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REPORT

Copper-induced non-monotonic dose response in Caco-2 cells

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Abstract

Copper is an essential dietary micronutrient in humans for proper cell function; however, in excess, it is toxic. The human cell line Caco-2 is popular as an in vitro model for intestinal absorption and toxicology. This study investigated the response of exponentially growing Caco-2 cells to prolonged copper exposure (120 h). An unexpected non-monotonic dose-response profile was observed in Caco-2 cells. Exposure to media supplemented with 3.125 μM $CuSO_4$ resulted in decreased cell yield vs. untreated. However, toxicity was progressively reduced from 90% at 3.125 μM to 60% at 25 μM. This effect was documented between 48 and 120 h continuous exposure ($p < 0.05$). This triphasic toxicity curve was observed to be specific to copper in Caco-2 cells, as iron, manganese and zinc displayed monotonic dose-response profiles. Two inorganic copper forms, copper sulphate and copper chloride, were shown to conserve the non-monotonic dose-response curve. The triphasic effect was shown to be specific to Caco-2 cells. These results have implications for research investigating the effect of copper and other micronutrients using Caco-2 cells.

Keywords Dose response · Copper · Caco-2 · Triphasic · Trace elements · Toxicity

Introduction

Copper is a redox-active transition metal required for survival by all aerobic eukaryotic organisms, yet causes toxicity when present in excess, due to catalytic Fenton and Haber–Weiss reactions leading to hydroxyl radical formation (Kozlowski et al. 2009; Linder 2012; Gaetke et al. 2014). Copper (Cu) is involved in numerous biological processes including embryogenesis, heme synthesis, iron absorption and mitochondrial respiration (Barceloux and Barceloux 1999), and is predominantly associated with proteins and the prosthetic groups of enzymes. Shuttling of intracellular Cu is strictly controlled by transporters and chaperone proteins (Nishito and Kambe 2018).

The intestinal epithelial barrier consists of a complex mix of cell types that work in tandem to regulate uptake for most nutrients following digestion. The adenocarcinoma-derived Caco-2 cell line exhibits similar characteristics to enterocytes of intestinal epithelia when cultured as a monolayer or in co-cultured differentiated systems (Hidalgo et al. 1989; Natoli et al. 2011). While recent developments including gut-on-a-chip (Kim et al. 2012), intestinal organoids (Spence et al. 2011) and ex vivo xenografts have shown potential, use of Caco-2 cells in a differentiated form has represented the workhorse of food and pharmaceutical industries to study toxicity, permeability and uptake of nutrients, pharmaceuticals and xenobiotics for over four decades (Fogh and Trempe 1975; Shah et al. 2006; Shao et al. 2017).

Non-monotonic dose responses (NMDR) are dose/effect phenomena which do not show linear or threshold dose-response relationships and their discovery precedes the 1900s (Schulz 1888). NMDR curves introduce paradoxical effects such as hormesis, an adaptive response in which stimulation is observed during exposure to low concentration of a stressor followed by a toxicity at higher ranges (Calabrese et al. 2001). These responses have shaped our understanding of pharmacology and toxicology to biological and environmental systems and have implications for understanding phenotypic plasticity and predicting dose-response effects during exposure to a wide variety of stressors (Michael Davis and Svendsgaard 1990; Vandenberg et al. 2012; Calabrese

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2014). Exposure of a stressor which results in a NMDR profile has been attributed to multiple factors, such as receptor-mediated interactions (Bartłomiejczyk et al. 2013) or via an overlay of two opposing monotonic profiles (Conolly and Lutz 2004; Calabrese 2013). Recently, fluctuating copper levels in basal media were demonstrated to have a notable influence on the expression of apoptosis- and autophagy-related proteins in Caco-2 cells (Keenan et al. 2018). Indeed, the expression or location of multiple transporters including divalent metal transporter 1 (DMT1) and copper transporter 1 (CTR1) and sequestration proteins (ATP7A/ATP7B, metallothionein) are also known to be modulated by Cu exposure (Tennant et al. 2002; Gao et al. 2014).

Focusing over a wide range of concentrations, a triphasic NMDR profile was observed in this study which has not been previously reported. The effect was seen in two copper formulations, was specific to Caco-2 and also appeared to be copper-specific as Fe, Mn and Zn did not display NMDR profiles.

