Copper Physiology in Ruminants: Trafficking of systemic copper, adaptations to variation in nutritional supply and thiomolybdate challenge

AH Clarkson[1], S Paine[1], J Martín-Tereso[2], NR Kendall[1]

[1]School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Leicestershire UK. LE12 5RD. Email: andrea.clarkson@notingham.ac.uk. Telephone: +44 (0) 115 951 6447

[2]Trouw Nutrition Research & Development, Amersfoort, Netherlands

ABSTRACT

Ruminants are recognised to suffer from copper responsive disorders. Present understanding of copper transport and metabolism is limited and inconsistent across vets and veterinary professionals. There has been much progress from the studies of the 1980s and early 90s in cellular copper transport and liver metabolism which has not been translated into agricultural practice. Copper metabolism operates in regulated pathways of copper trafficking rather in than pools of copper lability. Copper in the cell is chaperoned to enzyme production, retention within metallothionein or excretion via the Golgi into the blood. The hepatocyte differs in that copper-containing caeruloplasmin can be synthesized to provide systemic copper supply and excess copper is excreted via bile. The aim of this review is to improve understanding and highlight the relevant progress in relation to ruminants through the translation of newer findings from medicine and non-ruminant animal models into ruminants.

KEYWORDS: *Ruminant, copper transport, liver metabolism, thiomolybdate*

INTRODUCTION

Copper metabolism in ruminants remains poorly understood in practice [1–5]. Developments in the fundamental understanding of copper physiology have been insufficiently translated into livestock nutrition. While there is some awareness among industry professionals of the effects of 'copper deficiency' and of the potential nutritional effects by antagonists it is inconsistently understood [6]. Vets vary in their response to copper-related problems some may discourage supplementation in fear of toxicity problems, while others may continue to supplement [3,5–7].

There is considerable marketing pressure from mineral suppliers for their products and an inclination from producers to seek a 'quick fix' for trace element supplementation [8].

Recent surveys have found UK sheep and cattle are commonly affected by different forms of copper imbalance, including toxicity and deficiency [9,10]. Kendall et al. [10] reported as many as 40% of British dairy cattle may be accumulating excessive liver copper, with up to 52% of them above the Animal Health Veterinary Laboratories Agency (AHVLA) reference range of 300-8,000 µmol/kg DM [10]. Copper imbalance was the most common mineral problem reported between 2004 and 2014; with ~300 fatal occurrences each year reported for cattle and sheep combined for both toxicity and deficiency [11–13]. Indications from academic studies, government reports and industry suggest that copper imbalance is still highly prevalent [3,5,14,15]. Highlighting that copper supplementation remains a problem in ruminant production.

This review focusses on post-absorptive trafficking and systemic regulation of copper and describes the interference of thiomolybdates on these mechanisms. A review of the role of the rumen in thiomolybdate formation has been previously published [16].

COPPER METABOLISM AT CELLULAR LEVEL

Most recent fundamental knowledge generated on copper biology has been produced with models such as cell culture, *c.elegans,* laboratory animals and humans [17]. These selected species concentrate on a medical or nutritional perspective. The lack of emphasis on ruminants, and the limited overlap with human focused sciences, has prevented dissemination of this new understanding; resulting in a lack of progress from the classic ideas on copper in ruminants.

The copper chaperones and enzymes which exist in ruminants are the same as those studied in other mammalian species [17]. At cellular level, basic copper metabolism appears to be consistent throughout eukaryotic life and can be traced from laboratory animals to humans through their shared evolution [18]; demonstrating that copper in the systemic circulation is trafficked in the same manner in mammalian cells thus providing opportunities to expand our understanding of copper metabolism in ruminants [17].

Since 1966 radiolabelled copper, cell fractionation and isolation of intracellular membrane components have been used to develop mathematical models to describe copper movement in rat liver [19,20]. This led to the concept that separate pools, of varying availability existed [21]. Initially, the pools were designated as 'storage', 'synthetic' and 'excretory' [19]. The

relationship between the pools appeared complex, with no evidence of reversible movement between them. It was suggested the copper pools were able to become saturated, and the regulation or exchange between the pools was not determined [21,22]. The number and function of the pools was not easily apparent. Most studies agreed hepatocyte copper could be divided into at least two pools, one a readily available, extractable copper pool accounting for the majority of copper. The second, a less readily available pool containing the remainder of soluble copper and potentially a third, non-extractable, insoluble pool which could be considered a potential subset of the second pool [20,22]. By 1987 it was proposed that three separate pools existed within the liver representing bile, caeruloplasmin and 'storage' which was not further defined [21].

