Zn^0-Induced Cytotoxicity and Mitochondrial Stress in Microglia: Implications of the Protective Role of Immunoglobulin G In Vitro

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Background: Zinc (Zn), an essential micronutrient, regulates and maintains neurological functions. However, both Zn deficiency and excess can cause oxidative stress and neurodegenerative diseases. As previously reported, immunoglobulin G (IgG) can modulate oxidative stress in various disorders.

Aims: This study aimed to investigate whether IgG treatment can alleviate oxidative stress caused by Zn^0 on microglia in vitro.

Study Design: In vitro study.

Methods: The feasibility of Zn^0 treatment was evaluated using the MTS assay. Oxidative stress following treatment with Zn^0, either alone or with IgG supplementation, was determined with dihydrorhodamine 123 staining. Flow cytometry was employed to ascertain the intracellular protein levels of TRIM21, PINK, PARKIN, MFN2, Beclin-1, and active LC3B.

Results: In silico screening confirmed the association between Zn^0 cytotoxicity and apoptosis. Furthermore, oxidative stress was identified as a critical mechanism that underlies Zn^0 neurotoxicity. The in silico analysis revealed that Zn can interact with the constant region of the Ig heavy chain, suggesting a potential role for IgG in alleviating Zn^0-induced cytotoxicity. Experimental findings supported this hypothesis, as IgG administration significantly reduced Zn^0-induced mitochondrial stress in a dose-dependent manner. The upregulation of PINK1 levels by Zn^0 exposure suggests that mitochondrial injury promotes mitophagy. Interestingly, Zn^0 decreased TRIM21 levels, which is reversed by IgG administration.

Conclusion: These findings elucidate the cellular responses to Zn^0 and highlight the potential use of intravenous immunoglobulin in mitigating the adverse effects of acute Zn^0 exposure.

INTRODUCTION

Zinc (Zn) is the second most abundant trace element in the human body, following iron, and is essential for numerous biological processes.[1]

It serves as a cofactor for over 300 enzymes and is involved in the structural and regulatory functions of many proteins and DNA-binding transcription factors.[2] However, excess Zn can induce apoptosis and cause copper deficiency, which has been associated with various negative consequences, including the production of copper-dependent antioxidant enzymes.[3] In neural tissues, Zn imbalance can lead to impaired brain development and cognitive dysfunction, memory deficits and impaired learning ability, excitotoxic damage and death of postsynaptic neurons following traumatic brain injury, ischemia, and seizures. In addition, Zn imbalance is connected with an increased risk for depression and neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease.[1] Increased Zn levels in response to brain ischemia is proven to induce polarization of microglia to the proinflammatory M1 phenotype, which may result in deficiencies in object recognition memory.[4]

Zn exerts neurotoxicity by reducing mitochondrial adenosine triphosphate (ATP) production and increasing reactive oxygen species (ROS) levels.[5] Mitochondrial dysfunction results from increased ROS

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production along with decreased antioxidant activity, which in turn promotes further ROS production and leads to inflammation.[6] Thus, eliminating dysfunctional mitochondria is crucial for maintaining cellular viability. This is mainly achieved through mitophagy, a complex process that occurs through autophagy to eliminate damaged mitochondria.[7] One of the extensively studied stress-induced mitophagy pathways relies on the coordinated actions of key proteins, including Parkin, mitofusin 2 (MFN2), and PTEN-induced putative kinase 1 (PINK1).[8,9] When mitochondrial damage occurs, PINK1 accumulates on the outer mitochondrial membrane, where it phosphorylates both ubiquitin and MFN2. This enables the autophagic machinery to recognize mitochondrial proteins by ubiquitinating them through the recruitment of the E3 ubiquitin ligase Parkin.[10] The degradation of damaged mitochondria results from an interaction between ubiquitin on mitochondria and autophagy receptors, which is crucial for the maintenance of mitochondrial quality control. This process is essential for cellular homeostasis and the overall health of the organism. If mediated autophagy is impaired, cells can still participate in macroautophagy, which entails engulfing mitochondria and other cell organelles to form autophagosomes, which subsequently fuse with lysosomes for degradation.[11] Dysfunctions in mitophagy have been reported in older adults as well as those with age-related disorders.[7]

Previous studies have suggested that immunoglobulin G (IgG) administration can modulate oxidative stress in various diseases.[12,13] However, there is a lack of research examining the effect of IgG treatment on Zn-induced oxidative stress. The involvement of tripartite motif 21 (TRIM21), an E3 ubiquitin-protein ligase that takes part in intracellular IgG clearance, has been demonstrated to be involved in autoimmune and neurodegenerative diseases.[14-16] Hence, this study aimed to investigate whether IgG treatment can alleviate Zn-mediated oxidative stress in microglia in vitro and whether mitophagy plays a role in this protective effect.

