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Feeding nanoparticles of copper oxide coated with lysine with or without added antagonists affects the copper status but not performance of Holstein dairy cows

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ABSTRACT

The apparent absorption of copper (Cu) in ruminants is low, with between 0.01 and 0.07 g/g absorbed from sources such as copper oxide (CuO) under typical feeding conditions, resulting in high levels of excretion. Improving the bioavailability of Cu could reduce the supplemental amount required to maintain Cu status and reduce excretion, particularly in the presence of dietary antagonists such as sulfur (S) and molybdenum (Mo). The objective of our study was to determine the Cu status of cows when fed nanoparticle CuO coated with Lys compared with conventional CuO when fed without or in combination with antagonists to Cu absorption (S and Mo) in the diet of dairy cows. Fifty-six multiparous Holstein-Friesian cows that were 48 d ± 17.4 (mean ± SD) post calving and yielding 40.6 ± 6.9 kg milk/d were used in a 2 × 2 factorial design. The 4 treatment groups were; CuO (O-), CuO with added antagonists (O+), nano CuO with a lysine coating (N-), and nano CuO with a Lys coating with added antagonists (N+), fed for 16 wks. We formulated the diets to contain approximately 17 mg Cu/kg dry matter (DM) and diets with antagonists contained an additional 1 g S/kg DM and 6 mg Mo/kg DM, with Lys added to O- and O+ to provide the same daily supply as N- and N+. Blood samples were collected at wk 0, 2, 4, 6, 10 and 16, and liver biopsy samples at wk 0 and 16. We found no effect of dietary treatment on DM intake, milk yield, live weight or body condition score, with mean values of 23.3 kg/d, 40.1 kg/d, 646 kg and 2.68, but milk SCC was higher in cows fed conventional compared with non CuO, or with added antagonist. We also found no effect of treatment on blood activity of gamma glutamyl transferase, superoxide dismutase or ceruloplasmin, hematology profile, or plasma Cu and iron concentration. We found that plasma Mo concentration was increased from 0.36 µmol/L in cows fed O- or N- to 0.80 µmol/L in those receiving O+ or N+. Additional dietary antagonists also decreased the concentration of Cu in the liver of cows fed conventional CuO (C+) over the study period by 1.3 mg/kg DM/d, but in cows fed dietary antagonists and nano CuO coated with Lys (N+), liver Cu concentration was increased by 1.1 mg/kg DM/d. Our study is the first to demonstrate that reducing the particle size of CuO into the nano scale with a lysine coating improves the bioavailability of CuO in the presence of dietary antagonists in dairy cattle, and we did not observe any negative effects on performance or health.
KEY WORDS: antagonists, copper status, dairy cow, nanoparticles

INTRODUCTION

Copper (**Cu**) is a common trace element responsive disorder in dairy cows (Spears, 2023). In cattle Cu is required for the normal function of numerous enzymes such as cytochrome C oxidase, superoxide dismutase (**SOD**), and ceruloplasmin (**Cp**) (Cerone, et al., 2000). Consequently, the Cu status of the animal is vital for functions such as cellular respiration and protection from oxidants, and clinical deficiency can manifest itself in a variety of symptoms including loss of pigmentation, anemia, reduced immunity, poor growth rate, and reduced performance and fertility (Suttle, 2022).

There are 2 routes in which Cu responsive disorders can occur in ruminants (Suttle, 2022). A primary Cu responsive disorder is a result of suppling a diet that contains an insufficient concentration of Cu to meet the animal's requirements, with the consequence that Cu is mobilized from the liver to maintain blood levels until eventually the liver is depleted (Herdt and Hoff, 2011). A secondary Cu responsive disorder is more common, and is caused by high dietary concentrations of antagonists to Cu absorption and metabolism, with sulfur (**S**), molybdenum (**Mo**) and iron (**Fe**) being the most widely

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

studied (McCaughern et al., 2020; Sinclair et al., 2017; Spears et al., 2003). In the rumen, dietary S and Mo form thiomolybdates that combine with Cu to produce highly stable insoluble complexes that are not absorbed by the animal in the small intestine, and are consequently excreted (Gould and Kendall, 2011). Alternatively, under conditions of high dietary Mo and in the absence of sufficient dietary Cu, thiomolybdates may be absorbed by ruminants and subsequently inhibit Cu containing metalloenzymes (Suttle, 2022). If there are high levels of Fe in the diet there are 2 possible mechanisms that can reduce the availability of Cu to the animal (Gould and Kendall, 2011). First, a Fe-S complex may form, with Cu then displacing the Fe to form Cu-S (Suttle, 1974), or second Fe may react with sulfide 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 rendered unavailable (Suttle, 2022). In reality, there is not a clear distinction between primary and secondary responsive disorders.

To avoid negative effects on performance and health caused by a Cu responsive disorder, dairy cows are often supplemented (Grace and Knowles, 2015; Sinclair and Atkins, 2015; Spears, 2003). There are several forms of Cu available for cattle, and studies have demonstrated that they can vary in their efficacy, especially in the presence of high levels of antagonists (Ward et al., 1996; Spears 2023). The bioavailability of Cu has been defined as its potential to supply the physiological functions that depend on Cu within the animal's metabolism, with activity of Cu dependent enzymes such as ceruloplasmin or Zn/Cu superoxide dismutase, liver retention, serum or plasma Cu concentration, or apparent absorption proposed as the most suitable indicators (Brugger et al., 2022). Kegley and Spears (1994) reported that CuO was not very effective at maintaining the Cu status of cattle when challenged with a high level of antagonists in the diet, and dietary CuO has been shown to have a relatively low bioavailability, at approximately 15% of that of Cu sulfate (Parkins et al., 1994). Improving the bioavailability of CuO is of particular interest as it is one of the few forms of Cu that can be used in an intra-ruminal mineral bolus as it is relatively inert, has a high bulk density and a low solubility (SRL Ecoterm, 2017).

