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REVIEW

Consequences of trace metal variability and supplementation on Chinese hamster ovary (CHO) cell culture performance: A review of key mechanisms and considerations

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Abstract

Trace metals are supplied to chemically-defined media (CDM) for optimal Chinese hamster ovary (CHO) cell culture performance during the production of monoclonal antibodies and other therapeutic proteins. However, lot-to-lot and vendor-to-vendor variability in raw materials consequently leads to an imbalance of trace metals that are supplied to CDM. This imbalance can yield detrimental effects rooted in several primary mechanisms and pathways including oxidative stress, apoptosis, lactate accumulation, and unfavorable glycan synthesis. Recent research endeavors involve supplying zinc, copper, and manganese to CDM in excess to further maximize culture productivity and product quality. These treatments significantly impact critical quality attributes and furthermore highlight the degree to which trace metal availability can affect CHO cell culture performance. This review highlights the role of trace metal variability, supplementation, and interplay on key cellular mechanisms responsible for overall culture performance and the production and quality of therapeutic proteins.

KEYWORDS
CHO cell culture performance, raw material variability, therapeutic proteins, trace metal deficiency, trace metal supplementation, trace metals

1 | INTRODUCTION

Since the 1980s, monoclonal antibodies (mAbs) have emerged as the primary option for a variety of medical conditions including rheumatoid arthritis, multiple sclerosis, and various cancers (Singh et al., 2018). Global sales currently exceed $102 billion, accounting for approximately 48% of biopharmaceutical sales worldwide—a further indication of the success of mAb therapies (Dimitrov, 2012; Ecker, Jones & Levine, 2015). Due to their engineering versatility as well as their high productivity and robustness in cell culture, the industry has adopted the Chinese hamster ovary (CHO) cell line and its derivatives as the optimal hosts to produce these recombinant protein therapies (Fischer, Handrick & Otte, 2015). To correspond with the surge in mAb demand, primary research interests for industry involve the enhancement of cell culture performance to subsequently maximize the productivity of these therapeutic proteins. These efforts, which most notably include optimizing culture media feeding strategies, advanced clone selection methods, and cell line engineering techniques, have allowed the industry to achieve titers in excess of 10 g/L (Huang et al., 2010). CHO-K1 genomic sequencing, as well as complementary proteomic and transcriptomic analyses also provide substantial contributions to future cell line engineering efforts (Baycin-Hizal et al., 2012; Becker et al., 2011; Xu et al., 2011).

Trace metals facilitate a diverse range of intra- and extracellular functions in CHO cell culture necessary for optimal mAb productivity and quality. "Among these include: lactate consumption and energy metabolism, mAb productivity, and product quality (Kim & Park, 2016; Luo et al., 2012; Surve & Gadgil, 2015; Yuk et al., 2015)." Traditionally, trace metals (e.g., iron, copper, zinc, manganese) and other micronutrients were supplemented to culture media via 5–10% fetal bovine serum, or some alternative animal-based serum (Arigony

et al., 2013). However, lot-to-lot variability, irreproducibility, and concerns of microbial and viral contamination led to the advent of chemically-defined media (CDM), wherein prescribed concentrations of trace metals and nutrients are supplied de novo (Yao & Asayama, 2017). Although CDM provides much greater protection from such volatility, precise trace metal control, as well as comprehensive media characterization, are still difficult to achieve (Galbraith, Bhatia, Liu & Yoon, 2018; Lee, Christie, Liu & Yoon, 2012). And, while the source of variability in raw materials may be sometimes difficult to characterize, it is possible that any deficiency or overabundance of trace metals may disrupt the complex system of interacting mechanisms between metabolites.

Recent research endeavors involve further supplementing CHO culture media with excess concentrations of trace metals to subsequently maximize culture performance and productivity, as well as stabilize favorable glycosylation patterns and flux distributions of metabolites (Hutter et al., 2017; B. G. Kim & Park, 2016; Surve & Gadgil, 2015; Yuk et al., 2015). Conversely, any deficiency of trace metals due to raw material variability may consequently lead to undesired culture behavior. For example, zinc deficiency has long been known to induce early death of mammalian cells (Clegg, Hanna, Niles, Momma & Keen, 2005). Magnesium and calcium deficiency has shown to induce early apoptosis through the stress response of membrane scavenger receptor B1 in CHO cells (Feng, Guo, Gao & Li, 2011). Concerns of copper deficiency in relation to lactate metabolism in CHO culture have also been addressed (Yuk et al., 2015). Cell growth and mAb productivity have also shown to be significantly impacted in multivariate models by slight trace metal variability (Trunfio et al., 2017). These phenomena furthermore highlight the importance of trace metal availability and concentration repeatability in culture media.

