Heavy Metal Profiling in Human Cells Reveals a Synergistic Effect on Gene Expression During a Tandem Copper-Lead Exposure

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Abstract | BLUF

Service members face chronic exposure to "battlefield heavy metals" (such as Cu, Pb, Zn), yet the molecular mechanisms of exposure in humans and combined exposures remain unclear. Following 72h exposures in HEK293T cells, assays showed $CuCl_2$ and $ZnCl_2$ induced dose-dependent oxidative stress and cytotoxicity, whereas $PbCl_2$ did not. RNA-seq revealed a profound synergistic effect during combined Cu/Pb exposure, yielding 502 uniquely differentially expressed genes absent in single-metal treatments. Pathway analysis indicates these novel genes disrupt synaptic activity and universal signaling cascades. These findings suggest that tandem heavy metal exposures act synergistically, and that combined heavy metal exposures drive the distinct neurological and widespread multi-organ pathologies characteristic of chronic heavy metal toxicity.

Introduction

Uniformed service members are frequently exposed to "battlefield heavy metals", such as copper (Cu), lead (Pb), and zinc (Zn), through gunshot residue and munitions contamination during training and combat operations[1-6]. Chronic exposure to these elements disrupts cellular redox homeostasis, mitochondrial function, and gene regulation, leading to severe physiological consequences such as neurological disturbances and widespread, varied organ damage[8].

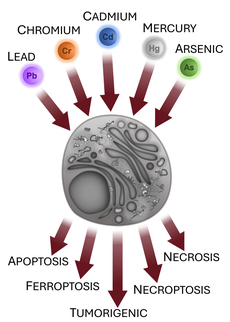


Figure 1. Heavy metal toxicity disrupts aerobic respiration, the cell cycle, and oxidative stress management resulting in cytotoxicity at the cellular level.

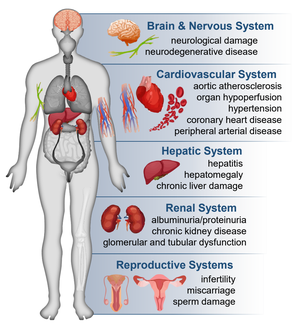


Figure 2. Heavy metal toxicity is associated with widespread and varied pathologies from the brain and nervous system to the cardiovascular system, hepatic system, renal system, and reproductive system.

While next-generation sequencing (NGS) has been utilized to evaluate heavy metal toxicity in various animal and plant models, its application to human cells, particularly regarding the effects of combined metal exposures, remains largely unexplored[9-16].

To address this critical gap and support the development of tailored medical interventions for military personnel, this study leverages NGS to comprehensively profile the global transcriptomic changes and toxicological pathways in human cells following individual and combined exposures to prevalent battlefield heavy metals.

Methods

Cellular Viability and ROS Measurements

To evaluate heavy metal toxicity, HEK293T cell viability was monitored over 72h using Propidium Iodide (PI)-coupled flow cytometry (Attune NxT). Intracellular reactive oxidative species (ROS) levels were measured using CellROX (ThermoFisher) fluorescent reporter-coupled flow cytometry in 72h-exposed HEK293T cells. Viability and ROS data were processed and normalized in R (flowCore)[17], then subject to statistical significance using ANOVA (GraphPad Prism).

Gene Expression Profiling (RNA-seq)

Total RNA from 72h-exposed HEK293T cultures were extracted using TRIzol, purified, and subject to rRNA depletion prior to cDNA library preparation using NEBNext kits (NEB). Libraries were pooled and sequenced on an Illumina NovaSeq X instrument, reads were aligned to the human genome (*hg38*), and differential gene expression analysis was performed using R (DESeq2)[18-20].

Gene Ontology and Pathway Analysis

To further characterize the functional impact of identified differentially expressed genes (DEGs), Gene Ontology (GO) enrichment was performed to categorize genes by biological process, molecular function, and cellular component. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was utilized to map these DEGs to established molecular pathways.

Results | Establishing Heavy Metal Toxicity

Cell viability assays revealed dose-dependent cytotoxicity in $CuCl_2$ and $ZnCl_2$ 72h-exposed HEK293T cells, whereas $PbCl_2$ exposure elicited no trend. $CuCl_2$ and $ZnCl_2$ exposures also induced dose-dependent increases in intracellular reactive oxygen species (ROS), with 200μM $ZnCl_2$ triggering a significant ROS spike that paralleled cell viability measurements.