There is no strict, regulated definition of nanoparticles, but they are generally regarded as particles with at least one dimension less than 100 nm (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 in a solution due to their increased surface area compared with other materials (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), potentially increasing paracellular absorption between enterocytes (Brugger et al., 2022). As a result of these changes it is hypothesized that nanoparticle trace elements will have an increased bioavailability when supplemented orally compared with conventional material of the same trace element compound. However, increasing the surface area may also increasing the potential for binding by antagonists such as thiomolybdates in the rumen, which may decrease bioavailability, particularly in situations of high dietary concentrations of S and Mo. Shen et al. (2021) and Min et al., (2022) reported that the supplementation of goats and sheep with nano Cu increased the concentration of Cu in blood more rapidly than Cu sulfate, and also improved the animals antioxidant status, as evidenced by elevated serum superoxide dismutase concentrations. Nano CuO may therefore be more effective than conventional CuO at maintaining the Cu status of cattle, although no studies have been undertaken in dairy cows.

Our hypothesis was that the Cu status of dairy cows would be improved by reducing the particle size of CuO into the nano scale, and that this effect would be greater when the concentration of dietary Cu antagonists were increased. Our primary objective was to determine the Cu status of cows fed CuO when supplied as nanoparticles compared with conventional CuO in Holstein-Friesian dairy cows when fed without or with added S and Mo by determining the accumulation of Cu in the liver, plasma mineral concentration and activity of Cu containing enzymes. The secondary objective was to monitor the effect on the performance of dairy cows.

MATERIALS AND METHODS

Animals, management, and treatments

All procedures involving animals were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (Amended 2012), and received local ethical approval. Fifty-six Holstein-Friesian dairy cows (12 primiparous and 44 multiparous) that were 48 ± 17.2 DIM (mean ± SD), yielding 41 ± 6.4 kg/d of milk per day, with a mean BCS of 2.8 ± 0.26 (where 1 = emaciated and 5 = obese, scored at 0.25 units; Ferguson et al., 1994) were used in a 2 × 2 factorial arrangement of treatments lasting 16 weeks. Based on recordings taken in the 2 weeks before commencing the study animals were blocked and randomly allocated to one of 4 dietary treatments according to calving date, lactation number and milk yield.

Cows were fed a TMR that was based on corn and alfalfa silages (0.35:0.65 ratio DM basis), concentrate feeds and minerals and vitamins to produce 40 kg of milk per day according to Thomas (2004; Table 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 for 10 min, and fed through electronic feed bins fitted with an automatic animal identification and forage weighing system (maximum of 1.5 cows per bin) that permitted access to the appropriate dietary treatment (Hokofarm Group B.V, Emmelrood, The Netherlands) calibrated to ± 0.1. Intake was recorded each time the animal accessed a feed bin, and data were downloaded on a daily basis. Fresh feed was offered at 0800 h at 1.05 of the previously recorded intake, with refusals collected 3 times a week on a Monday, Wednesday and Friday. The cows were housed in the same portion of a building containing free stalls. The passageways were scraped 6 times daily using automatic scrapers, and the stalls were bedded with sawdust and lime twice weekly. All cows had continual access to water.

To evaluate the effect of the form of Cu, the diets contained either CuO (O) or nano CuO with a lysine coating (N) to supply an additional 8 mg Cu/kg DM, resulting in a total dietary concentration of 17 mg Cu/kg DM. To determine the effect of antagonists the diets were either unsupplemented (-) or supplemented (+) with S and Mo to supply an additional 1.0 g S/kg DM and 6.0 mg Mo/kg DM, resulting in a total dietary concentration of approximately 4.4 g S/kg DM and 7.2 mg Mo/kg DM. A priori apparent Cu absorption calculations coefficients for supplemented diets (O+ and N+) were 0.013, nearly a third of the 0.036 for the unsupplemented diets (O- and N-; NRC, 2001, NASEM, 2021). There were therefore 4 dietary treatments; CuO (O-), CuO with added antagonists (O+), nano CuO with a Lys coating (N-), and nano CuO with a Lys coating with added antagonists (N+). The nano CuO was coated with hydrolyzed Lys (ratio of 1:1) to prevent agglomeration of the particles, and mixed using a metal stand mixer run at a slow speed to provide a uniform dispersion of the 2 materials. An equivalent amount of hydrolyzed Lys (Sigma-Aldrich, UK) was added to the diet of cows that received O- and O+. Characterization of the physical properties of the supplementary sources of Cu was conducted at Nottingham Trent University, Nottingham, UK before the start of the study (Table 2). Particle size was determined by transmission electron spectroscopy (JEOL JEM-2010, Michigan Tech, USA), and dynamic light scattering (Particulate Systems, NanoPlus HD, Kromtek, Malaysia). Elemental composition was determined using inductively coupled plasma optical emission spectrometry (ICPE-9820, Shimadzu, UK).