Trace metal interplay in CDM can differ significantly than from animal-based sources, wherein trace elements are regulated by naturally occurring proteins. Recent developments have furthered the understanding of trace metal impact on performance and quality of CHO cell processes cultured in CDM, both via supplementations and deficiencies of trace metals. This review aims to highlight the potential sources and consequences of metal variability in culture media, and furthermore explore the specific mechanisms of action responsible for shifts in mAb productivity and product quality via trace metal induced-metabolism. Here, we aim to provide an insightful look into the role of trace metal interplay in several key areas during the production of therapeutic proteins.

2 | VENDOR AND BATCH/LOT VARIABILITY OF METAL CONTENT IN CULTURE MEDIA

The trace metal content of culture media is subject to variation from both vendors and batches/lots alike. Although the appropriate metal content of media is predefined by each supplier, there still exist large variations from one supplier to the next. For example, one Food and Drug Administration survey of six commercially available CDMs for CHO culture points to wide ranges of variance in several key trace metals between vendors: iron (~437 μM range), zinc and copper, (~8 μM range), and manganese (~0.8 μM range; Mohammad et al., 2019). Examinations into metal content within multiple lots of media from the same supplier also show substantial variability. Analysis of two lots of serum-free CHO media from a leading commercial supplier revealed significant differences in iron, copper, zinc, selenium, and cobalt content (Keenan et al., 2018). An extension of this analysis also revealed that copper fluctuations from various lots of basal media were substantial enough to alter procaspase-3 expression in Caco-2 cells. A breakdown of copper content within 10 different lots of an undefined nutrient powder used in NS0 culture for mAb production showed similar fluctuations, offering a key avenue by which trace metals may be inadvertently introduced to culture media (Mondia et al., 2015).

Oftentimes, plant-based hydrolysates containing various vitamins, amino acids, and metabolites are provided to culture media to enhance CHO culture performance (Chun, Kim, Lee & Chung, 2007; Richardson et al., 2015). However, preparations of hydrolysates are subject to large fluctuations of content within various lots, further confounding efforts to characterize raw material and batch-to-batch variability (Li, Vijayasankaran, Shen, Kiss & Amanullah, 2010; Richardson et al., 2015; Trunfio et al., 2017). Hydrolysates commonly include various iron salts, therefore, providing additional incidental iron content to culture media (Kim & Lee, 2009). These incidental iron salts may also yield additional metal impurities as well. For example, ionic sulfates, which are provided to culture media to meet the prescribed metal demand, also contain added metal contaminants. Trace iron and other elemental impurities can be introduced to ionic sulfates at various stages of the refining process, including metal recovery from leaching solutions as well as precipitation operations (Crundwell, Moats, Ramachandran, Robinson & Davenport, 2011; Pakarinen & Paatero, 2011; Zhang & Cheng, 2007). Consequently, assorted lots of metal salts that are introduced to culture media are thus subject to these inconsistencies in trace metal content.

To eliminate any viral contamination that may be present during culture media preparations, batches of media and feeds often undergo high-temperature short-time pasteurization (HTST). These treatments elevate media and feed temperatures and pressures for designated periods of time for effective viral inactivation (Kiss, 2011; Weaver & Rosenthal, 2010). However, ionic salts can precipitate out of solution during this process. For example, metal ions such as calcium, magnesium, and iron can be supplemented to media by way of various chlorides, sulfates, nitrates, and citrates. After dissociating in solution, these ions can form small concentrations of ionic phosphates (Cao, Stimpfl, Wen, Frank & Hunter, 2013). During HTST treatments, elevated temperatures in excess of 100°C can drive these ionic phosphates and other inorganic salts out of solution (Cao, Loussaert & Wen, 2016; Cao et al., 2013; Floris, Curtin, Kaisermayer, Lindeberg & Bones, 2018; Pohlscheidt et al., 2014). Consequently, lot-to-lot variabilities of trace metal content are accentuated after

the precipitating solids are removed from the solution. In addition, precipitating salts have also shown to cause membrane fouling, which can further lead to ineffective media processing and compromise batch sterility as well (Cao et al., 2016; Pohlscheidt et al., 2014).

Trace metal leaching is an additional area of concern which can potentially impact CHO cell culture performance and mAb quality. Stainless steel and glass bioreactors can leach various amounts of trace metals which can subsequently affect CHO culture performance, as well as drug efficacy and stability. Several comprehensive reviews cover the breadth of leaching in biopharmaceutical processes through 2014 (Gilbert, Huang & Ryll, 2014; Kumar, Zhou & Singh, 2014). However, recently, this scope of impact has included mAb quality as well. Manganese leaching from glass bioreactors anywhere from 50 to 200 nM has shown to increase percentages of terminal galactosylation in mAbs (Williamson, Miller, McLaughlin, Combs & Chu, 2018). Magnetic stir bars have also shown to leach stainless steel-based metals (e.g., iron, chromium, nickel, and manganese) and significantly impact protein concentration and aggregation (Thompson et al., 2017). Among the many financial benefits of utilizing single-use technology, the decreased risk of microbial contamination is also advantageous. Yet even with single-use systems, metal leaching has shown to be a concern (Gao & Allison, 2016). As this occurs during culturing operations, efforts to control these trace metal variations CDM become more complex.

