, ZnT1 moves Zn to the interstitial fluid where it is bound to albumin or transferrin.
When Zn status is adequate pathway B commences:
B. ZIP4 is downregulated and enterocytes upregulate metallothionein which binds Zn that crosses the apical membrane therefore reducing Zn absorption (Goff, 2018).

Paracellular absorption of Zn also occurs, but the contribution this makes to overall Zn absorption in ruminants is unknown (Goff, 2018). It requires high concentrations of Zn in the gut lumen and therefore a high dietary intake (Condomina, et al., 2002). The process of paracellular transport moves ions through pores in the tight junction into the interstitial space (Goff, 2018). The potential difference in the interstitial space is approximately +5 mV and +30 mV in the gut lumen (Field, 2003). This electrical potential difference promotes absorption of anions but resists absorption of cations such as Zn (Goff, 2018). Resistance can only be overcome if there is a large concentration gradient of Zn between the gut lumen and interstitial space (Condomina, et al., 2002). Solvent drag may also be responsible for some absorption of Zn

particularly in the large intestine which is a site of net water absorption (Hoover, 1978). There has also been some suggestion that Zn when bound in an amino acid complex may be absorbed paracellularly across the epithelial wall with the bulk water movement (White, et al., 2001; Goff, 2018)

The Zn status of ruminants is under homeostatic control primarily by altering absorption in the duodenum (Herdt & Hoff, 2011; Goff, 2018). Homeostatic control monitors the pool of Zn in the gut lumen and intestinal epithelium, thus effectively monitors dietary Zn intake. When dietary intake is high, absorption of Zn is reduced by increased complexing with metallothionein, a metal binding protein (Herdt & Hoff, 2011). Zinc is sequestered in the intestinal epithelium, bound to metallothionein and is removed from the active Zn^{2+} metabolic pool within the cytosol (Herdt & Hoff 2011; Goff, 2018). The upregulation of metallothionein is a relatively slow process, whereby, days or weeks are needed to change the concentration (Taylor, et al., 1991). Absorption can also be altered by up and down regulation of the ZIP4 protein depending on the animals Zn status (Mao, et al., 2007). Following absorption into the cell the ZnT1 protein is not regulated by Zn status, therefore Zn that reaches the basolateral membrane not bound to metallothionein, will be absorbed and transported into the blood (Cousins, et al., 2006).

1.3.5. Zinc transport and storage

In the bloodstream Zn is transported bound to albumin or transferrin to the liver and other tissues, although there is no distinct storage pool for Zn (Herdt & Hoff, 2011). The arrival of Zn at the liver induces hepatic metallothionein synthesis which removes Zn from the bloodstream (Bremner, 1993). Some Zn is absorbed by cells and tissues outside the liver by endocytosis of albumin bound Zn (Rowe & Bobilya, 2000). As Zn is controlled primarily at the level of absorption, tissue concentrations of Zn remain relatively constant across a wide range of dietary intakes (Herdt & Hoff, 2011). If Zn in the diet becomes very low then mechanisms that reduce Zn loss from the pancreas and conserve Zn in cells are activated to maintain Zn status. For example when Zn is less than 1 mg/kg in the diet, expression of ZnT1 in pancreatic cells is reduced, and there is a reduction in metallothionein synthesis, and therefore concentration which reduces Zn losses by excretion (Lichten & Cousins, 2009).

1.3.6. Zinc excretion

Excretion of Zn is primarily via pancreatic secretions in the faeces and very little is voided in urine (Schryver, et al., 1980). A secondary mechanism by which Zn is excreted in faeces is that Zn that is bound to metallothionein when the enterocyte dies is sloughed off and incorporated in faeces (Chesters, 1997). Excretion via faeces is not a point of regulation, therefore, Zn retention is closely related to Zn absorption as discussed in section 1.3.4 (Herdt & Hoff, 2011). However, there is some change to excretion via milk, when dietary Zn is increased (Miller & Stake, 1974; Miller, 1975). For example increasing dietary Zn from 17 mg/kg DM to 40 mg/kg DM increased milk Zn from 3.3 mg/kg to 4.2 mg/kg (Miller & Stake, 1974).

1.3.7. Measuring Zn status

Zinc is abundant in the body but it is not stored to any great extent (Swain, et al., 2016) therefore Zn status is more challenging to investigate than Cu (Herdt & Hoff, 2011). The normal concentration of Zn in the liver is reported to be 75 to 300 mg Zn/kg DM (Puls, 1994), but Miller (1979) reported Zn responsiveness in cattle with liver Zn concentrations in excess of 100 mg Zn/kg DM. This is likely to be because Zn is not readily mobilised from the liver and therefore at times of dietary insufficiency even with an optimum liver Zn concentration, increased Zn in the diet can produce a response (Miller, 1979). Concentration of Zn in the liver is therefore of limited value in evaluation of Zn status but a concentration less than 75 mg Zn/kg DM can be used to indicate deficiency (Goff, 2018).

Serum Zn concentration is responsive to dietary Zn deficiency, but may not be as useful for evaluating animals of optimum Zn status, or where the diet has only just become deficient and low dietary intake has not been sustained for an extended period (Herdt & Hoff, 2011). Many factors, mainly related to overall animal health and sample preparation can affect serum and plasma Zn concentration, therefore sample handling and other measurements of animal health are critical to obtain accurate results (Taylor, 1997; Kincaid, 2000; Herdt & Hoff, 2011). For example, hypoalbuminemia can alter serum Zn concentration because approximately two thirds of Zn in serum is bound to albumin (Herdt & Hoff, 2011). Diseases that cause inflammation can reduce serum Zn because of hepatic Zn recruitment whereby Zn from extracellular fluids is redistributed into the liver and other organs causing serum Zn concentration to appear deficient despite an adequate dietary Zn intake (Hambrigge, et al., 1986; Goff & Stabel, 1990).

Sample preparation is vital because after blood sampling if the serum is left in contact with the blood clot Zn can leach from the red blood cells and increase serum concentrations, leading to an inaccurate result (Herdt & Hoff, 2011).

Measurements of animal performance, such as feed efficiency, can often indicate low dietary Zn intake before it is detected by serum Zn analysis (Herdt & Hoff, 2011). Plasma binding capacity, measured by calculating the percentage of unsaturated plasma Zn binding sites can accurately assess Zn intake (Kincaid & Cronrath, 1979). The enzyme alkaline phosphatase (ALP) can also be used as an indirect assessment of Zn status because it is a Zn dependent enzyme and in periods of dietary Zn deficiency activity declines (Herdt & Hoff, 2011).

1.3.8. Zinc supplementation of ruminants

Adequate Zn supply is vital for animal health and performance (Suttle, 2010), but Table 1.7 reports that forages and straights commonly fed to ruminants often have a shortfall of Zn compared to the requirement detailed in Section 1.3.2.

Table 1.7 The average Zn concentration (mg/kg DM) of feedstuffs commonly fed to ruminants.

Feedstuff	Average Zn concentration, mg/kg DM	Source
Grass	17	(Gralak, et al., 2006)
Hay	19	(Suttle, 2010)
Straw	14	(Suttle, 2010)
Maize	19	(MAFF, 1990)
Sorghum	14	(MAFF, 1990)

Additionally many of the feed sources for ruminants can also be highly variable in Zn concentration depending on environmental conditions and harvesting (Gralak, et al., 2006; Suttle, 2010). For example, Gralak et al. (2006) reported that pasture cut for forage can differ by 59% between different cuts. In order to prevent potential negative problems associated with Zn deficiency ruminants are often supplemented with Zn, but there are many different sources available which vary in their bioavailability (Baker & Ammerman, 1995b).

1.3.9. Bioavailability of Zn sources for ruminants

The relative bioavailability of supplemental Zn sources compared to Zn sulphate for cattle and sheep is presented in Table 1.8. Bioavailability and Zn status are often measured using different parameters between studies, thus comparing studies can be challenging. Bioavailability of Zn in the data presented in Table 1.8 has been measured by either bone Zn concentration, growth rate, liver Zn uptake or metallothionine synthesis, but does not include studies where there was no response to Zn supplementation (Baker & Ammerman, 1995b).

Table 1.8 Relative bioavailability of supplemental Zn sources[a] (Baker and Ammerman, 1995b).

	Species	
Source	Cattle	Sheep
Zn sulphate	1.00	1.00
Zn carbonate	0.60	-
Zn, chelated	-	1.10
Zn-methionine	-	1.00
Zn oxide	1.00	0.70
Zn, sequestered	-	1.05

[a]Average values rounded to the nearest "0.05" and expressed relative to response obtained with Zn sulphate. Terminology for sources is that of the author.

Baker & Ammerman (1995b) reported that in cattle Zn sulphate and Zn oxide had the same bioavailability and Zn carbonate was lower with a value of 0.60. A different result was reported in sheep whereby Zn sulphate, Zn methionine, Zn sequestered and Zn chelated were reported to have a similar bioavailability whereas Zn oxide was lower (Table 1.8). Similarly, in sheep Sandoval et al. (1997) also reported that Zn oxide had a lower bioavailability of 0.87 compared to Zn sulphate at 1.00 when they used tissue Zn uptake and metallothionein as indicators. They also reported that in sheep Zn carbonate had a higher relative bioavailability of 1.06 compared to Zn sulphate of 1.00.

In contrast Rojas et al. (1995) reported that the bioavailability of Zn sulphate, Zn methionine and Zn oxide were similar based on Zn accumulation in the kidney, liver and pancreas of crossbred wether lambs after a depletion, repletion study (Table 1.9).

Table 1.9 Mean Zn concentrations in tissues of lambs (mg/kg) supplemented with four sources of Zn (mg/kg DMB[a]) (Rojas, et al., 1995).

Tissue	Control [b]	Zn oxide[c]	Zn sulphate[d]	Zn methionine[c]	Zn lysine[c]	SEM
Bone[a]	83	98	97	96	91	13
Bone marrow[a]	63	79	79	84	73	21
Cornea[a]	7	6	6	7	8	2
Skin[a]	24	27	26	26	31	9
Hoof[a]	94	112	113	105	89	59
Kidney[a]	117[e]	137 [e]	234 [e]	226 [e]	581[f]	131
Liver[a]	127 [e]	140[ef]	195[f]	198[f]	389[g]	66
Muscle[a]	260	257	261	259	267	22
Pancreas[a]	86 [e]	107 [e]	139 [e]	135 [e]	340[f]	110

[a]DMB = dry matter basis; bone also on a fat free basis, cornea and skin on wet basis

[b] n = 6, [c]n = 8, [d]n = 7

[e, f, g] means with different superscripts within a row differ (P<0.05)

Rojas et al. (1995) also measured the accumulation of Zn in the organs of lambs supplemented with Zn lysine and found it to be higher than in those supplemented with Zn sulphate, Zn methionine or Zn oxide. It was theorised that there would have been differences between the Zn methionine and Zn sulphate groups compared to the Zn oxide and control groups if they had omitted the Zn lysine group from the results. There was no difference in Zn accumulation in the liver of the lambs supplemented with Zn oxide and the unsupplemented control animals (Rojas, et al., 1995) indicating that Zn methionine and Zn sulphate were more bioavailable than Zn oxide, as also concluded by Baker and Ammerman (1995b) for sheep. Similarly, it has been reported that plasma Zn concentration was increased when dairy cows were supplemented with chelated Zn compared to Zn sulphate (Kinal, et al., 2005). In contrast Cope et al. (2009) reported that there was no difference in plasma Zn concentration when dairy cows were fed either Bioplex Zn (organic source) or Zn oxide.

These contrasting results could be a result of differences in adequacy of Zn in the diet (Kincaid, 2000) or differences in metabolism of Zn sources (Spears, 1989). Spears (1989), reported that Zn oxide and Zn methionine were absorbed similarly but may be metabolised differently following a series of experiments conducted in lambs. When lambs were supplemented with Zn at 20 mg/kg DM (the basal diet contained 30 mg Zn/kg DM) absorption and retention were similar between Zn oxide and Zn methionine whereas when lambs were supplemented with Zn in a single dose of 300 mg, retention was higher for lambs supplemented with Zn methionine compared to Zn oxide (Spears,

1989). Similarly, the retention was higher for Zn deficient lambs (the basal diet contained 2.8 mg Zn/kg DM) supplemented with Zn methionine compared to Zn oxide at 5 mg Zn/kg diet (Spears, 1989). Cao et al. (2000) reported that organic sources of Zn (Zn amino acid chelate and Zn methionine) had a similar bioavailability in lambs compared to Zn sulphate whereas Zn proteinate A was higher based on the Zn accumulation in the liver, kidney, pancreas and liver metallothionein concentration in lambs (Table 1.10).

Table 1.10 Effect of dietary Zn on tissue Zn (mg/kg DM) and metallothionein (µg/gc) concentration of lambs (Cao, et al., 2000).

	None	Zn sulphate			Zn Pro	Zn AA	Zn Met	Pooled SE	
Zn mg/kg[b]	0	700	1400	2100	1400	1400	1400		P <
Liver	113	255	374	436	415	324	342	13.7	0.0001
Kidney	108	528	1164	1519	1394	1114	980	47.2	0.0001
Pancreas	84	305	1080	1157	1129	842	924	39.8	0.0001
Liver MT[c]	18	677	1924	2441	2332	1603	1749	84.1	0.0001

[a]Each value represents the mean of 6 lambs fed for 21 days.

bAdditional Zn - Basal diet contained 58 mg Zn/kg DM.

[c]Expressed as fresh tissue.

Similar findings have been reported in cattle, for example in a depletion, repletion study absorption and retention of Zn were similar for Zn sulphate and Zn methionine based on liver Zn concentrations, plasma Zn concentration, ALP concentration and urinary Zn excretion (Spears, et al., 2004). There was however, a tendency for an increased Zn absorption and retention for animals supplemented with a Zn glycine complex (Spears, et al., 2004).

In summary differences in Zn bioavailability between sources are difficult to detect, mainly because Zn is not stored in one specific location in the body (Swain, et al., 2016). In some studies organic sources of Zn have a similar bioavailability to Zn sulphate (Baker & Ammerman, 1995), whereas in other studies there is a trend for some organic Zn sources to have a slightly increased bioavailability (Rojas, et al., 1995; Cao, et al., 2000). The bioavailability of Zn oxide is, however, generally lower than Zn sulphate or organic Zn sources (Baker & Ammerman, 1995b).

1.4. Relationship between Cu and Zn

There is an antagonistic relationship between Cu and Zn, primarily with Zn interfering with the absorption of Cu (Goff, 2018). The alternative, where absorption of Zn can be

affected by Cu, occurs when the Cu to Zn ratio is greater than 50 to 1 (Van Campen, 1969). This situation is highly unlikely to occur in cattle or sheep because at this ratio death from Cu toxicity is more likely to occur than Zn deficiency (Goff, 2018).

The primary mechanism for Zn interference in Cu absorption is by upregulation of the synthesis of metallothionein. The concentration of liver metallothionein is dependent on the concentration of Zn in the liver (therefore dietary Zn concentration) with Cu as a secondary stimulus (Taylor, et al., 1991). Metallothionein is involved with the cellular detoxification of metals and has a role in storage of Zn and Cu so that they are not in a free ionised state in the cell (Bremner, 1993; Lopez-Alonso, et al., 2005). If there are high concentrations of dietary Zn then metallothionein is upregulated and this also sequesters Cu inside enterocytes (Lopez-Alonso, et al., 2005). When minerals are bound to metallothionein and the enterocytes die, they are sloughed off and the mineral is excreted in the faeces (Goff, 2018). A secondary mechanism is possible because the absorption of Zn and Cu from the intestine can be through the same DMT1 protein (Lutsenko, et al., 2007) but the effect of antagonism via this pathway is likely to be minimal because it is only a minor pathway for the absorption for both minerals (Goff, 2018).

1.5. Nanoparticles of trace elements

1.5.1. Nanoparticle technology

There is no strict, regulated definition of nanoparticles but they are generally regarded as particles with at least one dimension less than 100 nm (De Jong and Borm, 2008; Auffan, et al., 2009). Most nanoparticles have properties that differ from larger particles of the same material (Auffan, et al., 2009). One example, is the ability to suspend the particles in a solution due to their increased surface area compared to other materials (Kohane, 2007; Cardellini, et al., 2019). The primary cause for these properties is a large increase in the proportion of atoms at the surface relative to the total number in the material (Daniel and Astruc, 2004). As a result of these changes and reduced particle size it is hypothesised that nanoparticle trace elements will have an increased bioavailability when supplemented orally compared to conventional material of the same trace element compound (Sahoo, et al., 2014). In support of this, smaller particles have been shown to be more easily absorbed in the gastrointestinal tract of mice (Feng, et al., 2009) and uptake of particles in the gastrointestinal tract has been

shown to be inversely proportional to particle size (Desai, et al., 1997). Nanoparticles can also be transported directly through capillaries for absorption by cells (Sahoo & Labhasetwar, 2003)

Changes in physical properties, such as surface area to volume ratio and density, are a major reason why nanoparticles are of interest to a wide variety of industries ranging from electronics to pharmacy and animal nutrition (Landsiedel, et al., 2010). These different effects may also be a limiting factor in nanoparticle research because safety of the materials for different purposes is relatively unknown and consequently controversial (Yun, et al., 2015). In order to investigate this, research has focused on short term, high dose toxicity studies (Landsiedel, et al., 2010; Yun, et al., 2015) as opposed to longer term, low dose studies that may be more appropriate to accurately assess the effects of oral supplementation. Some preliminary work has been conducted to assess the safety of long term, low exposure, for example the safety of nano metal oxides has been modelled in fish. The concentration of Zn and titanium (Ti) did not differ in fish exposed to nano Zn oxide or Ti dioxide compared with controls (Johnston, et al., 2010). Minimal research has, however been conducted in more complex animals including ruminants, although this is currently an active research area. This part of the review will include nanoparticles of metal and metal oxides where they have been administered orally to ruminants and complex monogastrics with a focus on Cu, Zn and Se.

1.5.2. Nanoparticle trace elements in ruminants

To date the majority of nanoparticle trace element research in ruminants has focused on Se (Shi, et al., 2011; Kojuri, et al., 2012; Sadeghian, et al., 2012) and to a lesser extent Zn (Najafzadeh, et al., 2013; Zaboli, et al., 2013). There is no research that has investigated the supplementation of ruminants with nano Cu, although nano Cu has been investigated in studies with pigs with positive results (Section 1.5.3.1).

1.5.2.1. Selenium

The majority of nanoparticle trace element research in ruminants has been conducted using Se and has reported an improved bioavailability without Se toxicity (Sadeghian, et al., 2012; Pelyhe & Mezes, 2013). Plasma Se and glutathione peroxidase (GSHPx) were elevated by approximately 25% in goats when fed nano Se compared to either sodium selenite or Se yeast, which was associated with a 13% increase in growth (Shi

et al., 2011). In sheep Sadeghian et al. (2012) reported that Se nanoparticles were more effective than sodium selenite for increasing the count (Table 1.11) and improving survival duration and activity of neutrophils. Nano Se was also reported to have a greater antioxidative effect than sodium selenite (Sadeghian, et al., 2012)

Table 1.11 Neutrophil counts (means ± SD) for sheep fed either nano Se or sodium selenite or no Se on days 0, 10, 20 and 30 (Sadeghian, et al., 2012).

Day	Group 1 (nano Se)	Group 2 (sodium selenite)	Control
0	33.2 ± 1.92	32.4 ± 1.82	34.2 ± 2.86
10	43.2 ± 3.70*,***,****	37.8 ± 3.70*	35.0 ± 7.00
20	63.0 ± 10.84*.**,****	55.2 ± 8.29*,**,****	36.4 ± 1.52
30	53.8 ± 6.69*,**,****	44.6 ± 6.39*,****	34.4 ± 2.51
P value	< 0.05	< 0.05	> 0.05

*P < 0.05, significant increase to day 0; ** P < 0.05, significant increase to day 10; *** P < 0.05, significant increase to group 2 (sodium selenite); **** P < 0.05, significant increase to the control group

In sheep, the chemotactic activity of neutrophils and respiratory burst activities was increased following the inclusion of nano Se compared to selenite (Kojouri et al., 2012). The inclusion of nano Se has also been shown to increase whole tract digestibility and the proportion of propionate in the rumen compared to a yeast based Se source (Xun et al., 2012). It was suggested that the increase in digestibility and proportion of propionate was due to increased bacterial activity as a result of lowering rumen pH because of the improved absorption and availability of the nano Se (Xun et al., 2012). In addition to increased bioavailability (Shi, et al., 2011; Sadeghian, et al., 2012), improved immunity (Kojouri, et al., 2012; Sadeghian, et al., 2012) and improved digestibiltiy (Xun, et al., 2012) nano Se has also been reported to have a relatively low toxicity compared to inorganic Se sources (Pelyhe & Mezes, 2013). For example, the LD50 in mice was reported to be 92.1 mg Se/kg for nano Se compared to 25.6 mg Se/kg for selenomethionine (Wang, et al., 2007). Similarly, selenium-enriched yeast has been reported to have no adverse effects on animal performance or health when administered a ten times the EU maximum (Juniper, et al., 2008).

In contrast to other studies (Shi, et al., 2011; Sadeghian, et al., 2012) a study comparing sodium selenite and nano Se supplementated to lambs reported that there was no difference in glutathione peroxidase activity between the two groups (Yaghmaie, et al., 2018). Many of the studies conducted with nanoparticles, should be interpreted with caution because of their experimental design. For example, the study

of Yaghmaie et al. (2018) only used four lambs per treatment and therefore may not have been a large enough sample size to detect differences.

1.5.2.2. Zinc

Zaboli et al. (2013) did not find a difference in plasma Zn concentration between Zn oxide and nano Zn oxide when supplementing goat kids for 70 days (Table 1.12). At day 35 there was a difference in plasma Zn concentration between Zn doses, with concentrations in goats fed 40 mg Zn/kg DM Zn oxide being higher than in those fed 20 mg Zn/kg DM Zn oxide, 20 mg Zn/kg DM nano Zn oxide or the control group but not higher than goats fed 40 mg Zn/kg DM nano Zn oxide. By day 70 of feeding there was, however, no difference in plasma Zn concentration between any of the treatment groups.

Table 1.12 Plasma Zn concentration (μmol/l) in goat kids supplemented with Zn[1] for 70 days (Zaboli, et al., 2013)

Day	Control	Zn oxide(20)	Zn oxide(40)	nZn oxide (20)	nZn oxide(40)	P	SEM
0	3.79	3.02	9.94	10.40	10.09	0.633	0.642
35	14.68[b]	15.29[b]	18.81[a]	15.90[b]	17.28[ab]	0.019	0.826
70	15.59	15.44	16.21	17.74	17.13	0.205	0.765

[ab] Means with different superscript letters in rows are different (P<0.05).

[1]Treatments: Control: basal diet (Zn = 22.1 mg/kg DM), Zn oxide (20): basal diet + Zn oxide (added Zn = 20 mg/kg DM), Zn oxide (40): basal diet + Zn oxide (added Zn = 40 mg/kg DM), nZn oxide (20): basal diet + Zn nano oxide (added Zn = 20 mg/kg DM), nZn oxide (40): basal diet + Zn nano oxide (added Zn = 40 mg/kg DM).

Najafzadeh et al. (2013) supplemented lambs with 20 mg Zn/kg body weight as Zn oxide nanoparticles and reported that there was a numerical increase in serum Zn concentration from 0.79 μmol/L on day 0 to 2.02 μmol/L on day 25, although this was not significant (Table 1.13). In contrast, a different study in lambs reported that after 35 days of supplementation plasma Zn concentration was higher in lambs supplemented with nano Zn oxide compared to conventional Zn oxide when they were both supplemented in the diet at 60 mg/kg to provide a total dietary concentration of 90 mg/kg (Singh, et al., 2018). In the study of Najafzadeh et al. (2013) alanine transaminase (ATL), aspartate transaminase (AST), lactate dehydrogenase (LDH), and blood urea nitrogen (BUN) were measured but were not different between day 0 and day 25. There was a decrease in ALP and an increase creatinine (C) over time (Najafzadeh, et al., 2013).

Table 1.13 Enzymes[1] measured in serum in lambs supplemented with Zn oxide nanoparticles for 25 days (Najafzadeh, et al., 2013).

	Day 0	Day 25
Zn (µmol/L)	0.79 ± 0.09	2.02 ± 0.78
ATL (IU/L)	18.7 ± 3.71	16.0 ± 0.57
AST (IU/L)	86.7 ± 14.24	91.0 ± 29.7
ALP (IU/L)	470 ± 105.4*	248 ± 64.2
LDH (IU/L)	1056 ± 209.8	1008 ± 289.0
C (mg/dL)	1.00 ± 0.04*	1.85 ± 0.11
BUN (mg/dL)	15.0 ± 1.87	19.5 ± 3.57

[1]Plasma Zn, alanine transaminase (ATL), aspartate transaminase (AST), alkaline phosphate (ALP), lactate dehydrogenase (LDH), creatinine (C) and blood urea nitrogen (BUN).

*represents difference between before (day 0) and after (day 25) treatment with $P < 0.05$.

The decrease in serum ALP was suggested to be related to the lambs growing during the study (Najafzadeh, et al., 2013). Increased serum creatinine is indicative of renal dysfunction and the histopathological results indicated severe renal damage (Najafzadeh, et al., 2013). The histopathological results also reported minor liver damage, despite the relative stability of the liver enzymes, and Najafzadeh et al. (2013) concluded that Zn nanoparticles can be toxic to lambs. In contrast, Singh et al. (2018) reported serum creatinine levels that were within the normal reference range for lambs and not different between animals fed nano Zn oxide or conventional Zn oxide. It was concluded that there was no sign of toxicity in the lambs used in this study (Singh, et al., 2018).

The contrasting results are likely to be due to differences in doses of Zn supplementation, with 400 mg Zn/d in the study of Najafzadeh et al. (2013) in contrast to the 13 mg Zn/d in the study of Singh et al. (2018). Similarly to conventional trace elements, the toxicity of nano Zn oxide has been reported to be associated with dose and duration of exposure (Swain, et al., 2016). Najafzadeh et al. (2013) also used a drenching gun to administer the Zn supplement to the lamb and Smith et al. (1979) reported that the toxicity of Zn is enhanced when administered by drenching compared to intraruminal intubation because of the stimulation of the reticular groove reflex and therefore the solution can bypass the rumen and directly enter the abomasum (Smith, et al., 1979).

1.5.3. Nanoparticle trace elements in monogastric animals

1.5.3.1. Copper

Gonzales-Eguia et al. (2009) investigated the use of nano Cu in growing piglets when supplied in the diet a the same rate as Cu sulphate, 50 mg Cu/kg, to examine whether it reduced environmental excretion.Copper availability was increased by 29% and the concentration of Cu in faeces was reduced by 15% in the nano Cu group compared to the group supplemented with Cu sulphate. There was no difference in serum Cu concentrations, but SOD activity was higher in the nano Cu group 173.3 IU/mg protein compared to 109.9 IU/mg protein. It was concluded that there was an increased bioavailability of the nano Cu compared to Cu sulphate (Gonzalez-Eguia, et al., 2009).

1.5.3.2. Zinc

In a study where broilers were fed 20, 60 or 100 mg Zn/kg as either nano Zn oxide or 60 mg Zn/kg diet Zn oxide, at day 14 broilers fed nano Zn oxide had an increased weight gain than those receiving conventional Zn oxide with the exception of the broilers fed 100 mg Zn/kg diet nano Zn oxide (Zhao, et al., 2014). Serum SOD activity was also higher in the broilers fed 60 or 100 mg Zn/kg as nano Zn oxide on days 28 and 35, whilst catalase activity was higher in birds fed 20 or 60 mg Zn/kg nano Zn oxide compared to those fed 100 mg Zn/kg nano Zn oxide or 60 mg/kg conventional Zn oxide (Zhao, et al., 2014). In a similar study, Ahmadi et al. (2013) fed broilers 30, 60, 90 or 120 mg Zn/kg diet of nano Zn oxide for 21 days and reported that concentrations up to 90 mg Zn/kg improved growth performance (Table 1.14). The growth rate of broilers supplemented with 120 mg Zn/kg was, however, not different from the control group thus indicating a possible negative effect of over supplementation, a finding similar to Zhao et al. (2014). Overall, these two studies indicate that supplementing broilers with Zn oxide nanoparticles improved growth rate up to a maximum dose of 90 mg/kg diet. Supplementation of Zn oxide nanoparticles above this may be detrimental to animal performance and can have a toxic effect (Zhao, et al., 2014 and Ahmadi, et al., 2013).

Table 1.14 The effect of feeding nano Zn oxide at 0, 30, 60, 90 and 120 (mg/kg diet) for 21 days on the live weight (g) of broilers (Ahmadi, et al., 2013).

	Zn mg/kg diet					SEM	P value
	Control	30	60	90	120		
Live weight (g)	941[b]	1094[a]	1124[a]	1192[a]	1015[b]	128.2	0.013

[ab]Means with different superscript letters in rows are significantly different (P < 0.05).

Mohammadi et al. (2015) also reported a positive growth performance effect when supplementing broilers with Zn nanoparticles. Growth performance was higher when broilers were supplemented with nano Zn methionine or nano Zn-Max (nano chelating technology) at concentrations of 80 mg/kg diet compared to unsupplemented controls. In contrast, supplementation with nano Zn sulphate had a negative impact on performance reducing daily gain by 4.7 g/bird over 42 days compared to the unsupplemented controls (Mohammadi, et al., 2015). As an assessment of bioavailability, the concentration of Zn in the tibia was measured. In birds fed a form of nano Zn the concentration of Zn in the tibia was higher compared to those fed conventional Zn sulphate or unsupplemented control birds, with Zn methionine producing an intermediary result (Table 1.15). These results indicate an increased storage and therefore bioavailability of Zn from the nano sources compared to conventional Zn sulphate.

Table 1.15 The effect of different sources of Zn on Zn concentration in the tibia (mg/dl) of birds (Mohammadi, et al., 2015).

Source	Concentration (mg/kg diet)	Zn in tibia (mg/dl)
Control	0	275[c]
Zn sulphate	80	289[b]
Zn methionine	80	297[ab]
Nano Zn sulphate	80	303[a]
Nano Zn methionine	80	306[a]
Nano Zn-Max	80	304[a]
SEM		2.9
P value		< 0.0001

[abc]Means with different superscript letters in rows are significantly different (*P* < 0.05).

Overall, these studies indicate that supplementation of nano Zn as Zn oxide or Zn methionine at, less than 100 mg Zn/kg, is safe and has a positive effect on the performance of broilers.

1.6. Conclusions

Supplying the optimum concentration of Cu and Zn in the diet of sheep and cattle is vital to their health, productivity and profitability. A deficiency of either mineral, whether primary or secondary, has negative effects on performance and health. To avoid this sheep and cattle are often supplemented with minerals but the chemical form of the supplement has a large effect on its absorption and metabolism in the body and therefore its bioavailability. The forms of Cu and Zn that are often used in intra-ruminal boluses are usually oxides and they have a relatively low bioavailability compared to other forms of supplement available, for example sulphates.

There is a small volume of research which reports that changing the physical form of a trace mineral supplement, by reducing particle size into the nano scale, less than 100 nm in at least one dimension can improve bioavailability. This may offer an opportunity to improve the bioavailability of minerals in boluses. This could also reduce mineral excretion into the environment and decrease the dose of mineral to maintain animal health and performance.

1.7. Experimental hypothesis and objectives

The overall hypothesis of the experiments in this thesis was that nanoparticles of Cu and Zn oxide will have a greater bioavailability than conventional Cu and Zn oxide where the majority is in the micro scale. There were three main objectives:

1. To investigate the bioavailability of nano Cu and Zn oxide compared to conventional Cu and Zn oxide and Cu and Zn sulphate in growing lambs.
2. To investigate whether an improved bioavailability using nano minerals was effective in the presence of high levels of Cu antagonists (S and Mo) in the diet.
3. To determine the health and performance of animals supplemented with nano minerals.

2. General Material and Methods

2.1. Dry Matter (DM)

Lamb pellets, forage and total mixed ration (TMR) samples were oven dried at 105 °C for 24 h to a constant weight according to AOAC (2012; 943.01). Samples were cooled in a desiccator to room temperature and weighed. The following equation was used to calculate DM:

$$\text{Dry matter (g/kg)} = \frac{\text{Dried sample weight (g)}}{\text{Initial sample weight (g)}} \times 1000$$

Equation 2.1

A cyclone mill (Cycotec, FOSS, Warrington, UK) with a 1 mm screen was used to mill dry samples.

2.2. Crude Protein (CP)

Nitrogen content of dried and milled lamb pellets and TMR was determined using a Leco FP-528 (Leco Corporation, St Joseph, MI) operating the Dumas method according to AOAC (2012; 990.03). Approximately 0.15 g of sample were weighed into aluminium foil and placed in the Leco FP-528. The following equation was used to calculate CP:

$$\text{Crude protein (g/kg DM)} = \text{Total nitrogen (g/kg)} \times 6.25$$

Equation 2.2

2.3. Ash

Ash was determined by weighing approximately 2 g of dried and milled lamb pellets or TMR into a pre-weighed porcelain crucible. Samples were placed in a muffle furnace (Carbollte AAF 1100, Hope Valley, UK) at 550 ºC for 4 h. Samples were cooled in a desiccator to room temperature and weighed. The following equation was used to calculate ash:

$$\text{Ash (g/kg DM)} = \frac{\text{Ashed weight (g)}}{\text{Initial sample weight (g)}} \times 1000$$

Equation 2.3

2.4. Neutral Detergent Fibre (NDF)

Neutral detergent fibre (NDF) was determined according to Van Soest et al. (1991). Approximately 0.5 g of dried and milled lamb pellets or TMR was weighed into a pre-weighed glass crucible (porosity 1, Soham Scientific, Ely, UK) and placed onto Fibertec apparatus (1020, FOSS, Warrington, UK). To each crucible 25 ml of neutral detergent reagent was added. A solution was made up by dissolving 34 g of sodium tetraborate in approximately 3 L of hot distilled water and adding 150 g of sodium dodecyl sulphate (SDS) and 50 ml of tri-ethlylene glycol. A second solution was made up by dissolving 22.8 g of anhydrous disodium hydrogen phosphate in approximately 500 ml of hot distilled water. The second solution was added to the first, the contents were mixed together, and the volume was made up to 5 L. The pH was adjusted to be between 6.9 and 7.1 with either 0.1 M NaOH or 0.1 M HCl and 0.5 ml octan-1-ol was added.

Samples were boiled and digested for 30 minutes, then a further 25 ml of neutral detergent reagent was added to each crucible. Samples were boiled and digested for a further 30 minutes, filtered and washed with 3 x 25 ml of 80 °C distilled water to remove the neutral detergent reagent. A solution of alpha amylase was made up using 2 g of alpha amylase (80 EU/mg, Sigma, Gillingham, UK) dissolved in 90 ml distilled water and 10 ml tri-ethylene glycol. Following washing, 2 ml alpha amylase and 25 ml 80 °C distilled water was added to each sample and allowed to stand for 15 minutes. Samples were filtered and washed with 3 x 25 ml of 80 °C distilled water, crucibles removed from the Fibretec apparatus and oven dried at 105°C overnight. The crucibles were cooled in a desiccator and weighed before being placed in a muffle furnace at 550 °C for 16 h. Crucibles were cooled in a desiccator and reweighed. The following equation was used to calculate NDF:

$$\text{NDF (g/kg DM)} = \frac{\text{Dried (g)} - \text{Ashed weight (g)}}{\text{Initial sample weight (gDM)}} \times 1000$$

Equation 2.4

2.5. Starch

The starch content of the TMR was determined at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) by the polarimetric method as described by ISO 6493 (2000).

2.6. Blood Processing

2.6.1. Plasma Collection

Blood samples were collected into K_3EDTA and lithium heparin Vacutainer tubes (BD MidMeds Limited, Hertfordshire, UK). Vacutainer tubes were centrifuged at 1000 x g for 15 min and the plasma separated and stored at -20 ºC for subsequent analysis.

2.6.2. Serum Collection

Blood samples were collected into serum Vacutainer tubes (BD MidMeds Limited, Hertfordshire, UK). Blood samples were stored at 4 ºC for 24 h and then Vacutainer tubes were centrifuged at 1000 x g for 15 min and the serum separated and stored at -20 °C for subsequent analysis.

2.6.3. Whole Blood Collection

Blood samples were collected into K_2EDTA Vacutainer tubes (BD MidMeds Limited, Herfordshire, UK). Following analysis for haematology profile (section 2.9) blood samples were stored at -20 ºC for subsequent analysis.

2.7. Mineral analysis by ICP-MS

Plasma samples, dried and digested tissues and dried and milled lamb pellets and TMR were analysed for minerals by inductively coupled plasma mass spectroscopy (ICP-MS).

2.7.1. Plasma analysis

Plasma samples were defrosted overnight at 4 ^{0}C and then mixed using a MT-20 vortex mixer (Phillip Harris Ltd, Shenton, UK). Samples were diluted 1:20 for Experiment 1 (Chapter 3) and 1:50 for Experiment 2 and 3 (Chapter 4 and 5) in 1% HNO_3 (Trace element grade 70%; Fisher Scientific., UK), 1% methanol (Trace element grade; Fisher Scientific, UK) and 0.1% Triton X (Laboratory grade, Sigma-Aldrich, UK) in ICP-MS autosampler tubes. Following the method of Cope et al. (2009) gallium (Ga; QMX Laboratories, Essex, UK) was added to each sample at 10 µg/L as an internal standard. Samples were then thoroughly vortex mixed for 30 s. To determine the concentration of minerals in plasma for Experiment 1 (Chapter 3) samples were analysed by ICP-MS (Thermo-Fisher Scientific, Hemel Hempstead, UK) using a calibration at 0, 10, 50, 100, 200 and 400 ppb for Cu, Fe and Zn and 0, 1, 5, 10, 20 and 40 ppb for Mn and Mo. To determine the concentration of minerals in plasma for Experiment 2 and 3 (Chapter 4 and 5) samples were analysed by ICP-MS (NexION 2000, Perkin Elmer, Shelton, CT) using a calibration at 0, 5, 25, 50, 100 and 200 ppb for Cu, Fe and Zn and 0, 1, 2.5, 5, 10 and 20 ppb for Mn and Mo. The following equation was used to calculate the mineral concentration of the plasma:

$$\text{Mineral conc } (\mu\text{mol/L}) = \frac{\text{Sample (ppb)} - \text{Blank (ppb)} \times \text{dilution factor}}{\text{Molecular mass}}$$

Equation 2.5

2.7.2. Tissue analysis

To prepare the samples a 250 mg section of tissue was weighed in duplicate into two 50 ml tubes and dried for 24 h at 60 ºC to a constant weight. Samples were cooled in a desiccator to room temperature and weighed. Equation 2.1 was used to calculate DM.To digest the tissue 6 ml of concentrated HNO_3 (Trace element grade 70%; Fisher Scientific., UK) was added to each tube and empty tube (reagent blank) using an automated dispenser and digested in an oven at 60 ºC for 12 h. Tubes were cooled to room temperature in a fume cupboard and diluted to 50 ml mark with purite water and vortex mixed. Tissue samples were then prepared in the same way as plasma samples (2.7.1) for ICP-MS analysis. The following equation was used to calculate the mineral concentration of the tissue:

$$\text{Mineral conc (mg/kg DM)} = \frac{(\text{Sample (ppb)} - \text{Blank (ppb)}) \times \text{dilution factor} \times 50) / \text{sample weight} \times 1000}{1000}$$

Equation 2.6

2.7.3. Feed sample analysis

To digest the feed samples 0.5 g of dried and milled lamb pellets or TMR was weighed into a digiTUBE (50 ml, SCP Science, Canada) and 1 ml concentrated HCl (Trace element grade 37%; Fisher Scientific, UK) and 6 ml of concentrated HNO_3 (Trace element grade 70%; Fisher Scientific., UK), was added to each tube and two empty digiTUBEs (reagent blanks). The tubes were placed into a clear plastic rack with a plastic watch glass on top of each tube and inserted into the digiPREP heating block in a fume cupboard. The digiPROBE was inserted into one of the reagent blanks. In the digiPREP heating block the tubes were heated for 30 mins to 45°C, held for 1 minute, the temperature was then increased to 65 °C over a 25 minute period, held for 1 minute, increased to 100 °C over 15 minutes and then refluxed for 45 minutes. After digestion the samples were cooled to room temperature in a fume cupboard and then diluted to 50 ml with purite water and vortex mixed. A certified EU reference sample of hay (BCR-129) was also routinely digested and analysed. Feed samples were then prepared in the same way as plasma samples (2.7.1) for ICP-MS mineral concentration analysis. Equation 2.6 was used to calculate the mineral concentration of the feed.

2.8. Atomic absorption (Experiment 2, Chapter 4; Cu depletion phase)

To prepare the samples 1 ml of plasma and 9 ml of purite water was added to a 25 ml plastic tube and vortexed. Samples were analysed by atomic absorption spectrophotometry using a calibration for Cu of 0, 10, 20, 50, 100 and 200 ppb. Blanks and standards were prepared using Cu standard and diluting with purite water. The following equation was used to calculate the Cu concentration in plasma:

$$\text{Cu concentration } (\mu\text{mol/L}) = \frac{\text{Sample (ppm)} \times 10}{\text{Molecular mass} \times 1000}$$

Equation 2.7

2.9. Enzyme analysis

Blood samples were collected into Vacutainer tubes (BD MidMeds Limited, Herefordshire, UK) for serum, for analysis of ceruloplasmin (Cp) and gamma-glutamyl transferase (GGT), tubes containing K_2EDTA for analysis of superoxide dismutase (SOD) and lithium heparin for alkaline phosphatase (ALP). Blood samples were analysed for enzyme activity on a Cobas-Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK).

2.9.1. Ceruloplasmin (Cp)

Serum samples were analysed for Cp according to the method of Henry et al. (1974). Briefly, this method uses the ability of Cp as a general oxidase to oxidise p-phenylenediamine (PPD). When PPD is oxidised the products are purple with an absorption peak between 530 and 550 nm which can be measured on the Cobas-Mira Plus auto-analyser. Serum samples were pipetted into Cobas-Mira cups (ABX Diagnostics, Shefford, Bedfordshire, UK) and placed in the sample racks. A 0.1 M solution of PPD (BDH Laboratory Supplies, Poole, Dorset, UK) was prepared in 100 ml of 0.1M acetate buffer and adjusted to pH 6.0. Sodium azide (BDH Laboratory Supplies, Poole, Dorset, UK) was prepared using 0.1% solution in pH 6.0 acetate buffer. A test (CPT) reagent was prepared by adding 15 ml acetate buffer pH 6.0 and 15 ml PPD solution pH 6.0. A blank (CPB) reagent was prepared by adding 15 ml acetate buffer pH 6.0, 15 ml PPD solution pH 6.0 and 15 ml sodium azide solution. A blank reagent must be used because PPD is also oxidised by any Cu or Fe present in serum. In the blank sodium azide inhibits the Cu and Fe therefore the results subtracted from the test (CPT) indicates the Cp activity. The following equation was used to calculate Cp activity:

$$\text{Ceruloplasmin activity (mg/dl)} = \text{CPT} - \text{CPB}$$

Equation 2.8

2.9.2. Superoxide dismutase (SOD)

Whole blood samples were analysed for SOD activity using a kit catalogue no. SD 125, Randox Laboratories, Crumlin, Co. Antrim, UK. The method was adapted from Misra and Fridovich (1977), briefly, xanthine and xanthine oxidase are used to generate free radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium

chloride (I.N.T) to form a red formazan dye. The enzyme SOD inhibits generation of free radicals therefore the degree of inhibition of this reaction can be measured in order to indicate the activity of SOD. Before running the samples a control sample (Ransod, Randox laboratories, County Antrim, UK) was analysed to confirm that it was within the expected range. Frozen EDTA blood samples were defrosted (lysed cells) and vortexed using a MT-20 vortex-mixer (Philips Harris Ltd., Shenston, UK). A volume of 250 µl of whole blood was pipetted into a 1 ml micro-centrifuge tube (Sarstedt Ltd., Leicester, UK), and an additional 750 µl of purite water was added. The sample was vortexed and 10 µl of sample was then added to 490 µl of 0.01 mol/l phosphate buffer, pH 7.0 (Ransod, Randox laboratories, County Antrim, UK) into a micro-centrifuge tub and vortexed thoroughly. Samples were then transferred into Cobas-Mira cups (ABX, Diagnostic, Bedfordshire, UK), and placed into the sample racks and analysed by an automated method on Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). The following equation was used to calculate the SOD activity:

$$\text{SOD activity (U/g Hb)} = \frac{\text{SOD (units/ml of whole blood)}}{\text{g Hb/ml}}$$

Equation 2.9

2.9.3. Gamma-glutamyl transferase (GGT)

Serum samples were analysed for GGT using a kit catalogue no. GT553, Randox Laboratories, Crumlin, Co. Antrim. The method is adapted from Szasz and Bergmeyer (1974). In the presence of glycylglycine the substrate L-K-glutamyl-3-carboxy-4-nitroanilide is converted by GGT to 5-amino-2-nitrobenzoate and the absorption peak can be measured at 405 nm to indicate GGT activity. Serum samples were pipetted into Cobas-Mira cups (ABX Diagnostics, Shefford, Bedfordshire, UK) and placed in the sample racks and analysed by an automated method on Cobas Mira Plus (ABX Diagnostics, Bedfordshire UK). Before running the samples two control samples (Randox bovine assay control level 2 and level 3; Cat No. HN1530 and 1532) were analysed to confirm they were within the expected range.