Experimental procedure

Cows were milked twice daily at approximately 0600 and 1600 h through a 40-point internal rotary parlor (GEA AutoRotor Magnum 40, Germany). Milk yield was recorded at each milking and samples were collected fortnightly at consecutive am and pm milkings for subsequent analysis of fat, protein, lactose and **SCC**. The BW and BCS were recorded at 0900 h in the week before allocation, and then fortnightly. Forage samples were collected twice weekly and oven-dried at 105°C to

Table 1. Dietary composition and chemical analysis of the TMR fed to Holstein-Friesian dairy cows containing dietary CuO (O) or nano CuO (N), with (+) or without (-) added S and Mo

	Dietary concentration, g/kg DM			
Item	O-	O+	N-	N+
Ingredient, g/kg of DM				
Corn silage	394	393	394	393
Alfalfa silage	158	158	158	158
Sweet starch[1]	91	91	91	91
Rapeseed meal	74	74	74	74
Wheat distillers dark grains	74	74	74	74
Soybean meal, Hi-pro	31	31	31	31
Palm kernel	21	21	21	21
Molasses	6	6	6	6
Rapetec[2]	26	26	26	26
Soy hulls	84	83	84	83
Buffer[3]	5	5	5	5
Wheat straw	11	11	11	11
Limestone	0	1	0	1
Salt	3	4	3	4
Megalac[4]	13	13	13	13
Minerals and vitamins[5,6]	9	9	9	9
Hydrolyzed Lys[7]	0.01	0.01	0	0
Total	1,000	1,000	1,000	1,000
Chemical analysis				
DM, g/kg	482	472	468	472
CP, g/kg of DM	165	161	163	163
NDF, g/kg of DM	427	424	430	425
Starch, g/kg of DM	176	167	177	164
Ash, g/kg of DM	81	84	85	82
Ca, g/kg of DM	9.6	10.2	10.1	10.3
P, g/kg of DM	6.7	6.9	6.9	6.9
Mg, g/kg of DM	2.8	2.8	2.7	2.8
S, g/kg of DM	3.2	4.4	3.5	4.3
Cu, mg/kg of DM	16.6	16.8	16.5	17.0
Mo, mg/kg of DM	0.9	7.2	0.8	7.1
Zn, mg/kg of DM	75.9	78.4	75.3	77.2
Fe, mg/kg of DM	363	340	341	371

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

[1]A blend of bakery, confectionary, pastry and breakfast cereal, KW Feeds, Leeds, UK.

[2]Extruded natural meal derived from whole rape and wheat, KW Feeds, West Yorkshire, UK.

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

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

[5]Mineral/vitamin premix without additional antagonists (O- and N-): 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.

[6]Mineral/vitamin premix with additional antagonists (O+ and N+): 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

[7]Hydrolyzed Lys, reagent grade > 98%, Sigma-Aldrich, UK.

Table 2. Physical and chemical characteristics of the supplementary Cu fed to dairy cows

	CuO	Nano CuO
Coating	None	Hydrolyzed Lys
Coating ratio	N/A	1:1
Chemical formula	CuO	CuO
CAS Number	1317–38–0	N/A
Appearance color	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

a constant weight, and the amount of corn and alfalfa silage adjusted to achieve the desired ratio. Samples from each of the 4 TMR were collected once per week immediately after feeding and stored at −20°C for subsequent analysis. Blood samples were collected from 12 cows per treatment that had a representative performance of the whole group at wk 0, 2, 4, 6, 10 and 16 of the study at 1000 h via jugular venipuncture into Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing K_2EDTA for samples used for hematology profile and to determine superoxide dismutase (**SOD**) activity, silica for samples used to determine Cp, and K_3EDTA and sodium heparin for samples used to determine mineral concentration and gamma glutamyl transferase (**GGT**) activity, respectively. Vacutainer tubes containing K_3EDTA and lithium heparin were centrifuged at 1000 x *g* for 15 min at 4°C, and the plasma separated and stored at −20°C for subsequent analysis. Serum vacutainer tubes were stored at 4°C for 24 h and then centrifuged at 1000 x *g* for 15 min, and the serum separated and stored at −20°C for subsequent analysis. Samples collected in vacutainer tubes containing K_2EDTA were analyzed immediately for their hematology profile using a Vet Animal Blood Counter (Woodley Equipment Company Ltd., UK) calibrated using a control sample (ABX Minotrol 16; Horiba ABX Diagnostics, UK). Liver biopsy samples were obtained from all animals during wk 0 and 16 of the study by insertion of a 250 mm x 5 mm needle through the 11th intercostal space using the procedure described by Davies and Jebbet (1981), and stored at −80°C for subsequent analysis.

Analytical procedure

Samples of TMR for each dietary treatment were composited by month and sub-samples analyzed according to AOAC (2012) for DM (943.01; intra-assay CV of 2.5%), CP (990.03; intra-assay CV of 1.6%) using a Leco FP-528 (Leco Corporation, St Joseph, MI), **NDF** (intra-assay CV of 1.5%) according to Van Soest et al. (1991), and starch (intra-assay CV of 5.5%) according to ISO 6493 (2000) at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK). Samples of the TMR were also analyzed for calcium (**Ca**), Cu, Fe, magnesium (**Mg**), Mo, phosphorus (**P**), S and zinc (**Zn**) by inductively coupled plasma mass spectrometry (**ICP-MS**; NexION 2000, PerkinElmer, Shelton, CT). Certified European Union (EU) reference samples of hay (BCR-129) and dairy concentrate (BCR-185) were extracted and analyzed to determine the accuracy of the feed mineral analysis. Milk samples were analyzed for fat, protein, lactose and SCC by Eurofins Laboratories (Wolverhampton UK). Serum, plasma and whole blood samples were analyzed using a Cobas-Mira Plus autoanalyzer (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 catalog no. GT 553; intra-assay CV of 2.1%) and SOD (Randox, Crumlin, UK, Kit catalog no. SD 125; intra-assay CV of 3.5%). Plasma samples were also analyzed for Cu, Fe, and Mo by ICP-MS (intra-assay CV of 1.0%, 2.4% and 1.2% respectively). A 250 mg sample of the liver was dried for 24 h at 60°C and weighed to determine the DM content. Dried liver samples were then digested in 6 mL of concentrated HNO_3 at 60°C for 12 h, cooled to room temperature and diluted with purite water up to 50 mL before analysis for Cu by ICP-MS according to McCaughern et al., (2020), with the use of Ga as an internal standard. A ClinCheck certified lyophilized plasma control sample 2 (product no. 8885, RECIPE Chemicals and Instruments GmbH, Munich, Germany; certified concentration of Cu = 19.9 ± 4.2 µmol/L, analyzed concentration of 18.4 ± 0.08 µmol/L), and a certified EU reference bovine liver sample (BCR-185; certified concentration of Cu = 277 ± 5.0 mg/kg of DM, analyzed concentration = 274 ± 2.6 mg/kg of DM) were used to determine the accuracy of plasma and liver samples respectively.