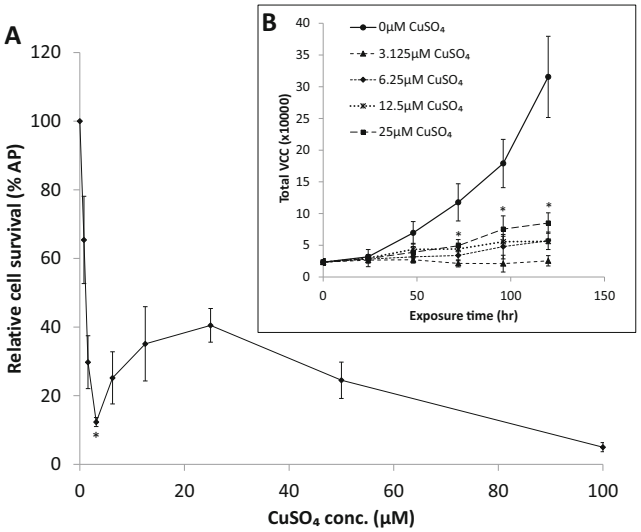

Figure 1. (*A*) Caco-2 cells exposed to increasing $CuSO_4$, cultured in 96-well plates, assayed by acid phosphatase after 120 h continuous exposure. (*B*) Time course of Caco-2 cells cultured in 6-well plates exposed to $CuSO_4$ at 0, 3.125, 6.25, 12.5 and 25 μM, assayed by daily viable cell counts (VCC) ($n = 5$). Results are presented as relative to untreated control ($n = 3$). *Asterisk* indicates significant difference from all ($p < 0.05$).

Results and Discussion

We identified a triphasic dose-response profile in proliferating Caco-2 cells (ATCC® HTB37™) during copper exposure not detected in other cell lines or micronutrients tested and not previously explored in the literature to the best of our knowledge.

In the course of this study, increasing Cu concentration to proliferating Caco-2 cells was shown to generate a triphasic non-monotonic dose response (NMDR) after 120 h (cultured in basal medium with serum, together containing 0.3 μM Cu). The curve consisted of a linear dose response from 0 to 3.125 μM added $CuSO_4$, followed by significantly reduced toxicity between 6.25 and 25 μM Fig. 1*A*, resulting in two points of inflexion within the range tested (1.56 to 100 μM). Above the first point of inflexion at 3.125 μM (cell survival was 12.33 ± 1.31%), progressively decreasing toxicity was identified up to 25 μM; cell toxicity was significantly reduced at 6.25 μM, 12.5 μM and 25 μM vs. 3.125 μM ($p < 0.05$). At the second point of inflexion, increased dose-response toxicity was generated with exposures above 25 μM $CuSO_4$ Fig. 1*A* up to 100 μM.

Caco-2 cell growth was measured every 24 h in Cu between 3.125 and 25 μM Fig. 1*B*. No change was observed during temporal measurements of viable cell counts for cells cultured in 3.125 μM supplemented $CuSO_4$ over the time course (coefficient of variation 10.9%); however, morphological observations showed clonal growth and much cell death at this concentration (not shown). Significant increases in growth were present in the other Cu concentrations added ($p < 0.05$). From 72 to 120 h exposure, 25 μM $CuSO_4$ presented a significantly increased yield every 24 h vs. 3.125, 6.25 and 12.5 μM ($p < 0.05$).

NMDR profiles are commonly reported in endocrine disruption (Vandenberg et al. 2012; Lagarde et al. 2015) and during heavy metal exposure (Helmestam et al. 2010; Chaube et al. 2010), but have not been reported previously for copper exposure in Caco-2 cells. However, profiles including stimulatory hormesis have been identified during exposure to cadmium in Caco-2 cells and genistein-exposed Caco-2 BBE (Chen and Donovan 2004; Mantha and Jumarie 2010). Metal nanoparticles (Mn, Sb, Ti) displayed a hormetic effect on Caco-2 cells which was attributed to the activation of growth factor receptors indirectly by ROS formed during low concentration exposure (Titma et al. 2016).

To investigate if the copper-induced triphasic toxicity curve was specific to Caco-2, cells derived from breast (HCC1954, BT-474), colon (HT29, HT29-MTX-E12, HCT116), intestine (porcine IPEC-J2), liver (Hep-G2) and pancreas (Mia-PaCa, BxPC3) were exposed to increasing $CuSO_4$ Fig. 2. The toxicity profiles obtained showed Caco-2 cells were considerably more sensitive to copper exposure; IC50 value for Caco-2 cells was 1.3 μM added $CuSO_4$ compared to lowest IC50 of 118.7 μM in HT29 cells.