3 | IMPACT OF TRACE METALS AND MEDIA ADDITIVES ON OXIDATIVE STRESS IN CHO CULTURE

The interplay between trace metals and other media additives introduces additional complexities with regard to oxidative stress. Variations of light exposure to different lots of culture media have shown to induce photodegradation of essential B vitamins, leading to stressed mammalian cell cultures and amino acid oxidation (McElearney, Ali, Gilbert, Kshirsagar & Zang, 2016; Schnellbaecher, Binder, Bellmaine & Zimmer, 2019; Zang et al., 2011). Here, the rate of photodegradation is exacerbated by trace metal ions, particularly zinc and copper (Ahmad, Anwar, Ahmed, Sheraz & Khattak, 2017). Vitamin C is also often added to culture media for defense against oxidative stress. However, xylosone, a biproduct of Vitamin C degradation, has shown to increases acidic charge variants on mAbs (Chumsae et al., 2015). Trace metals have also demonstrated both stabilizing and degrading impacts on Vitamin C in solution (Dolinska et al., 2012; Yao & Asayama, 2017). Thus, it is possible that an inadequate trace metal supply has the capacity to induce oxidative stress and negatively affect mAb quality, as lapses in productivity and destabilizing conformational changes of mAbs have been associated with oxidative stress (Burkitt, Domann & O'Connor, 2010; Ha, Hansen, Kol, Kildegaard & Lee, 2018; Handlogten, Zhu & Ahuja, 2018).

The detrimental effects of reactive oxygen species (ROS) such as super-oxides, peroxides, and hydroxyl radicals in mammalian cell culture are well established and are frequently tied to the availability of free transition metal ions (Halliwell, 2003). The accumulation of free hydroxyl radicals, which damage a diverse array of biomolecules, is catalyzed by a redox-cycling of copper (Cu) and iron (Fe) ions known as Fenton chemistry (Jomova, Baros & Valko, 2012; Park & Imlay, 2003; Winterbourn, 1995):

$$\text{Reductant} + Cu^{2+}/Fe^{3+} \rightarrow \text{Reductant} + Cu^{1+}/Fe^{2+} \quad (1)$$

$$Cu^{1+}/Fe^{2+} + H_2O_2 \rightarrow Cu^{2+}/Fe^{3+} + \cdot OH + OH^- \ (\text{Fenton reaction}) \quad (2)$$

The oxidation of a reductant molecule (e.g., cysteine) by free Cu^{2+}/Fe^{3+} ions can activate disulfide bond formation to cystine, causing a loss in oxidation state of the ions (Figure 1). The subsequent decomposition of hydrogen peroxide by Cu^{1+}/Fe^{2+} ions induces the formation of free hydroxyl radicals. Moreover, the regenerated oxidation state of Cu^{2+}/Fe^{3+} allows the ions to continuously partake in the Fenton mechanism.

Oxidative stress in serum-based media is regulated by a host of antioxidants and antioxidation qualities inherent to animal serum (Yao & Asayama, 2017). For example, bovine serum albumin proteins regulate oxidative stress via several primary mechanisms. Among the 17 disulfide bonds in albumin is one free cysteine residue (Cys34), which is known to scavenge free radicals while methionine residues simultaneously chelate redox-active transition metals (Francis, 2010). An additional mechanism of stress prevention includes disulfide formation at the Cys34 site. Here, free cysteine can form a disulfide complex to forestall its availability for Fenton reactions. The Cys34 site may also bind glutathione (GSH), an abundant and powerful antioxidant in its reduced form. Among the host of antioxidative mechanisms of GSH to scavenge ROS, its affinity to chelate Cu^{2+} further acts to inhibit the development of ROS species (Couto, Wood & Barber, 2016; Freedman, Ciriolo & Peisach, 1989). Many different strategies for supplementing medleys of nutrients in lieu of animal serum exist (van der Valk et al., 2010). Although it is commonplace to counterbalance trace metal supplementation with recombinant albumin and other antioxidants that are no longer supplied endogenously via animal serum, insufficient considerations here may yield pro-oxidant media and cause an array of dubious artifacts in culture (Halliwell, 2014; McGillicuddy, Floris, Albrecht & Bones, 2018).