Statistical analysis

Three cows were removed from the study for reasons unrelated to the dietary treatments; one each from N- (mastitis) and N+ (lame after injury) during wk 2, and one from O+ during wk 8 (mastitis). All data and residuals were tested for normality. Performance, plasma minerals and enzyme activity were analyzed by a generalized linear mixed model. Milk SCC was transformed to $\log_{10}$ before analysis. Treatment degrees of freedom were split into main effects of Cu form (conventional CuO (O) versus nano CuO (N)), antagonist (without (-) versus with (+)) and their interaction, and analyzed as:

$$Yijkl = \mu + Bi + Fj + Ak + Tl + F.Ajk + F.Tjl + A.Tkl + F.A.Tjkl + COV\ \varepsilon ijkl$$

Where Yijkl = dependent variable; μ = overall mean; Bi = random effect of cows nested within blocks, Fj = fixed effect of Cu form (j = conventional CuO or nano CuO); Ak = fixed effect of antagonists (S and Mo) (k = -, +); Tl = fixed effect of time; F.Ajk = interactions between Cu form and antagonist; F.Tjl = interaction between Cu form and time; A.Tkl = interaction between Cu form and time; F.A.Tjkl = interaction between Cu form, antagonist and time, COV = covariate measured during wk 0 as appropriate, and εijkl = residual error.

Liver mineral concentration, live weight and BCS and their change were analyzed a general linear mixed model as:

$$Yijk = \mu + Bi + Fj + Ak + F.Ajk + COV + \varepsilon ijk$$

Where Yijk = dependent variable; μ = overall mean; Bi = random effect of blocks; Fj = fixed effect of Cu source (j = conventional CuO or nano CuO); Ak = fixed effect of antagonists (S and Mo) (k = -, +); F.Ajk = interactions between Cu source and antagonist; COV = covariate and εijk = residual error. For liver mineral concentrations the concentration during wk 0 was used where appropriate as a covariate to determine the final liver mineral concentration and rate of mineral storage or mobilization. Statistical analysis was conducted using Genstat 23rd edition (VSNi, Hemel Hempstead, United Kingdom) and is presented as means with SED; $P < 0.05$ was used to indicate significance and a trend was considered when $P < 0.1$.

RESULTS

Dietary analysis and animal performance

We observed that all 4 diets had a similar DM, CP, NDF, starch and ash content with mean concentrations of 474 g/kg, 163, 427, 171 and 83 g/kg DM respectively (Table 1). The concentration of Cu was similar across all 4 diets with a mean concentration of 16.7 mg/kg DM, whereas diets O+ and N+ had a higher concentration of S and Mo (mean of 4.3 g S/kg DM and 7.2 mg Mo/kg DM) than O- and N- (mean of 3.4 g S/kg DM and 0.9 mg Mo/kg DM).

We did not detect an effect of form of Cu or presence of antagonists ($P > 0.05$) on milk yield, milk fat concentration or yield, milk lactose concentration or yield, or BW and BCS, with mean values of 40.1 kg/d, 37.6 g/kg, 1.50 kg/d, 45.2 g/kg, 1.82 kg/d, 646 kg and 2.68 (Table 3). We observed that milk protein concentration was 1g/kg lower ($P = 0.005$) in cows fed nano compared with conventional CuO, but there was no effect ($P > 0.05$) on daily milk protein yield, and that there was a trend ($P = 0.09$) for cows fed additional antagonists to have a 1.0 kg DM/d lower intake. We also observed that milk SCC concentration higher ($P < 0.05$) in cows fed conventional compared with nano CuO, or with added S and Mo.

Plasma mineral concentration, blood enzyme activity and hematology profile

We found that plasma Cu concentration did not change over time ($P = 0.592$; Figure 1), and there was no effect of form of Cu source ($P = 0.504$) or the presence of antagonists ($P = 0.904$) on plasma Cu concentration. We found an interaction between time and the presence of antagonists on plasma Mo concentration ($P = 0.001$; Figure 2), which was similar between treatments in wk 0 with a mean concentration of 0.33 μmol/L, but was higher in cows supplemented with antagonists (O+ and N+) at approximately 0.80 μmol/L compared with 0.36 μmol/L in those unsupplemented (O- and N-) thereafter. We also observed that plasma Fe concentration was affected by time ($P = 0.014$; Figure 3) but there was no effect of form of Cu source ($P = 0.455$), antagonists ($P = 0.654$) or interaction between Cu source and antagonists ($P = 0.603$).

We observed no effect ($P > 0.05$) of treatment on GGT, SOD or Cp activity (Table 4). We detected an interaction ($P = 0.033$) between the form of Cu supplementation and presence of antagonists on blood monocyte number, where cows fed CuO without antagonists (O-) had a lower count at 0.33 m/mm^3 than those fed nano CuO without antagonists (N-) at 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 no effect of treatment on any of the other hematological measurements.

Hepatic mineral concentration

We detected that the final hepatic Cu concentration was higher ($P = 0.040$) and the increase in Cu concentration over the study period was greater ($P = 0.044$) in cows supplemented with nano CuO compared with those supplemented with conventional CuO, with a mean final concentration and increase of 626 and 150 mg/kg of DM in N- and N+, compared with 478 and −8 mg/kg of DM in O- and O+ respectively (Table 5). We also observed that cows supplemented with additional antagonists (O+ and N+) had a lower final hepatic Cu concentration at 483 mg/kg of DM compared with 619 mg/kg of DM in those that were unsupplemented (O- and N-; $P = 0.036$), and mobilized 15 mg/kg of DM hepatic Cu over the study period compared with an increase of 154 mg/kg DM in cows that were not supplemented with antagonists (O- and N-; $P = 0.026$). There was no effect of treatment on final hepatic Mo or Fe concentration, or change over time ($P > 0.05$).