Subsequent research has mapped the intracellular movement of copper and improved our understanding of copper distribution in cells [23–27]. Fundamentally, this new knowledge does not contradict the description of copper as cellular pools, but it illustrates copper physiology in terms of copper trafficking. Free copper ions rarely exist within cells, thus copper is kept complexed to prevent intracellular damage [28]. Distinct intracellular pathways exist where copper is bound to chaperones and channelled across membranes rather than a series of storage compartments as the older model suggests. However, the persistence of the term 'pool', even in current literature, conjures images of discrete areas. It is perhaps better to update our terminology, and start discussing the 'pathways' of copper trafficking, rather than its 'pools' of availability to better reflect the process and improve understanding of the process as a continuous regulation instead of discrete compartments of varying lability.

OVERVIEW OF COPPER TRAFFICKING IN ENTEROCYTES

The one aspect of copper metabolism that differentiates ruminants from other species is their unique digestive system. Copper availability in the ruminant gastrointestinal tract presents peculiarities that are extensively reviewed elsewhere [16,29,30]. However, the process of absorption is well-preserved across the animal kingdom [31–33]. In order for copper to be absorbed, it must be reduced into its most reactive state (Cu^{+}). At the intestinal brush border a copper specific transporter (Ctr1) is responsible for ~70% of copper uptake into the enterocyte, the remainder is taken up by the non-specific transporter Divalent Metal Transporter 1 (DMT1) [34]. Where copper is trafficked through the DMT1 route direct competition for the transporter with dietary elements such as iron and zinc may be more biologically relevant [35]. Once inside

the cell, copper chaperone proteins bind copper and transport it to other specific proteins or incorporate it into enzymes. The pathway via the Golgi is known as the secretory pathway. Copper in excess of cellular requirements enters the secretory pathway to be bound to metallothionein by the Golgi and is stored in the lysosome, which acts as a buffer restricting free cellular copper. Once the metallothionein reaches its saturation capacity copper continues through the secretory pathway from the Golgi via its chaperone to the basolateral membrane for efflux from the cell.

The process in detail

Figure 1 below illustrates the process described.

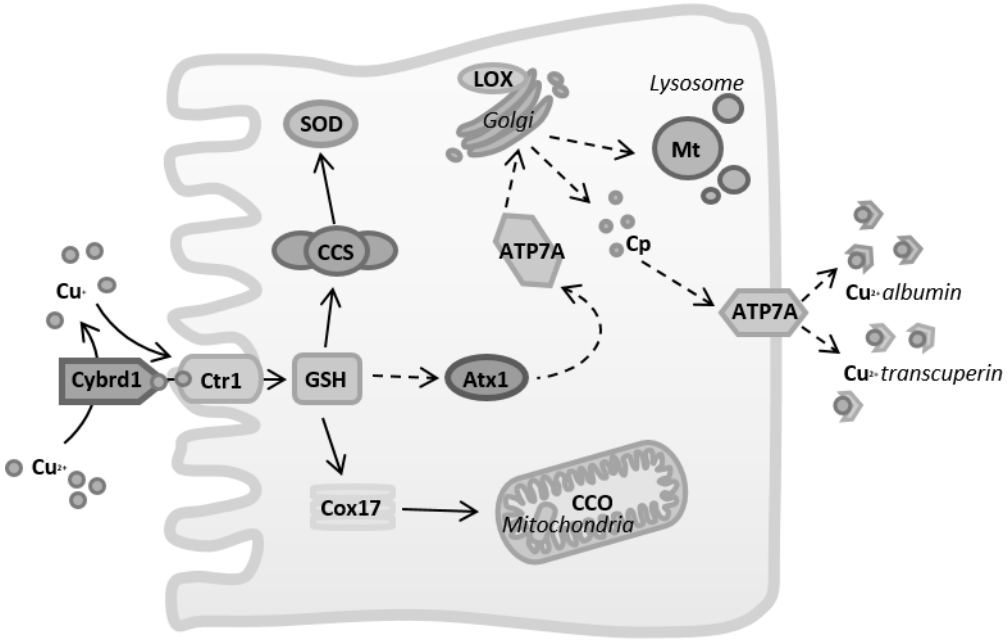

Figure 1: Copper trafficking pathways using the copper chaperones from the intestinal lumen. Atx1, antioxidant 1; CCO, cytochrome c oxidase; CCS, copper chaperone protein; Cox17, cyclo-oxygenase 17; Cp, caeruloplasmin; Ctr1, copper transporter 1; Cybrd1, cytochrome B reductase; GSH, glutathione; LOX, lysyl oxidase; Mt, metallothionein; SOD, superoxide dismutase.