2.9.4. Alkaline phosphatase (ALP)

Plasma samples were analysed for ALP (lamb study 1) using a kit catalogue no. AP307 (Randox Laboratories, Crumlin, Co. Antrim, UK). The method is adapted from Deutsche Gessellschaft Klinische Chemie (1927). Briefly, p-nitrohenylphosphate is

hydrolysed into phosphate and p-nitrophenol by ALP therefore conversion to phosphate and p-nitrophenol, indicated by a colour change is indicative of ALP activity. Plasma samples were pipetted into Cobas-Mira cups (ABX Diagnostics, Shefford, Bedfordshire), placed in the sample racks and analysed by an automated method. Before running the samples a factor was established with a calibrator (Randox calibretion serum level 3) and two control samples (Randox bovine assay control level 2 and level 3; Cat No. HN1530 and 1532) were analysed to confirm they were within the expected range.

2.10. Haematology profile

Vacutainers containing K_2EDTA with fresh whole blood samples were thoroughly mixed using a Spiramix 5, (Denley Instruments Ltd., West Sussex, UK) for a minimum of 15 minutes. The Vet Animal Blood Counter (Woodley Equipment Company Ltd., Bolton, UK) was calibrated using a haematology control sample (ABX Minotrol 16; Horiba ABX Diagnostics, Bedfordshire, UK) prior to the start of analysis to ensure the instrument results were accurate. Whole blood samples were analysed for white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin interaction (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV), plateletcrit (Pct), platelet distribution width (PDW) lymphocyte number/percentage, neutrophil number/percentage, basophil number/percentage and monocyte number/percentage.

2.11. Milk composition

For Experiment 3 (Chapter 5) milk composition of fat, protein, lactose, and somatic cell count (SCC) was determined by National Milk Laboratories (NML, Wolverhampton, UK). Milk samples were collected from consecutive am and pm milkings and preserved using broad spectrum microtabs II (Advanced Instruments, inc, Massachusetts, USA) and stored at 4 °C before analysis.

2.12. Manufacture of nanoparticles

Nanoparticles were manufacture at Nottingham Trent University, by Knowledge Partnership employee Joseph Thompson.

2.12.1. Copper oxide nanoparticles

A 0.1 M $CuCl_2 \cdot 2H_2O$ solution and 0.3 M NaOH solution are prepared using purite water. Solutions are heated to 90 °C on a hotplate with a magnetic stirrer. Foil is used as insulation to prevent evaporation. The two solutions were pumped at 60 ml/min through pre-heated tubes onto a spinning disk reactor which spins at 1000 rpm at a ratio of 1:1 ($CuCl_2 \cdot 2H_2O$: NaOH). The product from the spinning disk reactor was collected in large glass beaker before filtering through a sintered funnel (vacuum filtration). The product is rinsed from the funnel onto an evaporating dish using purite water. The product is dried in an oven at 120 °C for 12 h. After drying the black solid was ground to a fine powder. To coat, an equal mass of Cu oxide nanoparticles and hydrolysed lysine (Sigma-Aldrich, UK) were added to a mortar and pestle. The mixture was ground until a homogenous product was formed.

2.12.2. Zinc oxide nanoparticles

A 0.4 M $ZnCl_2$ solution dissolved in methanol and 0.8 M NaOH solution dissolved in acetone were prepared. The two solutions were pumped at 60 ml/min onto a spinning disk reactor at 1000 rpm a ratio of 1:3 ($ZnCl_2$ to NaOH). The product from the spinning disk reactor was collected in large glass beaker before filtering through a sintered funnel (vacuum filtration) and washed three times with purite water and twice with acetone. The product is dried in an oven at 50 °C for four hours. After drying the solid was ground to a fine powder. To coat, an equal mass of Zn oxide nanoparticles and hydrolysed histidine (Sigma-Aldrich, UK) were added to a mortar and pestle. The mixture was ground until a homogenous product was formed.

3. Experiment 1 – Determination of the bioavailability of nano Cu oxide and Zn oxide and its effect on performance and health in growing lambs

3.1. Introduction

Copper is a vital element for ruminants being a major component of many enzymes including SOD and CCO, and has a structural function in macromolecules (Herdt & Hoff, 2011). In ruminants deficient in Cu clinical signs such as decreased weight gain, decreased fertility, increased susceptibility to disease and hair depigmentation can be observed (Suttle, 2010). Young grazing animals are most likely to be affected; this can be either a primary or secondary Cu deficiency (Dargatz, et al., 1999). Primary Cu deficiency is caused by a low Cu content in the diet and is rare, whereas secondary Cu deficiency is caused by interference from other components in the diet, usually Mo, S or Fe, which reduce the availability of the Cu (Herdt & Hoff, 2011). As a result, Cu is frequently added to the diet of ruminant animals to meet requirements. One of the most common supplemental sources of Cu is in the form of an oxide, but absorption of Cu from this source in the diet is low; in sheep and cattle net absoprtion is approximately 5%, with 95% being excreted (Suttle, 2010).

Zinc is also a vital element for ruminants as a component of a number of critical enzymes as well as having a role in gene expression, appetite, fat absorption and an immune role as an antioxidant (Suttle, 2010). Signs of Zn deficiency include sub-optimal growth, skin conditions and reduced fertility (ARC, 1980). Similarly to Cu, Zn supplied in the form of an oxide is the least bioavailable source, with a bioavailabilty of 0.7 compared to Zn sulphate (Baker & Ammerman, 1995b).

Improving the absorption of minerals by using minerals of a smaller particle size with a larger surface area such as nanoparticles has been suggested (Rajendran, 2013). Several studies have reported a significant improvement in the bioavailability of trace minerals in ruminants when they have been supplied as nanoparticles but there is a deficit of literature on the effect of Cu nanoparticles, with most research to date focussing on Se and to a lesser extent Zn (Xun et al., 2012; Najafzadeh et al., 2013; Pelyhe and Mezes 2013). For example plasma Se and glutathione peroxidase were elevated by approximately 25% in goats when fed nano Se compared to either sodium selenite or Se yeast, which was associated with a 13% increase in growth (Shi, et al.,

2011). Similarly, in sheep Sadeghian et al. (2012) reported that the greater bioavailability of nano Se significantly upgraded selenoenzymes compared to selenite, whilst the chemotactic activity of neutrophils and respiratory burst activities was increased following the inclusion of nano-Se compared to selenite (Kojouri, et al., 2012). Rajendran (2013) also reported an increase in plasma Zn concentration (+39%) in cows receiving nano Zn compared to Zn oxide, with an improvement in milk yield. Nanoparticles of Cu have been investigated in pigs and Cu availability was increased by 29% and the concentration in faeces was reduced by 15% compared to the group supplemented with Cu sulphate (Gonzales-Eguia, et al., 2009).

The aim of this study was to determine the bioavailability of nano Cu oxide (with a hydrolysed lysine coating) and nano Zn oxide (with a hydrolysed methionine coating) compared to conventional inorganic Cu and Zn sources in lambs by analysing the accumulation of Cu and Zn in body tissue mineral stores, and determine the effect on perfomance.

3.2. Materials and Methods

3.2.1. Animals, management and treatments

Fifty, six month old, castrated male Welsh Mountain lambs were used. All of the lambs were sourced from the same farm where they were fed grazed grass only post-weaning. The lambs were gradually introduced to a pelleted diet (Table 3.1) formulated according to AFRC (1993) over a period of 3 weeks. The diet was based on oatfeed because of its low trace element content. Prior to the start of the study 10 lambs were slaughtered and their livers, kidneys and front legs were collected and stored at -20 ºC prior to analysis.

Table 3.1 Dietary and chemical composition of the basal diet.

Ingredient	Inclusion g/kg DM
Oatfeed	500
Barley	142
Wheat	61
Molassed sugar beet pulp	50
Molasses	43
Soya bean meal	90
Rapeseed meal	65
Urea	8
Megalac*	13
Minerals and vitamins**	29
Chemical analysis	
DM, g/kg	899
ME, MJ/kg	10.4
CP, g/kg DM	153
NDF, g/kg DM	430
Ash, g/kg DM	73.9
Cu, mg/kg DM	12.4
Zn, mg/kg DM	176
Co, mg/kg DM	2.2
Mo, mg/kg DM	0.6
Fe, mg/kg DM	226.4
S, g/kg DM	1.9

*Megalac rumen protected fat, Volac, Hertfordshire, UK

**Mineral/vitamin premix (Premier Nutrition, Peterborough, UK) Ca: 292 g/kg, Sodium: 99 g/kg, Chloride: 150 g/kg Manganese: 3200 mg/kg, Iodine: 160 mg/kg, Selenium: 20 mg/kg, Vit A: 320 IU, Vit D: 80 IU, Vit E: 2000 mg/kg, Vit B12: 4000 ug/kg.

The forty study lambs were weighed on two consecutive days at 1300 h and mean live weight was calculated. The lambs were stratified and blocked according to live weight and randomly allocated to one of four treatment groups (Table 3.2). Lambs were allocated to individual pens (1.3 m x 1.3 m) with *ad libitum* access to water. The lambs were evenly distributed by treatment group throughout the shed.

Table 3.2 Dietary treatments for lambs supplemented with Cu oxide, Zn oxide, nano Cu oxide, nano Zn oxide, Cu sulphate and Zn sulphate.

Treatment	Supplement	Approximate dose of element mg/kg DM
O	Cu oxide	8
	Zn oxide	40
NH	Nano Cu oxide (lysine coated)	8
	Nano Zn oxide (methionine coated)	40
NL	Nano Cu oxide (lysine coated)	4
	Nano Zn oxide (methionine coated)	20
S	Cu sulphate	8
	Zn sulphate	40

3.2.2. Characterisation of supplements

Characterisation of the supplementary sources of Cu was conducted at Nottingham Trent University before the start of the study (Table 3.3). Particle size was determined by analysing results from TEM (JEOL JEM-2010) and DLS (Particulate Systems, NanoPlus). Elemental composition was determined using ICP-OES (Shimadzu ICPE-9820).

Table 3.3 Physical and chemical characteristics of supplementary Cu and Zn sources fed to growing lambs.

	Cu oxide	Nano Cu oxide	Cu sulphate	Zn oxide	Nano Zn oxide	Zn sulphate
Coating	None	Hydrolysed lysine	None	None	Hydrolysed methionine	None
Coating ratio	N/A	1:1	N/A	N/A	1:1	N/A
Chemical formula	Cu oxide	Cu oxide	Cu sulphate.$5H_20$	Zn oxide	Zn oxide	Zn sulphate· $7H_2O$
CAS Number	1317-38-0	N/A	7758-99-8	1314-13-2	N/A	7446-20-0
Appearance colour	Black	Black	Blue	White	White	White
Appearance form	Powder	Fine powder	Crystals	Powder	Fine powder	Powder
Mean particle size (nm)	25000	60	N/A	N/A	27	N/A
Particle size range (nm)	40 – 33000	16 – 75	N/A	N/A	4 – 42	N.A
Elemental composition (%)	78.2	34.4	26.1	79.4	27.1	23.7
Solubility in H_20	<1%	<1%	100%	<1%	<1%	100%

3.2.3. Experimental procedure

Lambs were weighed weekly at 1300 h using a weigh crate (Shearwell Data Limited, Somerset, UK) which was calibrated using standard weights (Thornton and Co, Wolverhampton, UK). Feed intake was adjusted each week for each block based on the mean live weight and a target of 160 g/d live weight gain (AFRC, 1993). Lambs were fed twice daily at 0800 h and 1500 h and top dressed with the relevant supplement at the 1500 h feed. Feed refusals were collected and weighed daily at 0730 h. Feed was sampled weekly and frozen at -20 °C prior to proximate and mineral analysis.

For treatments O, NH and NL the supplement was delivered in sunflower oil (Henry Lamotte Oils, Bremen, Germany). For treatment S the supplement was delivered in water and sunflower oil was added to the feed at the same rate as treatments O, NH and NL. For the first three weeks of the study 8 ml of supplement was delivered; from week three to six 9 ml of supplement was delivered and from week six to nine 10 ml. Increase in the supplement rate was calculated as DMI intake increased with live weights.

Blood samples were collected weekly at 1100 h by jugular venepuncture using a 21G x 1” needle (Becton Dickinson and Company, Plymouth, UK) at 1100 h into Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing K_2EDTA for the determination of mineral concentrations in plasma. On weeks 0, 3, 6 and 9 blood samples were also collected into Vacutainers containing K_3EDTA, silica and sodium heparin. Bloods were processed as detailed in Section 2.6.

All sheep were slaughtered at a commercial abattoir (W & G Yates, Walsall, UK) at the start of week 9 and liver, kidney and front legs were collected from the animals at slaughter. Immediately after extraction of the liver a sub-section was biopsied and immersed in 1 ml of RNAlater (Ambion Inc.,Austin, TX) and stored at 4 °C for 24 hours and then frozen at -20 °C. Livers and kidneys were weighed immediately after extraction before whole organs were frozen at -20 °C. Front legs were frozen immediately at -20 °C.

3.2.4. Analytical procedure

Feed samples were analysed for cobalt (Co), Cu, Fe, Mn, Mo, S and Zn by ICP-MS (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) as described in Section 2.6, DM (943.01; intra-assay CV of 0.18%) as described in Section 2.1, CP (990.03; intra-assay CV of 2.8%) using a Leco FP-528 (Leco Corporation, St Joseph, MI) as described in Section 2.2, ash (intra-assay CV of 2.8%) as described in Section 2.3 and NDF (intra-assay CV of 3.5%) according to Van Soest, et al. (1991) and as described in Section 2.4. Whole blood samples were analysed for haematology profile using a Vet Animal Blood Counter (Woodley Equipment Company Ltd, Bolton, UK) as described in Section 2.9. Serum, plasma and whole blood samples were analysed using a Cobas-Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK) for Cp (intra-assay CV of 5.2%) according to the method of Henry, et al. (1974), GGT (Kit catalogue no. GT 553; intra-assay CV of 1.7%), SOD (Kit catalogue no. SD 125; intra-assay CV of 3.3%) and ALP Randox Laboratories, Crumlin, Co. Antrim, UK; kit catalogue no. AP307; intra-assay CV of 2.1%) as described in Section 2.7. Plasma samples were analysed for of Cu and Zn by ICP-MS (ICP-MS; Thermo Fisher Scientific Inc., Hemel Hempstead, UK; intra-assay CV of and 3.8% and 1.7% respectively) as described in Section 2.6. Livers and kidneys were analysed for Cu and Zn by ICP-MS (ICP-MS; Thermo Fisher Scientific Inc., Hemel Hempstead, UK; intra-assay CV of liver Cu 1.0%, liver Zn 8.3%, kidney Cu 1.0% and kidney Zn 1.0%) as described in Section 2.6.

3.2.5. Statistical analysis

3.2.5 Statistical analysis

Performance, plasma minerals and enzyme activity were analysed by repeated measures ANOVA as a randomised block design with 10 replicates per treatment using the data recorded in week 0 as a covariate where appropriate. Data was checked for normality by visual assessment of residual plots and analysed as:

$$Yijk = \mu + Bi + Mj + Tk + MTk + \varepsilon ijk$$

Where Yijk = dependent variable; μ = overall mean; Bi = fixed effect of blocks; Mj = effect of mineral; Tk = effect of time; MTk = interaction between mineral and time; and εijk = residual error.

Liver and spleen Cu concentration was analysed by ANOVA as a randomised block design and analysed as:

$Yijk = \mu + Bi + Mj + \varepsilon ijk$

Where Yijk = dependent variable; μ = overall mean; Bi = fixed effect of pairs; Mj = effect of mineral; and εijk = residual error.

All analysis was conducted using GenStat 17 and $P < 0.05$ was used to indicate significance. Data is presented as means with SED.

3.3. Results

3.3.1. Animal performance and intake

Initial live weight did not differ ($P = 0.807$) between treatments (Table 3.4). There was also no effect ($P > 0.05$) of form or level of Cu and Zn supplement on final live weight, live weight gain, DMI, liver or kidney weight.

Table 3.4 Effect of form and level of Cu and Zn supplement on the performance of growing lambs.

	Treatment[1]					
	O	NH	NL	S	SED	Significance
Initial live weight, kg	26.1	26.1	26.1	26.2	0.19	0.807
Final live weight, kg	31.6	31.8	32.4	31.4	0.38	0.619
Live weight gain, g/day	89	96	91	82	14.0	0.793
Mean DMI, g/day	1017	995	1025	1023	30.6	0.751
Liver weight, g	477	482	484	493	30.2	0.952
Kidney weight, g	53	57	57	56	3.9	0.636

[1]Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM

3.3.2. Blood parameters

There was no treatment x time effect ($P = 0.992$) or effect of form or level of Cu and Zn supplement ($P = 0.432$) or effect of time ($P = 0.426$) on plasma Cu concentration (Table 3.5). There was also no treatment x time effect ($P = 0.228$) or effect of form or level of Cu and Zn supplement ($P = 0.492$) or effect of time ($P = 0.833$) on serum GGT activity.

Table 3.5 The effect of treatment on plasma Cu (μmol/L) and GGT activity (U/l).

	Treatment[1]					Significance[2]		
	O	NH	NL	S	SED	Treatment	Time	Tr x T
Plasma Cu, μmol/L	15.5	14.9	14.3	14.8	1.99	0.432	0.426	0.992
GGT, U/l	51.5	56.7	54.3	55.3	5.83	0.492	0.833	0.228

[1]Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM

[2] Treatment = Effect of treatment, T = Effect of time and Tr x T = interaction between treatment and time.

There was no effect (P = 0.546) of form or level of Cu and Zn supplement on plasma Zn concentration (Figure 3.1). There was an increase in plasma Zn concentration in all lambs over time (P < 0.001). There was no treatment x time effect (P = 0.186) on plasma Zn concentration.

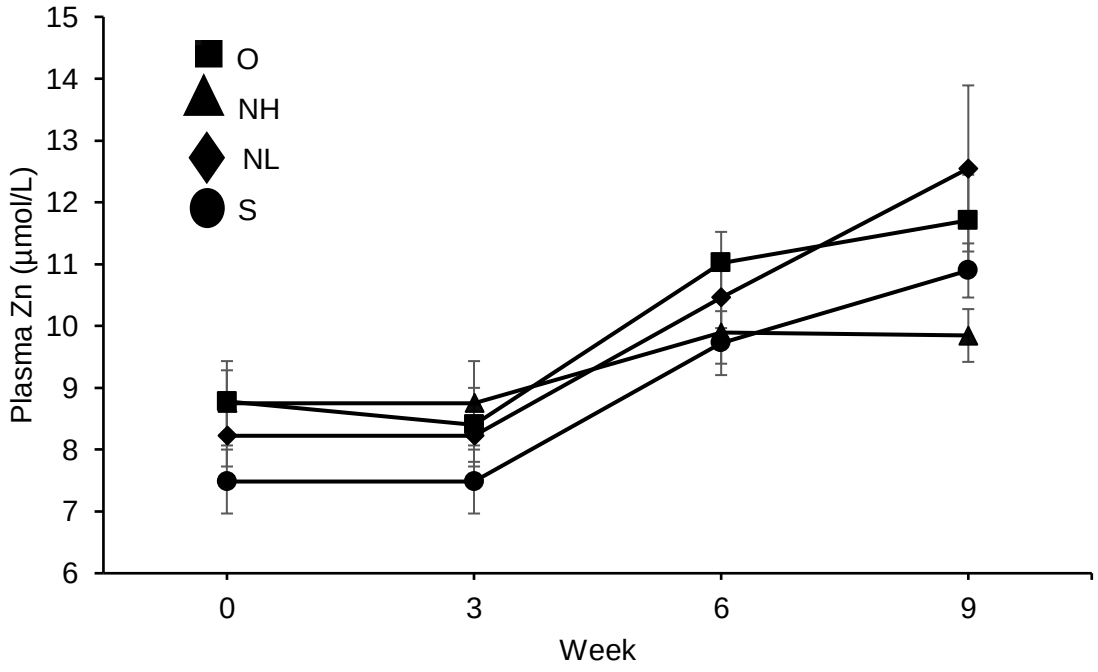

Figure 3.1 Effect of form and level of Cu and Zn supplement on mean plasma Zn concentration (μmol/L) over time in growing lambs. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM. Error bars represent the SED. Treatment, P = 0.546; time, P < 0.001; time x treatment, P = 0.186.

Plasma ALP activity was lower (P = 0.019) for lambs allocated to treatment S compared to treatment NH at week 0. Week 0 plasma ALP activity was therefore used as a covariate. There was no effect (P = 0.509) of form or level of Cu and Zn supplement on plasma ALP activity (Figure 3.2). There was an effect (P < 0.001) of time on plasma ALP activity which increased from week 0 to week 9 in lambs on all treatments. There was a trend for a treatment x time effect (P = 0.068) where lambs fed Cu sulphate had a faster rise in plasma ALP activity than lambs fed Cu oxide.

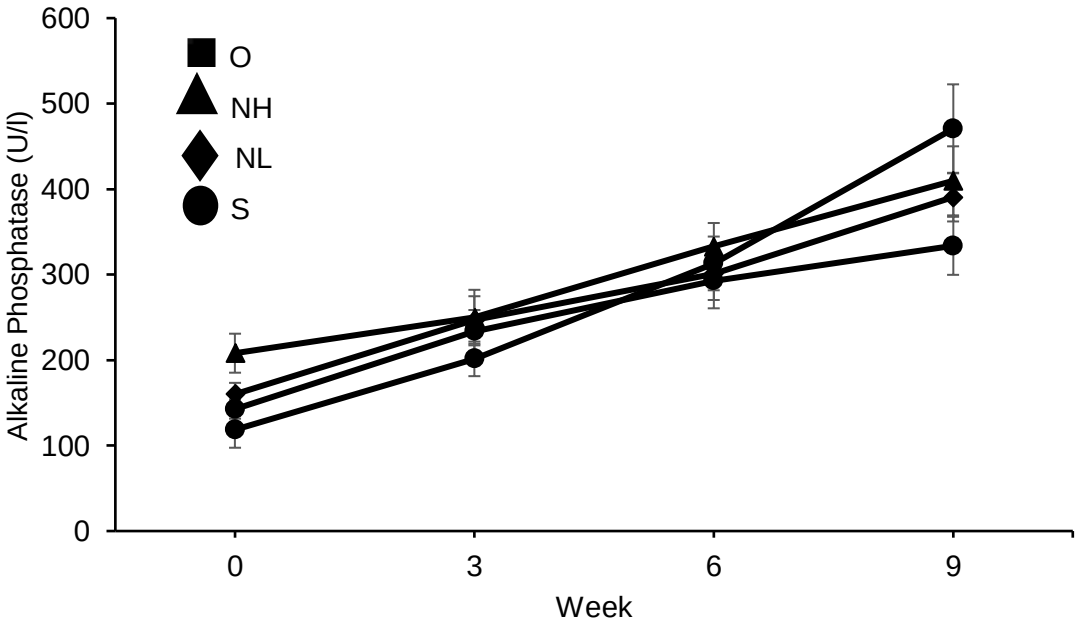

Figure 3.2 Effect of form and level of Cu and Zn supplement on mean plasma alkaline phosphatase concentration (U/l) over time in growing lambs. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM Error bars represent the SED. Treatment, P = 0.509; time, P < 0.001; time x treatment, P = 0.068.

Six lambs were treated for pneumonia prior to the study commencing or between week 0 and week 3. These lambs were removed from the serum Cp analysis. There was no effect of form or level of Cu and Zn supplement (P = 0.973) on Cp concentration but there was an effect of time (P < 0.001; Figure 3.3); Cp concentration declined between week 0 and week 3, increased at week 6 before declining again at week 9. There was no treatment x time effect (P = 0.334) on Cp concentration.

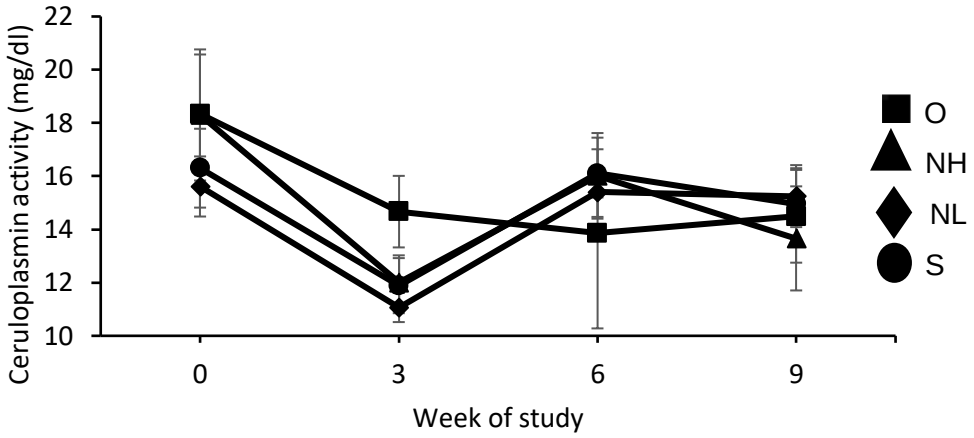

Figure 3.3 . Effect of form and level of Cu and Zn supplement on mean ceruloplasmin activity (mg/dl) over time in growing lambs. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM. Pooled SED = 0.903. Treatment, P = 0.973; time, P < 0.001; time x treatment, P = 0.334

There was also no effect of form or level of Cu and Zn supplement (P = 0.244) on blood SOD activity (Figure 3.4). There was a trend (P = 0.069) for SOD activity to increase in week 3 compared to week 0 in all groups. There was no treatment x time effect (P = 0.143) on SOD activity.

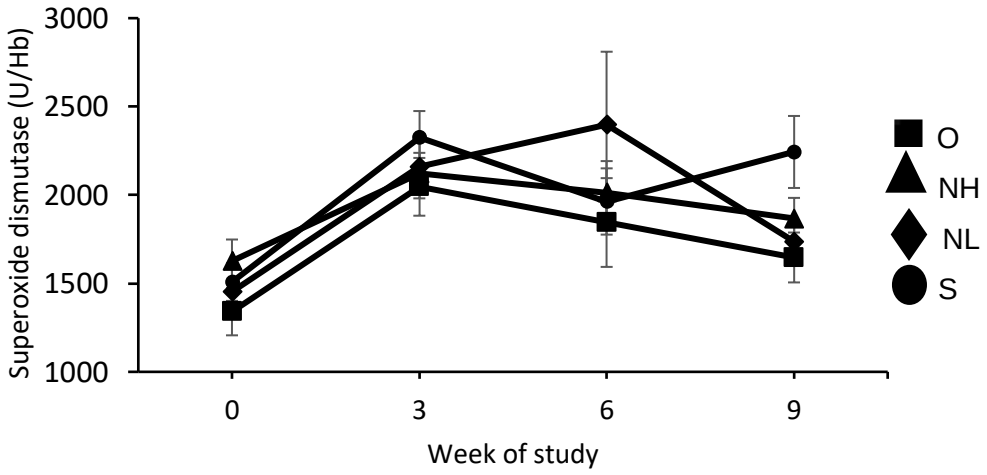

Figure 3.4 Effect of form and level of Cu and Zn supplement on mean superoxide dismutase activity (U/g Hb) over time in growing lambs. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM. Pooled SED = 0.109.5. Treatment, P = 0.244; time, P = 0.069; time x treatment, P = 0.143

3.3.3. Haematology profile

There was no effect of form and level of Cu and Zn supplement effect (P > 0.05) or treatment x time effect (P > 0.05) on the haematology profile of the lambs (Table 3.6). There was an effect of time on WBC, Mon No. and Neu No. which decreased (P < 0.001) from week 0 to week 6 and then increased at week 9 for lambs across all of treatment groups. Lym No. and Eo No. decreased (P < 0.002) from week 0 to week 6 and then increased at week 9. There was no effect (P > 0.05) of time on Ba No., Hb, RBC, or HCT for any treatment group.

Table 3.6 Effect of form and level of Cu and Zn supplement on haematology profile[2] in growing lambs.

	Treatment[1]					Significance[3]		
	O	NH	NL	S	SED	Treat	Time	Tr x T
WBC, $10^3/mm^3$	9.41	8.50	10.76	8.03	1.101	0.099	<0.001	0.987
Lym No, $10^3/mm^3$	6.42	5.99	7.64	5.73	0.807	0.114	<0.001	0.997
Mon No, $10^3/mm^3$	0.63	0.51	0.67	0.48	0.087	0.111	<0.001	0.764
Neu No, $10^3/mm^3$	2.14	1.78	2.25	1.66	0.301	0.212	<0.001	0.725
Eo No, $10^3/mm^3$	0.18	0.21	0.16	0.15	0.037	0.431	0.002	0.560
Ba No, $10^3/mm^3$	0.033	0.023	0.031	0.022	0.0048	0.437	0.900	0.963
Hb, g/dl	13.0	12.8	13.3	12.9	0.47	0.753	0.346	0.303
RBC, $10^6/mm^3$	12.8	12.6	13.2	12.9	0.41	0.573	0.788	0.423
HCT, %	40.0	38.0	39.4	38.3	1.24	0.351	0.876	0.534

[1]Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM

[2]white blood cells (WBC), lymphocyte number (Lym No.), monocyte number (Mon No.), neutrophil number (Neu No,), eosinophil number (Eo No.), basophil number (Ba No.), haemoglobin (Hb), red blood cells (RBC), and haematocrit (HCT),

[3] Treat = Effect of treatment, T = Effect of time and Tr x T = interaction between treatment and time.

3.3.4. Tissue mineral analysis

Final liver Cu concentration was increased (P < 0.05) in lambs supplemented with Cu sulphate with a mean of 529 mg/kg DM compared to lambs supplemented with conventional Cu oxide with a mean of 332 mg/kg DM (Figure 3.5). There was a trend (P = 0.11) for lambs supplemented with nano Cu oxide to have a higher liver Cu concentration than lambs supplemented with Cu oxide, with a mean liver Cu concentration of 428 mg/kg DM for low dose and high dose nano Cu oxide.

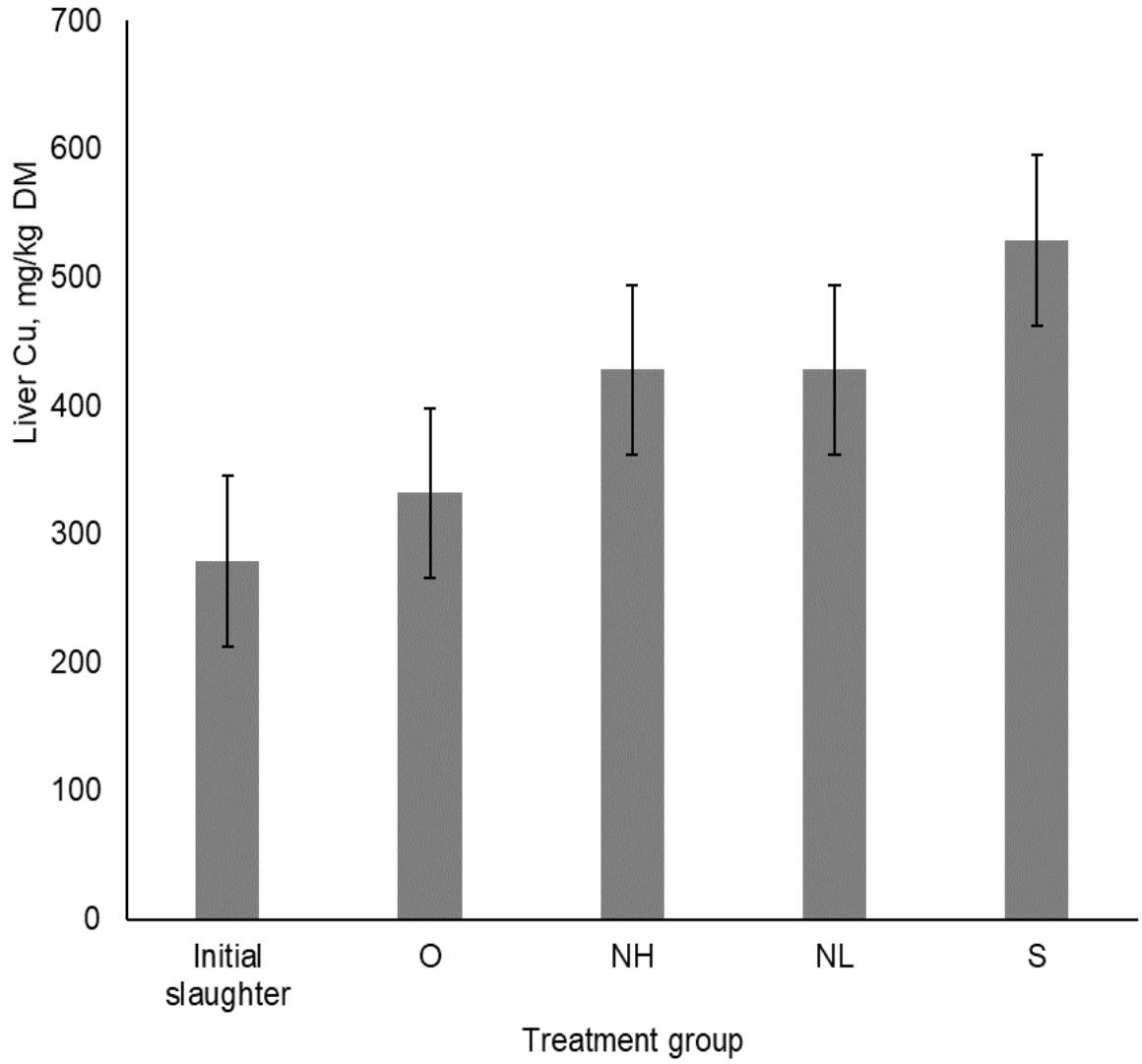

Figure 3.5 Final liver Cu concentration (mg/kg DM) of the initial slaughter group of lambs and in growing lambs fed a diet containing Cu oxide and Zn oxide (O), high dose nano Cu oxide and Zn oxide (NH), low dose nano Cu oxide and Zn oxide (NL) and Cu sulphate and Zn sulphate (S). Error bars indicate the SED. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM

There was no effect ($P > 0.05$) of treatment on final kidney Cu concentration with a mean of 8.2 mg/kg DM for lambs supplemented with Cu oxide, 7.7 mg/kg DM for lambs supplemented with low dose nano Cu oxide, 10.2 mg/kg DM for lambs supplemented with high dose nano Cu oxide and 7.1 mg/kg DM for lambs supplemented with Cu sulphate (Figure 3.6).

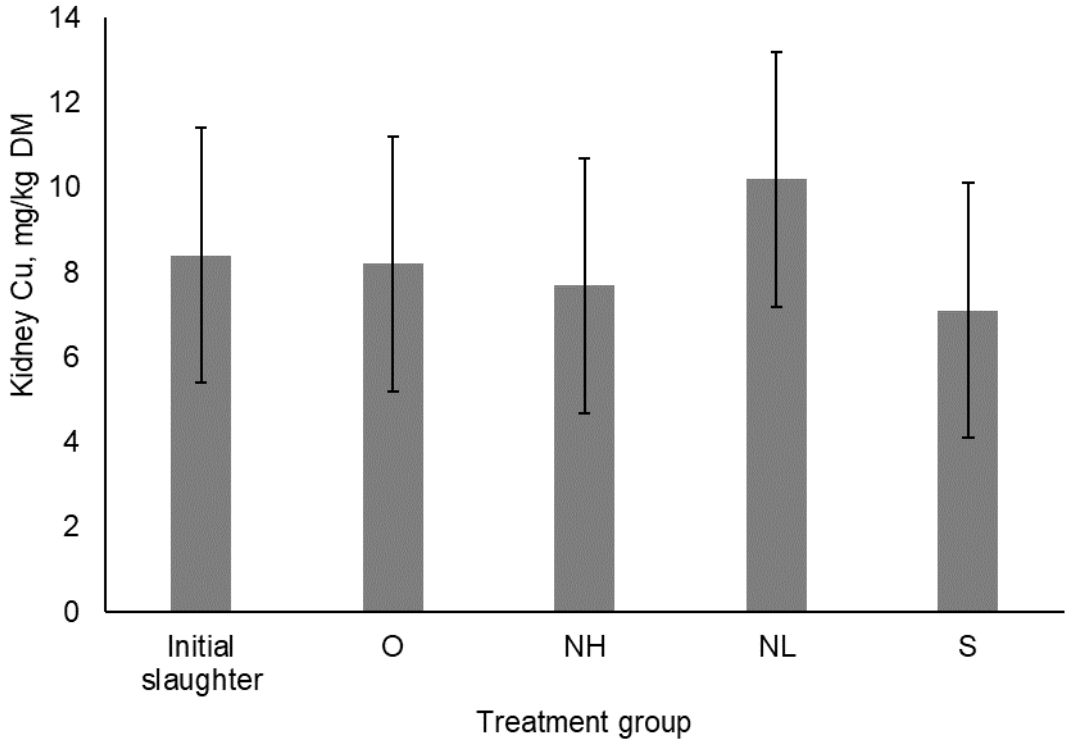

Figure 3.6 Final kidney Cu concentration (mg/kg DM) of the initial slaughter group of lambs and in growing lambs fed a diet containing Cu oxide and Zn oxide (O), high dose nano Cu oxide and Zn oxide (NH), low dose nano Cu oxide and Zn oxide (NL) and Cu sulphate and Zn sulphate (S). Error bars indicate the SED. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM

There was no effect ($P > 0.05$) of form or level on final kidney Zn concentration with a mean of 88 mg/kg DM for lambs supplemented with Zn oxide, 77 mg/kg DM for lambs supplemented with low dose nano Zn oxide, 66 mg/kg DM for lambs supplemented with high dose nano Zn oxide and 58 mg/kg DM for lambs supplemented with Zn sulphate (Figure 3.7). There was no effect ($P > 0.05$) of form or level on final liver Zn concentration with a mean of 89 mg/kg DM for lambs supplemented with Zn oxide, 67 mg/kg DM for lambs supplemented with low dose nano Zn oxide, 84 mg/kg DM for lambs supplemented with high dose nano Zn oxide and 76 mg/kg DM for lambs supplemented with Zn sulphate.

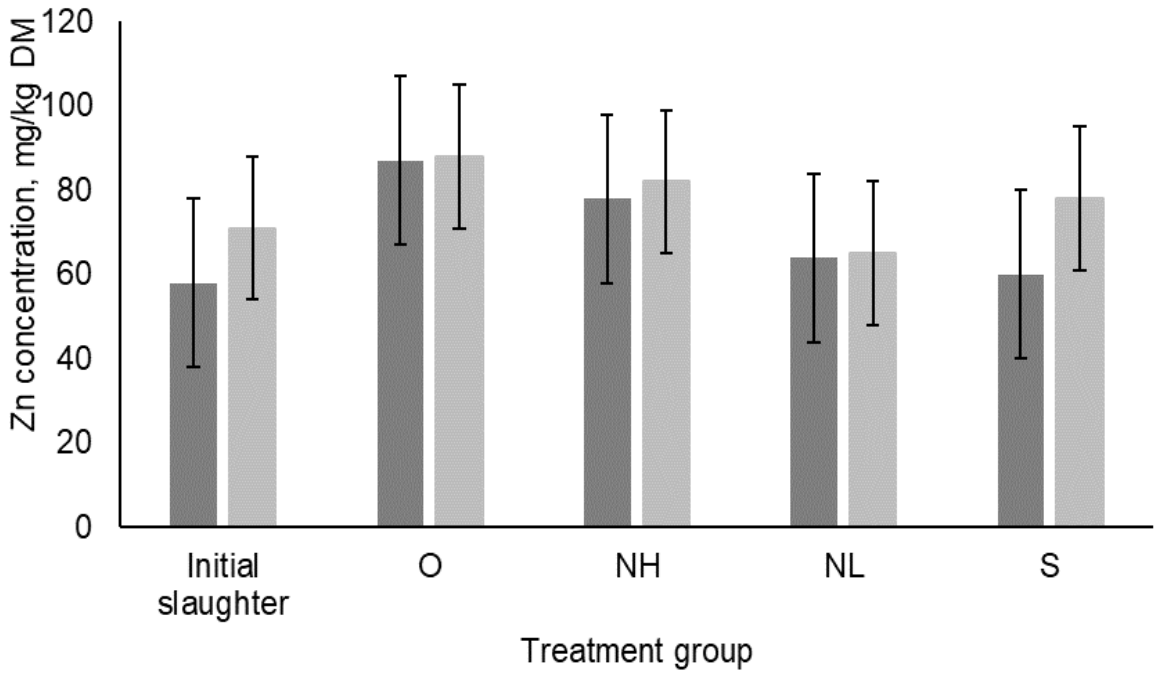

Figure 3.7 Final kidney (dark grey) and liver (light grey) Zn concentration (mg/kg DM) of the initial slaughter group of lambs and in growing lambs fed a diet containing Cu oxide and Zn oxide (O), high dose nano Cu oxide and Zn oxide (NH), low dose nano Cu oxide and Zn oxide (NL) and Cu sulphate and Zn sulphate (S). Error bars indicate the SED. Treatments were: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40 mg Zn/kg DM, NH; nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40 mg Zn/kg DM, NL; nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM

3.4. Discussion

3.4.1. Animal performance

The current study investigated the bioavailability of Cu and Zn oxide nanoparticles when added to the diet of growing lambs. Mineral nanoparticles are predicted to be more bioavailable than conventional mineral sources because of their small particle size (Swain, et al., 2016). Nanoparticles can have very different properties to the bulk conventional material because of a change in the surface area to volume ratio (Daniel & Astruc, 2004; Auffan, et al., 2009).

The four combinations of mineral supplements used in this study had different concentrations of Cu and Zn. Following ICP-MS analysis of the elemental content, the minerals were supplemented to ensure that Cu and Zn were provided at the same level

in each treatment group except NL, which was half the dose of the other three groups. A comparison between nano oxides and conventional oxides was also made with Cu sulphate and Zn sulphate because sulphates have a higher bioavailability than conventional mineral oxides and are often used by industry (Baker & Ammerman, 1995a; Baker & Ammerman, 1995b; Spears, 2003). The particle size of the conventional oxide and sulphate minerals that were used was in the micro scale thus the particle size of the nano minerals was approximately one thousand times smaller.

The basal diet was formulated to have a low Cu and Zn content and was provided to lambs in the form of a pellet at 0.95 *ad libitum* intake to avoid selective feeding and ensure that the lambs ate the top-dressed supplement. Feed refusals were collected, and feed intake did not differ between treatment groups. Consequently, there was no effect of form or level of Cu and Zn on DMI or live weight gain. Performance of lambs in response to Cu and Zn is rarely reported and Engle and Spears (2000) and Ward et al. (1993) reported that the form of Cu had no effect on live weight gain or DMI in growing steers. In contrast, Galyean et al. (1995) reported that supplementation with Cu-Lys (5 mg/kg DM) had a negative effect on live weight gain and DMI when supplemented to growing steers, but similarly to the current study form and level of Zn had no effect. Kessler et al. (2003) and Spears and Kegley (2002) also reported that the form of Zn had no effect on live weight gain or DMI of fattening bulls or steers.