METHODS

In silico screening

The main biological processes associated with neurodegenerative diseases, their relationship with Zn exposure, and the potential molecular functions implicated in neuroinflammatory responses were determined using the genetic data extracted from (Comparative Toxicogenomics Database; http://CTD.mdibl.org). The GeneMANIA web-based platform (http://geneMANIA.org/plug-in/) was used to predict the nature of regulatory gene interaction networks. The ToppGeneSuite portal (https://toppgene.cchmc.org) was utilized to acquire lists of the most significant biological processes related to the development of neurodegenerative diseases and linked to Zn exposure, as well as to predict the potential molecular functions involved in neuroinflammatory responses. Gene lists used for in silico analysis are provided in Supplementary Tables 1-4.

Cell culture conditions

Human immortalized microglia cell line HMC3 (American Type Culture Collection # CRL-3304™, passage no. 7-9) was cultured in Eagle's Minimum Essential Medium, (Sigma Aldrich #M4655) supplemented with 10% fetal bovine serum (Sigma Aldrich #M4655) and 100 U penicillin-100 µG/ml streptomycin antibiotic solution (Sigma Aldrich # P4333). Cell passaging was performed using trypsin-ethylenediaminetetraacetic acid (Thermo Fisher Scientific, #25200056).

The zero-valent Zn (Zn^0, Sigma Aldrich, #578002) was prepared by suspending the nanoparticles in complete culture medium and sonicating for half an hour to obtain a homogenous solution at 1 mg/ml. The human IgG (Sigma Aldrich #I4506) solution was prepared by dissolving lyophilized IgG in 150 mM NaCl solution at 10 mg/ml concentration. The IgG stock solution was aliquoted, stored at -80 °C for further use, and was not subject to freeze-thaw cycles. The IgG concentrations evaluated in this study were based on its physiological concentration, which was reported as 23.7 µg/ml.[17]

Determination of viability

The toxicity of Zn^0 on human microglia was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.[18] To evaluate viability, 5 x 10^3 cells/well were seeded into 96-well plates in triplicate and incubated overnight to allow attachment. The culture medium was then discarded, and the cells were incubated with Zn^0 (5, 2.5, 1, 0.5, 0.1, 0.05, 0.025 µg/ml) in fresh culture medium for 12, 24, 48, and 72 hours. Cellular viability was assessed by adding the MTS reagent (10% v/v) (Abcam, #ab197010) to culture wells and incubating the plates for an additional two hours. The optical density was measured at 490 nm with a spectrophotometer (Epoch, BioTek, USA). Untreated cells were used as a control, whereas the culture medium without cells was used as a blank. Relative viability was calculated according to the formula given below. Further studies were conducted with the lowest dose, which significantly decreased viability at the respective time points when compared to the control group.

$$Viability\ (\%) = \frac{Sample\ OD\ (490\ nm) - Blank\ OD\ (490\ nm)}{Control\ OD\ (490\ nm) - Blank\ OD\ (490\ nm)} \times 100$$

Evaluation of reactive oxygen species

Oxidative stress in response to Zn^0 treatment, either alone or in conjunction with IgG supplementation, was determined with dihydrorhodamine 123 (DHR123) staining.[19] For this purpose, 25 x 10^4 cells were seeded into 35 mm tissue culture plates in triplicate and incubated overnight to allow attachment. The cells were subjected to Zn^0 treatment, either alone or in conjunction with IgG, followed by trypsinization. They were then segregated using centrifugation at 300 g for three minutes. The cell pellet was washed once and suspended in 500 µl of Dulbecco's phosphate-buffered saline (DPBS). Following that, the cells were stained with DHR123 (5 µM, Santa Cruz Biotechnologies, #sc-203027) by incubating in the dark at room temperature for 40 minutes. Cells treated with tert-butyl hydroperoxide (Santa Cruz Biotechnology, #sc-251134) at a 100 µM concentration were used as a positive control.[20] The DxFLEX flow cytometry system (Beckman Coulter, USA) was promptly used to evaluate 25 x 10^3 events per tube following staining. The CytExpert for DxFLEX software was used for analyzing the data.