With the exception of HT29 and BT-474 cells, the other cell lines examined displayed monotonic dose-response curves with IC50 values greater than 50 μM. Low concentration $CuSO_4$ exposure (3.125–6.25 μM) to HT29 and BT-474 demonstrated a small but significant stimulation in cell growth (15 and 10%, respectively, $p < 0.05$). This slight hormetic response may indicate that Cu concentration in the growth media was too

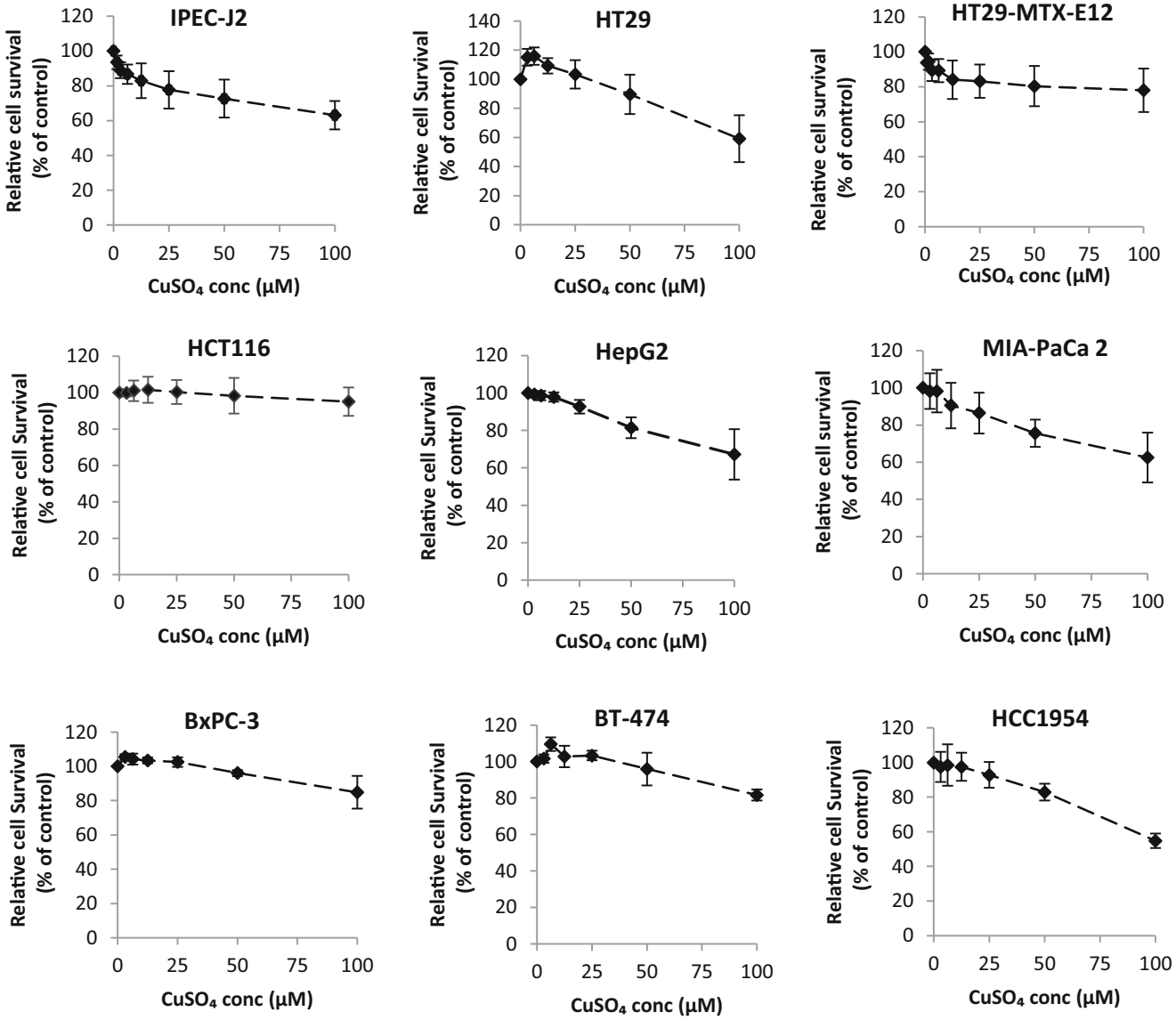

Figure 2. IPEC-J2, HT29, HT29-MTX-E12, HCT116, HepG2, MIA-PaCa 2, BxPC-3, BT-474 and HCC1954 cells exposed to increasing copper sulphate, assayed by acid phosphatase after 120 h continuous exposure. Results are presented as relative to untreated control ($n = 3$).

low or may suggest indirect growth factor receptor activation by ROS generation as described previously (Bartłomiejczyk et al. 2013). From the panel examined, this Cu-induced NMDR curve appeared specific to Caco-2 cells.