3.4.2. Zinc bioavailability

In the current study there was no effect of form or level of Zn on liver and kidney Zn concentration, with mean concentrations of 73 mg Zn/kg DM for the kidney and 78 mg Zn/kg DM for the liver. Liver and kidney weights did not differ between treatments therefore overall Zn in the liver was similar. This result is in agreement with Engle et al. (1997) who reported that there was no difference in liver Zn accumulation between calves fed Zn lysine, Zn methionine or Zn sulphate. Similarly, Rojas et al. (1995) reported that there was no difference in Zn accumulation in the liver and kidney when sheep were supplemented with Zn oxide, Zn sulphate or Zn methionine; the only Zn supplement that increased Zn accumulation was Zn lysine. Sandoval et al. (1997) reported that Zn accumulation in the kidney was increased when sheep were supplemented with Zn oxide compared to Zn sulphate but there was no difference in the accumulation in the liver. This finding may be different to the current study because Zn was supplemented at 1400 mg/kg DM which is 35 times higher than the current study. Differences in bioavailability of minerals are easier to detect when background

levels of the mineral are low and the supplement is a high proportion of dietary intake (Baker & Ammerman, 1995b). Oatfeed was 50% of the diet and was selected because of its low mineral content. Prior to feed manufacturing the oatfeed was analysed and the Zn concentration was 22 mg/kg DM and the minerals for the diet were specifically formulated without Zn so the basal diet was predicted to be 40 mg Zn/kg DM. However, subsequent analysis indicated a concentration of 170 mg Zn/kg DM in the basal diet. Increased Zn concentration of the basal diet meant that supplementary Zn sources were only 19% of total dietary intake. In studies which have reported differences in bioavailability of Zn supplements based on liver and kidney Zn concentration, the supplementary Zn was a greater proportion of total dietary Zn intake, for example 96% in the study of Sandoval et al. (1997) and 88% in the study of Hatfield et al. (2001). In studies that have not reported a difference in liver and kidney Zn concentration they have had a lower supplementary Zn level as a proportion of the total dietary Zn intake, for example 58% in the study of Engle et al. (1997).

Previous studies have also reported differences in Zn accumulation in liver and kidney depending on Zn dose (Henry, et al., 1997; Sandoval, et al.,1997) but these used supplementary doses of Zn of between 700 and 2100 mg/kg DM. In the current study there was no difference in liver or kidney Zn accumulation between lambs supplemented with 40 mg/kg DM Zn oxide nanoparticles (NH) and 20 mg/kg DM Zn oxide nanoparticles (NL). This is likely to be due to the small difference in Zn intake between the two groups (20 mg/kg DM), when total dietary Zn intake was 190 – 210 mg/kg DM.

Plasma Zn concentration and ALP activity did not differ between the form or level of Zn supplementation but there was an increase over time. The high level of Zn in the basal diet (total basal plus supplement; 210 mg/kg DM) was seven times greater than the NRC recommendation of 30 mg/kg DM for growing lambs and is likely the reason for the increase in plasma Zn concentration and ALP activity over time. Prior to the start of the study the lambs were grazing grass only, thus Zn intake may have been relatively low 21 – 48 mg Zn/kg DM (Nicholson, et al., 1999) and a change to a diet containing up to 210 mg/kg DM of Zn may have elicited a dose effect. Previous studies have shown that plasma Zn is responsive to Zn dose (Rojas, et al., 1995; Zaboli, et al., 2013), but not Zn form (Spears, 1989; Rojas, et al., 1995; Engle, 1997). Plasma ALP is a Zn dependent enzyme (Prasad & Oberleas, 1971) and similar to plasma Zn is responsive to Zn dose but not form (Ho and Hidiroglou, 1976). Spears (1989) observed that there was no difference between Zn source (Zn oxide or Zn methionine) on ALP activity in

growing lambs or growing cattle. Similarly, Berrie et al. (1995) reported increased ALP activity in lambs supplemented with additional Zn (35 or 70 mg/kg) compared to control on the basal diet (30 mg/kg Zn) but no difference between supplementation of Zn sulphate or Zn methionine. In the first published study to determine the effect of Zn oxide nanoparticles on ruminants, Najafzadeh et al. (2013) reported a decrease in serum ALP when lambs were supplemented with nano Zn oxide. The rate of supplementation of Zn oxide nanoparticles was 20 mg Zn/kg body weight (equivalent to 200 – 400 mg Zn/kg DM) by oral administration. Liver damage was reported in the lambs, and changes in ALP activity can be associated with liver damage and may have been the reason for the decline in ALP activity observed by Najafzadeh et al. (2013).

3.4.3. Copper bioavailability

Six lambs were treated for pneumonia prior to the study commencing or between week 0 and week 3. These lambs were removed from the Cp analysis because lambs with pneumonia may exhibit an acute phase response which increases Cp concentration (Pfeffer & Rogers, 1989). There was no difference in plasma Cu concentration or Cp concentration between lambs receiving any of the treatments. There were changes in Cp concentration over time, with a decline in Cp concentration between weeks 0 and 3 which may also have been linked to an acute phase response in the lambs at the start of the study due to the stress of a change in environment making the animals more susceptible to disease (Dickens, et al., 2010). The decline in WBC (13.1 to 7.5 $10^3/mm^3$), mon no. (0.96 to 0.45), neu no. (4.22 to 1.59) and lym no. (7.66 to 5.28) between weeks 0 and 6 also indicated a possible acute phase response at the start of the study (Pfeffer & Rogers, 1989)

Bioavailability of Cu by assessment of plasma Cu concentration is difficult because Cu concentration in the blood is under homeostatic control (Evans, 1973). Studies that have reported differences based on plasma Cu concentration and Cp activity have generally used a Cu depletion, repletion technique, or fed high levels of Cu absorption antagonists in the diet (Kincaid, et al., 1986; Kegley & Spears, 1994; Ward, et al., 1996). Following one experiment using Cu depletion and a second experiment feeding high levels of antagonists, Kegley & Spears (1994), reported that Cp and plasma Cu concentration was generally higher for animals supplemented with Cu sulphate compared to Cu oxide. In the current study the animals were not subject to depletion of their Cu stores prior to the start of the study or high levels of Cu antagonists in the diet

therefore similar plasma Cu concentration and Cp concentration across the treatment group was not surprising.

Blood SOD activity was not affected by Cu or Zn source, but there was a trend for an increase in SOD activity between week 0 and week 3 across all treatments. Previous studies have indicated that SOD activity is responsive to concentration of Cu in the diet (Ward & Spears, 1997; Nagalakshmi, et al., 2010; Cheng, et al., 2011) which could explain the increase in SOD activity in the current study because the concentration of Cu in the diet increased for all treatment groups compared to the pre-study diet from 12.4 mg Cu/kg DM to 20.4 mg/kg DM. Similar to the current study, Cheng et al. (2011) reported that there was no difference in SOD activity in lambs fed different Cu sources. In contrast, Nagalakshmi et al. (2010) reported increased SOD activity in lambs supplemented with Cu proteinate compared to Cu sulphate. Pal et al. (2010) also reported a significant increase in SOD activity in ewes supplemented with Cu methionine and Zn methionine compared to ewes supplemented with Cu sulphate and Zn sulphate.

When dietary supply exceeds requirement then Cu can be stored in the liver and therefore liver Cu concentration is reflective of the absorption and availability of the Cu source (Xin, et al., 1991; Sinclair, et al., 2013). Lambs supplemented with Cu sulphate (S) had a greater liver Cu concentration than lambs supplemented with conventional Cu oxide (O). This was expected because it is commonly reported that the bioavailability of Cu oxide is 0.2 of the bioavailability of Cu sulphate, thus when supplemented at the same level animals supplemented with Cu sulphate store more Cu than those supplemented with Cu oxide (Baker & Ammerman, 1995a). There was a trend for lambs supplemented with nano Cu oxide to have a higher liver Cu concentration than lambs supplemented with Cu oxide; indicating that nano Cu oxide may have a greater bioavailability than Cu oxide. The current study is the first one to attempt to differentiate the bioavailability of conventional Cu oxide and nano Cu oxide therefore the difference in liver Cu concentration was difficult to predict. In order to reach full statistical significance a greater number of replicates and reduced variation between individual animals may be required.

Source of Cu had no effect on the accumulation of Cu in the kidney. This finding is consistent with Ledoux et al. (1995) and Pal et al. (2010) who also reported that the accumulation of Cu in the kidney was not affected by Cu source. In studies where a difference in Cu accumulation in the kidney has been reported (Gopinath, et al., 1974; Theil & Calvert, 1978, Eckert, et al., 1999) animals had been fed extremely high dietary

concentrations of Cu so that liver Cu concentration was 2000 mg/kg DM or higher, indicative of Cu toxicity (Ishmael, et al.,1972; Gopinath, et al.,1974; Theil & Calvert, 1978). In contrast, Eckert et al. (1999) reported an interaction between dietary Cu concentration and source on final kidney Cu concentration when liver Cu concentration was less than 400 mg/kg DM. The interaction between dietary Cu concentration and source occurred because ewes fed Cu proteinate at a rate 20 mg Cu/kg DM had higher kidney Cu concentrations than ewes fed 10 or 30 mg Cu/kg DM but ewes fed Cu sulphate at a rate of 10 mg Cu/kg DM had higher kidney Cu concentrations than ewes fed 20 or 30 mg Cu/kg DM. For Cu sulphate there was an inverse relationship between liver and kidney concentrations, if liver Cu concentration was increased then kidney Cu concentration was decreased, this was not observed for ewes fed Cu proteinate (Eckert, et al., 1999).

3.4.4. Animal health

Studies investigating the use of nanoparticles in animal nutrition have reported a wide range of effects on animal health, from a lower toxicity compared to conventional sources (Pelyhe & Mezes, 2013) to renal and liver damage indicative of toxicity (Najafzadeh, et al., 2013). In the current study serum GGT activity was used to monitor liver damage. At week 0 GGT activity was higher than the normal range, 58.7 U/l (normal range for sheep is 33 – 55 U/l (Braun, et al., 1978)). Care was taken to ensure healthy lambs were purchased from a commercial farm. High levels are indicative of liver damage and are often caused by *Fasciola hepatica* (Piacenza, et al., 1999) and all lambs were wormed 3 weeks before starting the study. Three weeks may not have been sufficient time for GGT levels to decline if the lambs had a worm burden on arrival (Bulgin, et al., 1984). There was a numerical decline (58.7 to 53.1 U/l) in GGT activity through the study for all groups. The GGT activity in lambs supplemented with high dose or low dose nanoparticles (NH and NL) were similar to conventional Cu or Zn sources indicating they did not have a toxic effect as has been suggested by Najafzadeh et al. (2013). It is difficult however, to compare the current study with Najafzadeh et al. (2013) because they supplemented lambs with 200 – 400 mg Zn/kg DM as Zn oxide nanoparticles compared to 20 – 40 mg Zn/kg DM in the current study. There were no abnormal results in the haematology profile of lambs on any of the treatments further indicating that nanoparticles at the doses fed in this study did not cause an adverse effect on the health of the lambs.

3.5. Conclusions

Differences in Zn status were difficult to detect because the basal ration Zn concentration was higher than predicted and consequently the supplement was only a small proportion of total dietary intake. High dietary Zn intake is likely the reason for an increase in ALP activity and plasma Zn concentration for lambs in any of the treatment groups. Final liver Cu concentration indicated that Cu sulphate had a greater bioavailability than conventional Cu oxide and nano Cu oxide may have a greater bioavailability than conventional Cu oxide, but greater statistical power is required. This could be achieved by reducing variation between lambs and using a greater number of replicates in each treatment group. It has been speculated that nanoparticles may be toxic, but plasma GGT activity and haematology profile indicated that nanoparticles, at the doses fed in this study, did not cause liver damage in the lambs or elicit an immune response within the levels fed and for the duration employed in the current study.

4. Experiment 2 – Determination of the bioavailability of nanoparticles of Cu oxide using a Cu depletion and repletion technique in growing lambs

4.1. Introduction

Diets for growing lambs must contain sufficient levels of trace elements for optimal animal performance (Suttle, 2010). One trace element that is critical for performance is Cu, and therefore Cu is often supplemented to meet the demands of growing lambs (Herdt & Hoff, 2011). One form in which Cu can be supplemented is as Cu oxide but compared to other sources such as sulphate the bioavailability of Cu oxide is low; approximately 0.05 g/g is absorbed (Baker & Ammerman, 1995a). Improving the bioavailability of oxide could reduce the dose required to maintain or increase the Cu status of the animal and reduce excretion into the environment. One way in which bioavailability may be improved is by decreasing particle size. For example, Experiment 1 was the first study, to the authors knowledge, to compare the bioavailability of nano Cu oxide with conventional Cu oxide and showed that reducing the particle size of oxide into the nano scale may have the potential to increase bioavailability.

Accumulation of Cu in the liver is often used as a measure of the bioavailability of Cu sources (Suttle, 1974; Baker & Ammerman, 1995a). The liver Cu concentration results in Experiment 1 demonstrated that nanoparticles of oxide may have a greater bioavailability than oxide with a conventional particle size, but a greater number of replicates, and/or reduced variation in the initial liver Cu concentration between individual animals may be required to reach statistical significance. Reduced variation between individuals can be achieved by allocating animals to treatment groups based on their initial liver Cu concentration and using this as a covariate (Donald, et al., 1984). In order to gather this data lambs would need to be liver biopsied prior to the start of the trial. Liver biopsy of lambs is challenging and can result in high animal mortality rates and damage to the biliary tract which can affect subsequent animal performance (Suttle, 2012a). For this reason, liver biopsy was not considered a viable option for the current study.

Another option to reduce variability in Cu stores and to increase the sensitivity of assessing the difference in bioavailability between the two Cu sources is a Cu

depletion and repletion technique. This technique involves reducing the animal's Cu stores in the liver prior to assessment of the bioavailability of the Cu sources by feeding a diet with a low Cu content and high antagonist content which impairs the absorption of Cu from the diet (Suttle, 1974; Suttle, 2010). A reduction in liver Cu concentration is advantageous when trying to assess the bioavailability of a Cu source because accumulation of Cu in the liver is not linear; as liver Cu concentration increases the magnitude of accumulation in the liver is reduced (Figure 1.4 in Chapter 1.2.5; Wooliams, et al., 1983; Balemi, et al., 2010). This is likely because at higher liver Cu concentrations endogenous Cu losses are increased and the efficiency of Cu absorption is reduced (Suttle, 1974; Wooliams, et al., 1983). Thus, the bioavailability of a Cu source is more accurately assessed when liver Cu concentration is low and linear accumulation of Cu in the liver occurs (Suttle, 1974).

The primary aim of this study was to compare the bioavailability of nano Cu oxide (with a hydrolysed lysine coating) with conventional Cu oxide in growing lambs by analysing the accumulation of Cu in body tissue mineral stores, plasma mineral concentrations and enzyme activity using a Cu depletion and repletion technique. The secondary aim was to monitor the performance and health of lambs supplemented with nano Cu oxide and conventional Cu oxide.

4.2. Materials and Methods

4.2.1. Animals, management and treatments

Twenty eight, six month old, male Welsh Mountain lambs with a mean body weight of 20 kg (SD = 1.6) at the start of the depletion phase and 24 kg (SD = 2.3) at the start of the Cu repletion phase were used. Lambs were wormed with Zolvix^{TM} (Elanco, Basingstoke, UK), containing the active ingredient Monepantel, six weeks before commencement of the study. Prior to the study all lambs had been grazing swards containing predominantly ryegrass and received a six week adjustment period to adapt to a pelleted diet that was formulated according to AFRC (1993; Table 4.1). Following the adjustment period, the lambs were fed a depletion diet containing a high level of S, Mo and Fe, for six weeks until plasma Cu concentration was reduced to approximately 9 µmol/L (SD = 2.6).

Table 4.1 Dietary and chemical composition of the basal diet fed to growing lambs.

Ingredient	Inclusion g/kg DM	
	Depletion	Repletion
Oatfeed	299	299
Barley	220	220
Wheat	40	40
Dried beet pulp	120	120
Dried grass	100	100
Molasses	50	50
Hipro soy	50	50
Rapeseed meal	80	80
Urea	7	7
Megalac*	6	6
Sodium chloride	2.5	2.5
Minerals and vitamins**	25	25
Chemical analysis		
DM, g/kg	881	880
ME, MJ/kg	11.7	11.7
CP, g/kg DM	158	158
NDF, g/kg DM	400	410
Ash, g/kg DM	64.9	67.7
Cu, mg/kg DM	10.7	10.0
Zn, mg/kg DM	51.2	49.3
Co, mg/kg DM	0.1	0.1
Mo, mg/kg DM	5.9	0.5
Fe, mg/kg DM	792	232
S, g/kg DM	5.2	1.7

*Megalac rumen protected fat, Volac, Hertfordshire, UK

**Mineral/vitamin premix Rumenco Ca: 200 g/kg, Salt: 300 g/kg, Manganese: 2400 mg/kg, Zinc: 2000 mg/kg, Iodine: 200 mg/kg, Selenium: 10 mg/kg, Vit A: 320 IU, Vit D: 60 IU, Vit E: 1000 mg/kg, Vit B12: 2800 ug/kg

Prior to receiving the Cu repletion diet (Table 4.1) lambs were weighed on two consecutive days at 1300 h using a weigh crate (Shearwell Data Limited, Somerset, UK) which was calibrated using standard weights (Thornton and Co, Wolverhampton, UK). The lambs were then stratified and paired according to body weight and plasma Cu concentration, and randomly allocated to treatment group (Table 4.2). Lambs were housed in individual pens (1.3 m x 1.3 m), bedded on sawdust with *ad libitum* access to water. The lambs were evenly distributed by treatment group throughout the shed.

Table 4.2 Dietary treatments for lambs supplemented with Cu oxide or nano Cu oxide in the repletion period.

Treatment	Supplement	Cu, mg/kg DM
O	Cu oxide	9
N	Nano Cu oxide (lysine coated)	9

4.2.2. Characterisation of supplements

Characterisation of the physical properties of supplementary sources of Cu was conducted at Nottingham Trent University before the start of the study (Table 4.3). Particle size was determined by analysing results from TEM (JEOL JEM-2010, Michigan Tech, USA) and DLS (Particulate Systems, NanoPlus HD, Kromtek, Malaysia). Elemental composition was determined using ICP-OES (ICPE-9820, Shimadzu, UK).

Table 4.3 Physical and chemical characteristics of the supplementary Cu fed to growing lambs.

	Cu oxide	Nano Cu oxide
Coating	None	Hydrolysed lysine
Coating ratio	N/A	1:1
Chemical formula	Cu oxide	Cu oxide
CAS Number	1317-38-0	N/A
Appearance colour	Black	Black
Appearance form	Powder	Fine powder
Mean particle size (nm)	25000	45
Particle size range (nm)	40 – 33000	10 - 72
Elemental composition (%)	78.2	39.1

4.2.3. Experimental procedure

Lambs were weighed weekly at 1300 h. Feed intake was adjusted each week for each pair based on the mean live weight and a target of 160 g/d live weight gain (AFRC, 1993). Lambs were fed twice daily with equal amounts at 0800 h and 1500 h. The diets were supplemented with Cu by top dressing with the relevant supplement during the afternoon feed. The supplement was delivered in 10 ml of sunflower oil (Henry Lamotte Oils, Bremen, Germany) and the increase in the supplement rate was calculated based on increasing DMI and live weight over the study period. Feed refusals were collected daily at 0730 h. Feed was sampled weekly and frozen at -20 °C prior to proximate and

mineral analysis. Blood samples were collected weekly during the depletion phase at 1100 h by jugular venepuncture using a 21G x 1" needle (Becton Dickinson and Company, Plymouth, UK) into Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing K_2EDTA for the determination of mineral concentrations in plasma. During weeks 0, 4 and 8 of the repletion phase blood samples were also collected into Vacutainers containing K_2EDTA, K_3EDTA, silica and sodium heparin. Bloods were processed as detailed in Section 2.6. All sheep were slaughtered at a commercial abattoir (W & G Yates, Walsall, UK) by stunning and exsanguination at the end of week 8, and livers, kidneys and spleens were collected. Livers, kidneys and spleens were weighed immediately after extraction before being frozen at -20 °C.

4.2.4. Analytical procedure

Feed samples were analysed for Cu, Co, Fe, Mn, Mo, S and Zn by ICP-MS (NexION 2000, PerkinElmer, Shelton, CT) as described in Section 2.6, DM (943.01; intra-assay CV of 0.4) as described in Section 2.1, CP (990.03; intra-assay CV of 2.5%) using a Leco FP-528 (Leco Corporation, St Joseph, MI) as described in Section 2.2, ash (intra-assay CV of 2.5%) as described in Section 2.3 and NDF (intra-assay CV of 2.8%) according to Van Soest, et al. (1991) and as described in Section 2.4. Whole blood samples were analysed for haematology profile using a Vet Animal Blood Counter (Woodley Equipment Company Ltd, Bolton, UK) as described in Section 2.9. Serum, plasma and whole blood samples were analysed using a Cobas-Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK) for Cp (intra-assay CV of 3.8%) according to the method of Henry, et al. (1974), GGT (Kit catalogue no. GT 553; intra-assay CV of 2.0%) and SOD (Kit catalogue no. SD 125; intra-assay CV of 2.8%) as described in Section 2.7. During the depletion phase of the study plasma samples were analysed for Cu by atomic absorption (intra-assay CV of 1.0%) as described in section 2.8. Plasma samples were analysed for Cu, Fe, and Mo by ICP-MS (NexION 2000, PerkinElmer, Shelton, CT; intra-assay CV of 2.4%, 1.8% and 1.2% respectively) as described in Section 2.6. Livers, kidneys and spleens were analysed for Cu by ICP-MS (NexION 2000, PerkinElmer, Shelton, CT; intra-assay CV of 1.5%, 1.3% and 2.1% respectively) as described in Section 2.6.

4.2.5. Statistical analysis

Performance, plasma minerals and enzyme activity were analysed by repeated measures ANOVA as a randomised pair design with 14 replicates per treatment using

the data recorded in week 0 as a covariate where appropriate. Data was checked for normality by visual assessment of residual plots and analysed as:

$Y_{ijk} = \mu + B_i + M_j + T_k + MT_k + \varepsilon_{ijk}$

Where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of pairs; M_j = effect of mineral; T_k = effect of time; MT_k = interaction between mineral and time; and ε_{ijk} = residual error.

Liver, kidney and spleen Cu concentration was analysed by ANOVA as a randomised pair design and analysed as:

$Y_{ijk} = \mu + B_i + M_j + \varepsilon_{ijk}$

Where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of pairs; M_j = effect of mineral; and ε_{ijk} = residual error.

All statistical analysis was conducted using Genstat 18 and $P < 0.05$ was used to indicate significance. Data is presented as means with SED.

4.3. Results

4.3.1. Copper depletion phase

Lambs were depleted of their copper stores for 6 weeks until the mean plasma Cu concentration reached 9 μmol/L (Figure 4.1).

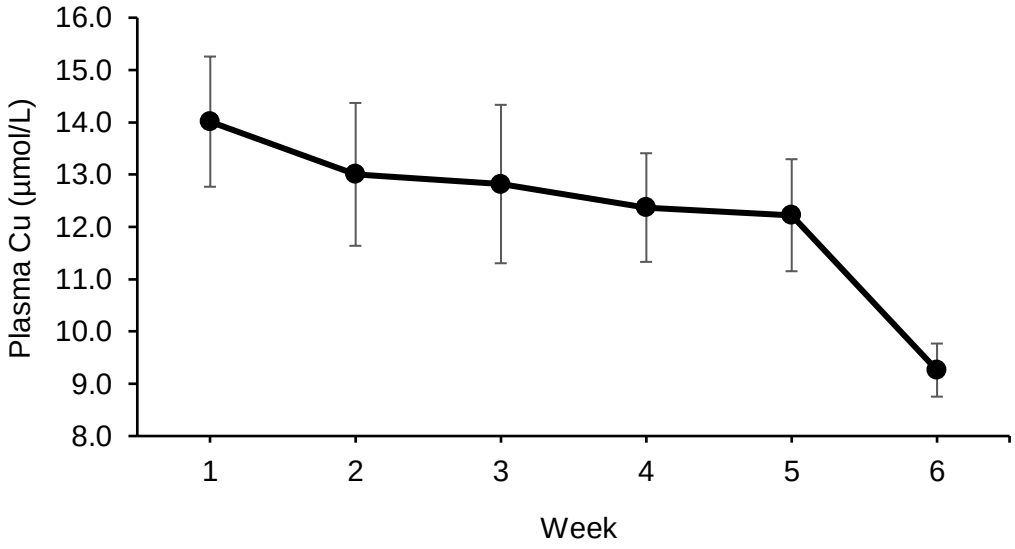

Figure 4.1 Mean plasma Cu concentration (µmol/L) over time in growing lambs fed a diet containing Cu antagonists during a 6 week Cu depletion phase. Error bars indicate the SED.

4.3.2. Copper repletion phase

4.3.2.1. Animal performance and intake during the repletion phase

Initial live weight did not differ (P = 0.438) between treatments (Table 4.4). There was no effect (P > 0.05) of supplement on, final live weight, live weight gain, DMI, liver, kidney or spleen weight during the Cu repletion phase.

Table 4.4 Performance and intake of growing lambs fed a diet containing Cu oxide (O) or nano Cu oxide (N) following Cu depletion.

	Treatment[1]			
	O	N	SED	Significance
Initial live weight at start of repletion phase, kg	24.3	24.5	0.50	0.438
Final live weight at end of repletion phase, kg	33.0	31.7	0.48	0.206
Live weight gain during repletion phase, g/d	161	140	11.1	0.172
Mean DMI during repletion phase, g/d	926	928	18.4	0.957
Liver weight, g	531	527	22.0	0.867
Kidney weight, g	44.7	42.4	1.35	0.111
Spleen weight, g	56.4	57.2	4.32	0.873

[1] Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM

4.3.2.2. Plasma mineral concentration

There was no effect (P = 0.138) of supplement on plasma Cu concentration but there was an effect of time (P < 0.001), with plasma Cu concentration increasing from week 0 to week 1, then plateauing between week 1 and week 8 (Figure 4.2). There was no time x treatment interaction (P = 0.544).

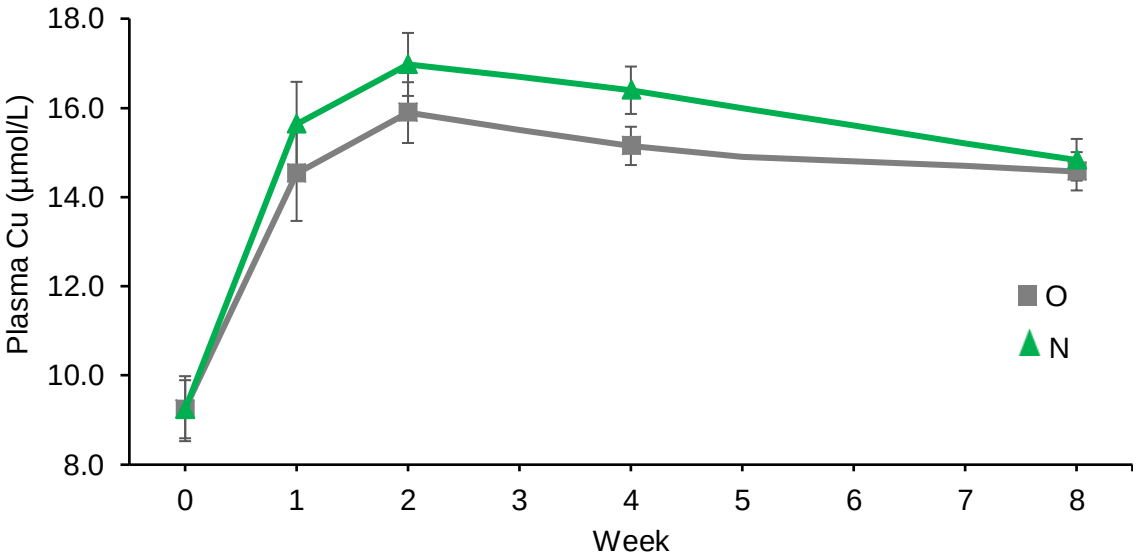

Figure 4.2 Mean plasma Cu concentration (µmol/L) over time in growing lambs fed a diet containing Cu oxide or nano Cu oxide following Cu depletion. Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM. Error bars indicate the SED. Treatment, P = 0.183; time, P < 0.001; time x treatment, P = 0.544.

There was no effect (P = 0.622) of supplement on plasma Mo concentration but there was an effect of time (P < 0.001), with plasma Mo concentration decreasing from week 0 to week 4, then plateauing between week 4 and week 8 (Figure 4.3). There was no time x treatment interaction (P = 0.571).

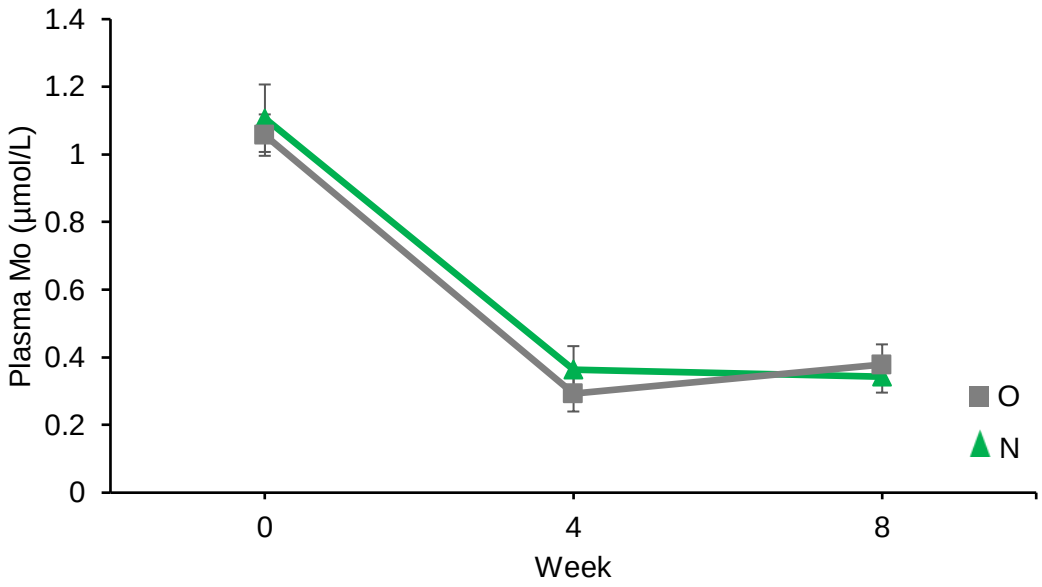

Figure 4.3 Mean plasma Mo concentration (µmol/L) over time in growing lambs fed a diet containing Cu oxide or nano Cu oxide following Cu depletion. Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM. Error bars indicate the SED. Treatment, P = 0.622; time, P < 0.001; time x treatment, P = 0.571.

There was no effect of supplement (P = 0.780) or time (P = 0.852) on plasma Fe concentration (Figure 4.4). There was no time x treatment interaction (P = 0.910).

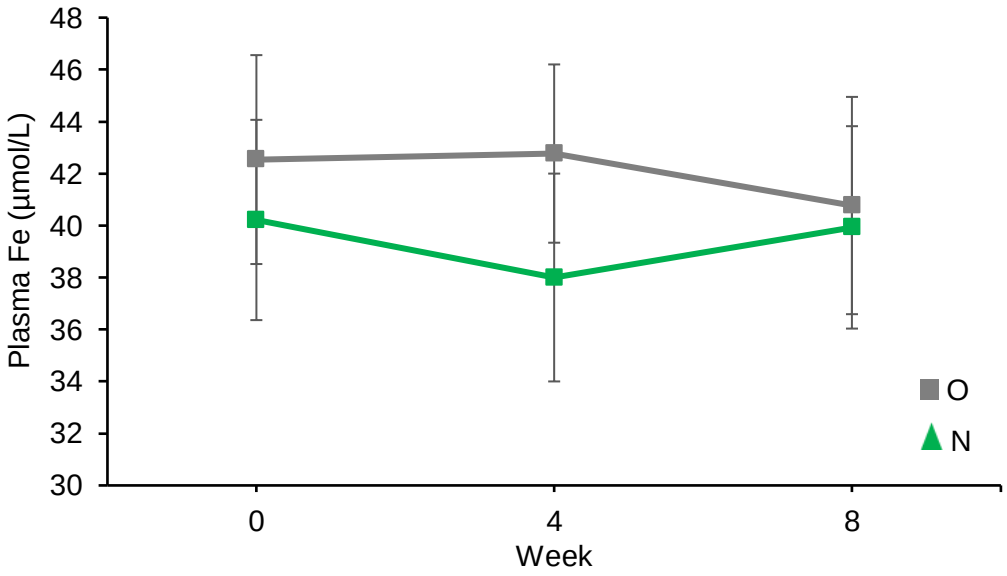

Figure 4.4 Mean plasma Fe concentration (µmol/L) over time in growing lambs fed a diet containing Cu oxide or nano Cu oxide following Cu depletion. Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM. Error bars indicate the SED. Treatment, P = 0.780; time, P = 0.852; time x treatment, P = 0.910

4.3.2.3. Blood enzyme activity

There was no effect (P = 0.361) of supplement on Cp concentration but concentration did increase over time (P < 0.001) from week 0 to week 8 (Figure 4.5). There was no time x treatment interaction (P = 0.266).

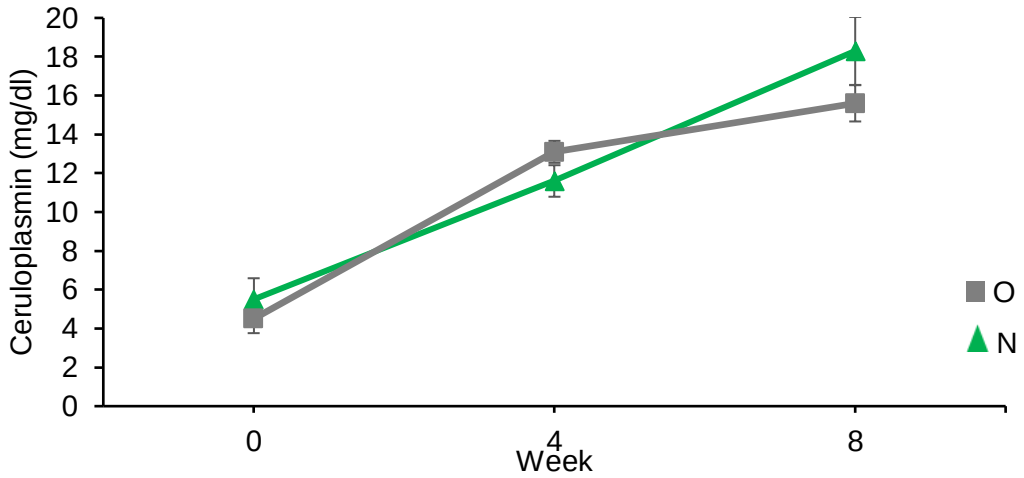

Figure 4.5 Mean ceruloplasmin activity (mg/dl) over time in growing lambs fed a diet containing Cu oxide or nano Cu oxide following Cu depletion. Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM. Error bars indicate the SED. Treatment, P = 0.361; time, P < 0.001; time x treatment, P = 0.266.

There was no effect of supplement (P = 0.701) on GGT activity but there was a trend (P = 0.057) for a decline in GGT activity over time (Figure 4.6). There was no time x treatment interaction (P = 0.717).

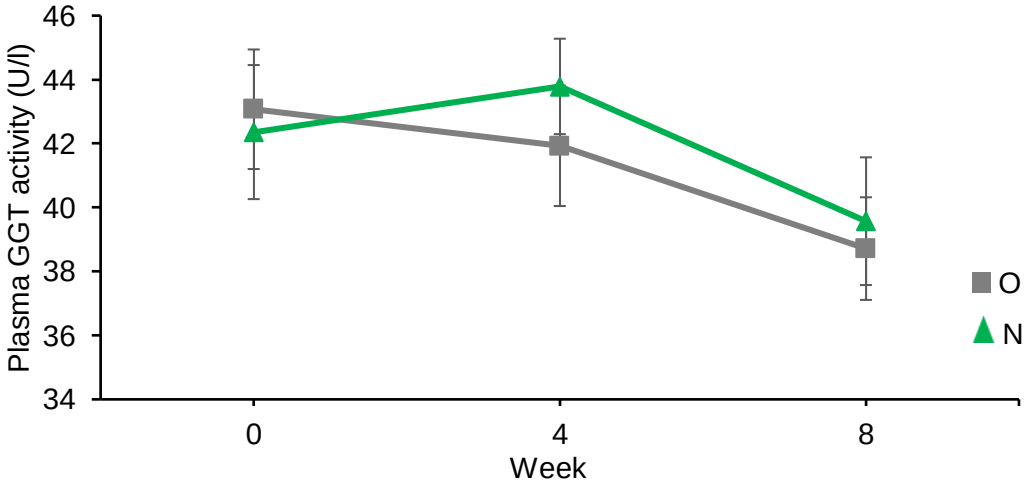

Figure 4.6 Mean plasma gamma glutamyl transferase activity (U/l) over time in growing lambs fed a diet containing Cu oxide or nano Cu oxide following Cu depletion. Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM. Error bars indicate the SED. Treatment, P = 0.701; time, P = 0.057; time x treatment, P = 0.717.

Whole blood SOD activity was higher (P = 0.033) in lambs supplemented with nano Cu oxide with a mean of 1310 U/g Hb, compared to lambs supplemented with conventional Cu oxide with a mean of 1074 U/g Hb (Figure 4.7). Whole blood SOD activity increased over time (P < 0.001) from week 0 to week 8. There was no time x treatment interaction (P = 0.335).

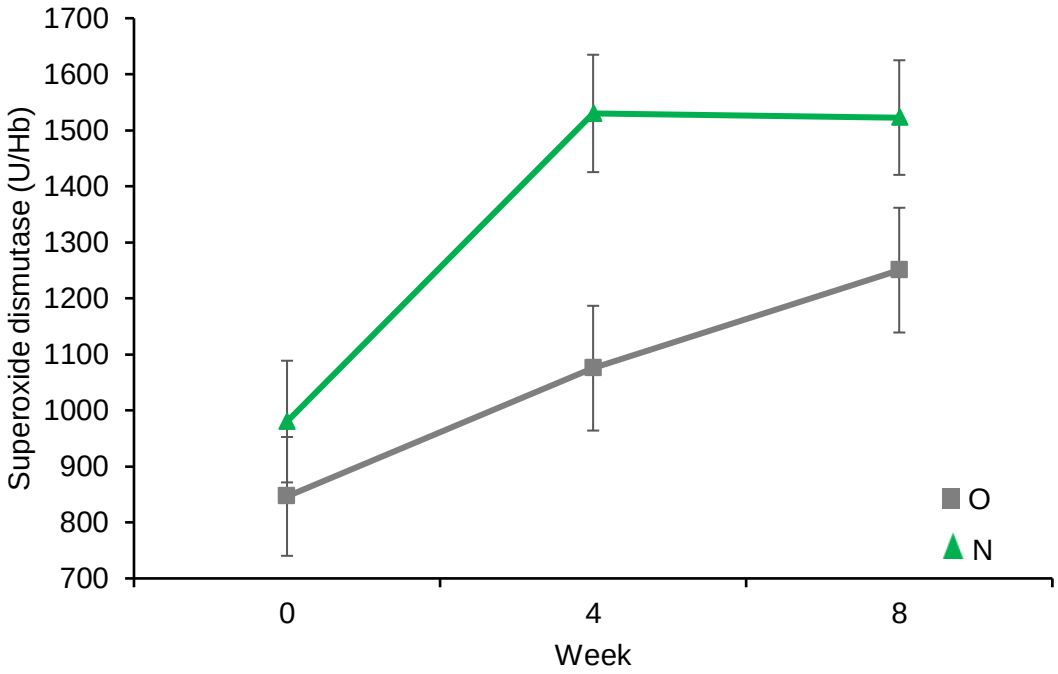

Figure 4.7 Mean superoxide dismutase activity (U/g Hb) over time in growing lambs fed a diet containing Cu oxide or nano Cu oxide following Cu depletion. Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM. Error bars indicate the SED. Treatment, P = 0.033; time, P < 0.001; time x treatment, P = 0.335.

4.3.2.4. Haematology profile

There was no treatment x time effect (P > 0.05) on the haematology profile of the lambs (Table 4.5). Levels of Hb were increased (P = 0.016) in lambs supplemented with nano Cu oxide compared to lambs supplemented with conventional Cu oxide. There was no other effect (P > 0.05) of form of Cu supplement on the haematology profile of lambs but for some parameters there was an effect (P < 0.05) of time.

Table 4.5 Effect of form of Cu supplement and time on haematology profile[2] in growing lambs.

	Treatment[1]			Significance[3]		
	O	N	SED	F	T	F x T
WBC,10^3/mm^3	8.38	8.23	0.665	0.789	0.003	0.695
Lym No, 10^3/mm^3	5.70	5.73	0.488	0.935	0.048	0.482
Mon No, 10^3/mm^3	0.43	0.44	0.0411	0.776	<0.001	0.352
Neu No, 10^3/mm^3	1.93	2.03	0.237	0.599	<0.001	0.468
Eo No, 10^3/mm^3	0.15	0.15	0.047	0.922	0.022	0.539
Ba No, 10^3/mm^3	0.028	0.029	0.0053	0.407	0.658	0.737
Hb, g/dl	11.4	12.2	0.453	0.016	<0.001	0.357
RBC, 10^6/mm^3	11.6	12.0	0.392	0.207	0.018	0.530
HCT, %	35.4	36.6	2.196	0.357	<0.001	0.153

[1]Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM

[2]white blood cells (WBC), lymphocyte number (Lym No.), monocyte number (Mon No.), neutrophil number (Neu No,), eosinophil number (Eo No.), basophil number (Ba No.), haemoglobin (Hb), red blood cells (RBC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

[3] F+L = Effect of form of Cu supplement, T = Effect of time and F x T = interaction between form of Cu supplement and time.

There was a decrease ($P < 0.05$) in WBC, Lym no., Mon no and Neu no. from week 0 to week 8 in lambs in both treatment groups. There was an increase ($P < 0.05$) in Eo no., RBC no., Hb and HCT from week 0 to week 8 in lambs in all treatment groups. There was no effect ($P > 0.05$) of time on Ba No.

4.3.2.5. Tissue Cu analysis

There was no effect of form ($P = 0.738$) of supplement on the final concentration of Cu in the kidneys with a mean of 8.5 mg/kg DM for lambs supplemented with nano Cu oxide and 8.2 mg/kg DM for lambs supplemented with conventional Cu oxide (Figure 4.8).

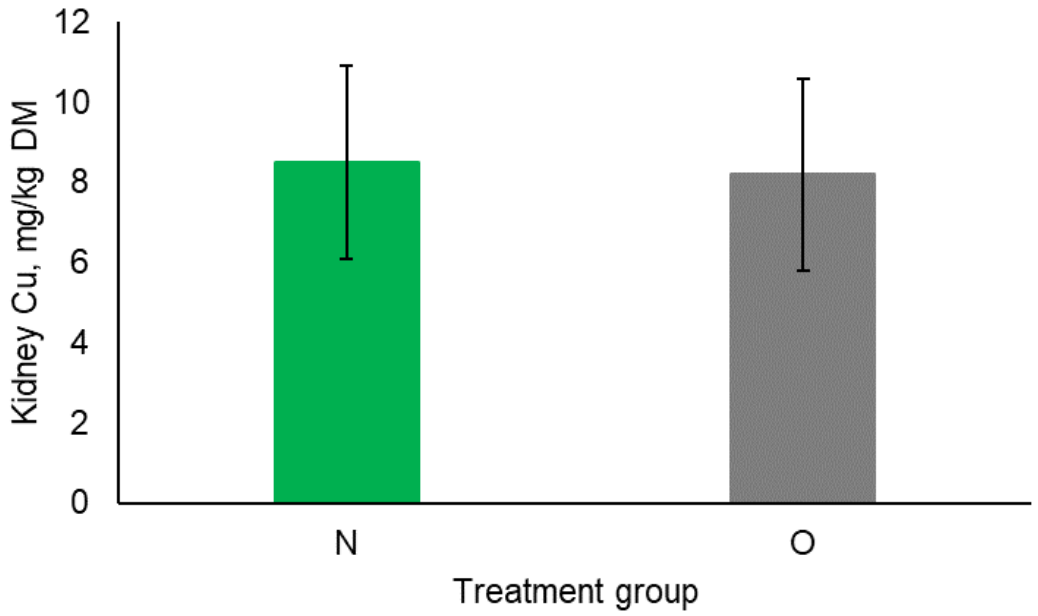

Figure 4.8 Kidney Cu concentration (mg/kg DM) in growing lambs fed a diet containing nano Cu oxide or Cu oxide following Cu depletion. Error bars indicate the SED.
Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM

There was no effect of form (P = 0.192) of supplement on the final concentration of Cu in the spleen with a mean of 15 mg/kg DM for lambs supplemented with nano Cu oxide and 10 mg/kg DM for lambs supplemented with conventional Cu oxide (Figure 4.9).