Evaluation of protein levels

Intracellular protein levels of TRIM21, PINK, PARKIN, MFN2, Beclin-1, and active LC3B (active microtubule-associated proteins 1A/1B light chain 3B) were determined by flow cytometry. For this purpose, cells were seeded on 35 mm dishes at a density of 25 x 10^4 cells per plate in triplicate and treated with Zn^0 either alone or in conjunction with IgG for 24 h. The cells were then detached and collected using centrifugation. Fixation was performed at 4 °C by suspending the cell pellet in a 1% paraformaldehyde (Sigma Aldrich, #158127, pH: 7.2-7.4) solution for 30 minutes. After fixation, the cells were permeabilized with a DPBS solution containing 0.1% Triton X-100 (Sigma Aldrich, #T9284) and 1% Blocker BSA (Thermo Fisher Scientific, #37525) by incubating for an additional 30 minutes at 4 °C. For staining the cells, the pellet was suspended in 500 µl of DPBS containing 1% Blocker BSA and labeled with Alexa Fluor 750-conjugated anti-Beclin 1 (Novus Biologicals, #NB500-249AF750, 1 µl/test), anti-active LC3B (Aviva Systems Biology, #OAAB13518, 2.5 µl/test), anti-TRIM21 (Novus Biologicals, #NBP1-87122 0.1 µl/test), anti-PINK (Biolegend, #846202, 0.25 µl/test), anti-Parkin (Biolegend, #870502, 0.25 µl/test) and anti-MFN2 (Novus Biologicals, #NBP2-66383, 0.25 µl/test) antibodies by 1 h incubation at 4 °C in the dark. Primary antibody solutions were discarded, and the cells were stained with the following secondary antibodies: PE-conjugated goat anti-mouse (Termo Fisher Scientific, #PA1-84395, final concentration: 0.1 mg/ml), PE-conjugated donkey anti-rabbit (Abcam, #ab7007, final concentration: 0.1 mg/ml), and DyLight 488-conjugated sheep anti-rabbit antibodies (Abcam, Cat. No.: ab96923, final concentration: 0.1 mg/ml) by incubating the cells in the dark at room temperature for 30 min. The cells were suspended in 500 µl of DPBS, and 25 x 10^3 events were analyzed using the DxFLEX flow cytometry system. Analysis was performed with the CytExpert for DxFLEX software.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed with the GraphPad Prism 8 software. The Shapiro-Wilk test was employed to assess normality. The Kruskal-Wallis and one-way ANOVA tests were applied for data with non-parametric and parametric data, respectively. *P* values lower than 0.05 were considered statistically significant.

RESULTS

In silico screening

Our in silico analysis was designed to elucidate the biological processes associated with Zn exposure, as disruptions in Zn homeostasis can impact neurodegeneration and neuroinflammation in humans.[1,21,22] We discovered that the cytotoxic effect of Zn exposure regulates apoptosis, programmed cell death processes, and cellular responses to oxygen-containing compounds (Table 1).

When considering the influence of Zn exposure on neurodegeneration, the most dominant biological processes included the response to oxidative stress. This demonstrated the significance of oxidative stress in Zn neurotoxicity[23] and guided us for further in silico analysis (Table 2). The analysis of regulatory gene interaction networks impacted by Zn exposure and associated with neurodegenerative disease development revealed that the primary types of gene interactions were physical interactions (32.53%; two gene products are linked if they were found to interact in a protein-protein interaction study), pathway interactions (30.39%; two gene products are linked if they participate in the same reaction within a pathway), and co-expression (20.60%; two genes are linked if their expression levels are similar across conditions in a gene expression study). The less prominent types of interactions included genetic interactions (11.59%; two genes are functionally associated if the effects of perturbing one gene were found to be modified by perturbations to a second gene), co-localization (3.53%; where the genes are expressed in the same tissue or proteins are found in the same location), and interactions predicted by the server (1.37%) (Figure 1).[24] Separate interactions affected by Zn exposure and linked with neurodegenerative disease development are presented in Supplementary Figures 1-6. Additional investigation into the molecular functions involved in neuroinflammatory diseases revealed that, apart from oxidoreductase activity, Ig (IgE and IgG) receptor activity and binding were the most significant activities (Table 3). Furthermore, the in silico analysis supported our hypothesis that IgG treatment can alleviate the toxic effects of Zn^0, as it can bind to IgG via the constant region of the Ig heavy chain. In conclusion, while the existing literature supports the findings of our in silico analysis,[25,26] additional comprehensive studies are required to elucidate the exact mechanism by which Zn ions bind to human IgG. Given the relationship between oxidative stress and neurodegeneration,[27] our in vitro evaluations focused on cell viability testing and mitochondrial ROS accumulation.