In addition to the copper- and iron-complexing proteins which inhibit the formation of ROS, zinc also serves as a very powerful antioxidant in culture. (Powell, 2000). A cofactor for over 300 different enzymes and transcription factors, sufficient concentrations of zinc are required for proper cellular functioning and metabolism (Marreiro et al., 2017). With regard to its antioxidation properties, several primary mechanisms help to demonstrate zinc's ability to most effectively combat redox-induced ROS. Zinc has a high affinity to stabilize and protect both intra- and extracellular sulfhydryl groups from oxidation and subsequent ROS formation (Eide, 2011). Zinc has just one oxidation state (Zn^{2+}) and thus will not participate

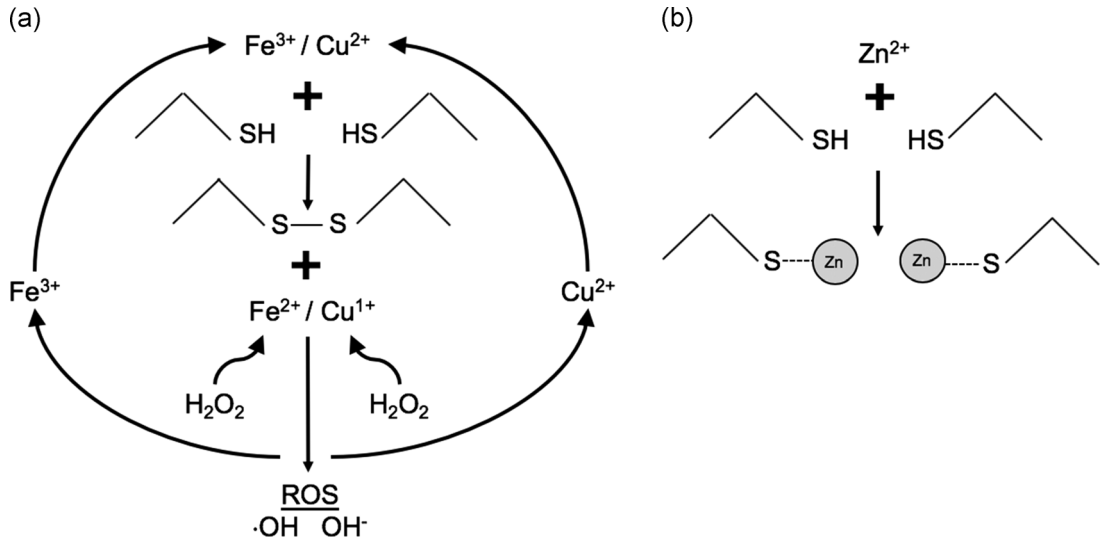

FIGURE 1 Visual representation of Fenton mechanisms on intracellular cysteine; (a) redox-active transition metals catalyze disulfide bond formation and subsequently induce ROS production via the Fenton reaction; (b) zinc stabilizes free sulfhydryl residues thereby inhibiting ROS formation. ROS, reactive oxygen species

in redox activity. Instead, it will bind onto the sulfhydryl group on cysteine molecules to inhibit Fenton mechanism otherwise caused by copper and iron (Pace & Weerapana, 2014; Truong-Tran, Carter, Ruffin & Zalewski, 2001).

Metallothioneins (MTs) are a group of thiol-rich proteins which play a key role in the intracellular maintenance and redistribution of zinc and copper throughout the cell. Comprised of approximately 30% sulfhydryl residues, these proteins maintain a very high affinity to bind heavy metals and subsequently work to prevent metal toxicity (Thirumoorthy, Manisenthil Kumar, Shyam Sundar, Panayappan & Chatterjee, 2007). Furthermore, the ability of MTs to scavenge free hydroxyl radicals provides key mechanisms for antioxidation interplay (Ruttkay-Nedecky et al., 2013; Valko, Jomova, Rhodes, Kuča & Musílek, 2016). However, due to gene methylation, CHO cells inherently express low levels of MT (Andersen et al., 1987; Lin, Chen, Lee & Lin, 2005; Yu, Chen & Lin, 1998). However, even in the case of overexpression, the extent to which MTs can combat cytotoxicity under a prearranged zinc surplus in CHO-K1 cultures is limited, as cell cycle progression may not be affected by an excess of MT-1 (Smith, Wiltshire, Furon, Beattie & Errington, 2008). This becomes especially important when considering strategies that involve supplementing cultures with zinc and/or copper in excess to enhance productivity and quality of mAbs. Here, counterbalancing a zinc or copper surplus by enhancing antioxidation affinities or mechanisms may be necessary. For example, Zn/Cu superoxide dismutase (SOD1) is a well-characterized enzyme in mammalian cells that binds zinc and copper to subsequently catalyze the decomposition of superoxide radicals (O_2^-; Fukai & Ushio-Fukai, 2011). Previously, messenger RNA sequencing of CHO cultures exposed to enhanced supplements of tryptophan, manganese, and copper revealed altered expression of both redox-controlling and copper-transport genes, including SOD1 (He et al., 2018). Human SOD1 transfection and upregulation have previously been applied to CHO cells for research in amyotrophic lateral sclerosis (Brotherton, Li & Glass, 2013). However, to date, there are no reports examining the impact of overexpressed SOD1 on mAb production or product quality, nor the impact of SOD1 regulation upon enhanced zinc supply to CHO culture. Tryptophan oxidation, which has shown to affect the antigen-binding capacity of certain mAbs, was also mitigated by tryptophan, manganese, and copper supplementation (Hazeltine et al., 2016; Z. Wei et al., 2007). This treatment similarly lowered free cysteine concentrations, minimizing the capacity for Fenton chemistry perhaps otherwise enhanced by copper supplementation. mAb conformation and immunogenicity are significantly affected by the oxidation of other amino acids on immunoglobulin G (IgG) side chains as well (Torosantucci, Schöneich & Jiskoot, 2014). Like cysteine, methionine similarly contains sulfur and is prone to oxidation in IgG antibodies (Kim, Weiss & Levine, 2014). With regard to trace metals, copper-induced stresses have shown to impact aggregation affinity of IgG2 mAbs via Met246, His304, and His427 oxidation on the Fc region as well (Luo et al., 2011).