Upon arrival at the intestinal brush border the membrane reductase Cybrd1 (Cytochrome B Reductase 1) and ascorbate (Vitamin C) reduce any dietary copper which is present as Cu^{2+} into Cu^{+} [36–38]. Reduced copper is carried across the membrane by high-affinity Copper transporter 1 (Ctr1) [34,39–41]. Once inside the cell it is immediately incorporated onto its specific

chaperones (CCS, Atx1 and Cox17) within the cytosol [42,43]. Copper chaperone protein (CCS) transports copper within the cytosol where the metalloenzyme Superoxide dismutase (SOD) is synthesised [17]. Cyclo-oxygenase 17 (Cox17) transports copper to proteins in the mitochondria where the metalloenzyme Cytochrome c oxidase (CCO) is synthesised [44,45]. Anti-oxidant 1 (Atx1) and ATP7A transport copper to the Golgi lumen where dopamine β-hydroxylase, peptidylglycine α-amidating monooxygenase, lysyl oxidase (LOX), SOD, tyrosinase, caeruloplasmin (Cp) and hephaestin vital for nerve and connective tissue function and for copper and iron transport are synthesised [18,46]. Surplus copper is bound to Metallothionein (Mt) and held in the lysosome after processing by the Golgi [18,44,47,48]. Upon reaching the metallothionein carrying capacity in the lysosome, surplus copper from the Golgi is transported using the ATP7A secretory pathway and effluxed from the enterocyte into circulation [17,18,45]. At the point of release from the cell membrane the oxygen tension of the interstitial fluid is sufficient to elicit spontaneous oxidation of the Cu^{+} to oxidised Cu^{2+} without the need for an oxidase in the membrane [49].

COPPER MOVEMENT IN THE BLOOD

Following efflux from the enterocytes copper is bound to albumin; an abundant plasma protein accounting for 15-20% of total copper transport, and transcuprein; a small protein which in contrast to albumin, is a specific copper carrier in plasma carrying 10-30% of total transported copper [34,50–53]. The concentration of albumin in blood plasma exceeds that of transcuprein, but transcuprein has a higher affinity for copper. Around a third of the copper entering the blood from the small intestine is bound to transcuprein [53]. These two proteins transport copper from the intestines through the systemic circulation to the liver. Metabolic studies have demonstrated that absorbed dietary copper from the portal circulation is cleared by the liver and appears in newly synthesised caeruloplasmin [54]. Caeruloplasmin is the predominant copper transporter in the systemic blood and is responsible for distribution of copper to the tissues after its synthesis in the liver [55,56]. In ruminants around 88% (range 86-90%) of total plasma copper is present bound to caeruloplasmin [57].

OVERVIEW OF HEPATIC COPPER TRAFFICKING

The liver has a major role in the regulation of copper [28]. This homeostatic control acts primarily through regulating the secretion of copper into bile [36,43,50,58]. Copper reaching the

liver is transported in a similar mechanism to the enterocytes. At the membrane the arriving copper is reduced and trafficked into the cell by the same copper transporter (Ctr1). Once inside the hepatocyte the chaperones fulfil their respective roles with one notable difference. The secretory pathway for efflux via the Golgi has a unique chaperone (ATP7B) which directs the majority of copper to be incorporated into caeruloplasmin which is then effluxed into circulation for distribution to other tissues. However, when caeruloplasmin bound copper from the peripheral tissues re-enters the circulation and returns to the liver the whole molecule of caeruloplasmin is absorbed for destruction and excretion through the biliary route.

The process in detail

Figure 2 illustrates the process described below.