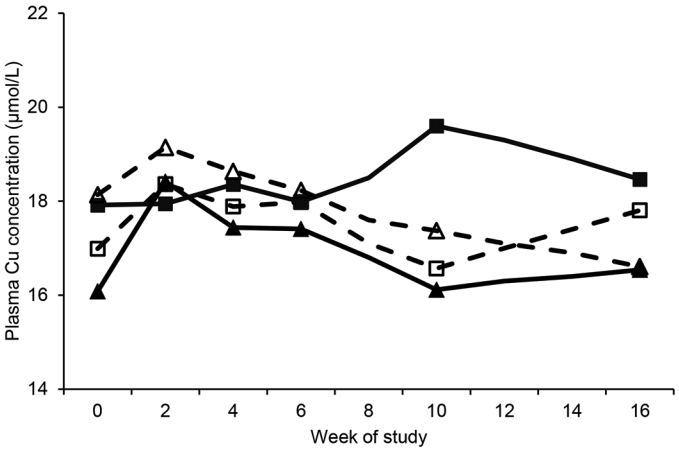

Figure 1. Effect of dieta[INSERT Figure 001][INSERT Figure 002][INSERT Figure 003][INSERT Figure 004]ry CuO (O) or nano CuO (N) fed with (+) or without (-) added S and Mo on plasma Cu concentration (µmol/L) in Holstein-Friesian dairy cows. Treatments were: O- (); CuO without antagonists, O+ (); CuO with antagonists, N- (); Nano CuO without antagonists and N+ (); Nano CuO with antagonists. Pooled SED = 1.28. Form of Cu, $P = 0.504$; Effect of antagonists, $P = 0.904$; Interaction effect between form of Cu and antagonists, $P = 0.154$; time, $P = 0.592$. n = 12 per treatment.

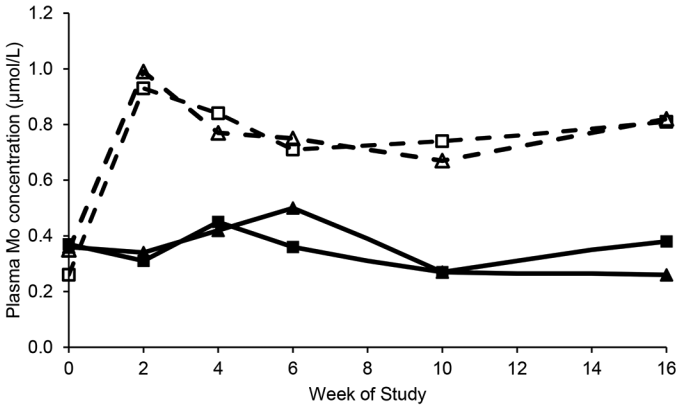

Figure 2. Effect of dieta[INSERT Figure 005][INSERT Figure 006][INSERT Figure 007][INSERT Figure 008]ry CuO (O) or nano CuO (N) fed with (+) or without (-) added S and Mo on plasma Mo concentration (µmol/L) in Holstein-Friesian dairy cows. Treatments were: O- (); CuO without antagonists, O+ (); CuO with antagonists, N- (); Nano CuO without antagonists and N+ (); Nano CuO with antagonists. Pooled SED = 0.527. Form of Cu, $P = 0.709$; Effect of antagonists, $P < 0.001$; Interaction effect between form of Cu and antagonists, $P = 0.189$; time, $P = 0.009$. n = 12 per treatment.

DISCUSSION

Our study is the first to report that reducing the particle size of CuO into the nano scale and coating with Lys improves the hepatic retention of Cu, both with or without high levels of antagonists included in the diet. The mean particle size of the conventional CuO that we fed was 25000 nm, whereas the nano CuO was approximately 5 hundred times smaller at 45 nm. The nanoparticle CuO also had a hydrolyzed Lys coating in a 1:1 ratio with the metal compound to prevent the agglutination of the fine particles, and we therefore added hydrolyzed Lys at the same rate to the diet of the cows fed conventional CuO to avoid possible effects of Lys supply on performance, although we calculate that the Lys added only 0.01% to the total crude protein content of the diet. It is possible however, that the particle size of the nano-treatments was not maintained either during the mixing process or within the intestinal tract, and that the lysine coating may have had a direct effect on altering Cu solubility and subsequent absorption.

Table 3. Effect of dietary CuO (O) or nano CuO (N) with (+) or without (-) added S and Mo on intake and performance in Holstein-Friesian dairy cows

	Treatment					Significance[1]		
Item	O-	O+	N-	N+	SED	Form	Ant	Int
DM intake, kg/d	24.1	23.1	23.5	22.4	0.79	0.342	0.087	0.927
Milk yield, kg/d	40.5	39.6	41.0	39.4	0.85	0.942	0.207	0.984
Milk fat, g/kg	39.8	36.2	37.2	37.0	1.67	0.353	0.181	0.175
Fat yield, kg/d	1.61	1.44	1.52	1.44	0.080	0.431	0.064	0.231
Milk protein, g/kg	31.1	30.6	29.3	30.2	0.29	0.005	0.675	0.071
Protein yield, kg/d	1.27	1.20	1.18	1.19	0.047	0.203	0.403	0.173
Lactose, g/kg	45.1	44.7	45.6	45.4	0.43	0.063	0.319	0.782
Lactose yield, kg/d	1.84	1.75	1.87	1.80	0.078	0.491	0.195	0.880
Milk SCC (log_{10})	1.69	1.86	1.36	1.71	0.166	0.044	0.028	0.490
BW, kg	654	642	653	635	18.5	0.788	0.254	0.769
BW change, kg/d	0.26	0.24	0.21	0.13	0.083	0.233	0.404	0.632
BCS	2.64	2.75	2.65	2.67	0.077	0.731	0.328	0.559
BCS change	−0.20	−0.13	−0.05	−0.08	0.100	0.217	0.551	0.537

[1]Form = main effect of form of Cu supplement, Ant = main effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists.