TABLE 1. Biological processes linked to the genes affected by Zn exposure (ToppGeneSuite portal).

ID		Name	p-value*
1.	GO:0042981	Regulation of apoptotic process	9.247E-98
2.	GO:0043067	Regulation of programmed cell death	2.362E-97
3.	GO:1901701	Cellular response to oxygen-containing compound	4.242E-85

*The default settings of the ToppGene Suite portal were used for this analysis, meaning that the p-value was set to 0.05 and false discovery rate corrected. GO, Gene ontology.

TABLE 2. Biological processes connected with neurodegenerative disease development and linked to the genes affected by Zn exposure (ToppGeneSuite portal).

ID		Name	p-value*
1.	GO:0006979	Response to oxidative stress	3.502E-14
2.	GO:0034599	Cellular response to oxidative stress	1.532E-10
3.	GO:1990000	Amyloid fibril formation	4.103E-10

*The default settings of the ToppGene Suite portal were used for this analysis, meaning that the p-value was set to 0.05 and false discovery rate corrected. GO, Gene ontology.

Determining viability of HMC3 cells after Zn^0 exposure

The MTS assay revealed that Zn^0 decreased the viability of the human microglia in vitro in a dose- and time-dependent manner (Figure 2a-d). After 12 hours, the lowest dose of Zn^0 that led to a significant decrease in microglia viability was 2.5 µg/ml ($p < 0.05$) (Figure 2a). Within a 24-hour period, Zn^0 promoted microglial proliferation at doses between 0.025 and 0.5 µg/ml ($p < 0.0001$ for 0.025 and 0.1 µg/ml; $p = 0.0003$ for 0.05 µg/ml), while it significantly decreased viability at 2.5 µg/ml ($p < 0.05$) (Figure 2b). At 48 hours (Figure 2c) and 72 hours (Figure 2d), the lowest doses of Zn^0 that significantly decreased the viability of HMC3 cells were determined to be 1 µg/ml ($p = 0.0039$) and 0.05 µg/ml Zn^0 ($p = 0.001$), respectively. To conduct additional evaluations, the lowest concentration that significantly reduced cellular viability for the respective time point was employed.

Evaluation of mitochondrial ROS accumulation

As revealed by the DHR123 assay, Zn^0 did not lead to ROS accumulation in HMC3 cells after 12 h (Figure 3a).

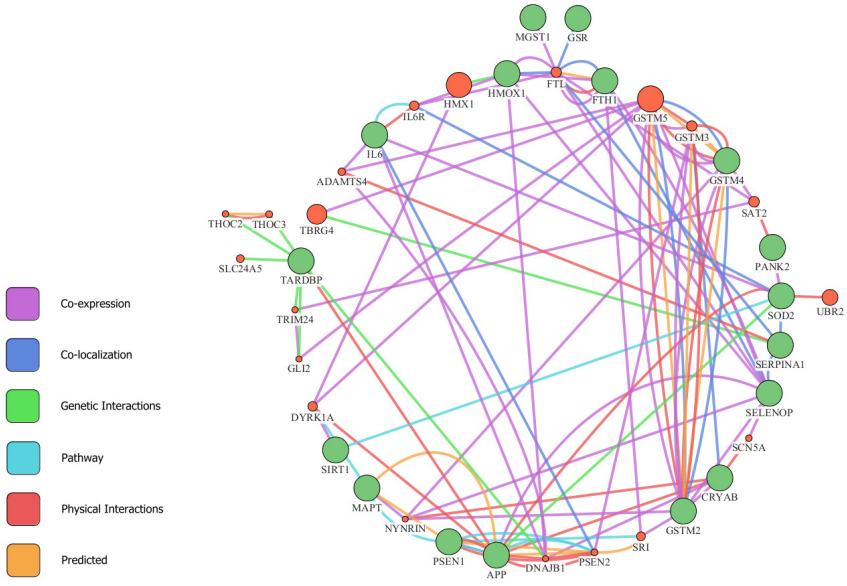

FIG. 1. Predicted interaction network of genes affected by Zn exposure and linked with neurodegenerative diseases development (GeneMANIA).