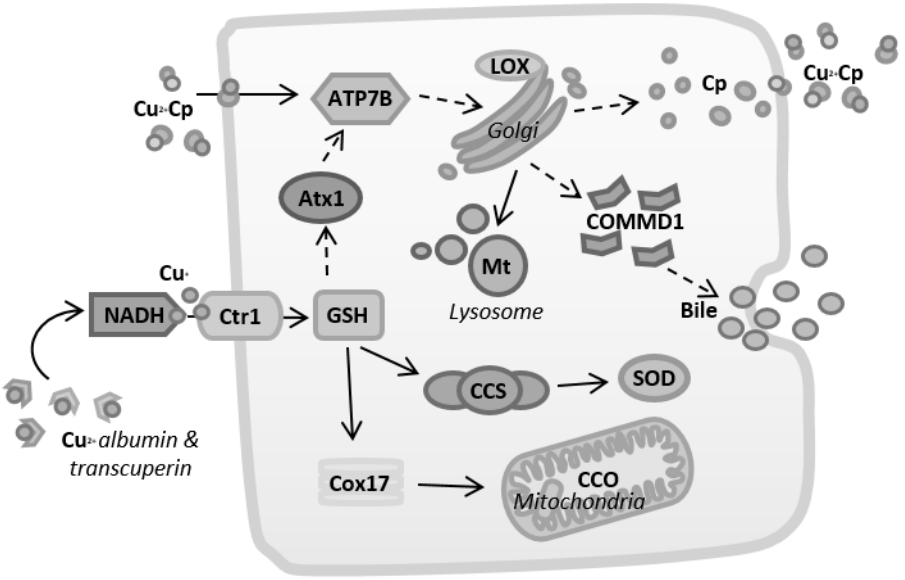

Figure 2: Copper trafficking pathways using the copper chaperones into hepatocytes and out into systemic circulation. Atx1, antioxidant 1; CCO, cytochrome c oxidase; CCS, copper chaperone protein; COMMD1, copper metabolism MURR1 domain; Cox17, cyclo-oxygenase 17; Cp, caeruloplasmin; Ctr1, copper transporter 1; Cybrd1, cytochrome B reductase; GSH, glutathione; LOX, lysyl oxidase; Mt, metallothionein; SOD, superoxide dismutase.

Copper reaches the liver bound to either transcuprein or albumin which are reduced on arrival by NADH oxidase [52]. Uptake of the reduced copper into the hepatocyte is mediated by Ctr1 [59]. Once inside, CCS and Cox 17 traffic their copper payload to the cytosol and mitochondria

respectively and Atx1 delivers copper to the Golgi body via ATP7B [60]. ATP7A is not expressed in the liver, instead hepatocytes express a unique version ATP7B [44]. ATP7B directs the majority of copper to be incorporated into caeruloplasmin to be subsequently returned to the circulation for distribution to other tissues [17,28,40,44,60]. When caeruloplasmin returns from systemic circulation to the hepatocytes the whole molecule is absorbed. The endothelial hepatocytes must first remove sialic acid residues from the caeruloplasmin to allow the underlying hepatocytes to absorb the caeruloplasmin molecule for proteolysis and destruction through the biliary route [58]. The excess hepatic copper is exported into the bile using the chaperones COMMD1 (copper metabolism MURR1 domain) and potentially also XIAP (X-linked inhibitor of apoptosis protein) [36,40,60]. COMMD1 binds to the N-terminal region of ATP7B but not to ATP7A, explaining the difference in ATPase channel expression between hepatocytes and other cells [60,61].

ADAPTATIONS TO CHANGING DIETARY COPPER SUPPLY

Under copper-limiting conditions the movement of copper into the secretory pathway (Atx1-ATP7A) is diminished in all tissues [25,50]. Copper bound to metallothionein is mobilised using the acidic pH of the lysosome to partially degrade the metallothionein held within the lysosome and release its copper into the cytosol [18,62,63]. The released copper is delivered, likely by glutathione (GSH), to the copper chaperones (cytosolic CCS and mitochondria targeting Cox17) equally, but not into the secretory pathway (Atx1) [25,63,64]. This redirection diminishes copper supply to the secretory pathway resulting in the production and secretion into the bloodstream of the copper-empty apo-caeruloplasmin, rather than its copper-containing holo form [63]. This process inhibits excretion and retains copper for intracellular use [65].