Table 4. Effect of dietary CuO (O) or nano CuO (N) with (+) or without (-) added S and Mo on GGT (U/l), SOD (U/g Hb) and Cp (mg/dl) activity and hematology profile in Holstein-Friesian dairy cows

	Treatment					Significance[2]		
Item[1]	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 activity, mg/dL	17.3	18.5	17.4	17.6	1.94	0.651	0.674	0.585
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]WBC = white blood cells; Lym No. = lymphocyte number; Mon No. = monocyte number, Neu No. = neutrophil number; Eo No. = eosinophil number; Ba No. = basophil number; Hb = hemoglobin; RBC = red blood cells; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

[2]Form = main effect of form of Cu supplement, Ant = main effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists.

Animal performance

We did not observe any differences in DMI between cows fed the 2 different Cu sources, a finding 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 sulfate or CuO had a reduced DMI compared with those that received a Cu injection or were unsupplemented. Sinclair et al. (2013 and 2017) reported that DMI was reduced in cows when fed additional S and Mo with either an inorganic Cu source or Cu sulfate, and in our current study there was a trend for DMI to also be lower in the cows fed additional antagonists. Under acidic rumen conditions dietary S is converted by sulfur-reducing bacteria into hydrogen sulfide (Drewnoski, et al., 2012). Hydrogen sulfide migrates to the gas cap where it can be absorbed across the rumen epithelium or eructated and absorbed in the lungs, resulting in neurological effects such as polio encephalomalacia, subsequently reducing DMI (Gould, 1998). McCaughern et al., (2020) reported that the reduction in DMI in cows fed high dietary S was greatest when a higher dietary starch level was fed that that resulted in a lower rumen pH. The dietary starch concentration fed in the current study was similar across all 4 dietary treatments and intermediate to the low and high dietary starch concentrations used by McCaughern et al., (2020), although neither rumen pH or ruminal H_2S concentration were measured in our study.

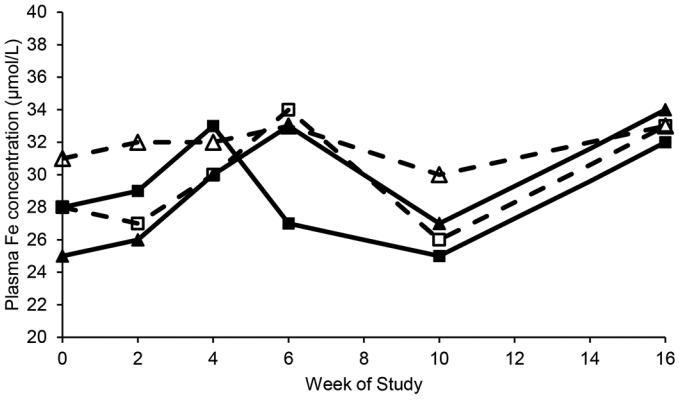

Figure 3. Effect of dieta[INSERT Figure 009][INSERT Figure 010][INSERT Figure 011][INSERT Figure 012]ry CuO (O) or nano CuO (N) fed with (+) or without (-) added S and Mo on plasma Fe concentration (µmol/L) in Holstein-Friesian dairy cows. Treatments were: O- (); CuO without antagonists, O+ (); CuO with antagonists, N- (); Nano CuO without antagonists and N+ (); Nano CuO 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, $P = 0.014$. n = 12 per treatment.

We did not observe a difference in milk yield or composition between cows fed the different Cu sources, or with additional antagonists, although milk protein content, but not daily yield, was lower in cows fed nano compared with conventional CuO. A meta-analysis by Rabiee et al. (2010) reported an increase in milk yield of 0.93 kg/cow/d when organic compared with inorganic trace minerals were fed, but the confounding effects of supplementing with several trace minerals does not allow a conclusion to be drawn on the direct effect of Cu source. Sinclair and Mackenzie (2013) reported little consistent effect of the level of Cu supplementation at up to 40 mg Cu/kg DM on milk yield or composition. Chase et al. (2000) also reported that milk composition was not affected by Cu source, although Engle et al. (2000) reported that

Table 5. Effect of dietary CuO (O) or nano CuO (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[1]

	Treatment					Significance[2]		
Item[1]	O-	O+	N-	N+	SED	Form	Ant	Int
Final Cu, mg/kg of DM	590	361	647	605	88.2	0.040	0.036	0.135
Cu change, mg/kg of DM	+132	−140	+175	+125	103.7	0.044	0.026	0.120
Final Mo, mg/kg of DM	2.4	5.4	3.6	3.9	2.04	0.915	0.105	0.262
Mo change, mg/kg of DM	+0.50	+1.6	+0.17	+1.0	2.03	0.575	0.305	0.737
Final Fe, mg/kg of DM	258	325	280	283	97.7	0.885	0.612	0.656
Fe change, mg/kg of DM	−11	+25	−24	+8	104.5	0.842	0.651	0.976

[1]Wk 0 value used as a covariate where appropriate.