It is proposed that zinc, copper, and iron will compete for sulfhydryl binding sites linked with oxidative stress in mammalian cells (Eide, 2011; Valko et al., 2016). Inherent trace metal variability may impact a series of stress-mechanisms. For example, excess zinc can induce a copper deficiency, while zinc deficiencies have previously been reported alongside excess copper buildup (Gaetke, Chow-Johnson & Chow, 2014; Marreiro et al., 2017). In either case, mechanisms of oxidative stress are activated. A relationship between the concentration of intracellular free zinc and copper ions and ROS may help to characterize the presence and effects of competition between metals. Because zinc can displace copper from binding sites, any slight imbalance in raw material content may generate a more substantial course of pro-oxidation within a culture (Gaetke & Chow, 2003). Although redox-active, manganese is needed as a cofactor for manganese SOD2 and, therefore, serves as an antioxidant as well.

However, manganese can also compete with iron for binding sites on SOD, constraining these manganese-activating antioxidation mechanisms (Aguirre & Culotta, 2012). With specific regard to CHO cell culture, there have been no studies to further elucidate how this competitive behavior may reflect on critical quality attributes of mAbs.

4 | TRACE METAL AVAILABILITY ON CHO CELL METABOLISM AND MAB PRODUCTION

Trace metals are often supplied to culture media in excess to maximize mAb productivity and quality profiles (Gilbert et al., 2014). For example, up to 60 μM of zinc sulfate supplemented to CHO-DG44 cultures has been shown to enhance mAb productivity by approximately two-fold (Kim & Park, 2016). Similarly, cultures inoculated with excess copper or exposed to daily copper provisions up to a necessary threshold exhibited an increase in mAb productivity as well as an increase in basic charge variants (Yuk et al., 2015). These copper treatments are also favorable to CHO cultures undergoing hypoxic stress, as enhanced copper can upregulate and stabilize the expression of hypoxia-inducible factor 1 alpha (Martin et al., 2005; Yuk et al., 2014). During process scale-up, when hypoxic conditions are oftentimes more profound due to the enhanced cellular demand for oxygen, additional copper and iron supplementation have shown to enhance cell growth and viability as well as productivity of a recombinant protein under otherwise hypoxic conditions (Qian et al., 2014).

Along with increasing mAb productivity, zinc supplementation may also help to achieve the necessary threshold for apoptosis suppression. Zinc is a known regulator of apoptosis in mammalian cells via a host of different mechanisms and pathways. The availability of zinc has shown to both influence and inhibit apoptosis in various cancer cells (Franklin & Costello, 2009). For example, in mice thymocytes, apoptosis induction/suppression by zinc sulfate was dose-dependent (Provinciali, Stefano & Fabris, 1995). Here, smaller concentrations of added zinc to serum-free media (7.5–15 μM) induced apoptosis, whereas more significant zinc supplementation (75–600 μM) suppressed apoptosis. Apoptotic sequences may occur via either the extrinsic or intrinsic pathway. Both pathways involve the activation of cysteine-aspartic acid protease 3 (caspase 3) to initiate the execution pathway towards programmed cell death (Elmore, 2007). The extrinsic pathway can be triggered by the interaction between a Fas ligand and a trio of Fas receptors (death receptors) on a target cell (Elmore, 2007; Eron, MacPherson, Dagbay & Hardy, 2018). A death-inducing signaling complex is subsequently completed by caspase 8 or caspase 10, which in turn cleaves caspase 3 and induces the execution pathway. A tumor necrosis factor model similarly induces the execution pathway via the caspase 8/10 and caspase 3 cascade (Elmore, 2007; Eron et al., 2018). The relationship between the extrinsic pathway and zinc availability has not been widely examined (Clegg et al., 2005; Eron et al., 2018). However, zinc has shown to prevent caspase 8 dimerization and activation by binding at two different sites (Eron et al., 2018). Specifically regarding CHO cells, previous examinations have noted evidence of apoptosis via the extrinsic pathway (Wei et al., 2011). However, proteomic analysis of apoptotic CHO cells subsequently pointed to the intrinsic pathway as the dominant apoptosis mechanism based on the abundance of caspase 9 in the early stages of cultures (Wei et al., 2011).