Under copper replete conditions in the tissues each of the copper transporters and proteins are down-regulated [25,48]. The down-regulation of copper transporter (Ctr1) in the membrane prevents any further copper uptake into the cell [66–68]. ATP7A (a chaperone in the secretory pathway) moves out of the trans-Golgi network into vesicles that move towards the membrane. These vesicles accumulate copper and intermittently fuse with the membrane to efflux the remaining excess copper from the cell into the blood before returning to the cytoplasm [69]. Increased metallothionein expression (regulated by Metal transcription factor MTF1) exerts intracellular homeostatic control through binding excess copper and acting as storage buffer protecting the cell [18,65].

When hepatocytes are exposed to increasing copper concentrations they behave similarly to other cells with one exception; ATP7B (from the hepatocyte secretory pathway) leaves the trans-Golgi network but instead of moving towards the membrane it moves towards the lysosome at the canalicular membrane [50,65]. Here, the ATP7B imports copper into the lysosomal lumen for temporary storage. Increasing intracellular copper concentrations induce exocytosis of the lysosome releasing the excess copper into the biliary canal (mediated by the secretory chaperones ATP7B and COMMD1) [25,36,60,70,71].

RUMINANT COPPER SENSITIVITY

When discussing the unique characteristics of ruminant copper handling it is important to first note that metallothionein knock-out animals, even from monogastric species, are hypersensitive to copper [72]. Sheep have a limited ability to synthesise metallothionein in response to rising copper concentration and they appear to have a restricted capacity to accumulate copper bound to metallothionein in the liver [56,73]. In comparison to rats, sheep reach a point where metallothionein synthesis is unable to keep up with rising copper at a much lower dietary inclusion resulting in less copper sequestering by the lysosome [73]. Additionally, sheep have a limited ability to increase biliary copper excretion in response to copper intake [74]. Cattle also have a lower capacity to store copper bound to metallothionein in comparison to monogastric species and a limited capacity to induce metallothionein in response to copper intake [56,75]. Furthermore, in cattle and sheep the copper-buffering capacity decreases as hepatic copper loading increases alongside the Cu:Zn ratio [76]. If the influx of copper exceeds the capacity of the metallothionein and lysosomal uptake, unbound copper will occur in the cytosol and begin to enter the nucleus, causing severe cell damage [76,77]. While, pigs and dogs have around 500-600 mg/kg, sheep and cattle have only ~200 mg/kg metallothionein in their livers [77]. Additionally, the metallothionein transcription in the lysosome of cattle and sheep does not effectively respond to rapid increases in copper [75,78], seemingly reaching a plateau of total copper concentration ~1,607 mg/kg DM (25,347 µmol/kg DM) in cattle and ~571-643 mg/kg DM (9,006- 10,142 µmol/kg DM) in sheep [74,75,77,78]. Potentially this plateau is linked to the limited production of metallothionein and an inhibited biliary copper excretion [74], theoretically explaining why cattle appear to be more copper tolerant than sheep and why both species appear sensitive in comparison to monogastric species such as pigs.

Further to species differences, breed differences among ruminants have also been documented. Texel sheep are more sensitive to copper than Landrace breeds [79,80]. In cattle, Holstein and Angus breeds are more copper tolerant than Jersey, Charolais and Simmental [81–83]. In cattle, the more copper tolerant breeds exhibit a greater expression of duodenal Ctr1 and ATP7A, and a higher hepatic expression of; Ctr1, Cox17, ATP7B, CCS and SOD where copper supply is inadequate [84,85]. These suggest the ability to increase expression of copper transporters and chaperones allows more effective uptake and utilisation where copper supply is insufficient; reducing the susceptibility of these breeds to deficiency in comparison to their counterparts [84,85]. This research highlights a potential mechanism for the observed breed differences, but further studies in a wider range of breeds and in sheep, under elevated and copper replete conditions would further clarify the role of transporter expression in copper sensitivity.

THIOMOLYBDATE DISRUPTION

Thiomolybdate is known to interact with copper. It naturally forms in the reducing environment of the rumen between dietary sulphur and molybdenum. Thiomolybdate poses a problem for copper availability and post-absorptive utilisation [29,86–88]. Thiomolybdates interact with available copper in the digestive tract forming an insoluble precipitate greatly reducing copper availability [29,86–89]. If there is insufficient copper where thiomolybdates form to 'de-toxify' them they can be absorbed into systemic circulation, where they exert their affinity for copper by complexing with copper contained in biological compounds rendering them biologically inactive [16,90]. Thiomolybdates are able to cross cell membranes but the mechanism by which this takes place is unknown. However, once inside the cell they have the potential to disrupt copper transport through binding to copper located on the copper chaperones, transporters and enzymes [17].