[2]Form = main effect of form of Cu supplement, Ant = main effect of antagonists, Int = interaction between form of Cu supplement and presence of antagonists. Initial mean hepatic Cu, Mo and Fe concentrations were 466, 3.0 and 287 mg/kg of DM respectively.

milk fat composition was altered with Cu dose. In our study both sources of trace mineral were inorganic, were supplemented at the same level, and animals had high hepatic reserves at the start of the study, and consequently a change in intake and milk yield was not expected even in animals with ongoing Cu deficiency (Daniel et al., 2022). Other studies have reported that dietary Cu concentration does not affect SCC (Chase et al., 2000; Scaletti and Harmon, 2012). In contrast, Scaletti et al. (2003) reported that when cows were challenged with *Escherichia coli* and fed 26.5 mg Cu/kg DM that there was a lower peak in milk SCC compared with those fed 6.5 mg Cu/kg DM. In our study we observed a lower SCC in cows receiving the nano CuO, which, in association with the greater Cu status as evidenced by the higher hepatic Cu concentration in cows receiving this treatment, may indicate a benefit to supplying nano-Cu coated with lysine. Sinclair et al. (2017) reported an interaction between basal diet and supplementation with Cu antagonists on milk SCC, although this was associated with a reduction in DMI which may have contributed to a greater metabolic stress. In our current study supplementation with S and Mo increased SCC with both sources of Cu and may therefore indicate a metabolic stress from the inclusion of these antagonists independent of Cu supply.

We did not observe any effect of form of Cu or added antagonists on the mean BW or BCS, with BW decreasing from wk 0 to wk 2 and then increasing from wk 2 to wk 16, while BCS decreased between wk 0 and wk 10 and then increased to wk 16. Several other authors have also reported no effect of Cu source or antagonists on BW or BCS in dairy cows (Sinclair et al., 2013; McCaughern et al., 2020). In contrast, Sinclair et al., (2017) reported a reduction in both BW and BCS in dairy cows when fed a grass silage based TMR following the addition of 2 g S/kg DM and 6.5 mg Mo/kg DM, which was associated with a reduction in DMI of 2.1 kg/d. In our current study S and Mo were added at 1 g/kgDM and 6 mg/kg DM respectively, which did not have a significant effect on DMI, and may explain the lack of an effect of antagonists on body tissue mobilisation.

Blood parameters

We observed that plasma Cu concentration was within the normal range (9 – 20 µmol/L) for all cows (McDowell, 1992), and there was no effect of dietary treatment or time. This finding is in agreement with others who have fed different levels of Cu (Chase et al., 2000), or levels of antagonists (Sinclair et al., 2017; McCaughern et al., 2020). Plasma Cu is under homeostatic control with excess dietary Cu being stored in the liver, which can then be released into the bloodstream if dietary supply is below requirements to maintain concentrations within the normal range (Suttle, 2022). As the hepatic Cu concentration of the cows in our study were all well above the lower level associated with clincial deficiency (19 mg/kg DM; Suttle, 2022), the cows had sufficient Cu to maintain their plasma concentration over the study period in all treatments. Previous studies have also been inconsistent in identifying differences in the bioavailability of Cu sources based soley on plasma concentration. For example, Rabiansky et al. (1999) did not detect a difference between Cu Lys and Cu sulfate when additional antagonists were added, while Ward et al. (1996) reported that plasma concentration was increased in cattle supplemented with Cu carbonate compared with Cu sulfate or Cu proteinate, but this was only observed when the diet contained added antagonists to Cu absorption. In contrast Sinclair et al. (2013) reported no effect of Cu sulfate or Cu proteinate on plasma Cu concentration when dairy cows were fed without or with added S and Mo, and concluded that plasma Cu concentration is not a reliable source to indicate the bioavailability of different Cu sources under normal feeding conditions.

We observed that plasma Mo concentration rapidly increased in the first 2 weeks of supplementation with the antagonists, and was then maintained, most likely because dietary Mo is readily absorbed and the concentration in the blood is reflective of dietary intake (Wittenberg and Devlin, 1987). Plasma Mo concentration was within the normal range, 0.02 to 0.4 µmol/L (Herdt and Hoff, 2011) for cows that were not fed additional antagonists, but it was approximately double in those fed antagonists. This magnitude of difference was similar to that of Sinclair et al. (2013; 2017), although the mean concentration in our study was higher. Plasma Fe concentration was also within the normal range of 14 to 37 µmol/L (Herdt and Hoff, 2011) for all cows throughout the study, although we observed that the concentration fluctuated over time. Fluctuations in plasma Fe concentration and Hb, which also changed over time but was within the normal range of 8–15 g/dL (Fielder, 2022), can be caused by several factors primarily linked to inflammatory diseases (Herdt and Hoff, 2011), but because the cattle in our study remained within the normal range and there was no other evidence of disease, no conclusions on health status can be made. Similarly, Rabiansky et al. (1999) reported that plasma Fe and Hb changed over time but there was no effect of different Cu sources, while Sinclair et al. (2017) reported that additional S and Mo in the diet did not have an effect on plasma Fe concentration.

We did not detect an effect of Cu source or additional antagonists on Cp activity, which was within the normal range of 15 to 45 mg/dL (Suttle, 2022). Ward et al. (1996) also reported that there was no difference in Cp activity between cattle supplemented with Cu proteinate, carbonate or sulfate. Differences in Cp activity are usually identified when there are also changes in plasma Cu concentration (Suttle, 2022), but we did not observe any effect of dietary treatment on the Cp:plasma Cu ratio. A change in this ratio can be indicative of the absorption of thiomolybdates which may interfere with the activity of Cu containing enzymes such as Cp or SOD (Suttle, 2010; Hussein and Staufenbiel, 2012), or inflammation, but there was no evidence for this in the current study. Similarly, Cheng et al. (2011), Mion et al. (2023), and Sinclair et al. (2013) also reported no difference in SOD activity in lambs or dairy cows when fed different Cu sources. There was also no effect of antagonists on plasma SOD activity in the studies of Sinclair et al. (2013) and Sinclair et al. (2017). In contrast, Pandey et al. (2023) reported an increase in Cp and SOD in young dairy calves supplemented with Cu nanoparticles compared with unsupplemented controls. 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 longer-term studies are therefore required to provide an opportunity for differences in the activity of this enzyme to be detected.