Efforts to elucidate the role of trace metals on apoptosis suppression commonly involve the intrinsic pathway (Figure 2). The intrinsic pathway is initiated by the phosphorylation of the Bad protein. If not phosphorylated, Bad will complex with Bcl-2/Bcl-XL on the outer mitochondrial membrane and allow for the release of cytochrome C and subsequent formation of the apoptosome, which activates caspase 9 and initiates the execution pathway

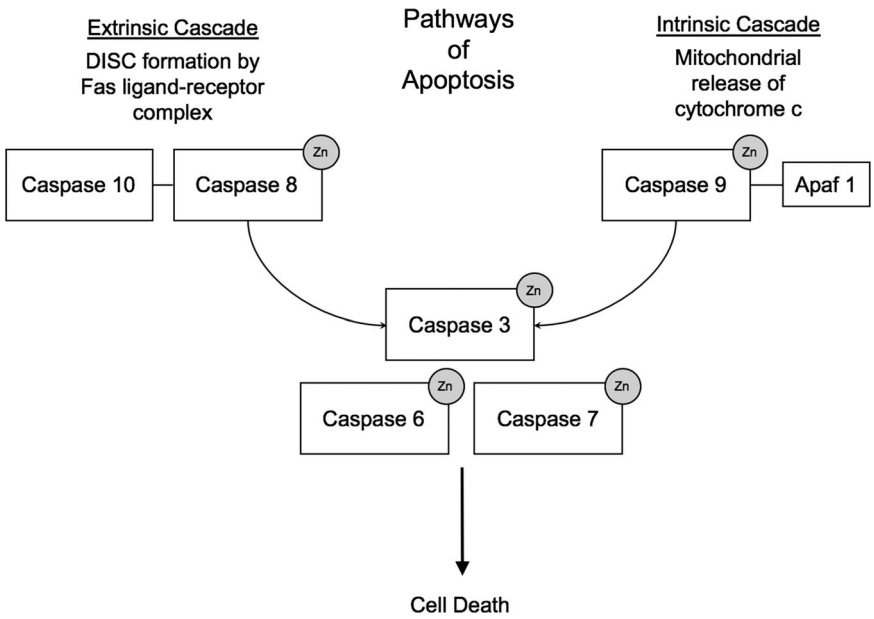

FIGURE 2 Simplified caspase cascade as affected by free zinc ions; caspase proteins in both intrinsic and extrinsic cascades can bind zinc on sulfhydryl residues as a reported mechanism for suppressing apoptosis

(Elmore, 2007). Zinc has shown to regulate caspase 9 activity by way of two cysteine-rich binding sites responsible for zinc-mediated inhibition (Huber & Hardy, 2012). Executioner caspase 3 is also regulated by zinc at thiol-rich sites (Eron et al., 2018; Perry et al., 1997). Successful efforts to inhibit caspase 3 activity via glutathionylation further help to draw a parallel between apoptosis and oxidative stress with regard to zinc availability (Z. Huang, Pinto, Deng & Richie, 2008). For example, apoptosis mechanisms are frequently tied to ROS via several reported mechanisms, including procaspase and cytochrome C release from mitochondria during oxidative stress (Anathy et al., 2012; Kannan & Jain, 2000; Zuo et al., 2009). Although specific mechanisms remain unclear, it is possible that enhanced zinc may protect caspase thiol residues from oxidation by Fenton-induced ROS and subsequently combat oxidative stress to suppress apoptosis (Huber & Hardy, 2012; Truong-Tran et al., 2001).

CHO cell-specific investigations into these phenomena help to expand the applicability of these mechanisms to mAb productivity. Apoptotic CHO cells may similarly demonstrate the overexpression of lactate dehydrogenase (LDH), which catalyzes pyruvate to lactate proceeding the glycolytic pathway (Wei et al., 2011). Lactate accumulation causes adverse effects in CHO cell culture, primarily on cell growth and productivity (Konakovsky et al., 2016; Zagari, Jordan, Stettler, Broly & Wurm, 2013). The effects of apoptosis-inhibited environments on lactate metabolism have been characterized. Here, efforts to modulate CHO metabolism to limit lactate build-up have included the overexpression of antiapoptotic genes, which demonstrated a 60% reduction in caspase 3 activity (Dorai et al., 2009). Cell line and metabolic engineering approaches to minimizing lactate accumulation have furthermore involved inducing a shift to lactate consumption during the stationary phase of CHO cultures (Toussaint, Henry & Durocher, 2016). This effect coincides with more optimal culture performance and higher protein titers and is thus a desirable characteristic for cell culture.