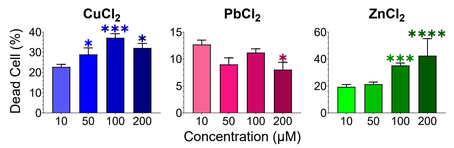


Figure 3. Heavy metal exposure compromises cell death in a dose-dependent manner. HEK293T cell death populations at 72h following exposure to increasing concentrations of $CuCl_2$, $PbCl_2$, $ZnCl_2$ using Propidium Iodide-coupled flow cytometry. Mean ± SEM shown for gated singlet events across 3 biological replicates (N=3, n=3,671,432). Significance calculated using two-way ANOVA relative to respective NaCl condition, *p ≤ 0.05, ***p ≤ 1×10^{-3}, ****p ≤ 1×10^{-4}.

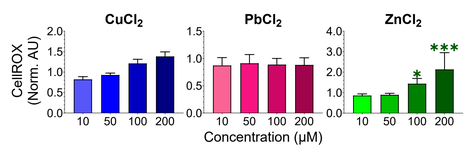


Figure 4. Heavy metal exposures increase intracellular oxidative stress levels. HEK293T cell whole-cell/intracellular reactive oxygen species (ROS) levels at 72h following exposure to increasing concentrations of $CuCl_2$, $PbCl_2$, $ZnCl_2$ using a whole-cell ROS reporter (CellROX, ThermoFisher) coupled with flow cytometry. Mean ± SEM shown for gated singlet events across 3 biological replicates (N=3, n=3,061,838). Significance calculated using two-way ANOVA, *p ≤ 0.05, ***p ≤ 1×10^{-3}.

Results | RNA-seq & Gene Ontology

Synergistic Effects of Combined Exposure

All combined heavy metal exposures tested revealed varying degrees of synergy on gene expression in human cells, suggesting that combined exposures to battlefield metals may be more dangerous than previously believed.

Tandem Copper-Lead Exposure Reveals Link to Heavy Metal-mediated Neurological Pathologies

This effect is vastly more pronounced during tandem copper-lead exposure in HEK293T cells. While individual $CuCl_2$ and $PbCl_2$ exposures caused moderate transcriptomic changes, their combined exposure triggered a profound synergistic effect. Over 70% of the 633 differentially expressed genes identified during co-exposure were novel and not triggered by exposure to either metal alone.

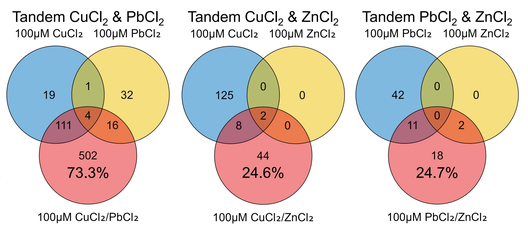


Figure 7. RNA-seq in human cells reveals a synergistic effect in tandem heavy metal exposures on gene expression. Venn diagrams identifying significant expression changes across tandem heavy metal exposures and corresponding single metal exposures. Numbers of differentially expressed genes are identified for each set with percentages reflecting the proportion of genes unique to the tandem heavy metal exposure not otherwise found in any other set.

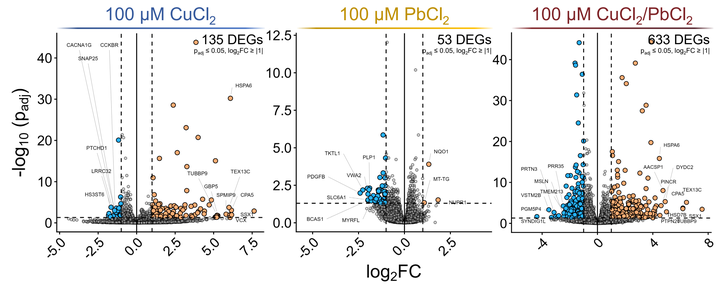


Figure 5. RNA-seq identifies differentially expressed genes (DEGs) in human cells exposed to $CuCl_2$, $PbCl_2$, or both $CuCl_2$/$PbCl_2$. Volcano plots show gene expression profiles in HEK293T cells exposed to heavy metals for 72h, with upregulated genes (p_{adj} ≤ 0.05, $log_2FC \geq 1$) shown in orange and downregulated genes (p_{adj} ≤ 0.05, $log_2FC \leq -1$) shown in blue. Top upregulated and downregulated DEGS labeled with their Gene Symbol, as determined by rank order of statistical significance and magnitude of expression change. Dashed lines indicate cut-offs used to determine statistical significance (p_{adj} ≤ 0.05) and expression change ($|log_2FC| \geq |1|$).

Gene Ontology (GO) analysis of these tandem exposure DEGs revealed a significant disruption to neurological processes, particularly in action potentials and synaptic activity. We subsequently isolated these neuro-associated genes for KEGG pathway analysis to map their broader physiological and systemic impacts to molecular and functional pathways.