Thiomolybdates can bind to copper in cuproenzymes including; caeruloplasmin, metallothionein, CCO, SOD [90–93], and Atx1 [94]. Binding does not remove the copper component but renders it unable to perform redox reactions (vital to its biological function) through the formation of a stable complex [16,29,95,96]. Superoxide dismutase has been shown to differ and copper may be partially stripped from this enzyme [97,98]. In the case of the chaperone Atx1, thiomolybdate supresses the incorporation of copper into the products of the secretory pathway disrupting the activity of the Atx1 [94]. Thiomolybdates have a high affinity for copper and they have no effect on other trace metals with similar properties such as iron, zinc or cadmium [99,100].

PRACTICAL IMPLICATIONS

Copper provision in ruminants requires a careful balance between intake and availability. The inhibited capacity of these species to adapt to copper influx explains their sensitivity to overloading. Routine calculation of copper intake at farm level is not routinely undertaken which can lead to over-supply [11,101]. Calculation of copper supply in combination with monitoring of biological parameters as part of routine management allows a more accurate assessment of copper status across the entire flock or herd to be made [102]. At present, liver sampling is an under-utilised as a measure of herd or flock copper status, especially where there is a history of oversupply. Annual monitoring of a representative sample, from cull animals or from biopsy, allows more effective long-term decisions to be made for copper provision. It has been recently demonstrated that a significant linear relationship exists between increasing hepatic copper concentrations and the abundance of rhodamine stained granules in hepatic tissue histology [15]. This staining technique detects the copper-filled lysosomes which occur as the cellular mechanism for copper storage becomes overwhelmed [15]. In effect, their presence has the potential to be used as an indicator that copper concentrations are in excess; although this technique is not yet used in practice. Little correlation exists between hepatic copper concentrations and copper concentrations in blood parameters [30,103]. It is useful to bear this in mind and employ both techniques in conjunction with each other to establish animal status [30,103].

The potential danger posed through absorption of thiomolybdate causing disruption to systemic copper chaperones and cuproenzymes should also not be neglected. The use of blood assay is of importance to help monitor changes in shorter-term copper status. Decreases in caeruloplasmin activity can be a useful indicator of systemic thiomolybdate presence or copper deficiency over and above the use of caeruloplasmin concentration [91]. Since the apo-protein will continue to be synthesised in the absence of adequate, available hepatic copper while its activity can be reduced to nil [104]. This measure is not without flaws, as caeruloplasmin is an acute phase protein and can be elevated by infection or stress leading to falsely elevated measures of copper status [30,105,106]. Unfortunately, a single, reliable measure for copper status does not yet exist. Therefore, it is important to use both blood and hepatic measures in monitoring ruminant copper status in addition to monitoring nutritional input [11,101]. Furthermore, it is important in practice to provide an appropriate copper source, or combination

of sources, which will be sufficient to 'de-toxify' thiomolybdate before it is absorbed and retain a sufficient supply of labile copper for absorption which does not provide an excess or exceed legal restriction [101].

CONCLUSION

Advances in understanding of the physiology of intracellular copper transport from fundamental biology have not effectively penetrated the field of ruminant nutrition leading to widespread misunderstanding and consequently widespread copper imbalance in practice. The pathways of copper transport are synonymous with other mammalian species and much information is available to underpin nutritional theory for ruminants. Greater understanding of the trafficking pathways and their response to over and under copper supply allows decisions for copper supply to be more informed. In ruminants and in particular sheep, these pathways have a limited ability to respond to changes in dietary copper supply which explains this species sensitivity to copper oversupply. Thiomolybdates formed under ruminal conditions have been shown to be able to interfere with the copper chaperone pathways leading to cellular disruption of their function, if they are not effectively 'de-toxified' preventing their entry into systemic circulation. Considering the cellular pathways for copper and their potential disruption through thiomolybdate absorption can help to better inform supplemental actions to remedy copper-related disorders in practice.

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CONFLICT OF INTEREST

None

AUTHORSHIP

Initial planning and selection of areas to review- AH Clarkson, NR Kendall, J Martin-Tereso, S Paine

Review of research and article writing- AH Clarkson

Proofing of concept and article content and wording- NR Kendall, J Martin-Tereso, S Paine

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