A wide variety of approaches to controlling this lactate shift exist, which include reducing glycolysis flux, limited amino acid supplementation, and a host of various cell line engineering techniques (Hartley, Walker, Chung & Morten, 2018; Kishishita et al., 2015; Mulukutla, Gramer & Hu, 2012). With specific regard to trace metals, copper supplementation to chemically-defined media has also demonstrated more favorable culture performance by constraining lactate accumulation in CHO cultures. At 5 μM concentrations of copper, downregulation of the LDH gene (*Ldha*) was noticed in an IgG producing CHO cell line—likely accounting for decreased lactate accumulation (Qian et al., 2011). Additional conclusions from this examination help to elucidate the potential role of copper supplementation on iron transport and ROS accumulation—a key area of trace metal interplay in cell culture. The downregulation of the transferrin receptor gene *Tfrc* demonstrates a possible effect of copper on limiting iron transport/accumulation. A downregulation of NADPH oxidase 4 (*Nox-4*) was also observed. A corresponding decrease in Nox-4 may help prevent the accumulation of ROS, perhaps countering the affinity of copper to oxidize free thiol residues and induce Fenton chemistry.

Gene regulation as documented by Qian et al. (2011) is not ubiquitous in CHO cultures exposed to comparable levels of copper treatments. On a different mAb-producing cell line, microarray, and RNA-seq characterizations of gene expression were unable to reveal a substantially up- or downregulated gene related to lactate metabolism line (Yuk et al., 2014). Differences in sampling times, cell lines, and inherent trace metal availability in media are possible reasons for the contrast in the degree to which copper-induced lactate shift is initiated at gene transcription. Upregulation of early growth factor 1 may be a response to copper-induced oxidative stress, yet this observation occurred after the lactate shift and hence the impact of oxidative stress is unclear (Yuk et al., 2014). However, it has been shown that CHO cultures undergoing a shift to net lactate consumption similarly demonstrate increased oxidative metabolism (Templeton, Dean, Reddy & Young, 2013; Zagari et al., 2013). Corresponding observations also include decreasing intracellular redox ratios and upregulated oxidative pentose phosphate pathway, likely as a combative response to ROS generated by accelerated oxidative metabolism (Sengupta, Rose & Morgan, 2011; Templeton et al., 2013). In fact, dissolved oxygen content at 50% air saturation similarly demonstrated a shift to net lactate consumption in GS-CHO cells, although redox activity is significantly affected by this treatment (Handlogten et al., 2018).

5 | IMPACT OF TRACE METAL SUPPLEMENTATION ON PRODUCT QUALITY

N-linked glycosylation of mAbs plays a pivotal role in protein folding as well as key quality metrics such as solubility, product half-life, and efficacy (Mimura et al., 2018; Solá & Griebenow, 2009; Zheng, Bantog & Bayer, 2011). The effects of different glycosylation patterns on product quality is a well-characterized focus in therapeutic drug production (Sha, Agarabi, Brorson, Lee & Yoon, 2016). Zinc, copper, and manganese have shown to affect mAb glycosylation to different degrees of significance. While maximizing mAb productivity via zinc supplementation, cultures on the threshold of zinc toxicity can decrease galactosylation on IgG mAbs, whereby a zinc/manganese imbalance triggers unfavorable galactosylation patterns (Prabhu, Gadre & Gadgil, 2018). Upon supplementing CHO cell cultures with excess copper up to the desired threshold, Yuk and coauthors noticed an increase in basic charge variants alongside increased culture productivity and shift to net lactate consumption (Yuk et al., 2015). Copper supplementation has also been reported to enhance the percentage of basic charge variants via deamidation on C-terminal prolines of an IgG1 mAb (Kaschak et al., 2011).

Manganese is a well-known cofactor for several glycosyltransferase enzymes involved in *N*-linked glycan synthesis, including galactosyltransferases which are responsible for the addition of galactose monosaccharides onto various glycoforms, such as asparagine 297 (Asn-297) residues on the side chains of IgG mAbs (Zheng et al., 2011). Further supplementation of CHO cell cultures with

excess manganese has demonstrated various results on glycan conformation. By supplementing 40 μM manganese salts to a CHO-K1 cell line, increases in G1F abundance along with a decrease in G0 and G0F glycans on an IgG1 mAb have been reported (St Amand, Radhakrishnan, Robinson & Ogunnaike, 2014). However, examinations into the effects of manganese on high mannose glycoforms have reported conflicting results. For example, Pacis et al (2011) reported a decrease in high mannose (M5) glycoforms upon the supplementation of 0.25–1.0 μM manganese on Day 3 of an IgG1-producing culture (Pacis, Yu, Autsen, Bayer & Li, 2011). In contrast, Surve and Gadgil (2015), demonstrated a 27% increase in high mannose glycans when supplementing 16 μM manganese to the 1 nM basal concentration. However, this result was predicated on the limited availability of glucose to the culture media, as it was exchanged for galactose in efforts to constrain lactate accumulation to nontoxic levels. Here, minimizing the presence of high mannose glycans by way of manganese supplementation was incumbent on a needed threshold of glucose available in the culture media. Enhanced copper supplementation has also shown to minimize glucose consumption, although no mention of the impact of high copper media on product quality was provided (Luo et al., 2012). Furthermore, there are no additional reports on the collaborative role of both copper and manganese supplementation on glucose metabolism and the subsequent impact on *N*-linked glycosylation patterns.