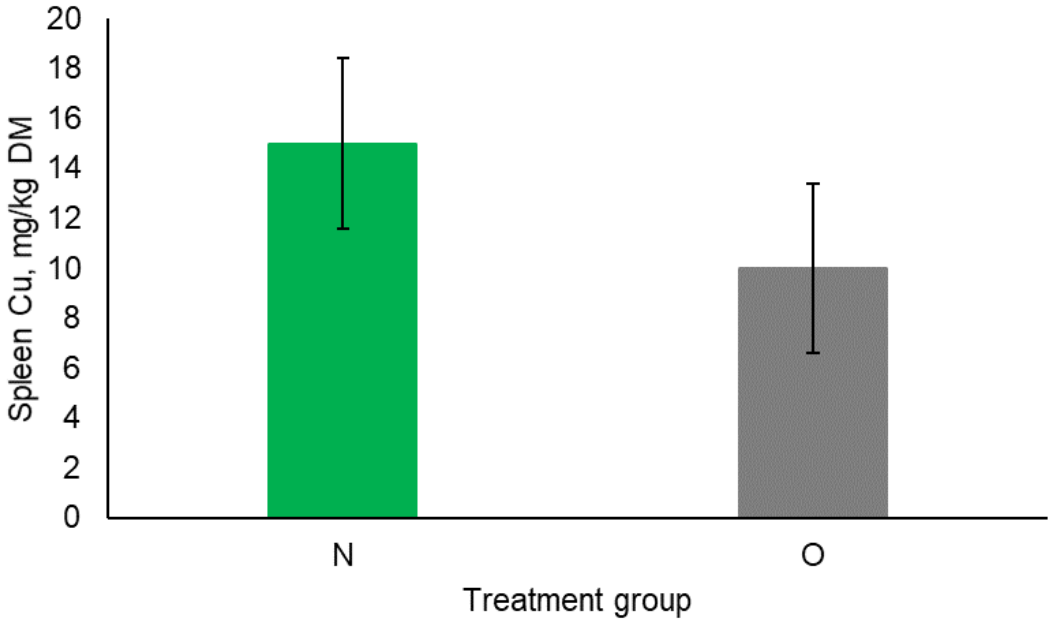

Figure 4.9 Spleen Cu concentration (mg/kg DM) in growing lambs fed a diet containing nano Cu oxide or Cu oxide following Cu depletion. Error bars indicate the SED.
Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM

Final concentration of Cu in the liver was increased (P < 0.05) in lambs supplemented with nano Cu oxide with a mean of 300 mg/kg DM, compared to those supplemented with conventional Cu oxide, with a mean of 200 mg/kg DM (Figure 4.10).

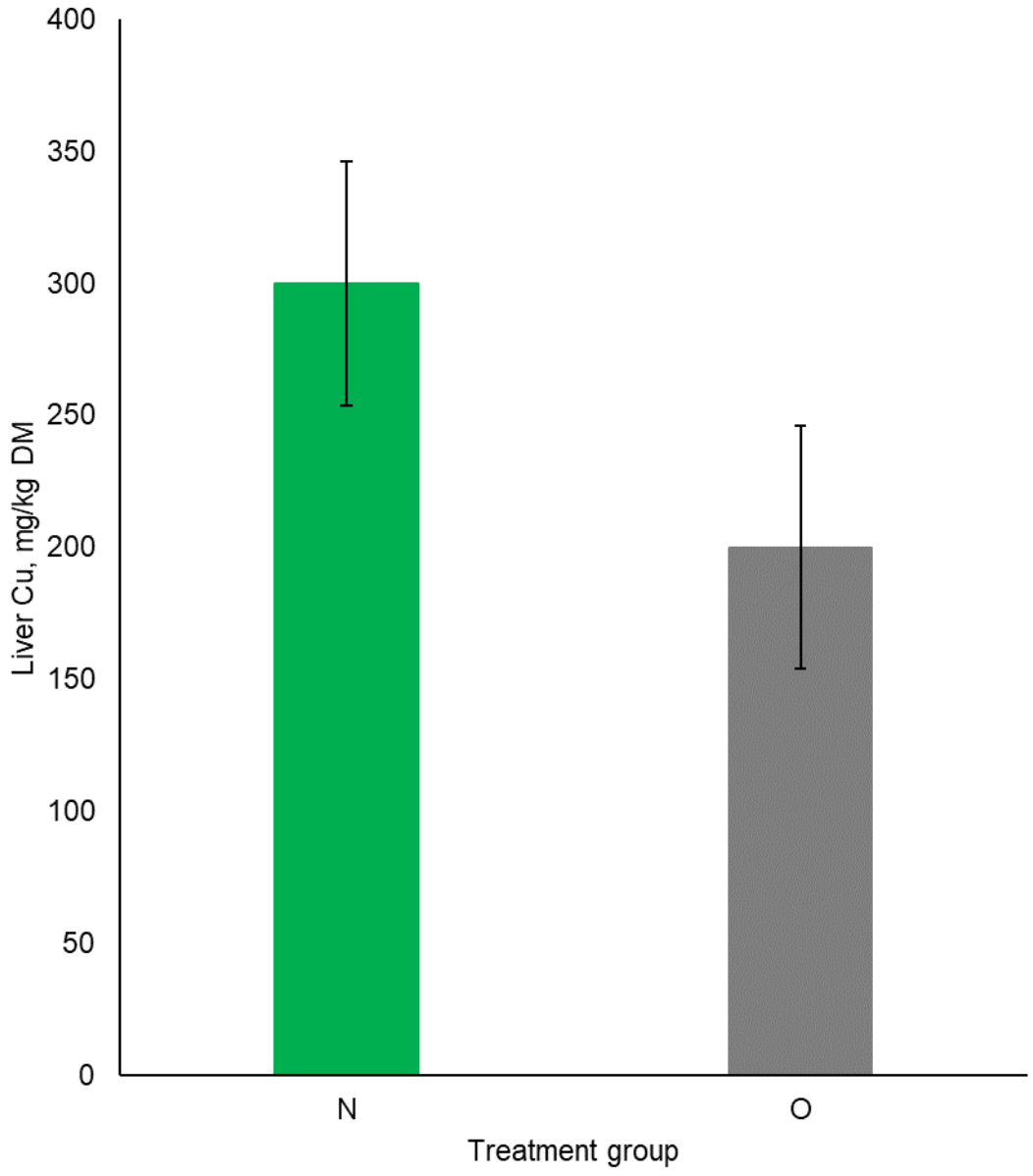

Figure 4.10 Liver Cu concentration (mg/kg DM) in growing lambs fed a diet containing nano Cu oxide or Cu oxide following Cu depletion. Error bars indicate the SED.

Treatments were: O; Cu oxide 9 mg/kg DM and N; Nano Cu oxide 9 mg/kg DM

4.4. Discussion

4.4.1. Introduction

Minerals in ruminant diets vary greatly in their bioavailability (Baker & Ammerman, 1995a). Mineral nanoparticles are predicted to be more bioavailable than conventional mineral sources because of their small particle size and associated large surface area (Sahoo, et al., 2014). Experiment 1 (Chapter 3) demonstrated that nanoparticles of Cu oxide may have a greater bioavailability than Cu oxide with a conventional particle size (in the micro range), but a greater number of replicates and reduced variation between individual animals were suggested to be required to reach statistical significance.

In the current study, variation was reduced by depleting the lambs of their Cu stores prior to commencement of the repletion phase of the study. This was achieved by feeding a diet high in the Cu antagonists S and Mo (5.2 g/kg DM and 5.9 mg/kg DM respectively) and moderate in Cu (10.7 mg/kg DM) until plasma Cu concentration reached 9 µmol/L (SD = 2.6). The plasma Cu concentration of 9 µmol/L was selected because values less than this are indicative of clinical Cu deficiency (Underwood, 1977). The nanoparticle Cu oxide and conventional Cu oxide supplements used in this study had different concentrations of Cu at 39.1% and 78.2% respectively. The supplements were therefore added at a rate that ensured that Cu was provided at the same concentration in each treatment group (9 mg Cu/kg DM). The basal diet was formulated to have a low Cu content at 10 mg/kg DM and was provided to lambs in the form of a pellet at 0.95 *ad libitum* intake to avoid selective feeding and to ensure that the lambs ate the top dressed supplement.

The current study aimed to investigate the bioavailability of nano Cu oxide and conventional Cu oxide when added to the diet of growing lambs to provide a direct comparison of the effect of particle size on mineral availability. The particle size of the conventional Cu oxide was 25000 nm whereas the particle size of the nano Cu oxide was 45 nm thus the particle size of the nano minerals was approximately five hundred times smaller.

4.4.2. Animal performance

Live weight gain was higher in the current study compared to Experiment 1; 150 g/d vs 93 g/d respectively, this is likely because the ME of the diet was increased between the

two studies from 10.4 MJ/kg to 11.7 MJ/kg. Similarly, to Experiment 1 there was no effect of form of Cu on DMI or live weight gain in the current study. The effect of supplementary Cu on live weight gain is rarely reported but Ward et al. (1993) and Engle & Spears (2000) reported that form of Cu had no effect on live weight gain or DMI in growing steers. In contrast, Galyean et al. (1995) reported that Cu lysine supplemented at 5 mg Cu/kg DM had a negative effect on live weight gain and DMI when supplemented to growing steers.

4.4.3. Plasma mineral concentration

In the current study, there was no difference between conventional Cu oxide (O) and nano Cu oxide (N) on plasma Cu concentration. In studies where a difference in the bioavailability of Cu sources has been reported based on differences in plasma concentration, initial concentrations were generally below the normal range for sheep and cattle (< 9 µmol/L; Suttle, 1974; Kegley & Spears, 1994; Ward et al., 1996). In the current study lambs started the Cu repletion phase with a mean plasma Cu concentration of 9.0 µmol/L (range 4 – 13 µmol/L) which may not have been consistently low enough to identify differences in bioavailability of Cu sources via plasma concentration. There could also be a smaller magnitude of difference in bioavailability between conventional Cu oxide and nano Cu oxide compared to the Cu sources used in previous studies. For example Kegley & Spears (1994) identified a difference in plasma Cu concentration between growing cattle supplemented with sulphate and oxide, but it is well documented that the bioavailability of Cu sulphate is at least 5 times greater than Cu oxide (Baker & Ammerman, 1995a).

In the current study there was an increase in plasma Cu concentration between week 0 and week 1 before plateauing, at 15.5 µmol/L, between week 1 and 8. Similarly, many Cu depletion and repletion studies have reported that when the Cu repletion phase begins plasma concentration increases rapidly before plateauing within the normal range (Suttle, 1974; Kegley & Spears, 1994; Rabiansky, et al., 1999). The Cu that is stored in the liver maintains plasma concentration within the normal range by homeostatic control, therefore if the animal is depleted of Cu then as soon as it is available in the diet it will immediately restore blood Cu levels into the normal range (Evans, 1973). Plasma concentration is maintained for as long as possible, until liver concentration is less than 27 mg/kg DM, indicative of Cu deficiency or more than 1000 mg/kg DM, indicative of toxicity (Suttle, 2010). In contrast to the current study Kegley & Spears (1994) reported that Cu oxide was essentially unavailable based on plasma

concentration and Cp concentration not differing from control animals that did not receive any Cu supplementation. This could indicate a difference in absorption of Cu oxide between the beef cattle used by Kegley & Spears (1994) and the Welsh Mountain lambs used in the current study or it could be a result of different experimental conditions. Kegley & Spears (1994) conducted two experiments, the first using calves depleted of Cu from birth and the second investigating Cu bioavailability while feeding high levels of antagonists. There are two major differences between the current study and that of Kegley & Spears (1994); species and different concentrations of antagnoists in the diet, which may have led to the contrasting results.

Plasma Mo concentration was measured to understand the effect of feeding high levels of antagonists in the diet and see if there was a response in the blood when the concentration of antagonists in the diet was decreased. Plasma Mo concentration decreased when the lambs were swapped from the Cu depletion diet (5.9 mg Mo/kg DM) to the Cu repletion diet (0.5 mg Mo/kg DM). Dietary Mo is readily absorbed therefore concentration in the blood is reflective of dietary intake (Wittenberg & Devlin, 1987). In contrast there were no differences or changes over time for plasma Fe concentration indicating that it was less responsive to dietary change. Iron is less responsive to dietary change because it is controlled at the level of absorption (Herdt & Hoff, 2011).

4.4.4. Enzyme activity

There was no difference in Cp concentration between lambs supplemented with nano Cu oxide or conventional Cu oxide, but there was an increase in Cp concentration over time. The increase in Cp concentration over time may be due to the depletion, repletion technique used; all lambs had an increased Cp concentration at week 4 and 8 compared to week 0 indicating a repletion of the bodies Cu stores and the availability of Cu for inclusion in enzymes. This pattern of increase in Cp concentration reflects the changes in plasma Cu concentration, analogous to the observations reported by Suttle (1974). Rabiansky et al. (1999) also reported that following Cu depletion Cp concentration increased over time but there was no difference between treatments when supplementing heifers with Cu lysine and Cu sulphate.

Previous studies have indicated that SOD activity is responsive to dietary Cu concentration (Ward & Spears, 1997; Nagalakshmi, et al., 2010; Cheng, et al., 2011). The current Cu depletion and repletion study produced a response in SOD activity over

time because Cu concentration in the diet increased from 10.7 mg Cu/kg DM to 19 mg Cu/kg DM and additional antagonists were removed from the diet. Lambs supplemented with nano Cu oxide had a higher SOD activity compared to those supplemented with conventional Cu oxide, with means of 1525 U/g Hb and 1163 U/g Hb respectively. Similarly, Nagalakshmi et al. (2010) and Pal et al. (2011) both reported differences in SOD activity in sheep with different Cu sources. In contrast, Cheng et al. (2011) reported that there was no difference in SOD activity in lambs fed different Cu lysine and tribasic Cu chloride at 10 or 20 mg Cu/kg DM. Gonzalez-Eguia et al. (2009) reported that SOD activity was increased in pigs supplemented with Cu sulphate compared to those supplemented with nano Cu oxide. Studies have shown that Cu sulphate has a much greater bioavailability than Cu oxide (Baker & Ammerman, 1995a) which could explain the results reported by Gonzalez-Eguia et al. (2009).

4.4.5. Liver Cu concentration

Accumulation of Cu in the liver of ruminants occurs when dietary supply exceeds requirements therefore liver Cu concentration is reflective of the absorption and availability of the Cu source (Xin, et al., 1991; McDowell, L.R., 1992; Sinclair, et al., 2013). Several previous studies have reported differences in the bioavailability of Cu sources as a result of liver Cu concentration (Kincaid, et al., 1986; Xin, et al., 1991; Ward, et al., 1993). In aquaculture it has been reported that whole body Cu concentration is greater in fish fed diets containing nano Cu oxide compared to conventional Cu sources, indicating a greater bioavailability (Wang, et al., 2018). Gonzalez-Eguia et al. (2009) reported that pigs supplemented with nano Cu oxide had reduced faecal Cu concentrations compared to those supplemented with Cu sulphate which indicated improved absorption and potentially greater storage. Similarly, in ruminants and monogastrics storage of Se has been reported to be increased by the provision of nano Se in the diet compared to conventional minerals (Shi, et al., 2011; Hu et al., 2012). In the current experiment, final liver Cu concentration was higher in lambs supplemented with nano Cu oxide (300 mg Cu/kg DM) compared to those supplemented with conventional Cu oxide (200 mg Cu/kg DM) indicating an increased bioavailability. Similarly to the current study Rabiansky et al. (1999) reported that an increase in liver Cu concentration in cattle could only be detected in those that had low initial liver Cu concentrations.

4.4.6. Haematology profile

There was a decrease in WBC, lym No., Mon No. and Neu No. over time for all lambs, but the values remained within the normal reference ranges for sheep (Etim, et al., 2014). Profile of white blood cells in blood can be affected by many variables and may not be a direct result of the treatments in this study. Most of the research examining changes in haematology profile of animals has been conducted in poultry but the decreases in white blood cells could be due to, but are not limited to, a change in environmental conditions (Vecerek, et al., 2002), stress (Schalm, et al., 1975), a change in diet including mineral supplementation (Tras, et al., 2000; Iheukwumere & Herbert, 2002) and limited availability for exercise (Swenson, 1970). Thus it is impossible to fully determine the most likely variables that are responsible for the changes in the haematology profile over time in the current study.

There was an increase over time in Hb, HCT and RBC levels for all lambs. This is most likely to be a result of the low Cu status in the depletion phase of the study followed by a restoration of Cu levels. Synthesis of Hb is reduced during Cu deficiency because mobilisation of Fe is required by the enzyme Cp, (Ward & Spears, 1997) thus Hb, HCT and RBC can be increased when Cu status is increased following a period of Cu deficit (Ward, 1978). Ward & Spears (1997) also reported increased Hb levels when growing cattle were supplemented with Cu. In the current study, lambs supplemented with nano Cu oxide compared to conventional Cu oxide had higher Hb levels. Suttle (1974) also reported an increase in Hb levels in hypocupremic ewes supplemented with Cu sulphate but not Cu monosulphide. Differences in the effect of Cu sources on Hb levels could therefore be related to the bioavailability of the Cu source and its ability to restore enzyme levels more rapidly.

4.4.7. Animal health

The limited research on nanoparticles in ruminants may be partially linked to caution over the potential negative effect of nanoparticles on both animal and human health (Radha, et al., 2014). Studies investigating the use of nanoparticles in animal nutrition have reported a wide range of effects on animal health, from a lower toxicity compared to conventional sources (Pelyhe & Mezes, 2013) to renal and liver damage indicative of toxicity (Najafzadeh, et al., 2013). Previous studies assessing safety of nanoparticles have primarily been conducted in rodents and have measured the distribution and accumulation of nanoparticles in the kidney and spleen, and in some cases reported a

long term accumulation (Balasubramaniam, et al., 2010; Sang, et al., 2012; Tassinari, et al., 2014). Balasubramaniam et al. (2010) reported that gold (Au) nanoparticles were accumulated in the spleen and liver of rat's immediately post intravenous injection, and that nanoparticles were retained for at least 2 months. In the kidneys there was a gradual increase in nanoparticle concentration which was inversely proportional to the nanoparticles present in urine (Balasubramaniam, et al., 2010). Similarly, there are studies that have measured the accumulation of titanium in the spleen as a result of administration of titanium dioxide nanoparticles and hypothesised that this could cause splenic injury (Sang, et al., 2012; Tassinari, et al., 2014).

In the current study there was no evidence that accumulation of Cu in the kidney or spleen was affected by Cu source. Ledoux et al. (1996) and Pal et al. (2010) also reported that the accumulation of Cu in the kidney was not affected by Cu source. In two studies where a difference in Cu accumulation in the kidney has been reported (Gopinath, et al., 1974; Theil & Calvert, 1978) the animals have been fed an extremely high Cu dose of 20 mg of Cu per kg body weight per day as Cu sulphate so that liver Cu concentration was 2000 mg/kg DM or higher, indicative of Cu toxicity (Ishmael, et al., 1972; Suttle, 2010). Eckert et al. (1999) reported an interaction between Cu source and level for kidney Cu concentration. Ewes fed 20 mg Cu/kg as Cu proteinate had greater concentrations than those fed 10 or 30 mg Cu/kg DM whereas ewes fed Cu sulphate at 10 mg Cu/kg DM had greater kidney Cu concentrations than those fed 20 or 30 mg Cu/kg DM. The authors reported that this may be because of different mechanisms of action for inorganic and organic sources (Eckert, et al., 1999).

Ledoux et al. (1996) also reported that the accumulation of Cu in the spleen was not affected by Cu source. Theil & Calvert (1978) reported that Cu accumulation in the spleen (39.2 mg/kg DM) occurred when sheep were supplemented with very high levels Cu sulphate (20 mg/kg LW/d). The increase in spleen Cu concentration was hypothesised to be due to the phagocytosis of erythrocytes containing high concentrations of Cu (Theil & Calvert, 1978). In the current study the accumulation of Cu in the kidney and spleen did not differ between nano Cu oxide and conventional oxide indicating that Cu was not supplied in excess in either of the diets. Liver Cu concentrations can also indicate excess Cu in the diet but they were also in the normal range (30 – 400 mg/kg DM) for sheep (Suttle, 2010). Excessive accumulation of Cu can cause hepatic damage and therefore serum GGT activity was measured as an indicator of liver damage (Bartholomew, et al., 1987). There was no difference in GGT activity between lambs supplemented with nano Cu oxide, with plasma concentration of

42 U/l compared to 41 U/l for lambs supplemented with conventional Cu oxide. The plasma GGT activity was within the normal range (20 – 52 U/l) for sheep indicating that nanoparticles did not cause liver damage (Radostits, et al., 2000). There were no abnormal results in the haematology profile of lambs on any of the treatments further indicating that nanoparticles did not cause an adverse effect on the health of the lambs within the 8 week period employed in the current study. Further research into the long term effects of nanoparticles on health and performance of sheep is still necessary.

4.5. Conclusions

The bioavailability of nano Cu oxide was greater than conventional Cu oxide as indicated by the increased storage of Cu in the liver and higher whole blood SOD activity. Nanoparticle Cu therefore offers the potential to improve Cu supply to ruminants. Supplying Cu oxide as nanoparticles did not have any effect on performance or indicators of health in the lambs, and the concentration of Cu in the kidneys and spleens did not differ between nano Cu oxide and conventional Cu oxide. This was, however a short term study, with the lambs receiving nanoparticles for 8 weeks, and longer term studies are required to ensure that the health and performance of animals are not negatively affected by nanoparticles.

5. Experiment 3 – Mineral status and performance of dairy cows fed nanoparticle copper, either without or in combination with antagonists

5.1. Introduction

Copper is a common trace element responsive disorder in dairy cows (Herdt & Hoff, 2011). In cattle Cu is crucial for many enzymes such as CCO, SOD, and Cp, therefore optimum Cu status of the animal is vital for cellular respiration and protection from oxidants. Clinical deficiency can manifest itself in a variety of signs which include loss of pigmentation, anaemia, reduced immunity, poor growth rates, reduced fertility and many others (Suttle, 2010).

There are two ways in which Cu responsive disorders can occur. A primary Cu deficiency is a result of diet with a low Cu concentration that is insufficient to meet requirements with the consequence that the animal has to mobilise Cu from its liver to maintain blood levels until eventually the liver is depleted (Herdt & Hoff, 2011). A secondary Cu responsive disorder is more common than primary and is caused by high dietary concentrations of Cu absorption and metabolism antagonists particularly Mo, S or Fe in the diet (Spears, 2003). In the rumen Mo and S form thiomolybdates that combine with Cu to produce highly stable insoluble complexes that are not absorbed by the animal with the Cu in these complexes being unavailable (Ward, et al., 1993). If there are also high levels of Fe in the diet there are two possible mechanisms which can reduce the availability of Cu to the animal (Gould & Kendall, 2011). Firstly, a Fe-S complex may form and then Cu may displace Fe to form Cu-S (Suttle, 1984), or secondly Fe may react with sulphide and Cu to produce a Fe-Cu-S complex (Suttle & Peter, 1985). Neither of these complexes can be absorbed by the animal and therefore the Cu is unavailable for absorption (Gould & Kendall, 2011).

To avoid negative effects on performance and health caused by a Cu responsive disorder, dairy cows are often supplemented with Cu (Spears, 2003). There are several different forms of Cu available for supplementation to cattle, and studies have shown that they can vary in their efficacy especially in the presence of high levels of antagonists (Kincaid et al., 1986; Kegley & Spears, 1994; Ward et al., 1996). For example, Kegley & Spears (1994) reported that Cu oxide was not very effective at

maintaining the Cu status of cattle when challenged with a high level of antagonists in the diet. This could be linked to the relatively low bioavailability of Cu oxide compared to other sources as the relative bioavailability has been reported to be 0.15 compared to Cu sulphate (Parkins et al., 1994; Baker & Ammerman, 1995a). The results of Experiment 2 (Chapter 4) demonstrated that reducing the particle size of Cu oxide into the nano from the micro scale increased bioavailability in growing lambs based on liver Cu concentration and SOD activity. Therefore, nano Cu oxide may be more effective than conventional Cu oxide at maintaining the Cu status of cattle, particularly when challenged by high dietary concentrations of Cu antagonists. This may be advantageous because Cu oxide is one of the only forms of Cu that can be used in an intra-ruminal mineral bolus because it is relatively inert, has a high bulk density and low solubility (SRL Ecoterm, 2017). Increasing the bioavailability of Cu oxide, especially when fed in the presence of high levels of antagonists, which is the most common reason for Cu responsive disorders, may therefore improve the efficacy of Cu containing mineral boluses.

The primary aim of this study was to compare the bioavailability of nano Cu oxide with conventional Cu oxide in Holstein-Friesian dairy cows when fed either without or in combination with S and Mo by determining the accumulation of Cu in the liver, plasma mineral concentration and enzyme activity. The secondary aim was to monitor the effect on the performance and health of dairy cows.

5.2. Materials and methods

5.2.1. Animals, management and treatments

Fifty-six Holstein-Friesian dairy cows that were 48 ± 17.2 days post calving, yielding 41 ± 6.4 kg/d of milk per day, weighing 655 ± 70.8 kg and an average body condition score of 2.8 ± 0.26 using the method of Ferguson et al. (1994) were used in a 2 x 2 factorial design for 16 weeks. Based on recordings taken in the two weeks prior to commencing the study animals were blocked and allocated to one of four dietary treatments according to calving date, lactation number, milk yield, condition score and live weight.

Cows were fed a TMR based on lucerne and maize silages (0.35:0.65 ratio DM basis) and straight feeds formulated to meet the requirement of a cow producing approximately 40 kg of milk per day according to Thomas (2004; Table 5.1). All dietary

ingredients were mixed using a Hi-Spec MixMax 10 diet forage mixer (Hi-Spec Engineering Ltd, Country Carlow, Ireland) calibrated to ± 1 kg. Diets were fed as a TMR through Insentec roughage intake feeders (RIC feeders, Insentec B.V, Marknessem Netherlands) fitted with an automatic animal identification and forage weighing system calibrated to ± 0.1 kg (Sinclair et al., 2005 and 2007). Fresh feed was offered at 1.05 of *ad libitum* intake at 0800 h with refusals collected three times a week on a Monday, Wednesday and Friday. The cows were housed in the same portion of a cubicle building containing Super Comfort free stalls. The passageways were scraped using automatic scrapers and the stalls bedded with sawdust and limed twice weekly. All cows had continual access to water.

Table 5.1 Dietary composition (g/kg DM) of the basal diet fed to Holstein-Friesian dairy cows on four dietary treatments*.

Ingredient	Dietary composition, g/kg DM
Maize silage	394
Lucerne silage	158
Sweet starch	91
Rape meal	74
Wheat distillers	74
Soya Hi-pro	31
Palm Kernel	21
Molasses	6
Rapetec[1]	26
Soya hulls	84
Acid Buffer[2]	5
Wheat straw	11
Limestone	0
Salt	3
Megalac[3]	3
Minerals and vitamins[4]	9
Hydrolysed lysine[5]	0.01

*Treatments are: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.

[1]Extruded natural meal derived from whole rape and whear, KW Feeds, West Yorkshire, UK

[2]Acid Buffer, calcareous marine algae, AB Vista, Wiltshire, UK

[3]Megalac rumen protected fat, Volac, Hertfordshire, UK

[4]Mineral/vitamin premix without additional antagonists: 180 g Ca/kg, 52.5 g P/kg 75 g Mg/kg, 75 g Na/kg, 120 g Cl/kg, 263 mg Se/kg, 30 mg Co/kg, 300 mg I/kg, 1500 mg Mn/kg, 4500 mg Zn/kg; with additional antagonists: 148 g Ca/kg, 52.5 g P/kg 75 g Mg/kg, 75 g Na/kg, 120 g Cl/kg, 140 g S/kg, 26.3 mg Se/kg, 30 mg Co/kg, 300 mg I/kg, 1500 mg Mn/kg, 4500 mg Zn/kg, 680 mg Mo/kg

[5]Hydrolysed lysine, reagent grade >98%, Sigma-Aldrich, UK

To evaluate the effect of form of Cu on Cu bioavailability the diets contained either conventional Cu oxide (O) or nano Cu oxide (N) to supply an additional 8 mg Cu/kg DM to the diet resulting in a total dietary concentration of 17 mg Cu/kg DM. To evaluate the effect of antagonists the diets were either unsupplemented (-) or supplemented (+) with additional Mo and S to supply an additional 6.0 mg Mo/kg DM and 1.5 g S/kg DM resulting in a total dietary concentration of 7.2 mg Mo/kg DM and 4.4 g S/kg DM. There were therefore four dietary treatments (Table 5.2).

Table 5.2 Dietary treatments for Holstein-Friesian cows supplemented with Cu oxide or nano Cu oxide and additional antagonists or no additional antagonists.

Treatment group	Dietary treatment
O-	Cu oxide
O+	Cu oxide and additional antagonists
N-	Nano Cu oxide
N+	Nano Cu oxide and additional antagonists

The nano Cu oxide had a hydrolysed lysine coating (Section 2.12) and therefore hydrolysed lysine (Sigma-Aldrich, UK) was added to the diet of cows that received conventional Cu oxide (O- and O+; Table 5.2). The coating on the nano Cu oxide was in a 1:1 ratio with the Cu oxide thus the amount of hydrolysed lysine added to the conventional Cu oxide diets (O- and O+) was calculated from the amount of nano Cu oxide to ensure that hydrolysed lysine was the same concentration in all four diets.

5.2.2. Characterisation of supplementary Cu

Characterisation of the physical properties of the supplementary sources of Cu was conducted at Nottingham Trent University before the start of the study (Table 5.3). Particle size was determined by analysing results from TEM (JEOL JEM-2010, Michigan Tech, USA) and DLS (Particulate Systems, NanoPlus HD, Kromtek, Malaysia). Elemental composition was determined using ICP-OES (ICPE-9820, Shimadzu, UK).

Table 5.3 Physical and chemical characteristics of supplementary Cu fed to dairy cows.

	Cu oxide	Nano Cu oxide
Coating	None	Hydrolysed lysine
Coating ratio	N/A	1:1
Chemical formula	Cu oxide	Cu oxide
CAS Number	1317-38-0	N/A
Appearance colour	Black	Black
Appearance form	Powder	Fine powder
Mean particle size (nm)	25000	45
Particle size range (nm)	40 – 33000	7 - 79
Elemental composition (%)	78.2	39.1

5.2.3. Experimental procedure

Cows were milked twice daily at approximately 06:00 h and 16:00 h through a Westaflia 40-point internal rotary parlour. Milk yield was recorded at each milking and samples were taken fortnightly at consecutive am and pm milkings for subsequent analysis of fat, protein, lactose and somatic cell count (SCC). The cows were weighed, and condition scored at 09:00 h in the week prior to allocation and then fortnightly. Forage samples were taken twice weekly: half the sample was oven dried at 105 ºC to a constant weight and the amount of lucerne and maize silage adjusted to achieve the desired ratio. Samples from each of the four diets were collected once per week immediately after feeding and stored at -20 ºC for subsequent analysis. Blood samples were collected at weeks 0, 2, 4, 6, 10 and 16 of the study at 10:00 h via jugular venipuncture into Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing K_2EDTA for samples used for haematology profile and to determine SOD activity, silica for samples used to determine Cp and K_3EDTA and sodium heparin for samples used to determine mineral concentration and GGT activity. Bloods were processed as detailed in Section 2.6. Liver biopsy samples were obtained from all animals during week 0 and 16 by insertion of a needle through the 11th intercostal space using the procedure described by Davies and Jebbet (1981) and stored at -80 ºC for subsequent analysis. Incidence of mastitis was recorded for each cow.

5.2.4. Analytical procedure

Samples of TMR for each dietary treatment were bulked each month. Sub-samples were analysed for Ca, Cu, Fe, Mg, Mo, P, S and Zn by ICP-MS (NexION 2000, PerkinElmer, Shelton, CT) as described in Section 2.6, for DM (943.01; intra-assay CV

of 2.5%) as described in Section 2.1, CP (990.03; intra-assay CV of 1.6%) using a Leco FP-528 (Leco Corporation, St Joseph, MI) as described in Section 2.2, NDF (intra-assay CV of 1.5%) according to Van Soest et al. (1991) as described in Section 2.4 and starch (intra-assay CV of 5.5%) according to ISO 6493 (2000) at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) as described in Section 2.5. Milk samples were analysed for fat, protein, lactose and SCC by Eurofins Laboratories (Wolverhampton UK) as described in Section 2.11. Serum, plasma and whole blood samples were analysed using a Cobas-Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK) for Cp (intra-assay CV of 3.9%) according to the method of Henry et al. (1974), GGT (Randox, Crumlin, UK, Kit catalogue no. GT 553; intra-assay CV of 2.1%) and SOD (Randox, Crumlin, UK, Kit catalogue no. SD 125; intra-assay CV of 3.5%) as described in Section 2.9. Plasma samples were analysed for Cu, Fe, and Mo by ICP-MS (NexION 2000, PerkinElmer, Shelton, CT; intra-assay CV of 1.0%, 2.4% and 1.2% respectively) as described in Section 2.7. Liver samples were analysed for Cu, Fe and Mo by ICP-MS (NexION 2000, PerkinElmer, Shelton, CT; intra-assay CV of 1.7%, 3.0% and 1.8%) as described in Section 2.7.

5.2.5. Statistical analysis

Performance, plasma minerals and enzyme activity were analysed by repeated measures ANOVA as a 2 x 2 factorial design. Milk SCC was not a normal distribution therefore it was transformed to $\log_{10}$ prior to analysis. Treatment degrees of freedom were split into main effects of Cu form (conventional Cu oxide (O) versus nano Cu oxide (N)), antagonist (without (-) versus with (+)) and their interaction, and analysed as:

$$Y_{ijkl} = \mu + B_i + F_j + A_k + T_l + F.A_{jk} + F.T_{jl} + A.T_{kl} + F.A.T_{jkl} + \varepsilon_{ijkl}$$

Where Y_{ijkl} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of Cu form (j = conventional Cu oxide or nano Cu oxide); A_k = effect of antagonists (S and Mo) (k = -, +); T_l = effect of time; $F.A_{jk}$ = interactions between Cu form and antagonist; $F.T_{jl}$ = interaction between Cu form and time; $A.T_{kl}$ = interaction between Cu form and time; $F.A.T_{jkl}$ = interaction between Cu form, antagonist and time, and ε_{ijkl} = residual error.

Liver mineral concentration, live weight and BCS change were analysed by ANOVA as a 2 x 2 factorial design as:

$$Y_{ijk} = \mu + B_i + F_j + A_k + F.A_{jk} + \varepsilon_{ijk}$$

Where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of Cu source (j = conventional Cu oxide or nano Cu oxide); A_k = effect of antagonists (S and Mo) (k = -, +); $F.A_{jk}$ = interactions between Cu source and antagonist; and ε_{ijk} = residual error. For liver mineral concentrations the concentration during week 0 was used where appropriate as a covariate to determine the final liver mineral concentration and rate of mineral storage or mobilisation.

Statistical analysis was conducted using Genstat 18 and is presented as means with SED; $P < 0.05$ was used to indicate significance and a trend was considered when $P < 0.1$.

5.3. Results

5.3.1. Dietary analysis

All four diets had a similar DM, CP, NDF and ash with mean concentrations of 474 g/kg, 163 g/kg DM, 427 g/kg DM and 271 g/kg DM respectively (Table 5.4). The Cu concentration was similar across all four diets whereas diets O+ and N+ had higher Mo and S concentrations (mean of 7.2 mg Mo/kg DM, and 4.3 g S/kg DM) than diets O- and N- (mean of 0.9 mg Mo/kg DM and 3.4 g S/kg DM).

Table 5.4 Chemical composition of the TMR for dietary treatments fed to Holstein-Friesian dairy cows containing dietary Cu oxide (O) or nano Cu oxide (N) with (+) or without (-) added S and Mo.

Chemical analysis	Dietary treatment[1]			
	O-	O+	N-	N+
DM, g/kg	482	472	468	472
CP, g/kg DM	165	161	163	163
NDF, g/kg DM	427	424	430	425
Starch, g/kg DM	176	167	177	164
Ash, g/kg DM	81	84	85	82
Cu, mg/kg DM	16.6	16.8	16.5	17.0
Zn, mg/kg DM	75.9	78.4	75.3	77.2
Mo, mg/kg DM	0.9	7.2	0.8	7.1
Fe, mg/kg DM	363	340	341	371
S, g/kg DM	3.2	4.4	3.5	4.3
Ca, g/kg DM	9.6	10.2	10.1	10.3
P, g/kg DM	6.7	6.9	6.9	6.9
Mg, g/kg DM	2.8	2.8	2.7	2.8

[1]Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.

5.3.2. Animal Performance and Intake

There was no effect of form of Cu or presence of antagonists on live weight, BCS, DMI, milk yield, milk fat concentration, milk fat yield, milk protein concentration, milk protein yield, milk lactose concentration and milk SCC (Table 5.5). There was a tendency ($P = 0.079$) for cows fed additional antagonists to have a reduced DMI and lower ($P = 0.071$) lactose yield. There was an interaction effect ($P = 0.029$) between the form of Cu and presence of antagonists for SCC with the addition of antagonists increasing SCC in cows fed nano Cu oxide but not conventional Cu oxide. There was an effect of time ($P < 0.001$) on live weight which decreased between week 0 and week 2 and then increased between week 2 and week 16. There was a time effect for BCS ($P < 0.001$) which decreased between week 0 and week 10 and then increased between week 10 and week 16. There was an effect of time ($P = 0.003$, $P = 0.024$ and $P = 0.012$, respectively) on DMI, milk yield and fat yield which fluctuated throughout the study and there was also a trend ($P = 0.062$) for milk fat content to change with time. There was a time effect ($P < 0.001$) for milk protein and yield which decreased between week 0 and week 2 and then increased between week 2 and week 16. There was a time effect ($P < 0.001$) for milk lactose concentration which increased over time.

Table 5.5 Effect of dietary Cu oxide (O) or nano Cu oxide (N) with (+) or without (-) added S and Mo on performance in Holstein-Friesian dairy cows.

	Treatment[1]					Significance[2]		
	O-	O+	N-	N+	SED	Form	Ant	Int
DMI, kg/d	24.1	23.1	23.1	22.6	0.79	0.441	0.079	0.932
Milk yield, kg/d	41.6	40.9	39.5	40.0	1.42	0.245	0.923	0.641
Milk fat, g/kg*	37.1	37.8	37.4	37.3	1.84	0.921	0.821	0.726
Fat yield, kg/d*	1.60	1.44	1.49	1.46	0.135	0.520	0.174	0.367
Milk protein, g/kg*	30.7	30.3	30.1	29.9	0.61	0.144	0.435	0.779
Protein yield, kg/d*	1.31	1.31	1.31	1.23	0.071	0.730	0.315	0.427
Lactose, g/kg*	30.6	30.2	30.2	29.9	0.61	0.293	0.223	0.823
Lactose yield, kg/d*	1.86	1.80	1.88	1.70	0.112	0.852	0.071	0.418
Milk SCC (log_{10}/ml)*	1.79	1.69	1.49	1.75	0.173	0.158	0.284	0.029
Final Lwt, kg	681	668	678	657	20.5	0.777	0.253	0.772
Lwt change, kg/d	0.25	0.22	0.28	0.18	0.741	0.932	0.331	0.599
Final BCS	2.74	2.75	2.74	2.74	0.114	0.803	0.439	0.931
BCS change	-0.20	-0.13	-0.04	-0.08	0.247	0.536	0.849	0.861

[1]Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.

[2]Significance: Form = Effect of form of Cu supplement, Ant = Effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists.

*Adjusted for week 0

5.3.3. Plasma mineral concentration

Plasma Cu concentration did not change over time (P = 0.592; Figure 5.1). There was no effect of form of Cu source (P = 0.504) or presence of antagonists (P = 0.904) on plasma Cu concentration andt here was no interaction between Cu source and presence of antagonists (P = 0.154).

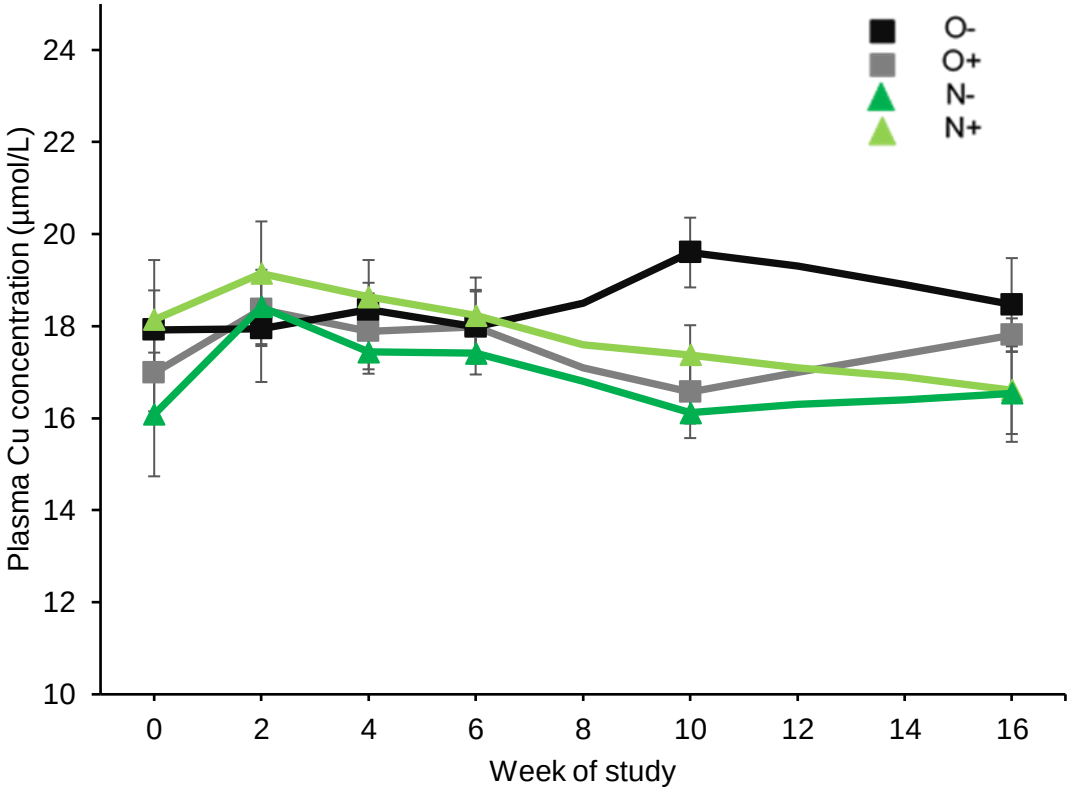

Figure 5.1 Effect of dietary Cu oxide (O) or nano Cu oxide (N) fed with (+) or without (-) added S and Mo on plasma Cu concentration (µmol/L) in Holstein-Friesian dairy cows. Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists. Error bars indicate the SED. Form of Cu, P = 0.504; Effect of antagonists, P = 0.904; Interaction effect between form of Cu and antagonists, P = 0.154; time, P = 0.592

Plasma Mo concentration changed over time (P = 0.009; Figure 5.2) and there was also an interaction effect between time and presence of antagonists (P = 0.001). Plasma Mo concentration was higher (P < 0.001) in cows supplemented with Cu antagonists (O+ and N+) compared to those not supplemented (O- and N-).There was no effect of Cu source (P = 0.709) or interaction between Cu source and presence of antagonists (P = 0.189),

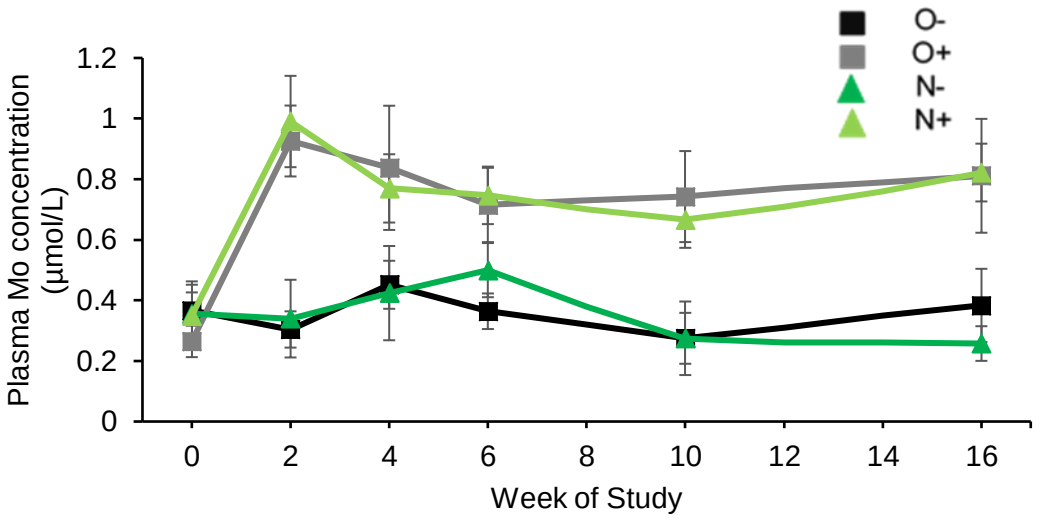

Figure 5.2 Effect of dietary Cu oxide (O) or nano Cu oxide (N) fed with (+) or without (-) added S and Mo on plasma Mo concentration (µmol/L) in Holstein-Friesian dairy cows.

Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists. Error bars indicate the SED . Form of Cu, P = 0.709; Effect of antagonists, P <0.001; Interaction effect between form of Cu and antagonists, P = 0.189; time, P = 0.009

Plasma Fe concentration fluctuated over time (P = 0.014; Figure 5.3). There was no effect of form of Cu source (P = 0.455) or antagonists (P = 0.654) and no interaction between Cu source and presence of antagonists (P = 0.603),

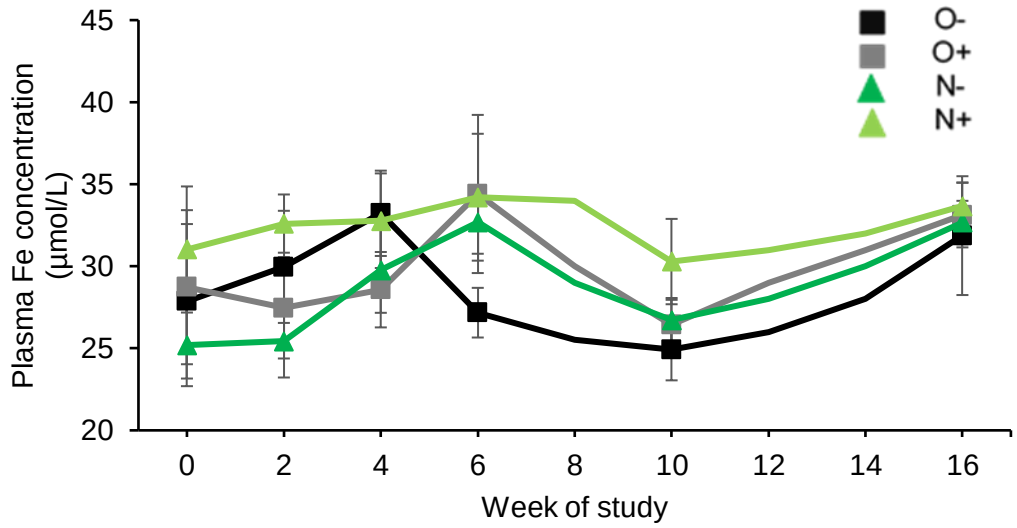

Figure 5.3 Effect of dietary Cu oxide (O) or nano Cu oxide (N) fed with (+) or without (-) added S and Mo on plasma Fe concentration (µmol/L) in Holstein-Friesian dairy cows.

Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists. Pooled SED = 3.966. Form of Cu, P = 0.455; Effect of antagonists, P = 0.654; Interaction effect between form of Cu and antagonists, P = 0.603; time, P = 0.014

5.3.4. Blood enzyme activity

There was no effect of form of Cu source (P = 0.200) or presence of antagonists (P = 0.263) and no interaction between Cu source and presence of antagonists (P = 0.823), on GGT activity (Table 5.6). The activity of GGT did not change over time (P = 0.542). There was no effect of form of Cu source (P = 0.414) or presence of antagonists (P = 0.171) and no interaction between Cu source and presence of antagonists (P = 0.712), on SOD activity. Activity of SOD did change over time (P < 0.001) increasing up to week four and then fluctuating (Figure 5.4). There was no effect of form of Cu source (P = 0.651) or presence of antagonists (P = 0.674) and no interaction between Cu source and presence of antagonists (P = 0.585) on Cp concentration. The concentration of Cp did not change over time (P = 0.691).

Table 5.6 Effect of dietary Cu oxide (O) or nano Cu oxide (N) with (+) or without (-) added S and Mo on GGT (U/l), SOD (U/g Hb) and Cp (mg/dl) activity in Holstein-Friesian dairy cows.

	Treatment[1]					Significance[2]		
	O-	O+	N-	N+	SED	Form	Ant	Int
GGT activity, U/l	32.0	35.0	27.2	31.5	5.86	0.200	0.263	0.823
SOD activity, U/g Hb	1753	1930	1872	1975	243.1	0.412	0.173	0.715
Cp concentration, mg/dl	17.3	18.5	17.4	17.6	1.94	0.651	0.674	0.585

[1]Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.
[2] Significance: Form = Effect of form of Cu supplement, Ant = Effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists.

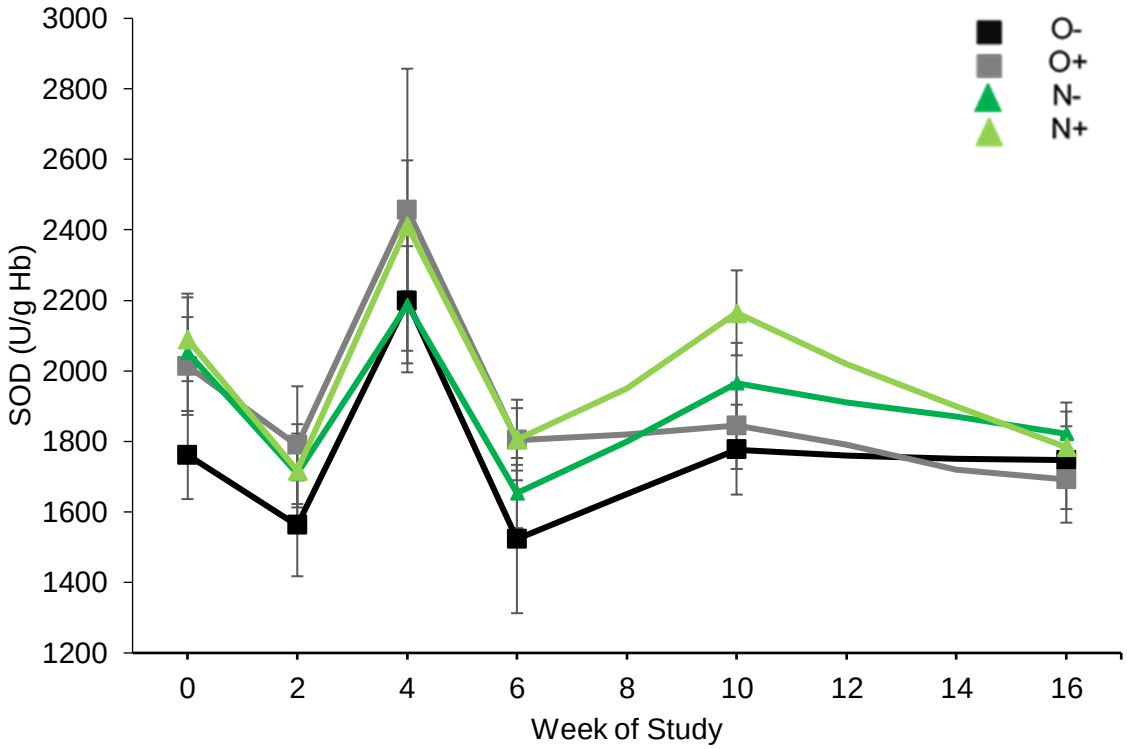

Figure 5.4 Effect of dietary Cu oxide (O) or nano Cu oxide (N) fed with (+) or without (-) added S and Mo on activity of SOD (U/g Hb) over time in Holstein-Friesian dairy cows. Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists. Error bars indicate the SED. Form of Cu, P = 0.414; Effect of antagonists, P = 0.171; Interaction effect between form of Cu and antagonists, P = 0.712; time, P < 0.001

5.3.5. Haematology profile

There was an effect of Cu source on Mon. No which was higher (P = 0.026) in cows fed nano Cu oxide (N- and N+) compared to cows fed conventional Cu oxide (O- and O+) with mean values of 0.40 m/mm^3 and 0.35 m/mm^3 respectively (Table 5.7). There was an interaction (P = 0.033) between the form of Cu supplementation and presence of antagonists on Mon. No, where cows fed conventional Cu oxide without antagonists (O-) had a lower count, 0.33 m/mm^3 than those fed nano Cu oxide without antagonists (N-), 0.42 m/mm^3 but those fed additional antagonists (O+ and N+) had similar counts, with a mean of 0.37 m/mm^3.

There was an effect of time (P < 0.001) on WBC which decreased between week 0 and week 2 and then increased up to week 16. There was also a trend for time (P = 0.096) for Lym. No. which fluctuated over the 16 week study. There were also fluctuations

over time (P = 0.010) for Hb. There was no effect of time (P > 0.05) or interaction (P > 0.05) on Neu. No., Eo. No., Ba No., RBC or HCT.

Table 5.7 Effect of dietary Cu oxide (O) or nano Cu oxide (N) with (+) or without (-) added S and Mo on haematology profile in Holstein-Friesian dairy cows.

	Treatment[1]					Significance[3]		
	O-	O+	N-	N+	SED	Form	Ant	Int
WBC, $10^3/mm^3$	8.0	9.2	10.1	9.2	1.33	0.131	0.858	0.129
Lym No, $10^3/mm^3$	3.12	3.35	3.68	3.42	0.396	0.113	0.914	0.212
Mon No, $10^3/mm^3$	0.33	0.37	0.42	0.37	0.044	0.026	0.992	0.033
Neu No, $10^3/mm^3$	4.69	5.52	6.16	5.45	0.957	0.196	0.911	0.156
Eo No, $10^3/mm^3$	0.13	0.08	0.08	0.08	0.047	0.474	0.263	0.278
Ba No, $10^3/mm^3$	0.037	0.049	0.047	0.044	0.0125	0.602	0.345	0.540
Hb, g/dl	10.2	10.2	10.2	10.4	0.41	0.489	0.791	0.497
RBC, $10^6/mm^3$	7.08	7.12	6.77	7.16	0.846	0.595	0.377	0.488
HCT, %	34.1	33.5	34.3	41.4	11.23	0.300	0.398	0.315

[1]Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.

[2]white blood cells (WBC), lymphocyte number (Lym No.), monocyte number (Mon No.), neutrophil number (Neu No,), eosinophil number (Eo No.), basophil number (Ba No.), haemoglobin (Hb), red blood cells (RBC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

[3] Significance: Form = Effect of form of Cu supplement, Ant = Effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists.

5.3.6. Liver mineral concentration

Final liver Cu concentration and the increase in Cu concentration was higher (P = 0.042 and P = 0.028, respectively) in cows supplemented with nano Cu oxide (N- and N+) compared to those supplemented with conventional Cu oxide (O- and O+; Table 5.8 and Figure 5.5). Cows supplemented with additional antagonists (O+ and N+) had a lower final liver Cu concentration (P = 0.036) and a lower change in hepatic Cu (P = 0.018) compared to those that were not supplemented with antagonists (O- and N-). There was no interaction between the form of Cu and presence of antagonists on liver Cu concentration (P > 0.05). There was no interaction or effect of form of Cu or supplementation of antagonists on liver Mo or liver Fe concentrations (P > 0.05).

Table 5.8 Effect of dietary Cu oxide (O) or nano Cu oxide (N) with (+) or without (-) added S and Mo on the final liver mineral concentrations (mg/kg DM) and the change in liver mineral concentrations (mg/kg DM) over 16 weeks in Holstein-Friesian dairy cows.

	Treatment[1]					Significance[2]		
	O-	O+	N-	N+	SED	Form	Ant	Int
Final Cu*, mg/kg DM	596	333	638	590	100.0	0.042	0.036	0.135
Final Mo*, mg/kg DM	2.4	5.4	3.6	3.9	2.04	0.915	0.105	0.262
Mo change, mg/kg DM	+0.50	+1.6	+0.17	+1.0	2.03	0.575	0.305	0.737
Final Fe*, mg/kg DM	258	325	280	283	97.7	0.885	0.612	0.656
Fe change, mg/kg DM	-11	+25	-24	+8	104.5	0.842	0.651	0.976

*Week 0 values were used as a covariate.

[1]Treatments are: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.

[2] Significance: Form = Effect of form of Cu supplement, Ant = Effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists.

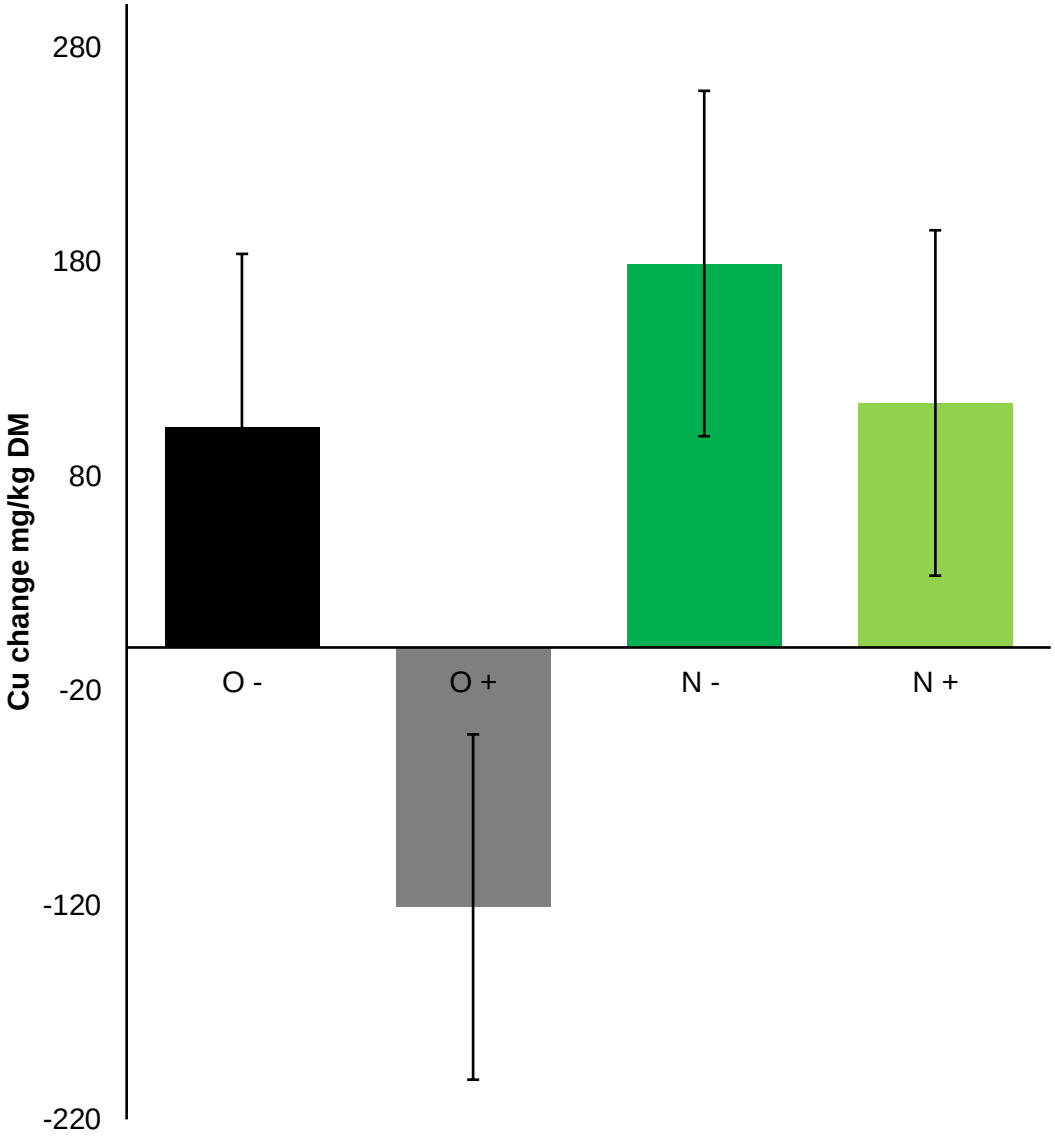

Figure 5.5 Effect of dietary Cu oxide (O) or nano Cu oxide (N) fed with (+) or without (-) added S and Mo on the change in liver Cu concentration (mg/kg DM) over 16 weeks in Holstein Friesian dairy cows. Error bars indicate the SED.

Treatments were: O-; Cu oxide without antagonists, O+; Cu oxide with antagonists, N-; Nano Cu oxide without antagonists and N+; Nano Cu oxide with antagonists.

5.4. Discussion

5.4.1. Introduction

The most commonly reported trace element disorder in cattle is Cu deficiency, which is most often due to diets which contain high levels of antagonists to Cu absorption such

as S, Mo and Fe (Phillippo, et al., 1987; Spears, 2003). To mitigate this problem cattle are often supplemented with Cu, one common form of supplementation is as an oxide, but this form is not always effective when the levels of antagonists are very high, probably because of its low bioavailability when compared to other sources (Baker & Ammerman, 1995a). Experiment 2 (Chapter 4) demonstrated that Cu oxide nanoparticles have a higher bioavailability than conventional Cu oxide based on liver Cu concentration and SOD activity. Improved bioavailability of a Cu source may mean that it is more effective at increasing or maintaining the Cu status of cattle in the presence of relatively high levels of antagonists in the diet. The current study investigated the effect of Cu oxide nanoparticles compared to conventional Cu oxide on the mineral status, performance and health of dairy cows when fed either with or without additional Cu absorption antagonists.

The nanoparticle Cu oxide and conventional Cu oxide supplements used in this study had different concentrations of Cu at 39.1% and 78.2% respectively. The compound minerals were therefore supplemented at a rate that ensured that Cu was provided at the same level of 8 mg Cu/kg DM to each treatment group. The particle size of the conventional Cu oxide was 25000 nm whereas the nano Cu oxide was 45 nm, with the consequence, that the particle size of the nano minerals was approximately five hundred times smaller. The basal diet was formulated to have a low Cu content of 9 mg/kg DM to allow a high dietary supplementation of Cu (8 mg/kg DM) without exceeding the UK recommended maximum level of 20 mg/kg DM (ACAF, 2010). The nanoparticle Cu oxide had a hydrolysed lysine coating in a 1:1 ratio with the metal compound. Hydrolysed lysine was therefore added at the same rate to the diet of cows fed conventional Cu oxide to avoid any effects of lysine supply on performance. The CP content of the diets was 163 g/kg DM and the added lysine supplied was calculated to supply only 0.01% of the total protein in the diet.

5.4.2. Diets

There was no difference in the formulation or the chemical composition including, CP, NDF, starch and Cu concentration of the four treatment diets, with the exception of the concentration of the antagonists S and Mo. Diets O+ and N+ were formulated to contain higher levels of antagonists compared to O- and N- by adding an additional 1.2g S/kg DM and 6 mg Mo/kg DM and this difference is reflected in the increased concentration in the diets; 7.2 mg Mo/kg DM and 4.4 g S/kg DM (O+ and N+) compared to 0.9 mg Mo/kg DM and 3.4 g S/kg DM (O- and N-).

5.4.3. Animal performance and intake

There was no effect of form of Cu supplementation or presence of antagonists on the cows live weight, body condition score, milk yield, milk fat or milk protein content. This finding is similar to Ward et al. (1993) and Engle & Spears (2000) who reported that the form of Cu had no effect on live weight gain in growing steers. Similarly, Sinclair et al. (2013) reported that form of Cu supplementation did not have an effect on body condition score, milk fat yield, energy corrected milk yield or milk protein in dairy cows but there was a trend for cows fed organic Cu to have a higher live weight gain than cows fed inorganic Cu. Milk yield was 5% higher in cows fed inorganic Cu compared to organic Cu but milk fat content was lower. In contrast, Galyean et al. (1995) reported that Cu lysine supplemented at 5 mg/kg DM had a negative effect on live weight gain and DMI when supplemented to growing steers. In the current study cow live weight decreased from week 0 to week 2 and then increased from week 2 to week 16, whilst BCS decreased between week 0 and week 10 and then increased to week 16. Cows commenced the study at 48 +/-17 d post-calving, approaching peak lactation and after week 2 of the study most cows had passed peak lactation and therefore a gradual gain in live weight was expected (Berry, et al., 2007). Cows in early lactation generally do not consume sufficient feed to meet the energetic requirements, leading to the catabolism of body energy reserves which are replaced after peak lactation when the cows start to increase their live weight (Roche, et al., 2006; Berry, et al., 2007).

There were fluctuations in DMI throughout the study, but the general trend was an increase in DMI up to week 12 of lactation and then a slow decline to week 21 of lactation. At week 7 of the study the cows were 97 +/- 17 d post-calving, and the pattern of increased and then decreased DMI fits in with the expected because peak DMI usually occurs after peak milk yield (Sondergaard, et al., 2002). There was no difference in DMI between cows fed the two different Cu sources. This is similar to Ward et al. (1996) who reported no effect of Cu source on DMI. In contrast Wittenberg and Boila (1988) reported that beef steers supplemented with Cu sulphate or Cu oxide had a reduced DMI compared to those that received a Cu injection or were unsupplemented. There was a trend for cows fed additional antagonists in the current study to have a lower DMI and lower milk lactose yield than those not supplemented. Sinclair et al. (2013 and 2017) also reported that DMI was reduced in cows fed S and Mo antagonists when fed an inorganic Cu source. A reduction in DMI could be due to interactions of the antagonists in the rumen. One theory is that under acidic rumen condition S in the diet would be present as hydrogen sulphide (Drewnoski, et al., 2012),

which when absorbed either across the rumen epithelium or in the lungs can have neurological effects, such as polioencephalomalacia (PEM), which decrease the DMI of the cow (Gould, 1998). Rumen pH was not monitored in this study therefore the presence of hydrogen sulphide in the rumen of cows fed antagonists cannot be predicted.

In the current study there was no difference in milk yield between cows fed different Cu sources and no effect of additional antagonists. A meta-analysis by Rabiee et al. (2010) reported that alterations to milk yield when cows were fed different trace mineral sources was attributable to organic versus inorganic minerals. In the current study both sources of mineral were inorganic with the addition of lysine, either coating the mineral particle (N) or mixed with the diet before feeding (O), and a change in milk yield was therefore not expected.

Some studies have reported that SCC is responsive to concentration of Cu in the diet. For example when cows were challenged with *Escherichia coli* and fed 26.5 mg Cu/kg DM there was a lower peak increase in SCC compared to those fed 6.5 mg Cu/kg DM (Scaletti, et al., 2003). In the current study the interaction between Cu source and antagonists indicated that SCC was increased in cows fed antagonists but only in the presence of nano Cu oxide and not conventional Cu oxide. This result is difficult to explain because other parameters in this study indicate that nano Cu oxide had a greater bioavailability than conventional Cu oxide. This result therefore indicates that SCC was not responsive to Cu status. Similarly, two studies indicated that dietary Cu concentration did not affect SCC (Chase, et al., 2000; Scaletti & Harmon, 2012). Sinclair et al. (2017) also reported an interaction between diet and antagonists on SCC but in contrast to the current study that was linked to a lower Cu status and DMI in grass silage compared to maize silage fed dairy cows

5.4.4. Plasma mineral concentrations

In the current study there was no effect of Cu source, or the presence of additional antagonists on plasma Cu concentration. The plasma Cu concentration was within the normal range (9 – 20 µmol/L) for all cows (McDowell, 1992). Plasma concentration is under homeostatic control with Cu stored in the liver released into the bloodstream to maintain concentrations within the normal range (Evans, 1973). In the current study the liver Cu concentrations were within or in excess of the normal range (19 – 508 mg/kg

DM; Livesey, et al., 2002) indicating that the cows were able to maintain their plasma concentration.

Previous studies have been variable in identifying differences in bioavailability of Cu sources based on plasma concentrations. Some studies have only been able to report differences between sources when there were low concentrations of antagonists in the diet. For example, Rabiansky et al. (1999) reported increased plasma Cu concentration in heifers supplemented with Cu lysine compared to Cu sulphate when the main antagonist added to the diet was Fe and the diet contained 50 mg Fe/kg DM. When the diet contained 5 g S/kg DM, 1000 mg Fe/kg DM and 5 mg Mo/kg DM there was no difference in plasma Cu concentration between Cu sources. Similarly Wittenberg et al. (1990) reported no difference in plasma concentration in Cu depleted steers fed Cu sulphate or Cu proteinate when fed a diet with 10 mg added Mo/kg DM. In contrast, Ward et al. (1996) reported that plasma concentration was increased in cattle supplemented with Cu carbonate compared to Cu sulphate or Cu proteinate, but this was only observed when the diet contained added antagonists to achieve a dietary concentration of 5 mg Mo/kg DM and 1.5 g S/kg DM. Other studies have reported no effect of dietary antagonist concentration on plasma Cu concentration between Cu sources. For example, Ward et al. (1993) reported that bioavailability of Cu lysine and Cu sulphate was similar regardless of whether Mo and S were added to the diet. Similarly, in a depletion study by Kegley & Spears (1994) it was concluded that cattle supplemented with Cu sulphate and Cu lysine had a higher plasma Cu concentration than those supplemented with Cu oxide when two experiments were conducted in growing cattle that had differing concentrations of antagonists. In contrast Sinclair et al. (2013) reported no effect of Cu sulphate or Cu proteinate on plasma Cu concentrations when dairy cows were fed without or with added S and Mo.

The expected difference between the bioavailability of Cu sources can also affect the ability of a study to identify differences based on plasma Cu concentrations. For example Kegley & Spears (1994) identified a difference in plasma Cu concentration between growing cattle supplemented with Cu sulphate and Cu oxide but it is well documented that bioavailability of sulphate is at least 5 times greater than oxide (Baker & Ammerman, 1995a). In the current study there could have been a smaller magnitude of difference in the bioavailability between conventional Cu oxide and nano Cu oxide therefore the plasma Cu concentration was similar for all cows.

Plasma Mo concentration was rapidly increased and then maintained in cows fed additional antagonists, most likely because dietary Mo is readily absorbed and

concentration in the blood is reflective of dietary intake (Wittenberg & Devlin, 1987; Kincaid & White, 1989). Plasma Mo concentration was within the normal range, 0.02 – 0.4 μmol/L (Herdt & Hoff, 2011) for cows that were not fed additional antagonists but it was approximately double in those fed additional antagonists. This magnitude of difference between cows fed additional antagonists was similar to that of Sinclair et al. (2013 and 2017) but the mean concentration in the current study was higher. In the current study there was no effect on the Cp:plasma Cu ratio. A change in the ratio of Cp:plasma Cu can be indicative of Mo toxicity (Hussein & Staufenbiel, 2012), and in the current study Mo toxicity was therefore unlikely.

Plasma Fe concentrations were within the normal range of 14 – 37 μmol/L (Herdt & Hoff, 2011) for all cows throughout the study but the concentration fluctuated over time. Fluctuations in plasma Fe concentration and Hb, which also changed over time, can be caused by a number of factors primarily linked to inflammatory diseases (Herdt & Hoff, 2011) but because the cattle in the current study remained within the normal range and there was no other evidence of disease this is likely to be a natural fluctuation. Similarly Rabiansky et al. (1999) also reported that plasma Fe and Hb changed over time but there was no effect of different Cu sources whilst Sinclair et al. (2017) reported that additional Mo and S in the diet did not have an effect on plasma Fe concentration.

5.4.5. Enzyme activity

There was no effect of the Cu source or additional antagonists on Cp concentration, which were within the normal range of 15 to 45 mg/dl (Telfer, et al., 2004). Ward et al. (1995) also reported that there were no differences in Cp concentration between cattle supplemented with Cu proteinate, carbonate or sulphate. Differences in Cp concentration are usually identified when there is also a difference in plasma Cu concentration (Suttle, 1974). For example, Kegley & Spears (1994) reported a difference in plasma concentration in Cu deficient growing cattle supplemented with Cu sulphate compared to Cu oxide in cattle. At the start of the current study the cattle already had a plasma Cu and Cp concentration that were within the normal range and an increase in these parameters was not expected. It is also likely that the magnitude of difference in bioavailability between nano Cu oxide and conventional Cu oxide was smaller than with Cu sulphate used by Kegley & Spears (1994).

There was no effect of the Cu source or additional antagonists on SOD activity. The dietary concentration of Cu was the same across all four treatments, with the only

difference being the form and presence of additional antagonists. Studies that have reported differences in SOD activity have tended to alter the concentration of Cu in the diet (Ward & Spears., 1997; Nagalakshmi, et al., 2010; Cheng, et al., 2011). Similarly, to the current study, Cheng et al. (2011) and Sinclair et al. (2013) also reported that there were no differences in SOD activity in lambs or dairy cows, respectively, when fed different Cu sources. In the study of Sinclair et al. (2013) there was also no effect of diets fed with or without additional Cu absorption antagonists. In contrast, Nagalakshmi et al. (2010) and Pal et al. (2010) both reported an increase in SOD activity in sheep fed organic Cu compared to inorganic Cu, but in both of these studies measurements of enzyme activity were taken over a longer period than the current study. Activity of SOD is measured in erythrocyte lysate and therefore to increase activity there has to be a turnover of red blood cells which takes approximately 160 d (Kerr, 1989) and therefore longer studies provide opportunity for differences in enzyme activity to be more easily identified.

5.4.6. Liver mineral concentration

The liver is the primary storage organ for Cu and accumulation in the liver is often used to indicate the bioavailability of a Cu source (Suttle, 2010). For the current study liver biopsy samples were taken at the start and the end of the study so that changes in liver Cu concentration could be measured. Hogan et al. (1971) reported that hepatic Cu concentration from a biopsy was an accurate estimate of the average Cu concentration in the liver. Mean liver Cu concentrations at the end of the study were 596, 333, 638 and 590 mg/kg DM for cows in treatment groups O-, O+, N- and N+ respectively. With the exception of cows in O+ all of these values were in excess of the 508 mg/kg DM limit suggested by Livesey et al. (2002) indicating that there was an excess dietary supply of Cu and potential risk of toxicity.

There was no difference in the hepatic accumulation of Cu between cows fed Cu oxide (O-) or nano Cu oxide (N-) without additional antagonists with mean values of 1.3 and 1.7 mg Cu/kg DM/d respectively. These values were similar to Engle et al. (2001) who reported that when dairy cows were supplemented with 10 mg Cu/kg DM as sulphate, Cu accumulated in the liver at a rate of 1.9 mg Cu/kg DM/d. In contrast, Du et al. (1996) reported that there was no difference in liver Cu concentration between dairy cows supplemented with Cu sulphate or proteinate at either 5 or 80 mg Cu/kg DM. Previous studies that have reported differences in bioavailability between Cu sources indicate that differences are usually more apparent when high levels of antagonists are

fed. For example, Ward et al. (1996) and Hansen et al. (2008) both reported that an increased bioavailability of organic Cu compared to Cu sulphate was more pronounced in cows that were also supplemented with additional S and Mo. In the current study when antagonists were added to the diet cows fed nano Cu oxide (N+) accumulated Cu in the liver at a rate of 1.2 mg Cu/kg DM/d whereas those fed conventional Cu oxide (O+) depleted Cu from their liver at a rate of -1.4 mg Cu/kg DM/d. The depletion of Cu stores when Cu oxide was fed in the presence of high Mo and S is similar to the findings of Sinclair et al. (2013) who reported that liver concentration decreased by 0.89 mg Cu/kg DM/d in cows supplemented with Cu sulphate and additional S and Mo. In the current study if depletion continued at the rate of -1.4 mg Cu/kg DM/d then cows would be expected to start showing clinical signs of deficiency after a further 224 d on treatment. To avoid this, they would require either a higher Cu inclusion rate or a source of Cu that was more bioavailable in the presence of antagonists. Cows fed nano Cu oxide in the presence of antagonists (N+) accumulated Cu in the liver indicating that this source of Cu was less subject to antagonism and was more bioavailable than conventional Cu oxide.

In the current study there was no difference in liver Mo or Fe concentrations between cows on any of the treatments, and all animals were within the normal range (1 – 4 mg Mo/kg DM (Herdt & Hoff, 2011) and 100 to 1000 mg Fe/kg DM; Suttle, 2010) with the exception of cows fed Cu oxide and additional antagonists that had a slightly raised liver Mo concentration of 5.4 mg/kg DM. There was a small increase in liver Mo concentration over the study period in all cows but this did not reflect the difference in plasma Mo concentration in cows fed additional antagonists (O+ and N+) compared to those not (O- and N-), with cows fed additional antagonists (O+ and N+) having approximately double the plasma Mo concentration compared to those that did not receive additional antagonists (O- and N-). This finding is in agreement with Sinclair et al. (2013) who suggested that the liver was not a major storage organ for Mo in cows. The lack of change of Fe in the liver is in contrast to other studies that reported increased liver Fe concentration due to additional antagonists (Phillippo, et al., 1987) or decreased by additional antagonists (Sinclair, et al., 2017).

5.4.7. Animal health

Research into the supplementation of ruminants, particularly cattle, with mineral nanoparticles is very limited. One reason for this could be the perception of potential negative effects on the animal. Studies investigating the use of nanoparticles in animal

nutrition have reported a wide range of effects on animal health, from a lower toxicity compared to conventional sources (Pelyhe & Mezes, 2013) to renal and liver damage indicative of toxicity (Najafzadeh, et al., 2013). In order to assess whether the accumulation of Cu in the liver from supplementation of Cu oxide nanoparticles resulted in liver damage serum GGT activity was monitored. The GGT enzyme is indicative of liver health and activity is raised when the liver is damaged usually by lethal cell necrosis (Gummow, 1996). There was no difference in GGT activity between cattle supplemented with nano Cu oxide (N+ and N-) or conventional Cu oxide (C+ and C-) and the results for all of the cows were within the normal range for cattle at less than 40 U/l (Johnston, et al., 2014), indicating that nanoparticles did not cause damage to the liver. The cows in the current study were however supplemented for a relatively short period of 16 weeks and commercial dairy cows often receive supplementary Cu in their diet throughout their life. Therefore, further work is required to determine the long term effects of supplementation of nano Cu oxide on liver health.

The haematology profile was also measured in the current study as an indicator of animal health. There was no effect of Cu source on any of the parameters measured, and all cows remained within the normal haematology range (Etim, et al., 2014) throughout the 16 week study. This indicated that supplementation of nanoparticles did not have any short term effect on the haematology of the cattle compared to a Cu source that is widely used as a supplement on commercial farms. There were fluctuations in some of the parameters over time such as WBC, Lym. No. Hb and MCV. Variation in haematology parameters can be caused by a range of factors and it is unlikely that it was a direct result of the treatments used in this study. For example, a change in environmental conditions (Vecerek, et al., 2002), stress (Schalm, et al., 1975), a change in diet including mineral supplementation (Tras, et al., 2000; Iheukwumere & Herbert, 2002) and limited availability for exercise (Swenson, 1970), can all alter the haematology profile of animals.

5.5. Conclusions

There were no performance effects between the two Cu sources. The addition of antagonists to the diet did not have any major performance effects, although there was a tendency for a reduced DMI and milk lactose yield. There was no effect of Cu source on plasma mineral concentrations or enzyme activity. Plasma Mo concentration was increased with the level of Mo in the diet with cows supplemented with additional antagonists having an increased concentration. Liver Cu concentration was increased

in cows fed nano Cu oxide compared to conventional Cu oxide and this effect was particularly pronounced when there were additional antagonists in the diet. In the presence of additional antagonists cows fed nano Cu oxide accumulated 1.2 mg Cu/kg liver DM/d whereas those fed conventional Cu oxide lost 1.4 mg Cu/kg DM/d, indicating that the nano Cu oxide was more bioavailable than the conventional Cu oxide. There was no evidence that accumulation of Cu in the liver in cows fed nanoparticles had any effect on health, with plasma GGT activity and haematology profile being within the normal range and not affected by Cu source, although longer term studies would be required to confirm this.

6. Experiment 4 – Developing a new technique for nanoparticle size analysis using single particle inductively coupled plasma - mass spectrometry (SP-ICP-MS)

6.1. Introduction

The growth in the application of nanoparticles in different fields has led to the pursuit of analytical techniques for measuring particles less than 100 nm accurately and quickly (Cornelius & Hassellov, 2014). Currently, there are few robust techniques for the analysis of nanoparticles (Lee, et al., 2014). An ability to analyse for nanoparticles in animal samples could be crucial to the regulatory demands that will be placed on nanoparticle products in the global market and thus have a crucial effect on the use of these products (Peters, et al., 2014).

One of the possible techniques is single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) because it is able to quantify the concentration and measure the size of nanoparticles (Lee, et al., 2014). Using an ICP-MS technique to measure particle size, as opposed to traditional techniques such as transmission electron microscopy (TEM) could offer time savings and improve accuracy as a greater number of readings per sample are used to calculate mean particle sizes. The manual process of TEM means that sample preparation has a large effect on the results obtained, as samples need to be dried, and the process can be lengthy if a large number of particles need to be measured to calculate the sample mean (Lee, et al., 2014). Techniques used for measuring the concentration of nanoparticles such as dynamic light scattering (DLS), and field flow fractionation (FFF) are often challenging because they require analyte concentration to be increased before they are conducted (Pace, et al., 2011). The DLS technique can only analyse particles within a very specific range and the presence of larger particles can inhibit the ability of the instrument to detect smaller particles (Schwamberger, et al., 2015). Compared to traditional methods SP-ICP-MS has advantages in that it has very low concentration detection limits, and size and concentration data can be gathered simultaneously (Lee, et al., 2014).

The aim of this series of method development experiments was to develop a technique for the identification of nanoparticles in feed, blood and milk. A sequence of mini experiments was conducted to meet this objective.

6.2. Method

6.2.1. The technique

The technique and methodology for SP-ICP-MS operates on the basis of traditional ICP-MS but it introduces metal nanoparticles individually into the instrument and tracks the resulting ion plumes with very short dwell times (Lee, et al., 2014). Particles are introduced as an aerosol of micro droplets from a colloidal suspension. The suspension is introduced via a peristaltic pump at a flow rate of 0.33 – 0.35 ml/min, aerosol is formed at the nebuliser, transported into a spray chamber which removes the larger droplets and into an argon plasma for ionisation. Each particle in the suspension is ionised and the ion plume produced is a function of the size of the original particle and the frequency of ion plumes produced is a function of the nanoparticle concentration. These ion plumes pass into the rest of the mass spectrometer where they can be detected. The instrument produces a series of peaks, where each peak represents the original particle that entered the plasma (Deguelfre & Favarger, 2003). The instrument software converts the peaks to produce an output which details the size of the particles analysed.

6.2.2. Methodology

All analyses were performed on an ICP-MS (NexION 2000, PerkinElmer, Shelton, CT) using the Syngistix™ Nano Application Software Module. Samples were prepared by dilution in purite water, the dilution factor and other sample preparation factors depended on the individual experiments which are detailed in Section 6.3.

To analyse for presence and size of nanoparticles the instrument was run under the conditions shown in Table 6.1.

Table 6.1 Instrument setup parameters for nanoparticle analysis by SP-ICP-MS.

Parameter	Value
Nebuliser	Glass concentric
Spray chamber	Glass cyclonic
Sample flow rate, ml/min	0.33 – 0.35
RF power, W	1600
Analysis mode	Standard
Quadrupole setting time, μs	0
Peristaltic pump, rpm	35

Sample flow rate was measured daily by weighing the amount of water that was delivered by the peristaltic pump for 10 min. Transport efficiency was determined using gold (Au) nanoparticle standards (BBI Scientific, Crumlin, UK) and Au dissolved standards (BBI Scientific, Crumlin, UK) in 1% HCl (Trace element grade 37%, Fisher Scientific, UK) to produce a particle and a dissolved calibration. For the particle calibration a blank, a 20 nm Au standard and a 40 nm Au standard (EMG20 and EMG40; BBI Scientific, Crumlin, UK) were used. For the dissolved calibration a blank, a 1 ppb, 2 ppb and 3 ppb Au standard from a certified Au solution (1000 ppm, BBI scientific, Crumlin, UK) were used to produce a calibration graph. Following calculation of transport efficiency, a sample read back of either the 20 or 40 nm standard was conducted to ensure the instrument was analysing the particle size accurately.

Method parameters including dwell time, data acquisition time, density and t1 mass fraction varied between the experiments because they formed a process of method development and thus method parameters are detailed below for each experiment. Dwell time, settling time and dilution rate are critical factors in the method for determination of particle size. There must be sufficient particles to enable the software to calculate a mean particle size, if there is an excess of particles the result will be incorrect because the software will “read” many single particles as one larger particle known as partial particle integration or coincidence (Cornelius & Hassellov, 2014; Lee, et al., 2014). Dwell time must be shorter than the particle transient time to avoid false signals generated from partial particle integration, particle coincidence, agglomerates and aggregates (Lee, et al., 2014).

6.3. Mini method development experiments

A step-wise series of experiments were conducted to optimise the method of analysing Cu oxide nanoparticles in feed and biological samples.

6.3.1. Au nanoparticle standards

6.3.1.1. Objective

To analyse the particle size of Au nanoparticles, of known size, by SP-ICP-MS in solution.

6.3.1.2. Introduction

Gold nanoparticles are relatively stable in solution and are used to calibrate the instrument for analysis of particle size of other elements and to calculate transport efficiency. Thus, accuracy and reliability of determining the particle size of Au is critical to the particle size results for all other nanoparticles that will be measured. Gold nanoparticle standards suspended in water (EM.GC20 and EM.GC40 BBI Solutions, Crumlin, UK) with technical datasheets detailing particle size were used to determine the accuracy of the method for measuring particle size by SP-ICP-MS using the NexION 2000 ICP-MS (PerkinElmer, Shelton, CT).

6.3.1.3. Method

The instrument was setup as described in Section 6.2 and in Table 6.2. Gold nanoparticle standards were diluted 1:1 billion in purite water. The particle size of each standard was measured in triplicate with acid and purite water washing between each sample.

Table 6.2 Method parameters for analysis of Au nanoparticle standards by SP-ICP-MS.

Parameter	Value
Analyte	Au
Dwell time, μs	100
Data acquisition time, s	100
Density, g/cm^3	4.23
T1 mass fraction, %	100

6.3.1.4. Results and discussion

For the 20 nm gold nanoparticles the reference range on the technical data sheet was 17 – 23 nm and for the 40 nm gold nanoparticles it was 37 – 43 nm with a coefficient of variation of < 8%. All of the measurements were within the ranges stated on the

technical data sheet and the coefficient of variation was 0.024% and 0.012% for 20 nm and 40 nm gold nanoparticles respectively (Table 6.3).

Table 6.3 Particle size of gold nanoparticles standards determined by SP-ICP-MS.

Standard	Particle size 1	Particle size 2	Particle size 3
20 nm (EM.GC 20)	19	20	20
40 nm (EM.GC 40)	40	41	40

6.3.1.5. Conclusions

To determine the particle size of Au nanoparticles in solution SP-ICP-MS is an appropriate technique to produce accurate and reliable results.