TABLE 3. Molecular functions involved in neuroinflammatory diseases (ToppGeneSuite portal).

ID		Name	p-value*
1	GO:0050664	Oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	2.613E-8
2	GO:0019767	IgE receptor activity	7.516E-8
3	GO:0019863	IgE binding	1.314E-7
4	GO:0004888	Transmembrane signaling receptor activity	6.239E-7
5	GO:0016175	Superoxide-generating NAD(P)H oxidase activity	8.213E-7
6	GO:0019763	Immunoglobulin receptor activity	1.067E-6
7	GO:0019772	Low-affinity IgG receptor activity	1.498E-5
8	GO:0016651	Oxidoreductase activity, acting on NAD(P)H	1.785E-5
9	GO:0001848	Complement binding	1.991E-5
10	GO:0019865	Immunoglobulin binding	2.181E-5
11	GO:0019770	IgG receptor activity	5.228E-5
12	GO:0005102	Signaling receptor binding	5.476E-5
13	GO:0009055	Electron transfer activity	6.454E-5
14	GO:0140375	Immune receptor activity	1.301E-4
15	GO:0019864	IgG binding	1.364E-4

*The default settings of the ToppGene Suite portal were used for this analysis, meaning that the p-value was set to 0.05 and false discovery rate corrected. GO, Gene ontology; IgE, immunoglobulin E; IgG, immunoglobulin G; NAD(P)H, nicotinamide adenine dinucleotide (phosphate).

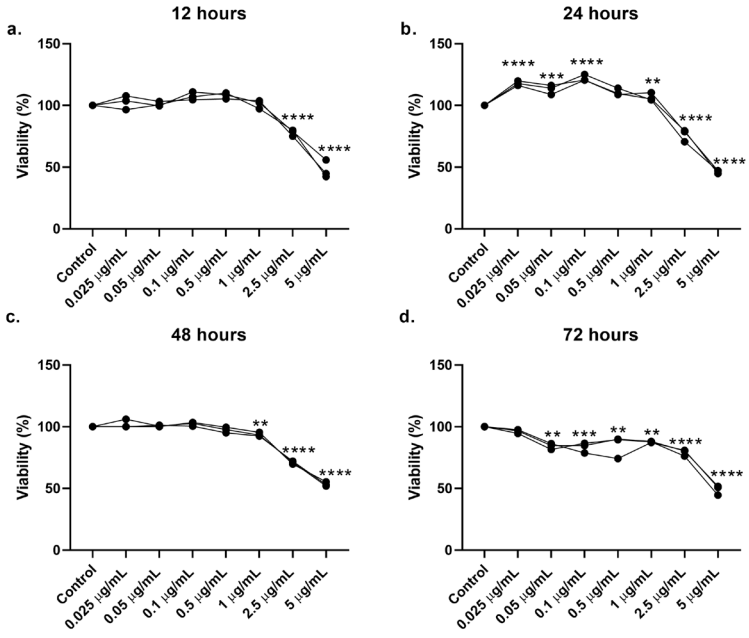

FIG. 2. Zn^0 decreased viability in a dose- and time-dependent manner. The lowest doses of Zn^0 that significantly decreased viability in the HMC3 cell line in comparison with the untreated (control) group were determined as (a) 2.5 µg/ml for 12 h, (b) 2.5 µg/ml for 24 h, (c) 1 µg/ml for 48 h and (d) 0.05 µg/ml for 72 hours. One-Way ANOVA followed by Dunnet's multiple comparison test was used for statistical analysis. Data are presented as mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