Batch and fed-batch cultures have been employed to characterize the effects of manganese on glycosylation, however, recent studies have also included perfusion processes. Here, galactose and manganese supplementation have shown to enhance the abundance of complex glycans through a 20-day run (Karst et al., 2017). The degree to which these supplements impact glycan synthesis is, however, governed by various culture parameters which may oscillate with time. Thus, manipulating feeding regimes of manganese and other nutrients can help to maintain a consistent glycosylation pattern throughout the duration of the process (Villiger, Roulet et al., 2016). Modeling efforts have furthermore allowed for the prediction of glycosylation patterns under these same feeding treatments (Villiger, Scibona et al., 2016). A separate study similarly concluded that timewise supplementation of manganese is critical for glycosylation control in mAbs (Radhakrishnan, Robinson & Ogunnaike, 2018). During both lag and exponential phases, additional manganese supplementation demonstrated a more significant impact on fucosylated glycans than during the stationary phase. This examination concurrently focused on metal chelation using ethylenediaminetetraacetic acid (EDTA) in a design of experiments to further demonstrate the extent of controllable glycosylation. When supplied in the presence of additional manganese, EDTA supplementation showed to enhance both cell growth and mAb titer alike, as well as alter the distribution of glycans (Radhakrishnan et al., 2018). Because EDTA can chelate any number of trace metals in cell culture media, a unique relationship between excess manganese and adjusted trace metal availability by EDTA is presented. However, there are no further examinations into metal-targeted specificity of EDTA in CHO culture alongside additional trace metal supplementations, Table 1.

TABLE 1 Trace metal availability on cell functionality and potential impact on mAb production

Zinc		**Copper**		**Manganese**	
Supplementation enhances specific productivity of IgG1	Kim & Park, 2016	Toxic conditions induce IgG2 aggregation	Luo et al. (2011)	High mannose glycans increased under limited glucose	Surve and Gadgil (2015)
Increase of availability in the cytoplasm upon the overexpression of MT-1	Smith et al. (2008)	Additional provisions increase IgG1 productivity, increase basic charge variants, shift to lactate consumption; upregulation of early growth Factor 1 (*EGR1*)	Yuk et al. (2015); Yuk et al. (2014)	Minimize tryptophan oxidation when supplemented with copper; alters the expression of redox- and copper transport-related genes	Hazeltine et al. (2016); He et al. (2018)
Characterization of two cysteine binding sites on caspase 9	Huber and Hardy (2012)	Supplementation downregulates lactate dehydrogenase (*Ldha*) expression	Qian et al. (2011)	Increase G1F glycan, decrease G0 and G0F glycans on IgG1	St. Amand et al. (2014)
Apoptosis suppression mechanisms on caspases 3, 6, 7, and initiator caspase 8	Eron et al. (2018)	Increase basic charge variants via deamidation on c-terminal prolines of IgG1	Kaschak et al. (2011)	Increase the percentage of high mannose glycan; decrease the percentage of high mannose glycan	Surve and Gadgil (2015); Pacis et al. (2011)
Supplementation decreases galactosylation of IgG	Prabhu et al. (2018)	Decreased glucose consumption rate	Luo et al. (2011)	Supplementation with galactose increases complex glycan in perfusion reactor	Karst et al. (2017)

Abbreviations: IgG, immunoglobulin G; mAbs, monoclonal antibodies; MT, metallothionein.

6 | FUTURE DIRECTIONS AND CONCLUSION

These research endeavors help to illustrate the role that traces metal presence has on CHO culture productivity and quality via oxidation/antioxidation mechanisms, apoptosis, glucose and lactate metabolism, glycosylation, and corresponding genomic and transcriptomic characterizations thereof. Furthermore, the degree of trace metal variability which exists on both a lot-to-lot and vendor-to-vendor basis adds additional complexities to this focus. More comprehensive quantification of metal ions in CHO culture can help to further elucidate the key areas by which trace metal variability is impactful. In this respect, it is important to continue consideration of the impact of trace metal variability on the multitude of CHO culture performance characteristics such as oxidative stress and mAb oxidation, apoptotic cascades, nutrient consumption, and glycosylation. Efforts to streamline trace metal analytics into process analytical technologies and subsequent modeling efforts may help to further understanding of the degree to which trace metal variability impacts overall culture performance. In doing so, in-line trace metal analysis and subsequent control strategies may be employed.

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