Hepatic mineral concentration

The liver is the primary storage organ for Cu and accumulation is often used to indicate Cu status when fed different sources (Suttle, 2022). The initial hepatic Cu concentration in the cows used in our study was comparatively high at 466 mg/kg DM, and the mean hepatic Cu concentration at the end of the study was 590, 361, 647 and 605 mg/kg DM for cows in treatment groups O-, O+, N- and N+ respectively. With the exception of cows receiving O+, all of these final values were in an excess of the 508 mg Cu/kg DM limit suggested by Livesey et al. (2002) to increase the risk of toxicity, although others have reported a greater susceptible range of 350–1500 mg Cu/kg DM (Suttle, 2022), but there were no cases of toxicity in our study. We did not observe any difference in the daily hepatic accumulation of Cu between cows fed CuO (O-) or nano CuO with a Lys coating (N-) in the absence of additional antagonists, with mean values of 1.2 and 1.6 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 sulfate, Cu accumulated in the liver at a rate of 1.9 mg Cu/kg DM/d. Previous studies that have reported differences in bioavailability between Cu sources have often fed high levels of antagonists. For example, Ward et al. (1996) and Hansen et al. (2008) both reported an increased bioavailability of organic Cu compared with Cu sulfate in cows that were also supplemented with additional S and Mo. In our study when antagonists were added to the diet, cows fed nano CuO (N+) accumulated Cu in the liver at a rate of 1.1 mg Cu/kg DM/d, whereas those fed conventional CuO (O+) depleted Cu reserves at the rate of 1.3 mg Cu/kg DM/d. This depletion of Cu stores when Cu was fed in the presence of high S and Mo is similar to the findings of Sinclair et al. (2013) who reported that hepatic concentrations decreased by 0.89 mg Cu/kg DM/d in cows supplemented with Cu sulfate and additional S and Mo. In our study if depletion continued at the rate of −1.3 mg Cu/kg DM/d then the cows would be expected to have reached a hepatic Cu concentration of 19 mg Cu/kg DM, the value generally considered to be the threshold below which clinical signs of deficiency are demonstrated (Suttle, 2022), after a further 263 d on treatment. To avoid this, these cows would require either a higher Cu inclusion rate or a source of Cu that was more bioavailable. In contrast, the cows fed nano CuO in the presence of antagonists (N+) accumulated Cu in the liver, indicating that this source of Cu was less subject to the antagonists and was therefore more bioavailable than conventional CuO. It should be borne in mind that

the additional Lys in our study was mixed with the nano-CuO in N- and N+, whereas it was added directly to the diet in O- and O+, and it is therefore possible that the Lys may have had a direct effect on reducing the solubility and therefore the interaction with antagonists, while retaining absorption and improving the availability of Cu in the nanoparticle treatments. Few studies have investigated the effect of CuO-Lys mixes, although Attaelmannan and Reid (1996) reported that Cu-Lys chelates disintegrated rapidly in the rumen, with the Cu present as phosphate and carbonate complexes, although Rabiansky et al., (1999) reported a greater bioavailability of Cu-Lys than $CuSO_4$.

In our study there was no difference in hepatic Mo or Fe concentrations between cows receiving 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, 2022) with the exception of cows fed CuO and additional antagonists that had a slightly higher liver Mo concentration of 5.4 mg/kg DM. A small increase in hepatic Mo concentration was observed over the study period in all cows but this did not reflect the large difference in plasma Mo concentration in those fed 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 cattle.

Hepatic enzymes and hematology

Research into the effects on the health of ruminants, particularly cattle, when feeding mineral nanoparticles is limited. Studies that have investigated the use of nanoparticles in animal nutrition have reported a wide range of effects on health, from a lower toxicity compared with conventional sources (Pelyhe and Mezes, 2013), to renal and liver damage indicative of toxicity (Najafzadeh, et al., 2013). To assess whether the accumulation of Cu in the liver from supplementation of CuO nanoparticles resulted in liver damage we monitored serum GGT activity. Plasma GGT is indicative of liver health, and activity is raised when the liver is damaged or there is biliary tract damage, usually by lethal cell necrosis (Gummow, 1996). In our study we did not observe a difference in GGT activity between cattle supplemented with nano (N+ and N-) or conventional CuO (O+ and O-), with the values for all of the cows being within the normal range for cattle of less than 40 U/l (Johnston, et al., 2014), indicating that nanoparticles did not cause damage to the liver, at least over the 16 week duration of this study.

We also measured the hematology profile as an indicator of animal health and found no effect of Cu source on any of the parameters measured, with all cows remaining within the normal range for all the parameters measured throughout the 16-wk study (Etim et al., 2014). We observed some fluctuations over time in aspects of the hematology profile such as WBC, Lym. No. Hb and MCV. Variation in hematology 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, stress, diet, and limited availability for exercise (Iheukwumere and Herbert, 2002; Etim et al., 2014) can all alter the hematology profile of animals.

CONCLUSIONS

We observed that nano CuO with a Lys coating was more bioavailable in dairy cows than conventional CuO. Cows fed nano CuO accumulated 1.3 mg Cu/kg liver DM/d whereas those fed conventional CuO lost 0.07 mg Cu/kg liver DM/d. All cows had sufficient hepatic reserves over the study period and there was no effect of dietary treatment on intake, milk performance or body weight in cows, and there was no evidence that nanoparticles had a detrimental effect on liver function or hematology, with plasma GGT activity and the hematology profile being within the normal range over the 16-wk study period. Future studies should investigate the dose response on nano-particles, and the effect of the lysine coating on absorption.

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