6.3.2. Stability of Au nanoparticle standards

6.3.2.1. Objective

To determine the stability of gold nanoparticle standards

6.3.2.2. Introduction

Gold nanoparticle standards are used to calibrate the instrument for particle size analysis alongside a dissolved metal calibration which is the standard method of using an ICP-MS (Yu, et al., 2001; Pace, et al., 2011). Section 6.3.1 showed that the SP-ICP-MS method described produced accurate and reliable results for analysis of the Au nanoparticle standards when they are analysed as fresh samples. The Au nanoparticle standards are suspended in purite water but in order to use them as a standard the particles are further diluted in purite water. The stability of these solutions is unknown, and it is possible that the particles may begin to agglomerate (Keene & Tyner, 2011). Peters et al. (2014) reported that in diluted samples silver nanoparticles increased in size over a period of 3 weeks indicating poor stability. The timeframe and conditions for Au agglomeration are unknown therefore they weretested over a number of days and at two temperatures; room temperature and < 4°C. If the particles do agglomerate, they may subsequently be split into their original individual nanoparticle form by sonication (Jiang, et al., 2009).

6.3.2.3. Method

The instrument was setup as described in Section 6.2 and the same method parameters were used as in Table 6.2. Gold nanoparticle standards were diluted 1:1 billion in purite water. The particle size of each standard was measured in triplicate with acid and purite water washing between each sample. Samples were then stored in the fridge or at room temperature and the particle size was measured 24 h, 48 h and 72 h later pre and post-sonication. To sonicate the standards they were placed in 50 ml plastic tubes in a sonicating bath for 15 mins.

6.3.2.4. Results and discussion

Table 6.4 shows that at 0 h the particle size is between 37 and 43 nm as predicted on the technical data sheet and sonicating the standard didn't have an effect on particle size. At 24 h the particle size had increased, and this increase continued at 48 h and 72 h indicating that the nanoparticles were agglomerating, similarly to the findings of Peters, et al. (2014).

Table 6.4 Particle size of gold nanoparticles standards determined by SP-ICP-MS over 3 d, pre and post-sonication when stored at room temperature or 4°C.

	Room temperature	4°C
0 h pre-sonication	40 ± 1	40 ± 1
0 h post-sonication	40 ± 1	40 ± 1
24 h pre-sonication	50 ± 3	49 ± 1
24 h post-sonication	51 ± 1	51 ± 2
48 h pre-sonication	55 ± 1	56 ± 2
48 h post-sonication	55 ± 2	55 ± 7
72 h pre-sonication	64 ± 5	66 ± 5
72 h post-sonication	63 ± 2	63 ± 2

6.3.2.5. Conclusions

Gold nanoparticles were not stable when they had been removed from the original storage bottle and diluted in purite water. In order to report an accurate particle size fresh gold nanoparticle standards must be prepared each time the instrument is run.

6.3.3. Wash out procedure between analysis of nanoparticle samples

6.3.3.1. Objective

To determine the most effective wash out procedure for analysis of particle size.

6.3.3.2. Introduction

The analysis of particle size calculates the size based on mass, charge and density of particles with a dissolved and particle calibration (Degueldre, et al., 2006; Degieldre, et al., 2006; Pace, et al., 2011). In order to accurately calculate the particle size of a sample it is crucial that the only particles that reach the detector for counting are the particles that are being investigated because the fundamental assumption is that each pulse of signal represents a single particle event (Pace, et al., 2011). Nanoparticles are highly reactive and readily dissolve in acidic conditions usually used to analyse samples by traditional ICP-MS techniques. In order to calculate the particle size the particles are suspended in purite water at a specific dilution. When analysis is changed from one sample to the next it is critical that the nanoparticles from the previous sample are completely washed out of the system. One way to do this is to put an acid wash through the system to dissolve the nanoparticles, followed by a purite water wash in order to remove all traces of acid. If there is any acid left over the next samples of particles will dissolve and a particle size analysis will not be possible. It is unknown how long the acid wash should be conducted for in order to remove previous samples of nanoparticles or how long the water wash should be conducted in order to remove any acid traces.

6.3.3.3. Method

A sample of nanoparticles was analysed on the SP-ICP-MS as per the method provided in Section 6.2 and Table 6.2. Following this an acid and water wash was conducted until the detection limit for nanoparticles was reached. The detection limit was less than 20 particles.

6.3.3.4. Results and discussion

The results show the declining number of particles detected as samples of purite water are analysed following analysis of a nanoparticle standard (Table 6.5). The results

indicate that the instrument should be washed for the minimum time it would take to analyse 10 samples after each sample; this was approximately 18 mins between each sample. Between sample 7 and 10 the number of particles detected increased. This was likely because the sampling system for the SP-ICP-MS is not a closed system so particles that were in the environment may accidentally contaminate the sample prior to sample uptake. A lack of a closed system and potentially low quality purite water could also be the reason why the particles detection count never reached zero. In future it may be useful to investigate using a higher grade of purite water to see if the particle count can be further reduced.

Table 6.5 Number of particles detected when purite water is analysed following analysis of a nanoparticle standard.

Sample Number	Number of particles detected
1	1990
2	425
3	169
4	66
5	50
6	44
7	32
8	80
9	213
10	21
11	13
12	13

6.3.3.5. Conclusions

Wash out is a critical step in the evaluation of particle size by SP-ICP-MS. If the instrument is not washed out appropriately particles from the previous sample may alter the mean particle size of the sample being analysed and the results will be inaccurate. A critical step is also to ensure that if an acid wash is conducted that all traces of acid are removed from the instrument to prevent the next sample from dissolving and consequently not being available for size analysis.

6.3.4. Sample concentration of Au nanoparticle standards

6.3.4.1. Objective

To determine the effect of sample concentration on particle size analysis for Au nanoparticle standards.

6.3.4.2. Introduction

The dilution rate is a critical factor in developing a method for analysis of particle size by SP-ICP-MS (Pace, et al., 2011; Laborda, et al., 2013). Dilution rate can alter the calculated particle size because of the number of particles entering the spectrometer during the assigned time period (Laborda, et al., 2013). If the sample is not diluted sufficiently this can result in an excess of particles entering the spectrometer. The software is unable to detect these particles as single events and the particle size reported will be larger than the true value because it is a measurement of more than one particle (particle coincidence) (Cornelius & Hassellov, 2014; Lee, et al., 2014). If the sample is too dilute there may be insufficient particles detected to accurately calculate the mean particle size (Linsinger, et al., 2013).

6.3.4.3. Method

A serial dilution method from 0.5 ppb to 50 ppb of Au nanoparticle standards with purite water as the diluent was undertaken. Each sample dilution of Au nanoparticles was analysed by SP-ICP-MS as per the method provided in Section 6.2 and Table 6.2. Particle size was then compared between each dilution factor and the true particle size value (from TEM results) to determine the optimum dilution for Au.

6.3.4.4. Results and discussion

Table 6.6 shows the effect that dilution factor has on calculated particle size. If the sample concentration was greater than 3 ppb then the calculated particle size was larger, indicating that there was partial particle integration or some of the particles were starting to agglomerate. The optimum concentration was between 1 and 3 ppb.

Table 6.6 The effect of dilution factor on the calculated particle size for 20 nm Au standards.

Sample concentration (ppb)	Particle size Au
0.5	15 ± 1
1	19 ± 1
2	19 ± 1
3	21 ± 0
5	32 ± 1
10	38 ± 2
20	32 ± 1
30	34 ± 2
40	38 ± 1
50	40 ± 3

6.3.4.5. Conclusions

Sample concentration had a large effect on the calculated particle size and therefore the optimum concentration and subsequent dilution factor must be identified in order to achieve accurate and reliable particle size results. The optimum sample concentration for Au was 1 to 3 ppb.

6.3.5. Comparison of TEM and SP-ICP-MS for Cu oxide nanoparticles

6.3.5.1. Objective

To determine the particle size of coated Cu oxide nanoparticles of known particle size by comparing with TEM results.

6.3.5.2. Introduction

There are two techniques, DLS and TEM that are conventionally used to measure particle size of nanoparticles (Pace, et al., 2011; Lee, et al., 2014). In order to evaluate the accuracy and precision of measuring nanoparticle size by SP-ICP-MS it can be compared to TEM which is a validated microscopy technique.

6.3.5.3. Method

Copper oxide nanoparticles were measured by SP-ICP-MS as describe in Section 2.2 using the method parameters in Table 6.7. Analysis by TEM was conducted at Nottingham Trent University by an experienced microscopy technician. The study was designed to be blind so neither operator knew the results from the other instrument.

Table 6.7 Method parameters for analysis of Cu oxide nanoparticle standards by SP-ICP-MS

Parameter	Value
Analyte	Cu
Dwell time, µs	100
Data acquisition time, s	100
Density, g/cm^3	4.23
T1 mass fraction, %	100

6.3.5.4. Results and discussion

When analysis was conducted by TEM a single value was produced for the particle size as a result of measuring more than 100 particles and calculating the mean (Table 6.8). When analysis was conducted by SP-ICP-MS the Syngistix software calculated the particle size and provided the mean and mode. Table 6.8 details that the two instruments produced similar results for the mean particle size and the mode size produced by SP-ICP-MS is different to the TEM size and mean size by SP-ICP-MS.

Table 6.8 A comparison of size of nanoparticle powders used in three animal studies measured by TEM and SP-ICP-MS.

	Size (nm)		
Study	TEM	SP-ICP-MS (mean)	SP-ICP-MS (mode)
Batch 1 Cow	38	35 ± 1	25 ± 3
Batch 2 Cow	50	48 ± 2	38 ± 3
Lamb study 2	45	45 ± 1	33 ± 4

6.3.5.5. Conclusions

The calculated mean particle size was similar for Cu oxide nanoparticles measured by TEM and SP-ICP-MS.

6.3.6. Sample concentration of Cu oxide nanoparticles

6.3.6.1. Objective

To determine the effect of sample concentration and the appropriate dilution factor and its effect on particle size analysis for coated Cu oxide nanoparticles

6.3.6.2. Introduction

Section 6.3.4 showed that dilution rate is a critical factor in developing a method for analysis of particle size by SP-ICP-MS. Dilution rate can alter the calculated particle size because of the number of particles entering the spectrometer. If the sample is insufficiently diluted this can result in an excess of particles entering the spectrometer (Laborda, et al., 2013). Due to the excessively high number of particles being delivered the software is unable to recognise each particle as a single event, The particle size may be overestimated due to particle coincidence (Cornelius & Hassellov, 2014; Lee, et al., 2014). If the sample is too dilute there will insufficient particles analysed to calculate an accurate mean particle size. Optimum dilution rates will vary depending on the element and type of sample being analysed but Section 6.3.4 showed that the optimum sample concentration for Au was between 1 and 3 ppb.

6.3.6.3. Method

A serial dilution method from 0.5 ppb to 50 ppb was used for Cu oxide powder samples with purite water as the diluent. Each sample dilution of Cu oxide nanoparticles was analysed on the SP-ICP-MS as per the method provided in Section 6.2 and Table 6.7. Particle size was then compared between each dilution factor and with the true particle size value (from TEM results) to determine the optimum dilution for Cu.

6.3.6.4. Results and discussion

Table 6.9 shows the effect that dilution factor had on the calculated particle size. If the dilution factor was greater than 3 ppb then the calculated particle size was larger, indicating that there were errors associated with counting individual particle events or some of the particles were starting to agglomerate. The optimum dilution rate was to achieve a sample that had a concentration of 1 to 3 ppb for Cu.

Table 6.9 The effect of dilution factor on calculated particle size for 40 nm Cu oxide powder.

Sample concentration	Particle size Cu
0.5	32 ± 0
1	41 ± 1
2	43 ± 1
3	45 ± 1
5	55 ± 2
10	68 ± 1
20	73 ± 3
30	75 ± 2
40	79 ± 3
50	80 ± 3

6.3.6.5. Conclusions

Sample concentration had a large effect on the calculated particle size with the optimum sample concentration for Cu being 1 to 3 ppb.

6.3.7. Analysis of TMR for Cu oxide nanoparticles

6.3.7.1. Objective

To extract coated Cu oxide nanoparticles that have been added to a TMR for dairy cows.

6.3.7.2. Introduction

During the dairy cow study (Experiment 3, Chapter 5) Cu oxide nanoparticles were added to the TMR, by mixing in by hand with the other minerals and then adding to the Hi-Spec mixer (Hi-Spec Engineering Ltd, Country Carlow, Ireland) before the TMR was dispensed into the Insentec RIC feeders (Insentec B.V, Marknessem Netherlands). Once a week, following feed out, samples of TMR were collected for analysis of chemical composition. These TMR samples should contain the nanoparticles that were originally added during the mixing process and the objective of the experiment was to extract them from the TMR and analyse their size using SP-ICP-MS. Conventional mineral analysis of a TMR sample would require drying, milling and digesting in acid. This method cannot be used for nanoparticle analysis because the nanoparticles would

dissolve in the acid. This means that the nanoparticles would not be detected because the technique relies on the production of signal pulses from discontinuous nanoparticles above the continuous background (Degueldre, et al., 2006; Navratilova, et al., 2015). A technique which keeps the nanoparticles intact but removes contaminants and dilutes the sample must therefore be employed. If the background level of other elements is too high then this can limit the ability of the software to calculate nanoparticle size (Cornelius & Hassellov, 2014). The optimum concentration of Cu in the nanoparticle suspension is between 1 and 3 ppb and therefore the TMR sample will be diluted until this concentration is achieved.

6.3.7.3. Method

A sample of TMR was collected immediately after feeding into the Insentec RIC feeders (Insentec B.V, Marknessem Netherlands) every Monday as described in Section 5.2.3. The fresh TMR was washed with purite water through a funnel containing filter paper. The solution was then washed through a second filter paper before being analysed for Cu concentration via ICP-MS (Section 2.7) and then further diluted with purite water to achieve a Cu concentration of 3 ppb. The solution was analysed for particle size by SP-ICP-MS using the method described in Section 6.2 and parameters in Table 6.10.

Table 6.10 Method parameters for analysis of Cu oxide nanoparticle by single particle ICP-MS to determine the particle size of Cu in a TMR fed to dairy cows.

Parameter	Value
Analyte	Cu
Dwell time, µs	100
Data acquisition time, s	500
Density, g/cm^3	4.23
T1 mass fraction, %	60

6.3.7.4. Results and discussion

The extraction of Cu oxide nanoparticles from a TMR was possible (Table 6.11). The nanoparticles had however, increased in size from the original particle size which could be because of agglomeration of the particles during the mixing and sampling of the TMR. An increase in size could also be an effect of a higher background level of other materials when the TMR solution was analysed compared to the original powder solution. It is challenging to remove these background levels because of the nature of

the sample. Conventional ICP-MS analysis would require acid to digest the sample and remove unwanted material but for SP-ICP-MS this technique cannot be employed because an acid digest would also digest the nanoparticles and the instrument would not be able to give an estimate of particle size (Degueldre, et al., 2006; Navratilova, et al., 2015). The results demonstrated that SP-ICP-MS may still be an effective method for measuring nanoparticle size from a feed but the concentration of the solution is critical and the results should be interpreted carefully along with the real time signal generated by Syngistix to ensure that they are an accurate representation of the particle size of the sample.

Table 6.11 Extraction of Cu oxide nanoparticles from a TMR and measurement of size by ICP-MS.

TMR sample collection	TMR particle size (nm)	Original particle size (nm)
February 2018	70 ± 2	35 ± 1
March 2018	81 ± 3	35 ± 1
April 2018	85 ± 3	35 ± 1
May 2018	90 ± 3	48 ± 2
June 2018	87 ± 3	48 ± 2
July 2018	95 ± 3	48 ± 2
August 2018	101 ± 4	48 ± 2

6.3.7.5. Conclusions

Particle size of minerals in a TMR can be measured by SP-ICP-MS but it is important to understand the limitations of this technique, whereby appropriate sample concentration is critical to achieve accurate results. The results of this experiment, show that the particles measured in the TMR are larger than the original particle size. This could be because the particles are agglomerating within the conditions of the TMR or it could be due to interference from other components of the TMR.

6.3.8. Analysis of spiked plasma and milk samples for Cu oxide nanoparticles

6.3.8.1. Objective

To spike plasma and milk samples with Cu oxide nanoparticles and determine the particle size

6.3.8.2. Introduction

The use of nanoparticles in animal feed is a relatively new field of research (Peters, et al., 2014). Some research has been conducted analysing the effect that nanoparticles have on the animal (Najafzadeh, et al., 2013; Zaboli, et al., 2013). For example, some studies have investigated renal function (Najafzadeh, et al., 2013), but very little work has been conducted to investigate the mechanism by which nanoparticle minerals are absorbed and whether they remain in the same physical and chemical form following absorption. When Cu is absorbed from the gut it is transported via the blood to the liver where the Cu is then released back into the bloodstream in order to maintain blood Cu concentration within a very narrow range (McDowell, 1992; Herdt & Hoff, 2011). Thus, characterisation of the Cu present in blood may be a useful indication of the mechanism by which the nanoparticles are absorbed. One of the concerns for nanoparticle technology is the effect they may have on human health. In the dairy cow study (Experiment 3, Chapter 5) milk samples were collected fortnightly. Characterisation of the Cu present in the milk samples may indicate whether the supplemented nanoparticles remained intact through the digestive tract, blood and Cu storage and also indicate whether there may be a concern in relation to human health. In order to establish whether it was possible to analyse plasma samples for nanoparticles, plasma and milk samples were spiked with nanoparticles of known particle size.

6.3.8.3. Method

Blood samples that had been collected into K_3EDTA tubes (BD MidMeds Limited, Hertfordshire, UK) and then centrifuged at 1000 x g for 15 min before removing plasma and storing at –20 ^{0}C from Experiment 2 (Chapter 4) were used. The plasma samples were defrosted overnight at 4 ^{0}C and then mixed using a MT-20 vortex mixer (Phillip Harris Ltd, Shenton, UK). Milk samples that were collected in Experiment 3 (Chapter 5) were used and defrosted overnight at 4 ^{0}C. Nanoparticles of Cu oxide were added to the plasma and milk samples. The samples were analysed for Cu concentration via ICP-MS (Section 2.7) and then diluted with purite water to achieve a Cu concentration of 2 ppb. The diluted samples were analysed for particle size by SP-ICP-MS using the method described in Section 6.2 and parameters in Table 6.12.

Table 6.12 Method parameters for analysis of Cu nanoparticle standards by SP-ICP-MS in plasma and milk samples.

Parameter	Value
Analyte	Cu
Dwell time, µs	100
Data acquisition time, s	200
Density, g/cm^3	4.23
T1 mass fraction, %	60

6.3.8.4. Results and discussion

Nanoparticles added to both plasma and milk were detected by SP-ICP-MS (Table 6.13). In order to use this technique, the background levels of Cu must be kept as low as possible and therefore appropriate dilution was a critical step. If the background levels were too high (Fig 6.1) then the signal produced by the instrument does not produce clear peaks and the software was unable to calculate a particle size. High background levels can be caused by another Cu isotope, such as Cu 65 or NaAr+ which can also be detected at the same mass/charge ratio as Cu 63. When the background levels were lower (Fig 6.2) then the signal produced shows very clear spikes for individual particles and the particle size could be calculated.

Table 6.13 Particle size of Cu detected by SP-ICP-MS in plasma and milk samples spiked with 40 nm Cu oxide nanoparticles.

Sample	Mean particle size	Mode particle size
Plasma 1	40 ± 0	35 ± 2
Plasma 2	41 ± 1	32 ± 3
Plasma 3	40 ± 1	37 ± 4
Milk 1	40 ± 1	31 ± 1
Milk 2	42 ± 0	36 ± 2
Milk 3	40 ± 1	35 ± 2

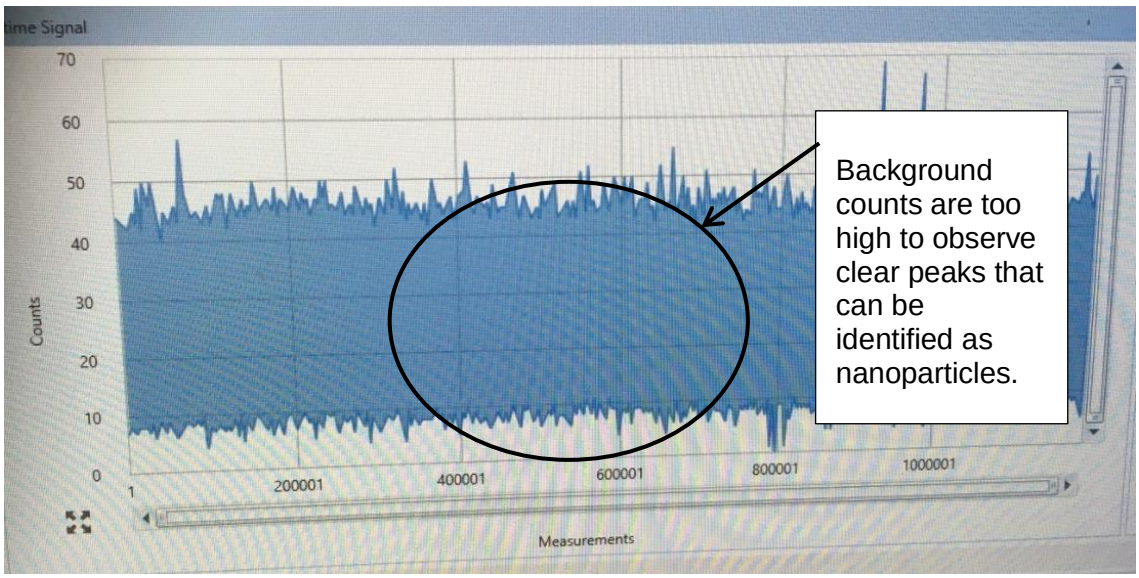

Figure 6.1 Realtime signal from SP-ICP-MS with high background levels.

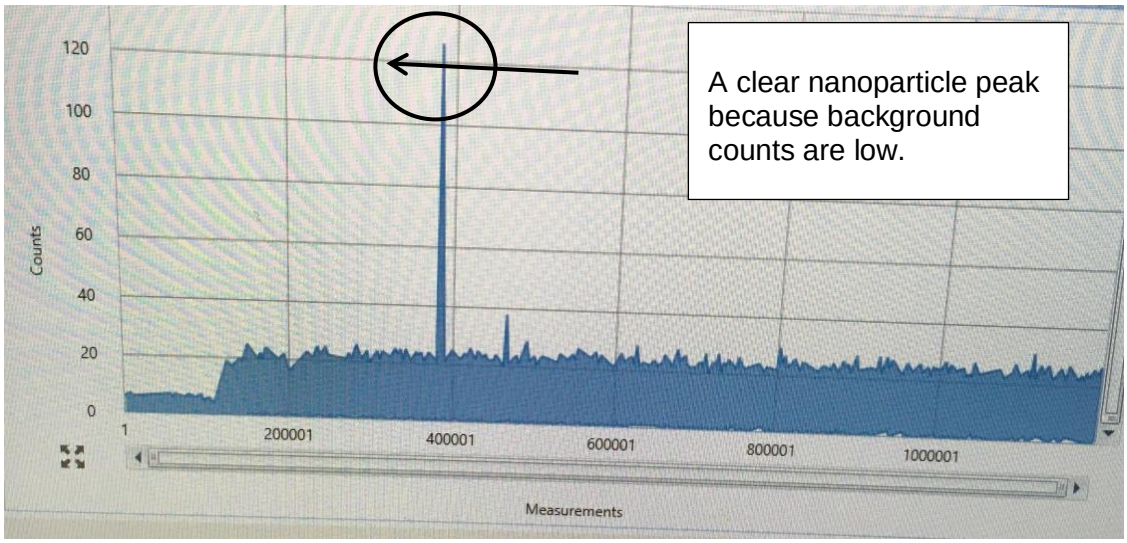

Figure 6.2 Realtime signal from particle size analysis by SP-ICP-MS with low background levels.

6.3.8.5. Conclusions

Particle size measurement by SP-ICP-MS was possible in biological samples that had been spiked with nanoparticles. The dilution rate was a critical factor because sample concentration was vital for accurate measurement of nanoparticles and low background counts.

6.3.9. Analysis of blood and milk of dairy cows for nanoparticle Cu oxide

6.3.9.1. Objective

To determine whether nanoparticles were present in the blood and milk of dairy cows that had been fed nanoparticles within a TMR.

6.3.9.2. Introduction

Section 6.3.8 demonstrated that nanoparticles could be recovered from plasma and milk spiked with nanoparticles if the sample was prepared at a concentration which is optimum for analysis of Cu nanoparticles and reduced the background interference. The aim of this experiment was to determine if Cu nanoparticles could be detected in the plasma and milk of dairy cows that were fed Cu oxide nanoparticles in the TMR.

6.3.9.3. Method

Blood samples that had been collected into K_3EDTA tubes (BD MidMeds Limited, Hertfordshire, UK) and then centrifuged at 1000 x g for 15 min before removing plasma and storing at –20 ^{0}C from Experiment 3 (Chapter 5) were used. The plasma samples were defrosted overnight at 4 ^{0}C and then mixed using a MT-20 vortex mixer (Phillip Harris Ltd, Shenton, UK). Milk samples that were collected in Experiment 3 (Chapter 5) were used and defrosted overnight at 4^{0}C. The samples were analysed for Cu concentration via ICP-MS (Section 2.7) and then diluted with purite water to achieve a Cu concentration of 2 ppb. The diluted samples were analysed for particle size by SP-ICP-MS using the method described in Section 6.2 and parameters in Table 6.14.

Table 6.14 Method parameters for analysis of Cu oxide nanoparticles by SP-ICP-MS in plasma and milk samples from dairy cows fed Cu oxide nanoparticles within the TMR.

Parameter	Value
Analyte	Cu
Dwell time, µs	100 or 500
Data acquisition time, s	500
Density, g/cm^3	4.23
T1 mass fraction, %	60

6.3.9.4. Results and discussion

The Cu oxide nanoparticles were unable to be detected in the plasma and milk samples of dairy cows that were fed nanoparticles in the TMR at dwell times of 100 and 500 µs. As nanoparticle detection in spiked plasma and milk samples were possible there could be two reasons why they could not be identified in the current samples. Firstly, a change in properties of the nanoparticles in the digestive tract of the cow may have occurred so they were not present in the same form in the plasma or milk of the cow. This is likely because the nanoparticles that were in the TMR were Cu oxide with a lysine coating, that was not chemically bound to the metal. It is not known whether the lysine coating remains intact with the Cu oxide as the particles pass through the digestive tract. If the coating and particle do not remain intact particles may agglomerate and would therefore no longer be nano in size. The process by which Cu is absorbed is complex and unless nanoparticle Cu is able to enter through tight junctions because of its small particle size it is likely that its chemical form changes in the digestive tract. The acidic environment in the abomasum usually causes Cu oxide to dissociate to Cu^{2+} ions and then the membrane brush border Cu metalloreductases reduce Cu^{2+} to Cu^{+} ions so they can be absorbed across the epithelial cells in the intestine in a process known as transcellular transport (Goff, 2018). This may mean that the supplemented nanoparticle were no longer in a nanoparticle form so could not be detected by SP-ICP-MS. Secondly, once the particles have been absorbed into the bloodstream the majority are bound to albumin and histidine and transported to the liver (Weiss & Linder, 1985; Goff, 2018). In order to maintain Cu homeostasis, the liver secretes Cu into the bloodstream to maintain plasma Cu concentration within a very tight range of 9 – 19 µmol/L (McDowell, 1992). Secretion of Cu is 80% as Cp, an enzyme synthesised in the liver (Herdt & Hoff, 2011). Thus, the majority of Cu in the bloodstream is bound to an organic which can affect the ability of SP-ICP-MS to identify and quantify nanoparticles. Therefore, further work is required to accurately assess whether any nanoparticles are present in their original form in blood and milk following nanoparticle ingestion.

An inability to detect nanoparticles in milk could also be because they had not entered the milk of the cow or they were in a concentration that was below the detection limit of the SP-ICP-MS. The nanoparticles may not have entered the milk because they have changed in their physical and chemical form in the digestive tract of the cow. The nanoparticles could also be at a concentration that is below the detection limit of the SP-ICP-MS because Cu is transported in very small amounts into milk. For example,

Puls (1994) reported that the normal range for Cu in milk was 0.1 – 0.9 μg/ml. Similarly, (Buckley, 1991) modelled Cu metabolism and reported that only 3.5% of Cu absorbed from the diet was transported to the milk. Thus, if the Cu oxide nanoparticles have behaved in a similar way to conventional Cu sources they will have been present in a very low concentration in the milk. The milk had to be diluted with purite water because if it is not diluted the high total dissolved solids (TDS) quenches the energy in the argon plasma and can lead to inaccurate data. This dilution may have reduced the concentration of Cu oxide nanoparticles below the detection limit of the instrument. For Cu the detection limit was 0.1 ppb which is approximately 10 nanoparticles per ml. Further work could identify a method of reducing other elements in the sample but maintaining the concentration of Cu.

A further analytical explanation for the inability to extract nanoparticles from plasma and milk samples is that the samples had been frozen prior to analysis. Freezing samples can alter the size of the nanoparticles (Choi, et al., 2004) possibly by agglomeration. Agglomeration indicates weakly bonded particles which may be broken by sonication whereas aggregation indicates strongly bonded or fused particles which are unlikely to be broken by sonication (Zook, et al., 2011). To try and resolve this the plasma and milk samples were sonicated for 15 min prior to analysis but this did not alter the results. Sonication works by using ultrasound energy to break agglomerates down into their original particles (Zook, et al., 2011) and these results therefore indicate that the bonds between the particles may have been too strong to be disrupted by sonication.

6.3.9.5. Conclusions

Nanoparticles of Cu oxide could not be identified in plasma or milk samples of cows that had been fed Cu oxide nanoparticles within the TMR. However, there were limitations to the methods used that may mean that nanoparticles in plasma and milk could not be definitively ruled out. The samples were frozen prior to analysis which can cause nanoparticles to agglomerate and aggregate and there was also a necessity to reduce background levels of other elements which may reduce the concentration of Cu nanoparticles below the detection limit of the instrument. It is also possible that nanoparticles of Cu are not present in plasma or milk because of alterations to their physical and chemical form in the digestive tract and binding to organic molecules in the blood and liver. Further work is required to improve the method of sample

preparation and detection to establish whether nanoparticles are present in the plasma or milk of dairy cows fed nanoparticle Cu oxide within the TMR.

6.4. Discussion

Conventionally the size of nanoparticles has been determined using DLS and microscopy techniques (Pace, et al., 2011; Lee, et al., 2014). A relatively new technique, SP-ICP-MS has been developed as a way of measuring and characterising nanoparticles (Pace, et al., 2011; Peters, et al., 2014). The analysis of particle size of nanoparticles by this ICP-MS technique is based on analysing the mass, charge and particle density of the chosen element and the Syngisitix software then uses this information to calculate the mean and mode particle size (Degueldre, et al., 2006; Degieldre, et al., 2006; Pace, et al., 2011). This technique offers advantages over other methods in terms of speed of analysis and lower cost when compared to microscopy techniques such as TEM. A large amount of data can be gained during a short acquisition period of 1-2 minutes. However, as it's a relatively new technique the accuracy, precision and reliability of the results needs confirmation.

In order to assess the accuracy, precision and reliability Au nanoparticle standards were analysed to confirm that particle size correlated with the declared values on the technical data sheet. The standards were also used to optimise sample concentration, wash out procedure for the instrument and stability of solutions. The results showed that the optimum concentration was 1 – 3 ppb Au, wash out should include at least 10 samples of purite water and that samples must be produced so they are fresh for each analysis. From this information, similar techniques were used to optimise the technique for Cu nanoparticles. The results showed that optimum sample concentration for Cu was also 1 – 3 ppb. In the animal studies, (Chapters 3, 4 and 5) TEM analysis was used to determine the particle size of the nanoparticles. Analysis of nanoparticle Cu oxide coated in hydrolysed lysine size by ICP-MS were very similar to results obtained by TEM. These nanoparticles could also be detected in a sample of TMR but there was some variation in particle size, most likely caused by agglomeration or possibly by higher background levels of Cu 65 and NaAr+ compared to the pure samples.

There are detection limits to measuring particle size via SP-ICP-MS. The minimum size that can be detected depends on the element and the calibration achieved, in these experiments Cu particles less than 10 nm were not detected and therefore if they were present in the sample they would not be included in the mean particle size.

Analysis of biological samples for nanoparticles was more challenging. One of the main challenges was that the extraction of nanoparticles from the sample was difficult. Impure samples could have higher background levels with a similar mass/charge ratio to the one being analysed, if the background levels are excessive, then this can hide the nanoparticle peaks that are produced so the software cannot extract and “read” the data to calculate particle size because the software subtracts the background signal from the pulse of signal (Pace, et al., 2011; Cornelius & Hassellov, 2014). In order to remove the high levels of background interference the samples must be extremely dilute. For the detection of Cu in samples the optimum concentration was 1 – 3 ppb. This presents a problem in terms of the working environment and reagents that can be used in sample analysis. The reagents must be ultra-pure, in terms of Cu and Na, to prevent them from contaminating the sample and adding to the number of particles detected. The environment in which the samples are prepared and analysed should also be as clean as possible and samples should be closed as much as possible to avoid contamination from particles in the environment. Dwell time can also be reduced to try and reduce background signal but in section 6.3.9 it was tested at 100 and 500 µs and this did not alter the detection of nanoparticles in the sample. There is also a risk that when increasing dwell time, it can increase the rate of particle coincidence (Hineman & Stephan, 2012).

A variety of further work is required to optimise the method of biological sample analysis for nanoparticles by SP-ICP-MS. Further work is also required on the optimal storage of samples prior to analysis, Section 6.3.9 demonstrated that analysis of frozen samples did not detect nanoparticles in the sample. Cryopreservation snap freezing may be a more appropriate method to keep original particles intact, but this requires further investigation. The wash out procedure between samples could also be optimised, with the use of a different chemical than purite water to speed up the process of cleaning the instrument between samples (Cornelius & Hassellov, 2014).

6.5. Conclusion

Detection and accurate analysis of the particle size of nanoparticles is possible by SP-ICP-MS. Analysis of standards, pure powders and feed is relatively straightforward whereas analysis of biological samples such as blood and milk is more challenging. Further work and method development is required to optimise the technique, particularly for the extraction from biological samples. It is possible that the detection of nanoparticle Cu in biological samples was not possible because the particles were not

retained in their original form in the digestive tract and therefore were not present in the blood or milk of animals.

7. General discussion

7.1. Introduction

Ruminants are often supplemented with Cu and one of the forms that is often used is oxide. Copper oxide has advantages over other supplementary forms of Cu because it has a high margin of safety compared to sources such as sulphate which are more likely to induce Cu toxicity (Suttle, 2010). The relatively high density of Cu oxide (SRL Ectotherm, 2017) also means that it can be added to intra-ruminal mineral boluses. There is one major drawback of Cu oxide and that is its low relative bioavailability compared to other Cu sources, for example the relative bioavailability of Cu oxide is 0.15 compared to Cu sulphate (Baker & Ammerman, 1995a). The current study is the first to investigate whether the bioavailability of Cu oxide for ruminants can be improved by using nanoparticles. The overall hypothesis of the current study was that the bioavailability of nano Cu oxide would be higher than conventional Cu oxide and there would be no negative effects on the performance or health of cattle and sheep.

7.2. Safety of nanoparticles

Research into mineral supplementation using nanoparticles is limited, and one of the reasons for this is likely to be concerns over safety. In the current thesis GGT activity, haematology profile and performance of the animals were used to indicate health status. For all the parameters measured the values were similar between animals fed nanoparticle minerals and those fed conventional minerals indicating that nanoparticles did not appear to have a negative effect. The current thesis provides a series of short term experiments of 56, 63 and 112 d and therefore it can only be concluded that short term supplementation of nano Cu oxide and Zn oxide does not affect animal health or performance. Ruminants are often supplemented with minerals throughout their lives, and therefore further longer term studies are required to measure the long term effects of nanoparticle supplementation.

It may also be useful to conduct a more in-depth assessment of minerals already fed to ruminants. In the current thesis, nano Cu oxide was fed and directly compared with conventional Cu oxide. The nano Cu oxide used was defined as nanoparticles because all particles in the sample were less than 100 nm (De Jong and Borm, 2008; Auffan, et al., 2009). The conventional Cu oxide that was used had a proportion of particles that

were less than 100 nm, with 40 nm being the smallest. Thus, a small proportion of particles in conventional Cu oxide could be defined as in the nano range. Many powdered minerals that are commercially fed to ruminants are likely to have a proportion of particles within the nano range, and therefore studies conducted using these supplements could provide indirect evidence of the safety of nanoparticles. Further research would still be required to examine the effect of higher doses of nanoparticles over the long term, but these studies may still be useful to provide a supporting body of evidence.

7.3. Relationship between liver Cu concentration and GGT activity

The primary storage organ for Cu is the liver (Suttle, 2010). Therefore, in order to assess whether accumulation of Cu in the liver from supplementation of nanoparticles resulted in liver damage, serum GGT activity was monitored throughout the three experiments (Chapter 3, Chapter 4 and Chapter 5). The GGT enzyme is indicative of liver health and activity is raised when the liver is damaged, usually by lethal cell necrosis (Gummow, 1996). Necrosis results in proliferation of biliary epithelial cells that begin to continually synthesise GGT (Ortolani, et al., 2003). Throughout the three experiments there was no difference in GGT activity between cattle or sheep supplemented with nano Cu oxide compared to conventional Cu oxide. All lambs and cattle were within the normal range (< 52 U/l and < 40 U/l respectively) with the exception of lambs at the start of Experiment 1 (Chapter 3) that had raised activity at the beginning of the experiment. The cause of the raised activity is unknown but may be linked to a parasitic infection such as *Fasciola hepatica* (Piacenza, et al., 1999). The results therefore indicate that liver necrosis had not occurred in any of the three experiments when Cu oxide nanoparticles were included at 8 – 9 mg/kg DM.

The data from the three experiments were combined to investigate the relationship between GGT activity and liver Cu concentration and whether GGT activity could potentially be used as a test of liver Cu to reduce the need for liver biopsy (Figure 7.1). A study investigating the predictive value of GGT for hepatic accumulation in cattle reported that the coefficient of determination (R^2) was 0.5838, therefore elevated serum GGT activity may be indicative of increased hepatic Cu (Antonio, et al., 2008). In contrast in the current combined experiments (Figure 7.1) there was no relationship (R^2 = 0.0065) between liver Cu concentration and GGT activity, therefore GGT cannot be used to predict liver Cu.

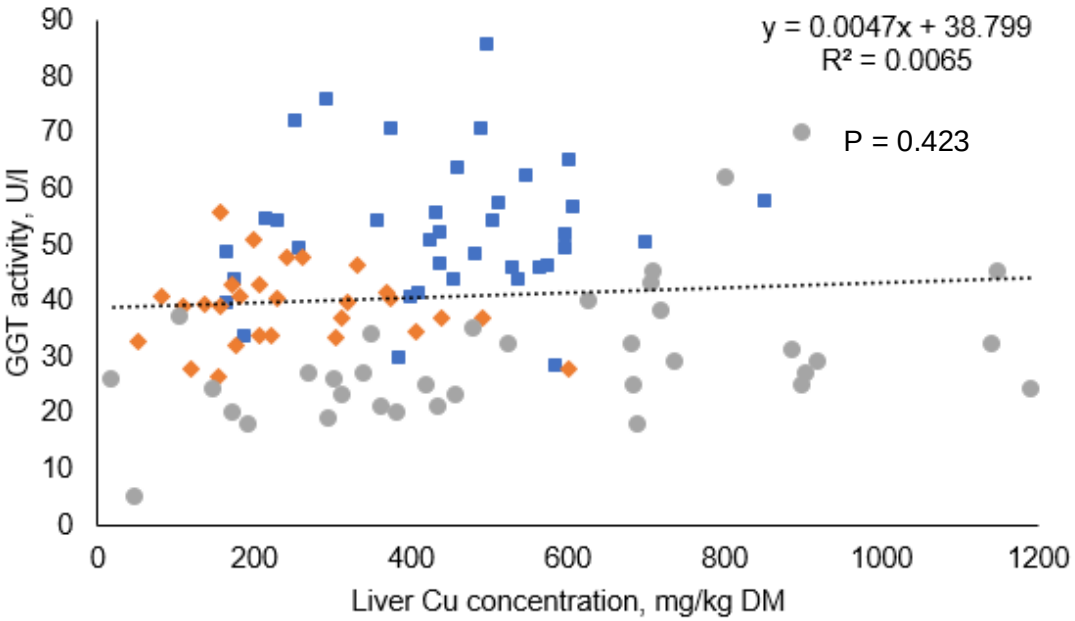

Figure 7.1 Relationship between liver Cu concentration (mg/kg DM) and GGT activity (U/l) in cattle and sheep supplemented with different forms of Cu.
Lamb study 1 (■), lamb study 2 (◆) and dairy cow study (●).

Similar, to the current study, Gummow (1996) reported that GGT could not be used as an indicator of liver Cu concentration because there was not a linear increase in GGT activity as liver Cu increased, instead spikes of high GGT activity occurred separated by periods of normal levels. These spikes are thought to coincide with episodes of severe cellular damage when individual hepatocytes are destroyed and replaced with fibrous tissue. Lopez-Alonso et al. (2006) and Humann-Ziehank et al. (2001) also reported that the diagnostic sensitivity of GGT for liver Cu concentration was too low for accurate prediction with an R^2 value of 0.228 in cattle, 0.238 in Suffolk sheep and 0.441 in mutton Merino. The even lower R^2 value of 0.0065 in the current thesis may be a result of more variables; two different Cu levels were used 8 and 9 mg/kg/DM, different forms of Cu were used for different treatments, there was addition of S, Mo and Zn to some diets, and some lambs were depleted of their Cu stores prior to the start of the study. Gummow (1996), Humann-Ziehank et al. (2001), Lopez-Alonso et al. (2006) and Antonio et al. (2008) all gave daily doses of Cu in the same form to the animals with no other variables. The results of the current study indicate that GGT activity would not be an appropriate diagnostic tool for predicting Cu status in commercial sheep flocks or cattle herds.

7.4. Relative bioavailability of nano Cu oxide

Bioavailability of Cu sources has been reported in many studies using a variety of parameters including, but not limited to, plasma Cu concentration, Cp activity, SOD activity, CCO activity and accumulation of Cu in organs (Baker & Ammerman 1995a). In the current study plasma Cu concentration, Cp activity, SOD activity and accumulation of Cu in the liver were measured in all experiments. Therefore, in the following discussion these values will be used to calculate the relative bioavailability of nano Cu oxide.

7.4.1. Plasma Cu concentration

Table 7.1 indicates that plasma Cu concentrations were similar for sheep and cattle fed different sources of Cu in the three experiments. If the values for the bioavailability of Cu sources are based on plasma Cu, then these data indicate that bioavailability was similar. The values for plasma Cu concentration are within the normal range (9 -20 µmol/L; McDowell, 1992) across all experiments and the inability to detect a difference in Cu sources using this parameter is not unexpected because of the homeostatic mechanisms that regulate plasma Cu concentration within a very tight range (Ward, et al., 1993).

Table 7.1 Mean plasma Cu concentration (µmol/L) in sheep and cattle fed conventional Cu oxide (Oxide), nano Cu oxide (Nano) or Cu sulphate (Sulphate) at an inclusion rate of 4, 8 or 9 mg/kg DM in three experiments.

Cu source	Inclusion rate, mg/kg DM	S, g/kg DM*	Mo, mg/kg DM*	Plasma Cu, µmol/L	Experiment
Oxide	8	1.9	0.6	16.5	1
Nano	8	1.9	0.6	17.4	
Nano	4	1.9	0.6	16.3	
Sulphate	8	1.9	0.6	17.2	
Oxide	9	1.7	0.5	13.9	2
Nano	9	1.7	0.5	14.6	
Oxide	8	3.2	0.9	17.6	3
Nano	8	3.5	0.8	18.0	
Oxide	8	4.4	7.2	18.4	
Nano	8	4.3	7.1	17.0	

*In phase where supplementary Cu was fed.