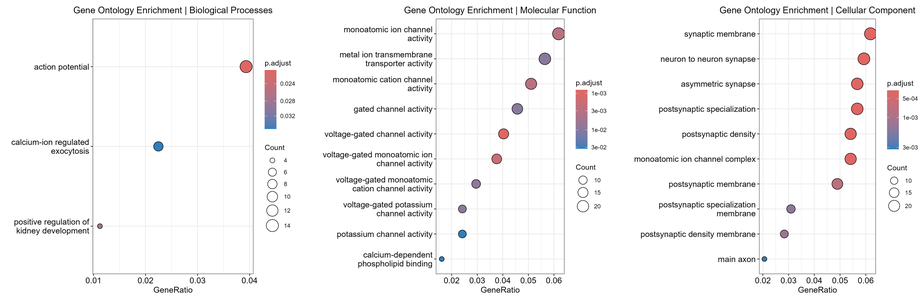


Figure 6. Gene Ontology (GO) analysis of tandem $CuCl_2$/$PbCl_2$ exposed human cells reveal enriched terms for neural functions and pathways. Cluster profile plots identify the most enriched GO terms for Biological Processes, Molecular Function, and Cellular Component corresponding to the 633 expression changes identified in tandem $CuCl_2$/$PbCl_2$ exposed HEK293T cells using RNA-seq.

Results | KEGG Pathway Analysis

Differentially Expressed Genes in Tandem Copper-Lead Exposures Perturb Major Cellular Signaling Pathways

KEGG pathway analysis of these neuro-associated DEGs revealed that many genes altered by tandem copper-lead exposure play important roles in major signaling cascades that regulate important biological functions in the body, including cAMP, circadian entrainment, and adrenergic signaling.

Many of the altered DEGs identified in our profiles serve as upstream regulators and receptors in their respective pathways, indicating heavy metal-mediated toxicity might inhibit proper function of these cellular signaling mechanisms altogether. Broadly, this profound dysregulation of universal signaling regulators may potential reveal why chronic heavy metal exposure drives characteristic widespread, multi-organ pathologies.

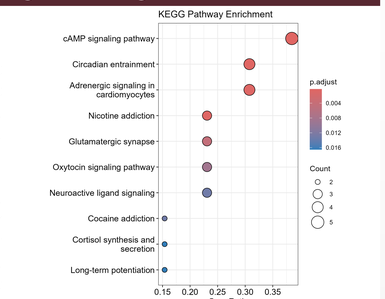
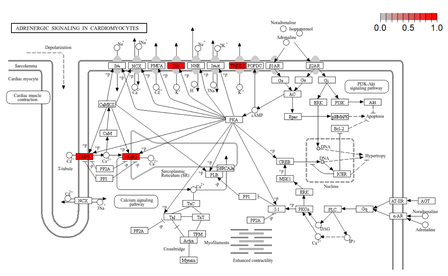


Figure 10. KEGG pathway analysis for DEGs altered in tandem $CuCl_2$/$PbCl_2$ exposures identifies major ubiquitous signaling pathways.



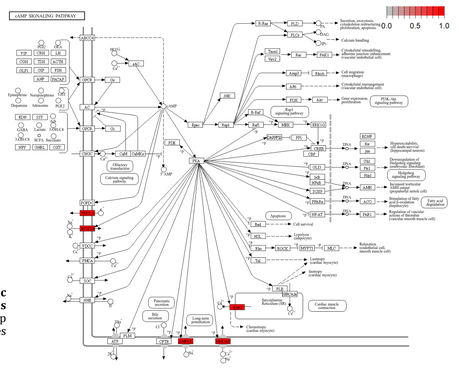


Figure 12. Tandem $CuCl_2$/$PbCl_2$ exposure in human cells perturb adrenergic signaling in cardiomyocytes and cAMP signaling, among other notable ubiquitous cellular signaling pathways. Functional protein-protein interaction maps for the top three pathways identified by KEGG pathway analysis for differentially expressed genes (DEGs) in tandem $CuCl_2$/$PbCl_2$ exposed HEK293T cells. DEGs labeled in red.

Discussion & Future Directions

While our *in vitro* expression profiles provide critical mechanistic insights into how tandem heavy metal exposures potentially drive multi-organ pathologies, this study has inherent limitations. Cell cultures cannot fully replicate the complex, systemic physiological responses of a whole organism and our focus on copper, lead, and zinc does not account for all trace metals present in actual gunshot residue (GSR) and military munitions. While next generation sequencing captures robust gene expression changes and provides a broad overview of expression profiles, it is ultimately agnostic to other mechanisms that are critical to the signaling cascades we identified, such as post-translational modifications like kinase activity and phosphorylation states.

Future studies will need to utilize animal models to better understand the systemic toxicological effects of heavy metal exposure in service members and translate our *in vitro* findings into *in vivo* models. These studies will be imperative for identifying targetable molecular pathways and developing effective medical countermeasures in our effort to preserve and protect the health of our warfighters. A manuscript detailing the synergistic effect of combined heavy metal exposures is currently in preparation for submission to the *Journal of Toxicology*.

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