Inorganic $CuCl_2$ also demonstrated a triphasic Cu-induced NMDR curve Fig. 3*A*, supporting the notion that toxicity was due to the copper component and not the chemical form of copper. Metal micronutrients other than copper including iron, manganese and zinc are also absorbed by intestinal epithelial cells and display interactions that may affect uptake and homeostasis (Goddard et al. 1997; Arredondo et al. 2006; Collins and Knutson 2010). Exposure of iron, manganese or zinc to Caco-2 cells did not cause a significant NMDR curve Fig. 3*B* between 3.125 and 800 μM. Increasing exposures of these micronutrients resulted in linear dose-response profiles after 120 h continuous exposure. Additionally, Cu exposure was noticeably more toxic to the cells; IC50 values for Fe, Mn and Zn were greater than 50 μM and reflects toxicity noted previously (Zödl et al. 2003; He et al. 2008). In Caco-2 cells, the induction of a triphasic toxicity curve appeared to be Cu-specific.

Conolly and Lutz 2004 established kinetic models which covered multiple mechanisms for NMDR responses in biological systems. They outlined mechanisms originating from fundamental biochemistry and others postulated on biological processes. The models are based

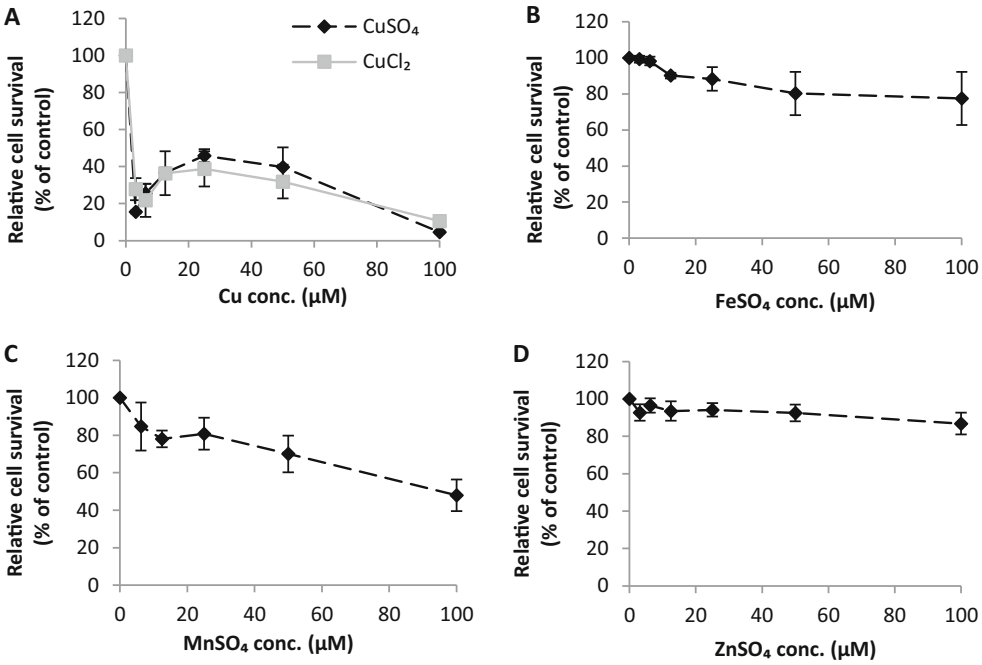

Figure 3. Caco-2 cell survival following exposure to (*A*) increasing $CuSO_4$ and $CuCl_2$ in 9.5cm^{2}wells and (*B*, *C*, *D*) iron sulphate, manganese sulphate and zinc sulphate in 96-well plates. Cells were subjected to continuous exposure after 24 h attachment for 120 h and analysed by acid phosphatase (AP) assay. Results are presented as relative to untreated control ($n = 3$).

on (a) opposing regulatory effects by membrane receptor subtypes, (b) modulation of gene expression by homodimers but not mixed dimers in increasing xenobiotic concentration (c) DNA damage repair induced by xenobiotic in a saturable manner and (d) concentration-dependant DNA damage leading to two opposing monotonic responses (cell cycle delay/cell cycle acceleration) which generate NMDR curves when superimposed. The recent publication by Keenan et al. identified that variation in levels of copper between different batches of media had a significant impact on autophagy and apoptosis-related proteins. Without this knowledge, we would not have been in a position to measure effects at such low levels, as the discrepancy between batches was observed at such a low level, thus prompting this investigation.

This study focussed on identifying a triphasic NMDR profile induced by copper in Caco-2 cells, with demonstration of metal and cell-type specificity. The observed NMDR profile demonstrates that lower concentrations of Cu result in greater toxicity than higher concentrations and has important practical implications in the modelling of Cu response with Caco-2 cells.

It is possible that this phenomenon represents a physiological response of some intestinal cells in vivo to copper deprivation, and that such cells "delay" switching on particular mechanisms such as Cu efflux or sequestration, until copper levels in the cells reach a critical level in order to replenish the bodies' stores of copper. More detailed studies will be required to determine the effect, if any, played by protein/DNA repair mechanisms and Cu-related oxidative stress-induced pathways in generating the Cu-specific NMDR profile.

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