In studies where a difference in plasma Cu concentration has been detected between Cu sources, the animals were usually subject to a Cu depletion and repletion

technique, as in Experiment 2 of the current thesis (Suttle, 1974; Kegley & Spears, 1994; Ward, et al., 1996). In Experiment 2, the plasma Cu concentration at the start of the repletion phase was between 4 and 13 µmol/L, which is in contrast to the other three studies where all the animals started below 9 µmol/L. Thus, in Experiment 2 of the current thesis the extent of depletion may not have been sufficient to identify differences in plasma Cu concentration in lambs fed different Cu supplements in the repletion phase. Measurement of plasma Cu concentration at more frequent intervals in the first two weeks of the repletion period may also have provided useful data to indicate the rate of repletion which may have allowed the calculation of the relative bioavailability of nano Cu oxide.

7.4.2. Ceruloplasmin activity

Table 7.2 indicates that Cp concentration was similar for sheep and cattle fed different sources of Cu in the three experiments. If the values for the bioavailability of Cu sources are based on Cp concentration, then these data indicate that the bioavailability was similar.

Table 7.2 Mean Cp concentration (mg/dl) in sheep and cattle fed conventional Cu oxide (Oxide), nano Cu oxide (Nano) or Cu sulphate (Sulphate) at an inclusion rate of 4, 8 or 9 mg/kg DM in three experiments.

Cu source	Inclusion rate, mg/kg DM	S, g/kg DM*	Mo, mg/kg DM*	Cp activity, mg/dl	Experiment
Oxide	8	1.9	0.6	15.5	1
Nano	8	1.9	0.6	14.9	
Nano	4	1.9	0.6	14.3	
Sulphate	8	1.9	0.6	14.8	
Oxide	9	1.7	0.5	14.1	2
Nano	9	1.7	0.5	15.2	
Oxide	8	3.2	0.9	17.3	3
Nano	8	3.5	0.8	17.4	
Oxide	8	4.4	7.2	18.5	
Nano	8	4.3	7.1	18.6	

*In phase where supplementary Cu was fed.

Blakley and Hamilton (1985) reported that Cp concentration correlated closely, R^2 = 0.60, with plasma Cu concentration and the relationship remained linear from the deficient to normal ranges of Cu. It is therefore not unexpected, that because there was no difference in plasma Cu, that a difference in Cp concentration was not identified.

Thus, similarly to plasma Cu, the relative bioavailability based on Cp concentration appeared the same between conventional and nano Cu oxide.

7.4.3. Superoxide dismutase activity

Table 7.3 indicates that SOD activity was similar for sheep and cattle fed different sources of Cu in the three experiments. If the values for bioavailability of Cu sources are based on SOD activity, then these data indicate that the bioavailability was similar.

Table 7.3 Mean SOD activity (U/g Hb) in sheep and cattle fed conventional Cu oxide (Oxide), nano Cu oxide (Nano) or Cu sulphate (Sulphate) at an inclusion rate of 4, 8 or 9 mg/kg DM in three experiments.

Cu source	Inclusion rate, mg/kg DM	S, g/kg DM*	Mo, mg/kg DM*	SOD activity, U/g Hb	Experiment
Oxide	8	1.9	0.6	1719	1
Nano	8	1.9	0.6	1907	
Nano	4	1.9	0.6	1938	
Sulphate	8	1.9	0.6	1966	
Oxide	9	1.7	0.5	1163	2
Nano	9	1.7	0.5	1526	
Oxide	8	3.2	0.9	1753	3
Nano	8	3.5	0.8	1872	
Oxide	8	4.4	7.2	1930	
Nano	8	4.3	7.1	1975	

*In phase where supplementary Cu was fed.

Activity of SOD is measured in haemoglobin, and to increase activity there has to be a turnover of red blood cells, which takes approximately 70 to 150 d in sheep and 160 d in cattle (Kerr, 1989). Small changes can be identified when approximately one quarter of the cells have been replaced. The experiments conducted as part of this thesis were relatively short term at 56, 63 and 112 d for experiments 1, 2 and 3 respectively. Experiment 3 was the longest, but plasma Cu, Cp activity and liver Cu concentration all showed that the cattle were never in Cu deficit, therefore there would never have been a shortfall of Cu for inclusion into SOD. Longer term studies would provide a better opportunity for differences in enzyme activity to be identified. For example, Nagalakshmi et al. (2010) and Pal et al. (2011) both reported increased SOD activity in sheep fed organic Cu compared to inorganic Cu, but these studies were conducted over a 180 d and 240 d period respectively.

7.4.4. Accumulation of Cu in the liver

The liver is the primary organ for Cu storage and is therefore one of the best indicators of Cu status (Ledoux, et al., 1995). Accumulation of Cu in the liver can be used as a measure of dietary Cu availability because it is representative of the Cu absorbed in excess of requirement (Zervas, et al., 1990).

In Experiment 1 (Chapter 3) the initial liver Cu concentrations were unknown because liver biopsy samples were not taken from the lambs because of the potential damage that can be caused to the biliary tract and high mortality rates from the procedure (Suttle, 2012a). To understand the variation in liver Cu concentration at the start of the study a group of 10 lambs were slaughtered for measurement of Cu concentration in the liver. Thus, for this experiment there are two ways to calculate the relative bioavailability of Cu from liver concentrations; firstly, using the control group mean as a base value and calculating estimated accumulation, or just using the final liver Cu concentration. In Experiment 1 there were four treatment groups, three of these groups fed Cu at a rate of 8 mg/lg DM, one group were fed Cu at a rate of 4 mg/kg DM. The calculations for the relative bioavailability in this experiment are based on the treatment groups that were fed 8 mg/kg DM with the group that were fed nano Cu oxide at 4 mg Cu/kg DM not included in the analysis.

Table 7.4 reports the relative bioavailability of nano Cu oxide (NH) compared to conventional Cu oxide (O). Based on the final liver Cu concentration the relative bioavailability of nano Cu oxide compared to conventional Cu oxide was 1.29. When relative bioavailability was calculated based on estimated Cu accumulation, then compared to conventional Cu oxide, nano Cu oxide was 2.81.

Table 7.4 Calculation of the relative bioavailability of nano Cu oxide compared to conventional Cu oxide in growing lambs calculated from final liver Cu concentration (mg/kg DM) and estimated liver Cu accumulation (Chapter 3, Experiment 1).

	Treatment group[1]	
	O	NH
Liver Cu, mg/kg DM	332 ± 135.6	428 ± 102.2
Relative bioavailability based on final liver Cu[2]	-	1.29
Estimated liver Cu accumulation, mg/kg DM	53 ± 135.6	149 ± 102.2
Relative bioavailability based on Cu accumulation[2]	-	2.81

[1]Treatments were: O; Cu oxide 8mg Cu/kg DM and NH; Nano Cu oxide 8 mg Cu/kg DM

[2]Compared to O at 1.

In order to compare the relative bioavailability to other published papers it is useful to convert them relative to Cu sulphate. Table 7.5 presents the relative bioavailability of Cu oxide in conventional (O) and nano (NH) form compared to Cu sulphate (S) for Experiment 1. When final liver Cu concentration is used to calculate the relative bioavailability to Cu sulphate at 1, the values were 0.62 for conventional Cu oxide and 0.81 for nano Cu oxide. When values were calculated from the estimated Cu accumulation in the liver, they were 0.21 for conventional Cu oxide and 0.60 for nano Cu oxide. Examining the values calculated compared to other publications shows that those calculated from estimated Cu accumulation were more similar to studies that have compared Cu oxide and Cu sulphate (Baker & Ammerman, 1995a; Ledoux, et al., 1995). For example, Baker and Ammerman (1995a) collated several studies and calculated bioavailability values for cattle and sheep and concluded that the relative bioavailability of Cu oxide was 0.15 of Cu sulphate. Similarly, Ledoux et al. (1995) used the accumulation of Cu in the liver to calculate the bioavailability of Cu and concluded that bioavailability of Cu sulphate was four times greater than Cu oxide. These values are similar to the relative bioavailability of 0.21 for Cu oxide calculated from accumulation in the liver, and much lower than 0.62 calculated from the final liver Cu concentration.

Table 7.5 Relative bioavailability of conventional Cu oxide (O) and nano Cu oxide (NH) to Cu sulphate (S) in growing lambs calculated from the final liver Cu concentration (mg/kg DM) and estimated liver Cu accumulation (Chapter 3, Experiment 1).

	Treatment group[1]		
	S	O	NH
Liver Cu, mg/kg DM	529 ± 162.8	332 ± 135.6	428 ± 102.2
Relative bioavailability based on final liver Cu[2]	-	0.62	0.81
Estimated liver Cu accumulation, mg/kg DM	250 ± 162.8	53 ± 135.6	149 ± 102.2
Relative bioavailability based on Cu accumulation[2]	-	0.21	0.60

[1]Treatments were: O; Cu oxide 8 mg Cu/kg DM, NH; Nano Cu oxide 8 mg Cu/kg DM and S; Cu sulphate 8 mg Cu/kg DM
[2]Compared to S at 1.

In Experiment 2 (Chapter 4) the lambs did not have biopsy samples taken nor was there an initial slaughter group and therefore the only values that can be used to calculate the relative bioavailability were the final liver Cu concentrations. Table 7.6 reports that the relative bioavailability of nano Cu oxide was 1.5 compared to conventional Cu oxide. This value is similar to the 1.29 reported in Experiment 1 based on the final liver concentration (Table 7.4).

Table 7.6 Relative bioavailability of nano Cu oxide (N) compared to conventional Cu oxide (O) in growing lambs calculated from final liver Cu concentration (mg/kg DM; Chapter 4, Experiment 2).

	Treatment group[1]	
	O	N
Liver Cu, mg/kg DM	200 ± 101.5	300 ± 131.6
Relative bioavailability based on final liver Cu[2]	-	1.50

[1]Treatments are: O; Cu oxide 9 mg Cu/kg DM; N; Nano Cu oxide 9 mg Cu/kg DM
[2]Compared to O at 1.

In Experiment 3 (Chapter 5) dairy cattle were used and it was possible to measure the accumulation of Cu in the liver because initial liver biopsy samples were taken. Table 7.7 presents the relative bioavailability of nano Cu oxide compared to conventional Cu oxide calculated from the final liver Cu concentration and Cu accumulation over 16 weeks. The values in Table 7.7 represent the relative bioavailability compared to conventional Cu oxide either with or without additional S and Mo in the diet. The bioavailability of nano Cu oxide was slightly higher than conventional Cu oxide with values of 1.08 – 1.30 when there was no additional S or Mo in the diet. When additional S and Mo were present the bioavailability rose to between 1.77 – 2.74.

Table 7.7 Relative bioavailability of nano Cu oxide (N- and N+) compared to convention Cu oxide (O- and O+) in dairy cows calculated from final liver Cu concentration (mg/kg DM; Chapter 5, Experiment 3).

	Treatment group[1]			
	O-	O+	N-	N+
Liver Cu, mg/kg DM	596 ± 217.2	333 ± 238.3	638 ± 197.8	590 ± 248.8
Relative bioavailability based on final liver Cu[2]	-	-	1.08[2]	1.77[3]
Liver Cu accumulation, mg/kg DM	+138 ± 264.3	-149 ± 168.2	+179 ± 305.1	+124 ± 235.5
Relative bioavailability based on Cu accumulation[2]	-	-	1.30[2]	2.74[3]

[1]Treatments are: O-; Cu oxide 8 mg Cu/kg DM without antagonists, O+; Cu oxide 8 mg Cu/kg DM with antagonists, N-; Nano Cu oxide 8 mg Cu/kg DM without antagonists and N+; Nano Cu oxide 8 mg Cu/kg DM with antagonists.
[2]Compared to O- at 1.
[3]Compared to O+ at 1.

Overall, based on liver Cu concentrations from the three experiments the relative bioavailability of nano Cu oxide compared to conventional Cu oxide was between 1.08 – 2.81 and relative to Cu sulphate is between 0.23 – 0.81 depending on the level of antagonists in the diet. The three experiments show that further work is required to

determine the bioavailability based on liver Cu concentration and that it may be affected by different factors. For example, Experiment 3 showed that nano Cu oxide may be more than twice as bioavailable as conventional Cu oxide in the presence of high levels of antagonists, but bioavailability was similar when there are no additional antagonists in the diet. Studies have shown that there are a number of experimental factors that alter bioavailability values including, but not limited to; calculating from final liver concentrations or measurement of liver accumulation, Cu status of animals prior to study, dose of Cu, breed of animals and other dietary factors (Suttle, 1974; Wooliams, et al., 1983). Suttle (1974 and 1978) reported that using a depletion and repletion technique may produce a more accurate prediction of bioavailability but there was risk of overestimating the value when fed to animals of normal Cu status. Thus, in terms of comparison to other sources Experiment 2 may be the best to indicate the bioavailability of nano Cu oxide but may overestimate the value if it was fed to sheep of normal Cu status. However, overestimation is likely to be minimal in the experiment because the sheep were not depleted to clinical conditions as has been induced in previous experiments (Suttle, 1974; Kegley & Spears, 1994; Ward, et al., 1996)

The drawback of Experiment 2 is that accumulation of Cu in the liver was not measured and variation on the initial liver Cu concentration may not have been the same between treatments. Experiment 1 showed that measuring the accumulation of Cu in the liver was more likely to provide an accurate assessment of bioavailability because the values produced were more similar to other published values (Baker & Ammerman, 1995a; Leudoux, et al., 1995). Measurement of the accumulation following a period of Cu depletion may be the best indication because Cu accumulation in the liver is only linear at low Cu concentrations (Figure 1.4; Wooliams, et al., 1983).

It is difficult to estimate the initial liver Cu concentrations from the lambs in Experiment 2 because it was not measured. Claypool et al. (1975) reported that plasma Cu levels were of little value in predicting liver Cu concentrations except for reporting that values less than 8 µmol/L are indicative of low concentrations in the liver. Similarly, Blakley & Hamilton (1985) reported a correlation coefficient of 0.35 between Cp activity and liver Cu concentration indicating that Cp cannot be used to predict liver Cu concentration.

The analysis of the bioavailability of Cu using liver concentrations shows that nano Cu oxide has a higher bioavailability than conventional Cu oxide but further work is required to identify a definitive bioavailability value. The improved bioavailability of Cu oxide by reducing particle size may be especially useful for feeding to animals when high levels of Cu antagonists are present in the diet.

7.5. Relative bioavailability of nano Zn oxide

The bioavailability of Zn sources has been reported in many studies using a variety of parameters including, but not limited to, plasma Zn concentration, ALP activity and accumulation of Zn in organs (Baker & Ammerman 1995b). Plasma Zn concentration, ALP activity and the accumulation of Zn in liver and kidney (Table 7.8) were analysed in Experiment 1 (Chapter 3) with the intention of calculating the relative bioavailability of nano Zn oxide compared to conventional Zn oxide and Zn sulphate.

Table 7.8 Plasma Zn µmol/L, ALP activity, U/L liver Zn and kidney Zn (µmol/L) measured in growing lambs fed different sources of Cu and Zn over 8 weeks.

	Treatment[1]				
	O	NH	NL	S	Significance of F+L[2]
Plasma Zn, µmol/L	11.7	10.0	11.3	10.9	0.546
ALP activity, U/L	287	331	313	328	0.509
Final liver Zn, µmol/kg DM	89	84	67	76	0.611
Final kidney Zn, µmol/kg DM	88	66	77	58	0.314

[1]Treatments are: O; Cu oxide 8 mg Cu/kg DM and Zn oxide 40mg Zn/kg DM, NH; Nano Cu oxide 8 mg Cu/kg DM and nano Zn oxide 40mg Zn/kg DM, NL; Nano Cu oxide 4 mg Cu/kg DM and nano Zn oxide; 20 mg Zn/kg DM and S; Cu sulphate 8 mg Cu/kg DM and Zn sulphate 40 mg Zn/kg DM
[2]Form and Level

Table 7.8 shows that there was no effect of form or level of Zn on plasma Zn concentration, ALP activity, final liver Zn or kidney Zn concentration indicating that the bioavailability of Zn oxide, nano Zn oxide and Zn sulphate were similar. The current finding is in contrast to the data collated by Baker & Ammerman (1995b) which reported that in sheep the relative bioavailability of Zn oxide was 0.7 compared to Zn sulphate. Increased bioavailability of Zn sulphate compared to oxide was also reported by Spears (1989) and Rojas et al. (1995).

The most likely reason for the lack of a difference in the bioavailability between Zn sources was because the concentration of Zn in the basal diet was higher than predicted at 170 compared to the 40 mg/kg DM that was formulated. Differences in bioavailability are easier to detect when background levels of the mineral are low and the supplement is at a high proportion of dietary intake (Baker & Ammerman, 1995b). In the current study the supplementary Zn was 19% of total dietary intake. In studies where bioavailability differences have been identified between Zn sources the supplementary Zn has provided a higher proportion of dietary intake, for example 96% (Sandoval, et al., 1997) and 88% (Hatfield, et al., 2001). Further work is required to

quantify the bioavailability of nano Zn oxide when Zn is included at a higher proportion of total intake.

7.6. Potential mechanisms for improved bioavailability

The data from the current thesis indicate that the bioavailability of nano Cu oxide is greater than conventional Cu oxide. There are very few studies that have been conducted to investigate the bioavailability of nanoparticles in ruminants and there is a lack of evidence for the mechanisms by which bioavailability is increased, although there are a number of theories. Feng et al. (2009) reported that smaller particles were more easily absorbed in the gastrointestinal tract of mice, and Desai et al. (1997) reported that the uptake of particles in the gastrointestinal tract was inversely proportional to particle size. Similarly, Sahoo and Labhasetwar (2003) concluded that nanoparticles are small enough to be transported directly from capillaries for absorption by cells. In ruminants improved absorption of nano Cu may be because the major pathway for Cu absorption is across the epithelial cells in a process called transcellular transport (Goff, 2018). This would mean that the nanoparticles must remain in a smaller form than conventional Cu oxide as they pass through the rumen, reticulum, omasum and abomasum to the site of Cu absorption in the small intestine (Gooneratne, et al., 1989). To establish this further research on the stability of nanoparticles in the conditions of the gastro-intestinal tract is required.

A second mechanism by which the nanoparticles may have a greater bioavailability than conventional minerals is the change in physical properties. Nanoparticles have an increased percentage of atoms at the surface relative to the total number of atoms in the material compared to conventional materials (Daniel and Astruc, 2004) which could mean they have an increased bioavailability compared to the same chemical compound in a conventional particle size (Sahoo, et al., 2014). The change in properties of nanoparticles in the current study is also linked to solubility. Conventional Cu oxide is insoluble in water, but when nano Cu oxide (with an amino acid coating) is in water it remains suspended for at least four hours. This suspension of the particles could increase absorption because it may affect transit of the Cu through the gastrointestinal tract. It is thought that acid solubilisation of Cu supplements in the abomasum is required for dissociation to Cu^{2+} which is required for absorption of Cu in the small intestine (Goff, 2018). Slower transit times and increased solubility can increase the supplement solubilisation in the abomasum, and therefore absorption is increased (Judson, et al., 1982; Kegley & Spears, 1994; Spears, 2003)

A third mechanism by which nano Cu oxide may be more bioavailable than conventional Cu oxide is the amino acid coating. Some studies have shown that organic minerals have a greater bioavailability than inorganic minerals (Surai, et al., 2019). For Cu, this has been more pronounced in the presence of high levels of Cu antagonists (Kincaid, et al., 1986; Ward, et al., 1996). For example, Kincaid et al. (1986) fed 0.6 mg Mo/kg concentrate and 5 mg Mo/kg hay and Ward et al. (1996) fed 5 mg Mo/kg DM. Both studies reported that Cu proteinate had a greater bioavailability than Cu sulphate (Kincaid, et al., 1986; Ward, et al., 1996). One theory for the improved bioavailability in the presence of antagonists is related to solubility. If a supplement is relatively insoluble in the rumen but soluble in the abomasum it may be more resistant to formation of insoluble compounds with thiomolybdate because the interaction between Cu, S and Mo occurs in the rumen (Ward, et al., 1996). The stability of nanoparticles and whether the amino acid coating remains attached to the particles in the gastrointestinal tract is unknown, and an understanding of the stability and solubility in different parts of the gastrointestinal tract would help to identify the mechanism that increases bioavailability.

7.7. Conclusions

The series of experiments, in this thesis indicates that nano Cu oxide has a greater bioavailability than conventional Cu oxide when assessed using the concentrations of Cu in the liver of sheep and cattle. The increased bioavailability was more pronounced when there were high levels of S and Mo in the diet or animals had a low initial Cu status. The metabolism and distribution of Cu in the body is under homeostatic control and, in the current experiments none of the animals were deficient in Cu (with the exception of lambs in the depletion period of Experiment 2) and therefore there were no differences identified between Cu sources for plasma Cu concentration, Cp or SOD activity. Nano Zn oxide did not have a greater bioavailability than conventional Zn oxide but this was likely to be due to high levels of Zn in the basal diet resulting in the supplementary Zn providing a low proportion of total dietary intake. Further work is required to evaluate the bioavailability of nano Zn oxide, and further long term studies are required to evaluate the long term effects of nanoparticle supplementation particularly with regard to the health of the animals. Several mechanisms have been proposed for the increased bioavailability of nanoparticles, and in ruminants the most likely is increased absorption in the digestive tract due to small particle size and increased solubility. There is considerable speculation regarding the safety of

nanoparticles but many powdered minerals already fed to animals will have a proportion of particles that are below 100 nm and could therefore be classified as being in the nano scale. The safety of nanoparticle minerals requires assessment by thorough, rigorous scientific methods when these supplements are fed at the intended dose and directly compared with conventional supplements.

References

ACAF, 2010. Copper in Animal Feed DISCUSSION PAPER, s.l.: Advisory committee on animal feedingstuffs.

AFRC, 1993. Energy and protein requirements of ruminants. CAB International, Wallingford.

Allen, J. & Gawthorne, J., 1987. Involvement of the solid phase of rumen digesta in the interaction between copper, molybdenum, and sulphur in sheep. British Journal of Nutrition, Volume 58, pp. 265 - 276.

Allen, J.G., Masters, H,G., Peet, R.L., Mullins, K.R., Lewis, R.D., Skirrow, S.Z. & Fry, J., 1983. Zinc toxicity in ruminants. Journal of comparative pathology, Volume 93, pp. 363 - 377.

Andrewartha, K. & Caple, I., 1980. Effects of changes in nutritional copper on erythrocyte superoxide dismutase activity in sheep. Research in Veterinary Science, Volume 28, pp. 101 - 104.

Antonio, H.H., Minervino, R.A., Barreto, F.J.G., Queiroz, S., Headley, A. & Ortolani, E.L., 2008. Predictive values of aspartate aminotransferase and gamma-glutamyl transferase for the hepatic accumulation of copper in cattle and buffalo. Journal of Veterinary Diagnostic Investigation, Volume 20, pp. 791 - 795.

AOAC., 2012. Official methods of analysis, Association of official analytical chemist 19th edition, Washington D.C., USA.

Arredondo, M., Munoz, P., Mura, C. & Nunez, M., 2003. DMT1, a physiologically relevant apical Cu1+ transporter of intestinal cells. American Journal of Physiology Cell Physiology, Volume 284, pp. 1525 - 1530.

Auffan, M., Rose, J., Bottero, J-Y., Lowry, G., Jolivet, J-P., Wiesner, M., 2009. Towards a definition of inorganic nanoparticles from an environmental health and safety perspective. Nature Nanotechnology, Volume 4, pp. 634-641.

Baker, D. & Ammerman, C., 1995a. Copper Bioavailability. In: C. Ammerman, D. Baker & A. Lewis, eds. Bioavailability of Nutrients for Animals Amino acids, Minerals and Vitamins. London: Academic Press, pp. 127-156.

Baker, D. & Ammerman, C., 1995b. Zinc Bioavailability. In: C. Ammerman, D. Baker & A. Lewis, eds. Bioavailability of nutrients for animals amino acids, minerals and vitamins. London: Academic Press, pp. 367-398.

Balasubramaniam, S.K., Jittiwat, J., Manikandan, J., Ong, C.N, Yu, L.E. & Ong, W.Y., 2010. Biodistribution of gold nanoparticles and gene expression changes in the liver and spleen after intravenous administration in rats. Biomaterials, Volume 31, pp. 2034-2042.

Balemi, S.C., Grace, N.D., West, D.M., Smith, S.L & Knowles, S.O., 2010. Accumulation and depletion of liver copper stores in dairy cows challenged with a Cu-deficient diet and oral and injectable forms of Cu supplementation. New Zealand Veterinary Journal, Volume 58, pp. 137 - 141.

Barlow, R., Purves, D., Butler, E. & MacIntyre, I., 1960. Swayback in south-east Scotland II Clinical, pathological and biochemical aspects. Journal of Comparative Physiology, Volume 70, pp. 411 - 428.

Bartholomew, M., Willet, L., Liu, T-T. & Moorhead, P., 1987. Changes in hepatic function tests to induced toxicity in the bovine liver. Journal of Animal Science, Volume 64, pp. 201-209.

Beattie, J. & Trayhurn, P., 2002. Metallothioneins and oxidative stress. Nutritional Sciences, Volume 5, pp. 228 - 233.

Berrie, R.A., Hallford, D.M., Galyean, M.L., Brundage, A.L. & White, T.W., 1995. Effects of zinc source and level on performance, carcass characteristics, and metabolic hormone concentrations of growing and finishing lambs. The Professional Animal Scientist, Volume 11, pp. 149 – 156.

Berry, D.P., Lee, J.M., Macdonald, K.A., Matthews, L. & Roche, J.R., 2007. Associations Among Body Condition Score, Body Weight, Somatic Cell Count, and Clinical Mastitis in Seasonally Calving Dairy Cattle. Journal of Dairy Science, Volume 90, pp. 637 – 648.

Bidewell, C., VLA, W., Livesey, C. & VLA, W., 2002. Copper poisoning: an emerging disease in dairy cattle, s.l.: VLA.

Blakley, B.R. & Hamilton, D.L., 1985. Ceruloplasmin as an indicator of copper status in cattle and sheep. Canadian Journal of Comparative Medicine, Volume 49, pp. 405 – 408.

Bone, P., Payne, J. & Twigge, J., 2011. Guidance note for supplementing copper to bovines, s.l.: ACAF & FSA.

Bonham, M., O'Connor, J., Hannigan, B. & Strain, J., 2002. The immune system as a physiological indicator of marginal copper status. British Journal of Nutrition, Volume 87, pp. 393 - 403.

Braun, J.P., Rico, A.G., Benard, P., Thouvenot, J.P. & Bonnefis, M.J., 1978. Tissue and blood distribution of gamma-glutamyl transferase in the lamb and in the ewe. Research in Veterinary Science, Volume 25, pp. 37-40.

Bremner, I., 1970. Zinc, copper and manganese in the alimentary tract of sheep. British Journal of Nutrition, Volume 24, pp. 769 - 783.

Bremner, I., 1993. Metallothionein in copper deficiency and copper toxicity. In: M. Anke, D. Meissner & C. Mills, eds. Proceedings of the Eighth International Symposium on Trace Elements in Man and Animals. Gersdorf, Germany: Verlag Media Touristik, pp. 507 - 515.

Bremner, I., 1998. Manifestations of copper excess. American Journal of Clinical Nutrition, Volume 67, pp. 1069S - 1073S.

Bremner, I., Humphries, W., Phillippo, M. & Walker, M., 1987. Iron-induced copper deficiency in calves: dose-response relationships and interactions with molybdenum and sulphur. Animal Science, Volume 45, pp. 403 - 414.

Brugger, D. & Windisch, W., 2015. Environmental responsibilities of livestock feeding using trace mineral supplements. Animal Nutrition, Volume 1, pp. 113 - 118.

Buckley, W., 1991. A kinetic model of copper metabolism in lactating dairy cows. Canadian Journal of Animal Science, Volume 71, pp. 155 - 166.

Bulgin, M., Anderson, B., Hall, R. & Lang, B., 1984. Serum gamma glutamyl transpeptidase in cattle with induced fascioliasis. Research in Veterinary Science, Volume 37, pp. 167 - 171.

Campbell, A., Coup, M., Bishop, W. & Wright, D., 1974. Effect of elevated iron intake on the copper status in growing cattle. New Zealand Journal of Agricultural research, Volume 17, pp. 393 - 399.

Cao, J., Henry, P.R., Guo, R., Holwerda, R.A., Toth, J.P., Littell, R.C., Miles, R.D. & Ammerman, C.B., 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. Journal of Animal Science, Volume 78, pp. 2039 – 2054.

Cardellini, A., lberghini, M., Rajand, A.G., Misra, R.P., Blankschtein, D. & Asinari, P., 2019. Multi-scale approach for modeling stability, aggregation, and network formation of nanoparticles suspended in aqueous solutions. Nanoscale, Volume 11, pp. 3979 - 3992.

Chase, C.R., Beede, D.K., Van Horn, H.H., Shearer, J.K., Wilcox, C.J, & Donovan, G.A., 2000. Responses of lactating dairy cows to copper source, supplementation rate and dietary antagonist (Iron). Journal of Dairy Science, Volume 83, pp. 1845 - 1852.

Cheng, J., Ma, H., Fan, C., Zhang, Z., Jia, Z., Zhu, X. & Wang, L., 2011. Effects of Different Copper Sources and Levels on Plasma Superoxide Dismutase, Lipid Peroxidation, and Copper Status of Lambs. Biological Trace Element Research, Volume 144, pp. 570 – 579.

Chester, J., 1992. Trace element- gene interactions. Nutrition reviews, Volume 50, pp. 217 – 223.

Chidambaram, M.V., Barnes, G. & Frieden, E., 1984. Inhibition of ceruloplasmin and other copper oxidases by thiomolybdate. Journal of Inorganic Chemistry, Volume 22, pp. 231 – 2393.

Choi, M.J., Briancon, S., Andrieu, J., Min, S.G. & Fessi, H., 2004. Effect of freeze-drying process conditions on the stability of nanoparticles. Drying Technology, Volume 22, pp. 335 - 346.

Claypool, D.W., Adams, F.W., Pendell, H.W., Hartmann, N.A. & Bone, J.F., 1975. Relationship between the level of copper in the blood plasma and liver of cattle. Journal of Animal Science, Volume 41, pp. 911 - 914

Collins, J., Proshaska, J. & Knutson, M., 2010. Metabolic crossroads of iron and copper. Nutrition reviews, Volume 68, pp. 133 - 147.

Condomina, J., Zornoza-Sabina, T., Granero, L. & Polache, A., 2002. Kinetics of zinc transport in vitro in rate small intestine and colon: Interaction with copper. European Journal of Pharmaceutical Sciences, Volume 16, pp. 289 - 295.

Cope, C.M., Mackenzie, A.M., Wilde, D. & Sinclair, L.A., 2009. Effects of level and form of dietary zinc on dairy cow performance and health. Journal of Dairy Science, Volume 92, pp. 2128 – 2135.

Cornelius, G. & Hassellov, M., 2014. A single deconvolution method to discriminate smaller nanoparticles in single particle ICP-MS. Journal of Analytical Atomic Spectrometry, Volume 29, pp. 134 - 144.

Cousins, R., 2010. Gastrointestinal factors influencing zinc absorption and homeostasis. International Journal of Vitamin Nutrition Research, Volume 80, pp. 243 - 248.

Cousins, R.J., Blanchard, R.K., Moore, J.B., Cui, L., Green, C.L., Liuzzi, J.P., Cao, J. & Bobo, J.A., 2003. Regulation of zinc metabolism and genomic outcomes. Journal of Nutrition, Volume 133, pp. 1521S - 1526S.

Cousins, R., Liuzzi, J. & Lichten, L., 2006. Mammalian zinc transport, trafficking and signals. Journal of biological chemistry, Volume 281, pp. 24085 - 24089.

Cunningham, I., 1950. Copper and molybdenum in relation to diseases of cattle and sheep in New Zealand. In: W. McElroy & B. Glass, eds. Copper Metabolism: A symposium on animal, pant and soil relationships. Baltimore, USA: Johns Hopkins Press, pp. 246 - 273.

Daniel, M.-C. & Astruc, D., 2004. Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. Chemistry Reviews, Volume 104, pp. 293-346.

Dargatz, D., Garry, F. & Clark, G., 1999. Serum copper concentrations in beef cows and heifers. Journal of the veterinary medical association, Volume 215, pp. 1828 - 1832.

Davies, D. & Jebbet, I., 1981. Liver biopsy of cattle. The Veterinary Record, Volume 109.

De Jong, W. & Borm, P., 2008. Drug delivery and nanoparticles: Applications and hazards. International Journal of Nanomedicine, Volume 3, pp. 133-149.

Degieldre, C., Favarger, P.-Y., Rosse, R. & Wold, S., 2006. Uranium colloid analysis by single particle inductively coupled plasma-mass spectrometry. Talanta, Volume 68, pp. 623 - 628.

Degueldre, C., Favarger, P.-Y. & Wold, S., 2006. Gold colloid analysis by inductively coupled plasma-mass spectrometry in a single particle mode. Analytica Chimica Acta, Volume 555, pp. 263 - 268.

Deguelfre, C. & Favarger, P., 2003. Colloid analysis by single particle inductively coupled plasma-mass spectroscopy: a feasability study. Colloids and Surfaces A: Physicochemical and Engineering aspects, Volume 217, pp. 137 - 142.

Desai, M.P., Labhasetwar, V., Walter, E., Levy, R.J. & Amidon, G.L., 1997. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. Pharm Res, Volume 14, pp. 1568-1573.

Dickens, M., Delehanty, D. & Romero, L., 2010. Stress: An inevitable component of animal translocation. Biological Conservation, Volume 143, pp. 1329 - 1341.

Donald, G., Paull, D. & Langlands, J., 1984. Liver biopsy as a technique for assessing copper status of sheep. Australian Veterinary Journal, Volume 61, pp. 121 – 122.

Drewnoski, M.E.S., Ensley, S.M., Beitz, D.C., Shoonmaker, J.P., Loy, D.D., Imerman, P.M., Rathje, J.A. & Hansen, S.L., 2012. Assessment of ruminal hydrogen sulfide or urine thiosulphate as diagnostic tools for sulfur-induced polioencephalomalacia. Journal of Veterinary Diagnostic Investigation, Volume 24, pp. 702 - 709.

Du, Z., Hemken, R. & Harmon, R., 1996. Copper metabolism of Holstein and Jersey cows and heifers fed diets high in cupric sulphate or copper proteinate. Journal of Dairy Science, Volume 79, pp. 1873 - 1880.

Eckert, G., Greene, L., Carstens, G. & Ramsey, W., 1999. Copper status of ewes fed increasing amounts of copper from copper sulfate or copper proteinate. Journal of Animal Science, Volume 77, pp. 244 - 249.

Engle, T.E., Fellner, V. & Spears, J.W., 2001. Copper status, serum cholesterol, and milk fatty acid profile in Holstein cows fed varying concentrations of copper. Journal of Dairy Science, Volume 84, pp. 2308 – 2313.

Engle, T.E., Nockels, C.F., Kimberling, C.V., Weaber, D.L. Johnson, A.B., 1997. Zinc repletion with organic or inorganic forms of zinc and protein turnover in marginally zinc-deficient calves. Journal of Animal Science, Volume 75, pp. 3074 - 3081.

Engle, T.E. & Spears, J.W., 2000. Effects of dietary copper concentration and source on performance and copper status of growing and finishing steers. Journal of Animal Science, Volume 78, pp. 2446 – 2451.

Etim, N., Williams, M., Akpabio, U. & Offiong, E., 2014. Haematological parameters and factors affecting their values. Agricultural Science, Volume 2, pp. 37 - 47.

Etzel, K., Swerdel, M., Swerdel, J. & Cousins, R., 1982. Endotoxin-induced changes in copper and zinc metabolism in the Syrian hamster. Journal of Nutrition, Volume 112, pp. 2363 - 2373.

Evans, G., 1973. Copper homeostasis in the mammalian system. Physiological Reviews, Volume 53, pp. 535-570.

Evans, G. & Winter, T., 1975. Zinc transport by transferrin in rat portal blood plasma. Biochemical and Biophysical Research Communications, Volume 66, pp. 1218 - 1224.

Fell, B., Dinsdale, D. & Mills, C., 1975. Changes in enterocyte mitochondria associated with deficiency of copper in cattle. Research in Veterinary Science, Volume 18, pp. 274 - 281.

Fell, B.F., Farmer, L.J., Farquharson, C., Bremner, I. & Graca, D.S., 1985. Observations on the pancreas of cattle deficient in copper. Journal of Comparative Pathology, Volume 95, pp. 573 - 590.

Feng, M., Wang, Z., Zhou, A. & Ai, D., 2009. The effects of different sizes of nanometer zinc oxide on the proliferation and cell integrity of mice duodenum-epithelial cells in primary culture. Pakistan Journal of Nutrition, Volume 8, pp. 1164 - 1166.

Ferguson, J.D., Galligan, D.T. & Thomsen, N., 1994. Principal descriptors of body condition core in Holstein cows. Journal of Dairy Science, Volume 77, pp. 2695 – 2703.

Ferguson, J., 1996. Diet, production and reproduction in dairy cows. Animal Feed Science and Technology, Volume 59, pp. 173 - 184.

Field, M., 2003. Intestinal ion transport and the pathophysiology of diarrhoea. Journal of Clinical Investigation, Volume 111, pp. 931 - 943.

Galyean, M.L., Gunter, S.A., Berrie, R.A., Malcolm-Callis, K.J., Brazle, F.K. & Essig, H.W., 1995. Effects of Zinc Source and Level and Added Copper Lysine in the Receiving Diet on Performance by Growing and Finishing Steers. The Professional Animal Scientist, Volume 11, pp. 139 – 148.

Gambling, L., Kennedy, C. & McArdle, H., 2011. Iron and copper in fetal development. Seminars in Cell & Developmental Biology, Volume 22, pp. 637 - 644.

Gengelbach, G. & Spears, J., 1998. Effects of dietary copper and molybdenum on copper status, cytokine production and humoral immune response of calves. Journal of Dairy Science, Volume 81, pp. 3286 - 3292.

Gengelbach, G., Ward, J. & Spears, J., 1994. Effects of dietary copper, iron and molybdenum on growth and copper status of beef cows and calves. Journal of Animal Science, Volume 72, pp. 2722 - 2727.

Ghodrat, A., Yaghobfar, A., Ebrahimnezhad, Y., Shahryar, H.A. & Ghorbani, A., 2015. In vitro binding capacity of organic (what bran and rice bran) and inorganic (perlite) sources for Mn, Zn, Cu, and Fe. Journal of applied animal research, Volume 45, pp. 80 - 84.

Goff, J., 2018. Invited review: Mineral absorption mechanisms. mineral interactions that affect acid-base and antioxidant status, and diet considerations to improve mineral status. Journal of Dairy Science, Volume 101, pp. 1 - 51.

Goff, J. & Stabel, J., 1990. Decreased plasma retinol, alpha-tocopherol and zinc concentration during the periperturient period: Effect of milk fever. Journal of Dairy Science, Volume 73, pp. 3195 - 3199.

Gonzales-Eguia, A., Chao-Ming, F., Fu-Yin, L. & Tu-Fa, L., 2009. Effects of nanocopper on copper availability and nutrients digestibility, growth perfomance and serum traits of piglets. Livestock Science, Volume 126, pp. 122 - 129.

Gooneratne, S., Bailey, J., Symonds, H. & Christensen, D., 1987. Effect of dietary Cu, Mo and S levels on bile and urine Cu excretion in 2 breeds of cattle. Canadian Journal of Animal Science, Volume 67, pp. 1188.

Gooneratne, S., Buckley, W. & Christensen, D., 1989. Review of copper deficiency and metabolism in ruminants. Canadian Journal of Animal Science, Volume 69, pp. 819 - 845.

Gooneratne, S., Christensen, D., Bailey, J. & Symonds, H., 1988. Influence of breed, and dietary Cu, Mo and S levels on biliary Cu excretion in cattle. In: L. Hurley, C. Keen, B. Lonnerdal & R. Rucker, eds. Proceedings of the 6th Symposium. Trace elements in man and animals (TEMA-6). New York: Plenum Press, pp. 313 - 314.

Gooneratne, S., Howell, J. M. & Gawthorne, J., 1979. Intracellular hepatic distribution of copper in normal and copper loaded sheep. Research in Veterinary Science, Volume 27, pp. 30 - 37.

Gooneratne, S., Symonds, H., Bailey, J. & Christensen, D., 1994. Effects of dietary copper, molybdenum and sulfur on biliary copper and zinc excretion in Simmental and Angus cattle. Canadian Journal of Animal Science, Volume 74, pp. 315 - 325.

Gopalasmy, G.L., Alpers, D.H., Binder, H.J., Tran, C.D., Ramakrishna, B.S., Brown, I., Manary, M., Mortimer, E. & Young, G.P., 2015. The relevance of the colon in zinc nutrition. Nutrients, Volume 7, pp. 572 - 583.

Gopinath, C., Hall, G.A. & McHowell, J.C., 1974. The Effect of Chronic Copper Poisoning on the Kidneys of Sheep. Research in Veterinary Science, Volume 16, pp. 57 – 69.

Gould, D., 1998. Polioencephalomalacia. Journal of Animal Science, Volume 76, pp. 309 - 314.

Gould, L. & Kendall, N., 2011. Role of the rumen in copper and thiomolybdate absorption. Nutrition Research Reviews, Volume 24, pp. 176 - 182.

Grace, N. & Knowles, S., 2012. Trace element supplementation of livestock in New Zealand: Meeting the challenges of free-range grazing systems. Veterinary Medicine International, Volume 133, pp. 1 – 8.

Grace, N.D., Knowles, S.O., Rounce, J.R., West, D.M. & Lee, J., 1988. Effect of increasing pasture copper concentrations on the copper status of grazing Romney sheep. New Zealand Journal of Agricultural Research, Volume 41, pp. 377 - 386.

Gralak, M., Bates, D., Von Keyserlingk, M. & Fisher, L., 2006. Influence of species, cultivar and cut on the microelement content of grass forages. Slovakian Journal of Animal Science, Volume 39, pp. 84 - 88.

Gummow, B., 1996. Experimentally induced chronic copper toxicity in cattle. Oderstepoort Journal of Veterinary Research, Volume 63, pp. 277 - 288.

Hambrigge, K., Casey, C. & Krebs, N., 1986. Trace elements in human and animal nutrition. New York: Academic Press.

Hansen, S. L., Schlegel, P., Legleiter, L.R., Lloyd, K.E. & Spears, J.W., 2008. Bioavailability of copper from copper glycinate in steers fed high dietary sulfur and molybdenum. Journal of Animal Science, Volume 86, pp. 173 - 179.

Hashimoto, A. & Kambe, T., 2015. Mg, Zn and Cu transport proteins: A brief overview from physiological and molecular perspectives. Journal of Nutritional Science, Volume 61, pp. S116 - S118.

Hatfield, P.G., Swenson, C.K., Kott, R.W., Ansotegui, R.P., Roth, N.J. & Robinson, B.L., 2001. Zinc and copper status in ewes supplemented with sulfate and amino acid-complexed forms of zinc and copper. Journal of Animal Science, Volume 79, pp. 261 – 266.

Heisler, L. & Lam, D., 2017. An appetite for life: brain regulation of hunger and satiety. Current opinions in pharmacology, Volume 37, pp. 100 - 106.

Henry, R. J., Cannon, D. C. & Winkelman, J. W., 1974. Clinical Chemistry. Principles and Techniques, 2nd edn. New York, Harper and Row. pp 458 - 460

Henry, P.R., Little, R.C. & Ammerman, C.B., 1997. Effect of high dietary zinc concentration and length of zinc feeding on feed intake and tissue zinc concentration in sheep. Animal Feed Science and Technology, Volume 66, pp. 237 – 245.

Herdt, T. & Hoff, B., 2011. The use of blood analysis to evaluate trace mineral status in ruminant livestock. Veterinary Clinics of North America: Food Animal Practice, Volume 27, pp. 255 - 283.

Hineman, A. & Stephan, C., 2012. Effect of dwell time of single particle inductively coupled plasma mass spectrometry data acquisition quality. Journal of Analytical Atomic Spectrometry, Volume 29, pp. 1252 – 1257.

Ho, S.K. & Hidiroglou, M., 1977. Effects of dietary chelated and sequestered zinc and zinc sulfate on growing lambs fed a purified diet. Canadian Journal of Animal Science, Volume 57, pp. 93 – 99.

Hogan, K., Money, D., White, D. & Walker, R., 1971. Weight responses of young lambs to copper and connective tissue lesions associated with grazing pasture of high molybdenum content. New Zealand Journal of Agricultural Research, Volume 14, pp. 687 - 701.

Hoover, W., 1978. Digestion and absorption in the hindgut of ruminants. Journal of Animal Science, Volume 46, pp. 1789 - 1792.

Howell, J., 1968. Observations on the histology and possible pathogenesis of lesions in the central nervous system of sheep with swayback. Proceedings of the Nutrition Society, Volume 27, pp. 85 - 87.

Hu, C.H., Li, Y.L., Xiong, L., Zhang, H.M., Song, J. & Xia, M.S., 2012. Comparative effects of nano elemental selenium and sodium selenite on selenium retention in broiler chickens. Animal Feed Science and Technology, Volume 177, pp. 204 – 210.

Humann-Ziehank, E., Coenen, M., Ganter, M. & Bickhardt. K., 2001. Long-term observation of subclinical chronic copper poisoning in two sheep breeds. Journal of Veterinary Medicine. A, physiology, pathology, clinical medicine, Volume 48, pp. 429 – 439.

Humphries, W., Phillippo, M., Young, B. & Bremner, I., 1983. The influence of dietary iron and molybdenum in copper metabolism in calves. British Journal of Nutrition, Volume 49, pp. 77 - 86.

Hurley, L., 1981. Teratogenic effects of manganese, zinc and copper in nutrition. Physiological reviews, Volume 61, pp. 249 - 295.

Hussein, H. & Staufenbiel, R., 2012. Variations in copper concentration and ceruloplasmin activity of dairy cows in relation to lactation stages with regard to ceruloplasmin to copper ratios. Biological Trace Element Research, Volume 146, pp. 47 - 52.

Ighodaro, O. & Akinloye, O., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine, Volume 54, pp. 287 - 293.

Iheukwumere, F.C. & Herbert, U., 2002. Physiological Responses of Broiler Chickens to Quantitative Water Restrictions: Haematology and Serum Biochemistry. International Journal of Poultry Science, Volume 2, pp. 117 – 119.

Ishmael, J., Gopinath, C. & McC.Howell, J., 1972. Experimental Chronic Copper Toxicity in Sheep: Biochemical and Haematological Studies during the Development of Lesions in the Liver. Research in Veterinary Science, Volume 13, pp. 22 – 29.

Ivan, M., Viera, D. & Kelleher, C., 1986. The alleviation of chronic copper toxicity in sheep by ciliate protozoa. British Journal of Nutrition, Volume 118, pp. 718 - 724.

Jiang, J., Oberdorster, G. & Biswas, P., 2009. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. Journal of Nanoparticle Research, Volume 11, pp. 77 - 89.

Johansson, M.E.V., ; Ambort, D., Pelaseyed, T., Schutte, A., Gustafsson, J.K., Ermund, A., Subramani, D.B., Holmen-Larsson, J.M., Thomsson, K.A., Bergstrom, J.H., van der Post, S., Rodriguez-Pineiro, A.M., Sjovall, H., Backstrom, M. & Hansson, G.C., 2011. Composition and function role of the mucus layers in the intestine. Cellular and molecular life sciences, Volume 68, pp. 3636 - 3641.

Johnston, B., Scown, T.M., Moger, J., Cumberland, S.A., Baalousha, M., Linge, K., Van Aerle, R., Jarvis, K., Lead, J.R. & Tyler, C.R., 2010. Bioavailability of nanoscale metal oxides, TiO2, CeO2 and Zn oxide to fish. Environmental Science & Technology, Volume 44, pp. 1144 - 1151.

Johnston, H., Beasley, L. & MacPherson, N., 2014. Copper toxicity in a New Zealand dairy herd. Irish Veterinary Journal, Volume 67, pp. 1 – 6.

Jones, D. & Suttle, N., 1987. Copper and disease resistance. In: Trace substances in environmental health - XXI. Columbia: University of Missouri, pp. 514 - 524.

Jones, M. & Jones, G., 2008. Advanced Biology. 10th ed. Cambridge: Cambridge University Press.

Judson, G., Brown, T.H., Gray, D., Dewey, D.W., Edwards, J.B. & McFarlane, J.D., 1982. Oxidized copper wire particles for copper therapy in sheep. Australian Journal of Agricultural Research, Volume 33, pp. 1073 - 1083.

Juniper, D.T., Phipps, R.H., Givens, D.I., Jones, A.K., Green, C. & Bertin, G. 2008. Tolerance of ruminant animals to high dose in-feed administration of a selenium-enriched yeast. Journal of Animal Science, Volume 86, pp. 197 - 204.

Kaplan, J. & Lutsenko, S., 2009. Copper transport in mammalian cells: Special care for a metal with special needs. Journal of biological chemistry, Volume 284, pp. 25461 - 25465.

Keene, A. & Tyner, K., 2011. Analytical characterization of gold nanoparticle primary particles, aggregates, agglomerates, and agglomerated aggregates. Journal of nanoparticle research, Volume 13, pp. 3465 - 3481.

Kegley, E. & Spears, J., 1994. Bioavailability of feed-grade copper sources (oxide, sulfate, or lysine) in growing cattle. Journal of Animal Science, Volume 72, pp. 2728 - 2734.

Kendall, N.R., Holmes-Pavord, H.R, Bone, P.A., Ander, E.L. & Young, S.D. 2015. Liver copper concentrations in cull cattle in the UK: are cattle being copper loaded? The Veterinary Record, Volume 177, pp. 493 - 496

Kendall, N.R., Mackenzie, A.M. & Telfer, S. 2012. The trace element and humoral immune response of lambs administered a zinc, cobalt and selenium soluble glass bolus. Livestock Science, Volume 148, pp. 81 - 86.

Kerr, M.G. Veterinary Laboratory Medicine: Clinical Biochemistry and Haematology. 1989. Blackwell Science, Horsham, UK.

Kessler, J., Morel, I., Dufey, P.A., Gutzwiller, A., Stern, A. & Geyer, H., 2003. Effect of organic zinc sources on performance, zinc status and carcass, meat and claw quality in fattening bulls. Livestock Production Science, Volume 81, pp. 161 – 171.

Kim, B.-E., Nevitt, T. & Theile, D., 2008. Mechanisms for copper acquisition, distribution and regulation. Nature chemical biology, Volume 4, pp. 176 - 185.

Kim, E.-S., Noh, S. & Koo, S., 1998. Marginal zinc deficiency lowers the lymphatic absorption of alpha-tocopherol in rats. Journal of Nutrition , Volume 128, pp. 265 - 270.

Kinal, S., Korniewiez, A., Jamroz, D., Zieminiski, R. & Slupezynska, M., 2005. Dietary effects of zinc, copper and manganese chelates and sulphates on dairy cows. Journal of Food Agriculture and Environment, Volume 3, pp. 168 - 172.

Kincaid, R., 2000. Assessment of trace mineral status of ruminants: A review. Journal of Animal Science, Volume 77, pp. 1 - 10.

Kincaid, R., Blauwiekel, R. & Cronrath, J., 1986. Supplementation of copper as copper sulfate or copper proteinate for growing calves fed forages containing molybdenum. Journal of Dairy Science, Volume 69, pp. 160 - 163.

Kincaid, R. & Cronrath, J., 1979. Effects of dietary zinc upon tissue zinc and percent unsaturated plasma-zinc binding capacity. Journal of Dairy Science, Volume 62, pp. 572 - 576.

Kohane, D., 2007. Microparticles and nanoparticles for drug delivery. Biotechnology and bioengineering, Volume 96, pp. 203 - 209.

Kojouri, G., Sadeghian, S., Mohebbi, A. & Mokhber Dezfouli, M., 2012. The effects of oral consumption of selenium nanoparticles on chemotactic and respiratory burst activities of neutrophils in comparison with sodium selenite in sheep. Biological Trace Element Research, Volume 146, pp. 160 - 166.

Kwun, I.-S., Cho, Y-E., Lomeda, R. A-R., Kwon, S-T., Kim, Y. & Beattie, J.H., 2007. Marginal zinc deficiency in rats decreases leptin expression independently of food intake and corticotrophin-releasing hormone in relation to food intake. British Journal of Nutrition, Volume 98, pp. 485 - 489.

Laborda, F., Jimenez-Lamana, J., Bolea, E. & Castillo, J., 2013. Critical considerations for the determination of nanoparticle number concentrations, size and number size distributions by single particle ICP-MS. Journal of Analytical Atomic Spectrometry, Volume 28, pp. 1220 - 1232.

Landsiedel, R., Ma-Hock, L., Kroll, A., Hahn, D., Schnekenburger, J., Wiench, K. & Wohlleben, W., 2010. Testing metal-oxide nanomaterials for human safety. Advanced Materials, Volume 22, pp. 1 - 27.

Ledoux, D.R., Pott, E.B., Henry, P.S., Ammerman, C.B., Merritt, D.V.M. & Madison, J.B., 1995. Estimation of the relative bioavailability of inorganic copper sources for sheep. Nutrition Research, Volume 15, pp. 1803 - 1813.

Ledoux, D.R., Henry, P.S. & Ammerman, C.B., 1996. Response to high dietary copper and duration of feeding time on tissue copper concentration of sheep. Nutrition Research, Volume 16, pp. 69 – 78.

Lee, S., Bi, X., Reed, R.B., Ranville, J.F., Herckes, P. & Westerhoff, P., 2014. Nanoparticle size detection limits by single particle ICP-MS for 40 elements. Environmental Science and Technology, Volume 48, pp. 10291 - 10300.

Lichten, L. & Cousins, R., 2009. Mammalian zinc transporters: nutritional and physiological regulation. Annual review of nutrition, Volume 29, pp. 153 - 179.

Linsinger, T.P., Chaudhry, Q., Dehalu, V., Delahaut, P., Dudkiewicz, A., Grobe, R., von der Kammer, F., Larsen, E.H., Legros, S., Loeschner, K., Peters, R., Ramsch, R., Roebben, G., Tiede, K. & Weigel, S., 2013. Validation of methods for the detection and quantification of engineered nanoparticles in food. Food Chemistry, Volume 138, pp. 1959 - 1966.

Livesey, C., Bidwell, C., Crawshaw, T. & David, G., 2002. Investigation of copper poisoning in adult cows by the veterinary laboratories agency. Cattle Practice, Volume 10, pp. 289 - 294.

Lopez-Alonso, M., 2012. Trace minerals and livestock: Not too much not too little. ISRN Veterinary Science, pp. 1 - 18.

Lopez-Alonso, M., Crespo, A., Miranda, M., Castillo, C., Hernandez, J. & Benedito., 2006. Assessment of some blood parameters as potential markers of hepatic copper accumulation in cattle. Journal of Veterinary Diagnostic Investigation, Volume 18, pp. 71 – 75.

Lopez-Alonso, M., Prieto, F., Miranda, M., Castillo, C., Hernandez, J., Benedito, J.L., 2005. The role of metallothionein and zinc in hepatic copper accumulation in cattle. The Veterinary Journal, Volume 169, pp. 262 - 267.

Lutsenko, S., Barnes, N., Bartee, M. & Dmitriev, O., 2007. Function and regulation of human copper-transporting ATPases. Physiology Reviews, Volume 87, pp. 1011 - 1046.

Lutsenko, S. G. A., Burkhead, J. & Zuzel, V., 2008. Cellular multitasking: The dual role of human Cu-ATPases in cofactor deliver and intracellular copper balance. Archives of Biochemistry and Biophysics, Volume 476, pp. 22 - 32.

MAFF, 1990. UK Tables of the Nutritive Value and Chemical Composition of Foodstuffs. In: D. Givens, ed. Aberdeen: Rowett Research Services.

Mao, X., Kim, B.E., Wang, F., Eide, D.J. & Petris, M.J., 2007. A histidine-rich cluster mediates the ubiquitination and degradation of the human zinc transporter hZIP4, and protects against zinc cytotoxicity. Journal of Biological Chemistry, Volume 282, pp. 6992 - 7000.

Markossian, K. & Kurganov, B., 2003. Copper chaperones, intracellular copper trafficking proteins. Function, structure and mechanism of action. Biochemistry, Volume 68, pp. 827 - 837.

Marques, R.S., Cooke, R.F., Rodrigues, M.C., Cappellozza, B.L., Mills, R.R., Larson, C.K., Moriel, P. & Bohnert, D.W., 2016. Effects of organic or inorganic cobalt, copper, manganese, and zinc supplementation to late-gestating beef cows on productive and physiological responses of the offspring. Journal of Animal Science, Volume 94, pp. 1215 - 1226.

McDonald, M., Mila, I. & Scalbert, A., 1996. Precipitations of metal ions by plant polyphenols: optimal conditions and origin of precipitation. Journal of Agricultural Food Chemistry, Volume 44, pp. 599 - 606.

McDowell, L., 1992. Minerals in Animal and Human Nutrition. London: Academic.

Miller, M., 1975. New concepts and developments in metabolism and homeostasis of inorganic elements in dairy cattle. A review. Journal of Dairy Science, Volume 58, pp. 1549 - 1560.

Miller, W., 1979. Dairy Cattle Feeding and Nutrition. New York: Academic Press.

Miller, W. & Stake, P., 1974. Use and limitation of biochemical measurements in diagnosing mineral deficiencies. In Proceedings of the Georgia Nutrition Conference for Feed Industry, pp. 25.

Mills, C., Delgarno, A. & Wenham, G., 1976. Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency. British Journal of Nutrition, Volume 35, pp. 309 - 311.

Miranda, M., Cruz, J., Lopez-Alonso, M. & Benedito, J., 2006. Variations in liver and blood copper concentrations in young beef cattle raised in north-west Spain:

associations with breed, sex, age and season. Animal Science, Volume 82, pp. 253 - 258.

Misra, H.P. & Fridovich, I., 1977. Superoxide dismutase and peroxidase: A positive activity stain applicable to polyacrylamide gel electropherograms. Archives of biochemistry and biophysics, Volume 183, pp. 511 – 515.

Mohammadi, V., Ghazanfari, S., Mohammadi-Sangcheshmeh, A. & Nazaran, M., 2015. Comparative effects of zinc-nano complexes, zinc-sulphate and zinc-methionine on performance in broiler chickens. British Poultry Science, Volume 56, pp. 486 - 493.

Mullis, L., Spears, J. & McGraw, R., 2003. Effects of breed (Angus v Simmental) and copper and zinc source on mineral status of steers fed high dietary iron. Journal of Animal Science, Volume 81, pp. 318 - 322.

Nagalakshmi, D., Gururaj, K. & Senthilkumar, P., 2010. Effect of dose and source of copper supplementation on antioxidant status, humoral immune response and carcass characteristics of lambs. Indian Journal of Animal Science, Volume 80, pp. 162 – 167.

Najafzadeh, H., Ghoreishi, S.M., Mohammadian, B., Rahimi, E., Afzalzadeh, M., Kazemivarnamhkhasti, M. & Ganjealidarani, H., 2013. Serum biochemical and histopathological changes in liver and kidney in lambs after zinc oxide nanoparticles administration. Vet World, Volume 6, pp. 534 - 537.

National Research Council., 2007. Nutrient requirements of small ruminants sheep, goats, cervids and new world camelids, The National Academies Press, Washington DC.

Navratilova, J., Praetorius, A., Gondikas, A., Fabienke, W., von der Kammer, F. & Hofmann, T., 2015. Detection of engineered copper nanoparticles in soil using single particle ICP-MS. International Journal of Environmental Research and Public Health, Volume 12, pp. 15756 - 15768.

Nellans, H., 1991. Mechanisms of peptide and protein absorption (1) paracellular intestinal transport: Modulation of absorption. Advanced Drug Delivery Reviews, Volume 7, pp. 339 - 364.

Nicholson, F., Chambers, B. & Williams, J. & Unwin, J. R., 1999. Heavy metal contents of livestock feeds and animal manures in England and Wales. Bioresource Technology, Volume 70, pp. 23 - 31.

Noh, S. & Koo, S., 2001. Intraduodenal infusion of lysophosphatidylcholine restores the intestinal absorption of vitamins A and E in rats fed a low-zinc diet. Experimental Biology and Medicine, Volume 226, pp. 342 - 348.

Ortolani, E., Machado, C. & Sucupira, M., 2003. Assessment of some clinical and laboratory variables for early diagnoses of cumulative copper poisoning in sheep. Veterinary and Human Toxicology, Volume 45, pp. 289 - 293.

Ott, E., Smith, W., Harrington, R. & Beeson, W., 1966a. Zinc Toxicity in Ruminants. II. Effect of High Levels of Dietary Zinc on Gains, Feed Consumption and Feed Efficiency of Beef Cattle. Journal of Animal Science, Volume 25, pp. 419 - 423.

Ott, E., Smith, W., Harrington, R. & Beeson, W., 1966b. Zinc Toxicity in Ruminants. I. Effect of High Levels of Dietary Zinc on Gains, Feed Consumption and Feed Efficiency of Lambs. Journal of Animal Science, Volume 25, pp. 414 - 418.

Overton, T. & Yasui, T., 2014. Practical applications of trace minerals for dairy cattle. Journal of Animal Science, Volume 92, pp. 416 - 426.

Pace, H.E., Rogers, N.J., Jarolimek, C., Coleman, V.A., Higgins, C.P. & Ranville, J.F., 2011. Determining transport efficiency for the purpose of counting and sizing nanoparticles via single particle inductively coupled mass spectrometry. Analytical chemistry, Volume 83, pp. 9361 - 9369.

Pal, D.T., Gowda, N.K.S., Prasad, C.S., Amarnath, R., Bharadwaj, U., Suresh Babu, G. & Sampath, K.T., 2010. Effect of copper- and zinc-methionine supplementation on bioavailability, mineral status and tissue concentrations of copper and zinc in ewes. Journal of Trace Element Medical Biology, Volume 24, pp. 89 – 94.

Patterson, D., Foulkes, J. S. D., Glancy, E. & Tfrlecki, S., 1974. A neurochemical study of field cases of the delayed spinal form of swayback (enzooticataxia) in lambs. Journal of Neurochemistry, Volume 23, pp. 1245 - 1253.

Patterson, D., Sweasey, P. & Hebert, C., 1971. Changes occuring in the chemical composition of the central nervous system during foetal and post-natal development of sheep. Journal of Neurological Chemistry, Volume 18, pp. 2027 - 2040.

Parkins, J.J., Hemingway, R.G., Lawson, D.C. & Ritchie, N.S., 1994. The effectiveness of copper oxide powder as a component of a sustained-release multi-trace element and

vitamin rumen bolus system for cattle. British Veterinary Journal, Volume 150, pp. 547 – 553.

Pelyhe, C. & Mezes, M., 2013. Myths and facts about the effect of nano-selenium in farm animals - Mini Review. European chemical bulletin, Volume 2, pp. 1049 - 1052.

Peters, R.J.B., Rivera, Z.H., Bemmel, G.V., Marvin, H.J.P., Weigel, S. & Bouwmeester., 2014. Development and validation of single particle ICP-MS for sizing and quantitative determination of nano-silver in chicken meat. Analytical and bioanalytical chemistry, Volume 406, pp. 3875 – 3885.

Pfeffer, A. & Rogers, K., 1989. Acute phase response of sheep: changes in the concentrations of ceruloplasmin, fibrinogen, haptoglobin and the major blood cell types associated with pulmonary damage. Research in Veterinary Science, Volume 46, pp. 118 - 124.

Phillippo, M. & Grace, D., 1983. Biliary copper secretion in cattle. Proceedings of the Nutrition Society, Volume 42, p. 46A.

Phillippo, M., Humphries, W., Atkinson, T. & Henderson, G., 1987. The effect of dietary molybdenum and iron on copper status, puberty and oestrus cycles in cattle. The Journal of Agricultural Science, Volume 109, pp. 321 - 336.

Piacenza, L., Acosta, D., I, Basmadjian., Dalton, J.P. & Carmona, C., 1999. Vaccination with Cathepsin L Proteinases and with Leucine Aminopeptidase induces high levels of protection against Fascioliasis in sheep. Infection and Immunity, Volume 67, pp. 1954 - 1961.

Pott, E.B., Henry, P.R., Ammerman, C.B., Merritt, A.M., Madison, J.B. & Miles, R.D., 1994. Relative bioavailability of copper in a copperlysine complex for chicks and lambs. Animal Feed Science and Technology, Volume 45, pp. 193 - 203.

Prasad, A. & Oberleas, D., 1971. Changes in activities of zinc-dependent enzymes in zinc deficient tissues of rats. Journal of Applied Physiology, Volume 31, pp. 842 - 846.

Price, J. & Chesters, J., 1985. A new bioassay for assessment of copper availability and its application in the study of the effect of molybdenum on the distribution of available Cu in ruminant digesta. British Journal of Nutrition, Volume 53, pp. 323 - 336.

Price, J., Will, A., Paschaleris, G. & Chesters, J., 1987. Identification of thiomolybdates in digesta and plasma from sheep after administration of 99Mo-labelled compounds into the rumen. British Journal of Nutrition, Volume 58, pp. 127 - 138.

Prohaska, J., 2006. Copper. In: L. Filer & E. Ziegler, eds. Present knowledge in nutrition. Washington DC: International Life Science Institute - Nutrition Foundation.

Puls, R., 1994. Mineral levels in Animal Health: Diagnostic data. Clearbrook, BC, Canada: Canada Sherpa International.

Rabiansky, P. A., McDowell, L.R., Valsquez-Pereira, J., Wilkinson, N.S., Percival, S.S., Martin, F.G., Bates, D.B., Johnson, A.B., Batra, T.R. & Salgado-Madriz, E., 1999. Evaluating copper lysine and copper sulfate sources for heifers. Journal of Dairy Science, Volume 82, pp. 2642 - 2650.

Rabiee, A.R., Lean, I.J., Stevenson, M.A, & Socha, M.T., 2010. Effects of feeding organic trace minerals on milk production and reproductive performance in lactating dairy cows: A meta-analysis. Journal of Dairy Science, Volume 93, pp. 4239 – 4251.

Radha, K., Thomas, A. & Sathian, C., 2014. Application of nano technology in dairy industry: prospects and challenges - A review. Indian Journal of Dairy Science, Volume 67, pp. 367 - 374.

Radostits, O., Gay, C., Blood, D. & Hinchcliff, K., 2000. Appendix 3 Laboratory Reference Values: Biochemistry. In: Veterinary Medicine, pp. 1819 - 1822.

Rajendran, D., 2013. Application of Nano Minerals in Animal Production System. Research Journal of Biotechnology, Volume 8, pp. 1 - 3.

Reid, R. & Attaelmannan, M., 1998. NMR studies of copper speciation in the bovine rumen environment. Journal of inorganic biochemistry, Volume 69, pp. 59 - 65.

Roche, J.R., Berry, D.P. & Kolver, E.S., 2006. Holstein-Friesian Strain and Feed Effects on Milk Production, Body Weight, and Body Condition Score Profiles in Grazing Dairy Cows. Journal of Dairy Science, Volume 89, pp. 3532 – 3543.

Rojas, L., McDowell, L.R., Cousins, R.J., Martin, F.G., Wilkinson, N.S., Johnson, A.B. & Velasques, J.B., 1995. Relative bioavailability of two organic and two inorganic zinc sources fed to sheep. Journal of Animal Science, Volume 73, pp. 1202 - 1207.

Rosa, I., Ammerman, C. & Henry, P., 1986. Interrelationships of dietary copper, zinc and iron on performance and tissue mineral concentration in sheep. Nutritional Reports International, Volume 34, pp. 893 - 902.

Rowe, D. & Bobilya, D., 2000. Albumin facilitates zinc acquisition by endothelial cells. In Proceedings of the Society for Experimental Biology and Medicine, Volume 224, pp. 178 – 186.

Sadeghian, S., Kojouri, G. & Mohebbi, A., 2012. Nanoparticles of selenium as species with stronger physiological effects in sheep in comparison with sodium selenite. Biological Trace Element Research, Volume 146, pp. 302 - 308.

Sahoo, A., Swain, R. & Kumar Mishra, S., 2014. Effect of inorganic, organic and nano zinc supplemented diets on bioavailability and immunity status of broilers. International Journal of Advanced Research, Volume 2, pp. 828 - 837.

Sahoo, S. & Labhasetwar, V., 2003. Nanotech Approaches to Drug Delivery and Imaging. Drug Discovery Today, Volume 73, pp. 1112 - 1120.

Sandoval, M., Henry, P.R., Littell, R.C., Cousins, R.J. & Ammerman, C.B., 1997. Estimation of the relative bioavailability of zinc from inorganic zinc sources for sheep. Animal Feed Science and Technology, Volume 66, pp. 223 - 235.

Sang, X., Zheng, L., Sun, Q., Li, N., Cui, Y., Hu, R, Gao, G., Cheng, Z., Cheng, J., Gui, S., Liu, H., Zhang, Z. & Hong, F., The chronic spleen injury of mice following long-term exposure to titanium dioxide nanoparticles. Journal of Biomedical Materials Research, Volume 100A, pp. 894 – 902.

Scaletti, R. & Harmon, R., 2012. Effect of dietary copper source on response to coliform mastitis in dairy cows. Journal of Dairy Science, Volume 95, pp. 654 - 662.

Scaletti, R., Trammell, D., Smith, B. & Harmon, R., 2003. Role of copper in enhancing resistance to Escherichia coli mastitis. Journal of Dairy Science, Volume 86, pp. 1240 - 1249.

Schalm, O., Jain, N. & Carroll, E., 1975. Veterinary haematology. 3rd ed. Philadelphia, USA: Lea and Fabiger.

Schryver, H., Hintz, H. & Lowe, J., 1980. Absorption excretion and tissue distribution of stable zinc and 65zinc in ponies. Journal of Animal Science, Volume 51, pp. 896 - 902.

Shi, L., Xun, W., Yue, W., Zhang, C., Ren, Y., Liu, Q., Wang, Q. & Shi, L., 2011. Effect of elemental nano-selenium on feed digestibility, rumen fermentation, and purine derivatives in sheep. Animal Feed Science and Technology, Volume 163, pp. 136-142.

Sinclair, L., Hart, K., Johnson, D. & Mackenzie, A., 2013. Effect of inorganic or organic copper fed without or with added sulfur and molybdenum on the performance, indicators of copper status, and hepatic mRNA in dairy cows. Journal of Dairy Science, Volume 96, pp. 4355 - 4367.

Sinclair, L.A & Atkins, N.E., 2015. Intake of selected minerals on commercial dairy herds in central and northern England in comparison with requirements. The Journal of Agricultural Science, Volume 153, pp. 743 – 752

Sinclair, L., Johnson, D., Wilson, S. & Mackenzie, A., 2017. Added dietary sulfur and molybdenum has a greater influence on hepatic copper concentration, intake, and performance in Holstein-Friesian dairy cows offered a grass silage-rather than corn silage-based diet. Journal of Dairy Science, Volume 100, pp. 4365 - 4376

Sinclair, L. A. & Mackenzie, A. M., 2013. Mineral nutrition of dairy cows: supply vs. requirements. In Recent Advances in Animal Nutrition (Eds, Garnsworthy, P. C. & Wiseman, J.) pp. 13 – 30. Ashby de la Zouch, UK: Context Products Ltd

Singh, K., Maity, S. & Maity, A., 2018. Effect of nano zinc oxide on zinc bioavailability and blood biochemical changes in pre-ruminant lambs. Indian Journal of Animal Sciences, Volume 88, pp. 805 - 807.

Small, J., 1997. Serum mineral concentrations in relation to parturition in beef heifers and cows fed conserved forage. Canadian Journal of Animal Science, Volume 77, pp. 63 - 68.

Small, J., Charmley, E., Rodd, A. & Fredeen, A., 1997. Serum mineral concentrations in relation to estrus and conception in beef heifers and cows fed conserved forage. Canadian Journal of Animal Science, Volume 77, pp. 55 - 62.

Smith, B., 1977. Toxicity of zinc is ruminants in relation to facial eczema. New Zealand Veterinary Journal, Volume 25, pp. 310 - 312.

Smith, B., Field, A. & Suttle, N., 1968. Effect of intake on copper, molybdenum and sulfate on copper metabolism in the sheep. III Studies with radioactive copper in male castrated sheep. Journal of Comparative Pathology, Volume 78, pp. 449 - 461.

Smith, B., Reynold, G. & Embling, P., 1977. Zinc solutions and closure of the reticular groove in sheep. New Zealand Journal of Experimental Agriculture, Volume 5, pp. 231 - 263.

Smith, B., Reynolds, G. & Embling, P., 1979. Effect of method of oral administration of zinc sulphate on acute zinc toxicity in the sheep. New Zealand Journal of Experimental Agriculture, Volume 7, pp. 107 - 110.

Soli, N. & Rambaek, J., 1978. Excretion of intravenously injected copper-64 in sheep. Acta Pharmacologica et Toxicologica (Copenhagen), Volume 43, pp. 205 - 210.

Sondergaard, E., Sorensen, M., Mao, I. & Jensen, J., 2002. Genetic parameters of production, feed intake, body weight, body composition, and udder health in lactating dairy cows. Livestock production Science, Volume 77, pp. 23 - 34.

Spears, J.W., 1989. Zinc methionine for ruminants: relative bioavailability of zinc in lambs and effects of growth and performance of growing heifers. Journal of Animal Science, Volume 67, pp. 835 - 843.

Spears, J.W., 2003. Trace mineral bioavailability in ruminants. Journal of Nutrition, Volume 133, pp. 1506S - 1509S.

Spears, J.W., Schlegel, P., Seal, M. & Lloyd, K., 2004. Bioavailability of zinc from zinc sulfate and different organic zinc sources and their effects on ruminal volatile fatty acid proportions. Livestock Production Science, Volume 90, pp. 211 - 217.

Spears, J.W. & Kegley, E.B., 2002. Effect of zinc source (zinc oxide vs zinc proteinate) and level on performance, carcass characteristics, and immune response of growing and finishing steers. Journal of Animal Science, Volume 80, pp. 2747 – 2752.

Spears, J.W & Weiss, W., 2014. Invited Review: Mineral and vitamin nutrition in ruminants. The Professional Animal Scientist, Volume 30, pp. 180 - 191.

Srinivasan, S. & Avadhani, N., 2012. Cytochrome c Oxidase Dysfunction in Oxidative Stress. Free Radical Biology & Medicine, Volume 53, pp. 1252 - 1263.

SRL Ecoterm, 2017. Product technical data sheep copper oxide black, Italy: Ecoterm SRL.

Stahlhut, H., Whishant, C. & Spears, J., 2005. Effect of chromium supplementation and copper status on performance and reproduction of beef cows. Animal Feed Science and Technology, Volume 128, pp. 266 - 275.

Surai, P.F., Kochish, I.I., Fisinin, V.I. & Juniper, D.T. 2019. Revisiting oxidative stress and the use of organic selenium in dairy cow nutrition. Animals, Volume 9 pp. 462

Suttle, N., 1974. Effects of organic and inorganic sulfur on the availability of dietary copper to sheep. British Journal of Nutrition, Volume 32, pp. 559 - 568.

Suttle, N., 1978. Determining the copper requirements of cattle by means of an intravenous repletion technique. Trace element metabolism in man and animals. Volume 3, pp. 473 - 480.

Suttle, N., 1987. The nutritional requirement for copper in animals and man. In: J. Howell & J. Gawthorne, eds. Copper in animals and man. Volume I. Florida: CRC Press, pp. 21 - 44.

Suttle, N., 1991. The interactions between copper, molybdenum and sulfur in ruminant nutrition. Annual Review of Nutrition, Volume 11, pp. 121 - 140.

Suttle, N., 2010. The Mineral Nutrition of Livestock. 4th ed. Wallingford: CABI.

Suttle, N., 2012a. Control of hepatic copper retention in Texel ram lambs by dietary supplementation with copper antagonists followed by a copper depletion regimen. Animal Feed Science and Technology, Volume 173, pp. 194 - 200.

Suttle, N., 2012b. Copper imbalances in ruminants and humans: unexpected common ground. Advanced Nutrition, Volume 3, pp. 666 - 674.

Suttle, N., 2013. Rates of change in liver copper concentration in cattle given a copper-deficient diet, with or without pre-treatment with tetrathiomolybdate, for evaluation of two parenteral copper supplements. New Zealand Veterinary Journal, Volume 61, pp. 154 - 158.

Suttle, N. & Angus, K., 1978. Effects of experimental copper deficiency on the skeleton of the calf. Journal of Comparative Pathology, Volume 82, pp. 137 - 145.

Suttle, N. & Field, A., 1968. The effect of intake of copper, molybdenum and sulfate on copper metabolism in sheep 1. Clinical condition and blood copper distribution in the pregnant ewe. Journal of Comparative Pathology, Volume 78, pp. 351 - 363.

Suttle, N. & Field, A., 1983. Effects of dietary-supplements of thiomolybdates on copper and molybdenum metabolism in sheep. Journal of Comparative Physiology, Volume 93, pp. 379 - 389.

Suttle, N.F. & Peter, D.W., 1985. Rumen sulphide metabolism as a major determinant of copper availability in the diets of sheep. In Proceedings of the Fifth International Symposium on Trace Elements in Man and Animals, pp. 367 – 370 [CF Mills, I Bremner and JK Chesters, editors]. Farnham Royal, UK: Commonwealth Agricultural Bureaux.

Suttle, N.F. & Price, J., 1976. The potential toxicity of copper-rich animal excreta to sheep. Animal Production, Volume 23, pp. 233 – 241.

Swain, P.S., Rao, S.B.N., Rajendran, D., Dominic, G. & Selvaraju, Sl., 2016. Nano zinc, an alternative to conventional zinc as animal feed supplement: a review. Animal Nutrition, Volume 2, pp. 134 - 141.

Schwamberger, A., De Roo, B., Jacob, D., Dillemans, L., Bruegemann, L., Seo, J. W., & Locquet, J. P., 2015. Combining SAXS and DLS for simultaneous measurements and time-resolved monitoring of nanoparticle synthesis. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, Volume 343, pp. 116 – 122.

Swenson, M., 1970. Physiological properties, cellular and chemical consituents of blood. In: M. Swenson, ed. Dukes' physiology of domestic animals. London: Cornstock Publishing Associates, pp. 75 - 83.

Szasz, G. 1974. Gamma glutamyl transpeptidase, Methods of enzymatic analysis. Edited Bergmeyer, H.U., 2nd Eds., Academic press, pp. 715 – 720.

Tassinari, R., Cubadda, F., Moracci, G., Aureli, F., D'Amato, M., Valeri, M., De Berardis, B., Raggi, A., Mantovani, A., Passeri, D., Rossi, M. & Maranghi, F., 2013. Oral, short-term exposure to titanium dioxide nanoparticles in Sprague-Dawley rat: focus on reproductive and endocrine systems and spleen. Nanotoxicology, Volume 8, pp. 654 – 662.

Taylor, A., 1997. Measurement of zinc in clinical samples. Annals of Clinical Biochemistry, Volume 34, pp. 142 - 150.

Taylor, C., Bacon, J., Aggett, P. & Bremner, I., 1991. Homeostatic regulation of zinc absorption and endogenous losses in zinc deprived men. The American Journal of Clinical Nutrition, Volume 53, pp. 755 - 763.

Telfer, S., Kendall, N., Illingworth, D. & Mackenzie, A., 2004. Molybdenum Toxicity in cattle: an underestimated problem. Cattle Practice, Volume 12, pp. 259 - 263.

Theil, E. & Calvert, K., 1978. The effect of copper excess on iron metabolism in sheep. Biochemistry Journal, Volume 170, pp. 137 - 143.

Thomas, C., 2004. Feed into Milk: A new applied feeding system for dairy cows. Nottingham University Press.

Thornton, I., Kershaw, G. & Davies, M., 1972a. An investigation into copper deficiency in cattle in the southern Pennines. 1. Identification of suspect areas using geochemical reconnaissance followed by blood copper surveys. Journal of Agricultural Science, Cambridge, Volume 78, pp. 157 - 163.

Thornton, I., Kershaw, G. & Davies, M., 1972b. An investigation into copper deficiency in cattle in the southern Pennines. 2. Response to copper supplementation. Journal of Agricultural Science, Cambridge, Volume 78, pp. 165 - 171.

Tras, B., Inal, F., Bas, A.L., Altunok, V., Elmas, M. & Yazar, E., 2000. Effects of continuous supplementations of ascorbic acid, aspirin, vitamin E and selenium on some haematological parameters and serum superoxide dismutase level in broiler chickens. British Poultry Science, Volume 41, pp. 664 – 666.

Turner, J. S. V., Osborn, P. & Gower, S., 1987. Copper absorption in sheep. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, Volume 86, pp. 147 - 150.

Underwood, E., 1977. Trace elements in human and animal nutrition. 4th ed. New York: Academic Press.

Van de Berghe, P. & Klomp, I., 2009. New developments in the regulation of intestinal copper absorption. Nutrition reviews, Volume 67, pp. 658 - 672.

Van Soest, P.J., Robertson, J.B, & Lewis, B.A., (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharies in relation to animal nutrition. Journal of Dairy Science, Volume 74, pp. 3583 – 3597.

Vecerek, V., Strakova., E., Suchy, R. & Voslarova, E., 2002. Influence of high environmental temperature on production and haematological and biochemical indexes in broiler chickens. Czech Journal of Animal Science, Volume 47, pp. 176 – 182.

Wang, H., Zhang, J. & Yu, H., 2007. Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: comparison with selenomethionine in mice. Free Radical Biology and Medicine, Volume 42, pp. 1524 - 1233.

Wang, H., Zhu, H., Wang, X., Li, E., Du, Z., Qin, J. & Chen, L., 2018. Comparison of copper bioavailability in copper-methionine, nano-copper oxide and copper sulfate additives in the diet of Russian sturgeon Acipenser gueldenstaedtii. Aquaculture, Volume 482, pp. 146 - 154.

Wapnir, R., 1998. Copper absorption and bioavailability. American Journal of Clinical Nutrition, Volume 67, pp. 1054 - 1060.

Ward, G., 1978. Molybdenum toxicity and hypocuprosis in ruminants: a review. Journal of Animal Science, Volume 46, pp. 1078 - 1085.

Ward, J. & Spears, J., 1993. Comparison of copper lysine and copper sulfate as copper sources for ruminants using in vitro methods. Journal of dairy science, Volume 76, pp. 2994 - 2998.

Ward, J. & Spears, J., 1997. Long-term effects of consumption of low-copper diets with or without supplemental molybdenum on copper status, performance, and carcass characteristics of cattle. Journal of Animal Science, Volume 75, pp. 3057 - 3065.

Ward, J., Spears, J. & Kegley, E., 1993. Effect of copper level and source (copper lysine vs copper sulfate) on copper status, performance and immune response in growing steers fed diets with or without supplemental molybdenum and sulfur. Journal of Animal Science, Volume 71, pp. 2748 - 2755.

Ward, J., Spears, J. & Kegley, E., 1996. Bioavailability of copper proteinate and copper carbonate relative to copper sulfate in cattle. Journal of Dairy Science, Volume 79, pp. 127 - 132.

Weiss, K. & Linder, M., 1985. Cooper transport in rats involving a new plasma protein. American Journal of Physiology, Volume 12, pp. 77 - 78.

Whitelaw, A., Armstrong, R., Evans, C. & Fawcett, A., 1979. A study of the effects of copper deficiency in Scottish Blackface lambs on improved hill pasture. Veterinary Record, Volume 104, pp. 455 - 460.

Whitelaw, A., Fawcett, A. & McDonald, A., 1984. Cupric oxide needles for the prevention of bovine hypocuprosis. Veterinary Record, Volume 115, p. 357.

White, S.D., Bourdeau, P., Rosychuk, R.A., Cohen, B., Bonenberger, T., Fieseler, K.V., Ihrke, P., Chapman, P.L., Schultheiss, P., Zur, G., Cannon, A. & Outerbridge, C., 2001. Zinc-responsive dematosis in dogs: 41 cases and literature review. Veterinary dermatology, Volume 12, pp. 101 - 109.

Wiener, G., Suttle, N., Field, A. & Herbert, J., 1978. Breed differences in copper metabolism in sheep. The Journal of Agricultural Science, Volume 91, pp. 433 - 441.

Wittenberg, K.M. & Boila, R.J., 1988. Supplementary copper for growing cattle consuming diets high in molybdenum or molybdenum plus sulfur. Canadian Journal of Animal Science, Voume 68, pp. 1143 – 1154.

Wittenberg, K.M. & Devlin, T.J., 1987. Effects of dietary molybdenum on productivity and metabolic parameters of lactating beef cows and their offspring. Canadian Journal of Animal Science, Volume 67, pp. 423 – 431.

Wittenberg, K.M., Boila, R.J. & Shariff, M.A., 1990. Comparison of copper sulfate and copper proteinate as copper sources for copper-depleted steers fed high molybdenum diets. Canadian Journal of Animal Science, Volume 70, pp. 895 – 904.

Wooliams, J., Suttle, N. W. G., Field, A. & Wooliams, C., 1983. The long-term accumulation and depletion of copper in the liver of different breeds of sheep fed diets of differing copper content. The Journal of Agricultural Science, Volume 100, pp. 441 - 449.

Wooliams, J.A., Suttle, N.F., Wiener, G., Field, A.C. & Wooliams, C., 1982. THe effect of breed of sire on the accumulation of copper in lambs, with particular reference to copper toxicity. Animal Production, Volume 35, pp. 299 - 307.

Wooliams, J.A., Wooliams, C., Suttle, N.F., Jones, D.G. & Wiener, G.I., 1986. Studies on lambs from lines genetically selected for low or high plasma copper status. 2. Incidence of hypocuprosis on improved hill pasture. Animal Production, Volume 43, pp. 303 - 317.

Xin, Z., Waterman, D.F., Hemken, R.W. & Harmon, R.J., 1991. Effects of Copper Status on Neutrophil Function, Superoxide Dismutase, and Copper Distribution in Steers. Journal of Dairy Science, Volume 74, pp. 3078 – 3085.

Xun, W., Shi, L., Yue, W., Zhang, C., Ren, Y. & Liu, Q., 2012. Effect of High-Dose Nano-selenium and Selenium–Yeast on Feed Digestibility, Rumen Fermentation, and Purine derivatives in sheep. Biological Trace Element Research, Volume 150, pp. 130 - 136.

Yaghmaie, P., Ramin, A., Asri-Rezaei, S. & Zamani, A., 2018. Evaluation of glutathione peroxidase activity, selenium, copper, zinc, iron concentration and weight gain following administration of selenium nano-particles in lambs. Iranian Journal of Veterinary Clinical Sciences, Volume 12, pp. 81-89.

Yun, J.-W., Kim, S-H., You, J-R., Kim, W.H., Jang, J-J., Min, S-K., Kim, H.C., Chung, D.H., Jeong, J., Kang, B-C. & Che, J-H., 2015. Comparative toxicity of silicon dioxide, silver and iron oxide nanoparticles after repeated oral administation to rats. Journal of Applied Toxicology, Volume 35, pp. 681 – 693.

Yu, Z., Robinson, P. & McGoldrick, P., 2001. An evaluation of methods for the chemical decomposition of geological materials for trace element determination using ICP-MS. Geostandards and geoanalytical research, Volume 25, pp. 199 - 217.

Zaboli, K., Hassan, A., Asghar, B. & Roghieh, A., 2013. Role of dietary nano-zinc oxide on growth performance and blood levels of mineral: A study on in Iranian Angora (Markhoz) goat kids. Journal of pharmaceutical and health sciences, Volume 2, pp. 19 - 26.

Zago, M. & Oteiza, P., 2001. The antioxidant properties of zinc: interactions with iron and antioxidants. Free radicals in biology and medicine, Volume 31, pp. 266 - 274.

Zervas, G., Nikolaou, E. & A, Mantzios. 1990. Comparative study of chronic copper poisoning in lambs and young goats. Animal Production, Volume 50, pp. 497 - 506.

Zhao, C.Y., Tan, S-X., Xiao, X.Y., Qui, X-S., Pan, J-Q., Tang, Z.H ., 2014. Effects of dietary zinc oxide nanoparticles on growth performance and antioxidative status in broilers. Biological Trace Element Research, Volume 160, pp. 361-367.

Zimnicka, A., Ivy, K. & Kaplan, J., 2011. Acquisition of dietary copper: a role for anion transporters in intestinal apical copper uptake. American Journal of Physiology Cell Physiology, Volume 300, pp. 588-599.

Zook, J.M., MacCuspie, R.I., Locascio, L.E., Halter, M.D. & Elliott, J.T., 2011. Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity. Nanotoxicology, Volume 5, pp. 517 - 530.