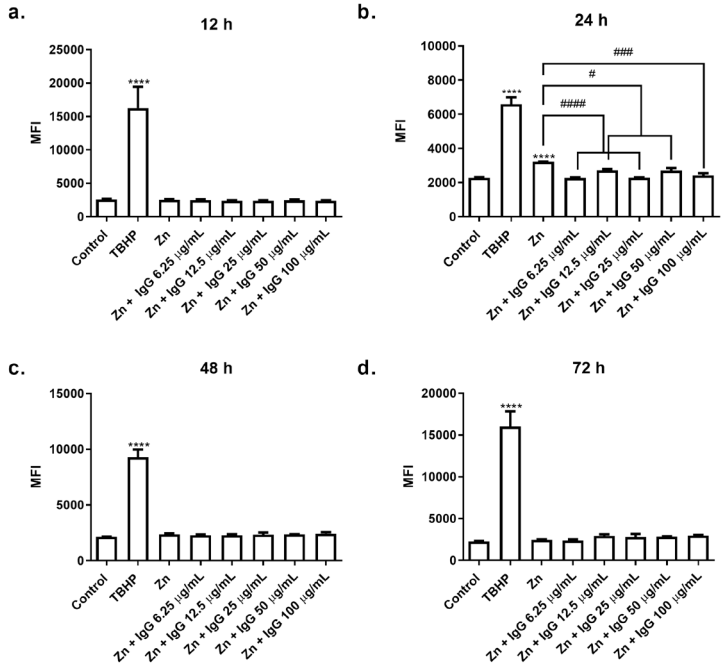

FIG. 3. Zn^0 significantly enhanced ROS accumulation in the human microglial cell line HMC3 after 24 hours, though this effect of the metal is not observed on longer incubation. (a) Zn^0 did not promote ROS accumulation at the 12-hour timepoint. (b) Zn^0 promoted ROS accumulation after 24 hours while simultaneous IgG administration successfully decreased ROS to the level in the control group. (c) Zn^0 didn't promote ROS accumulation at the 48-hour timepoint. (d) Zn^0 did not promote ROS accumulation at the 72-hour timepoint. *Indicates differences between the test and control groups while # indicates differences from the only Zn^0 group. Kruskal-Wallis test followed by Dunn's multiple comparison test was used for the 12-hour comparisons, while One-Way ANOVA followed by Tukey's multiple comparison test was used for 24, 48, and 72-hour comparisons. Data are presented as mean ± standard deviation. $^{****}p < 0.0001$; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

However, the increased DHR123 median fluorescence intensity (MFI) at 24 hours suggests that Zn^0 induced mitochondrial stress was observed at this time point ($p < 0.0001$) (Figure 3b) Representative histogram plots regarding the DHR123 assay are presented in Supplementary Figure 7. Moreover, IgG administration at concentrations ranging from 6.25 to 100 µg/ml in conjunction with Zn^0 treatment significantly decreased DHR123 MFI ($p < 0.05$) after 24 hours of incubation ($p < 0.0001$ for 6.25 and 25 µg/ml; $p = 0.0004$ for 100 µg/ml; $p = 0.023$ for 50 µg/ml; and $p = 0.027$ for 12.5 µg/ml), revealing that IgG administration can suppress ROS accumulation. Since ROS accumulation was observed after 24 hours of Zn^0 exposure, evaluations regarding the proteins implicated in mitophagy and macrophagy were evaluated at this time point. However, ROS accumulation was not observed when the cells were incubated either with Zn^0 or IgG along with Zn^0 for more than 24 hours (Figure 3c-d). This implies that microglia may compensate for Zn-induced mitochondrial stress in a time-dependent manner in vitro. In addition, the administration of IgG at concentrations ranging from 6.25 to 100 µg/ml in combination with Zn^0 did not promote mitochondrial ROS accumulation.

Evaluation of mitophagy

The Zn^0 exposure did not upregulate Beclin-1 ($p > 0.999$) (Figure 4a) or active LC3B ($p > 0.291$) protein levels (Figure 4b), suggesting that macro-mitophagy is not induced by Zn^0 exposure at concentrations that lead to oxidative stress. Supplementary Figure 8, 9 illustrate the flow cytometry histogram plots for Beclin-1 and active LC3B, respectively. No changes in MFN-2 levels were observed in relation to mitochondrial fusion (Figure 4c) (Supplementary Figure 10) ($p > 0.946$). Although the Parkin levels were comparable (Figure 4d) (Supplementary Figure 11) ($p > 0.445$), Zn^0 alone significantly upregulated PINK1 levels (Figure 4e) (Supplementary Figure 12), which indicates the Parkin-independent activation of mitophagy. Additionally, Zn^0 exposure also led to a significant decrease in TRIM21 levels in comparison with the control group ($p < 0.05$) (Figure 4f) (Supplementary Figure 13) ($p = 0.006$).

The results of the study indicate that macro-mitophagy is not the primary pathway through which IgG exerts its protective effects when administered in combination with Zn^0 at concentrations ranging from 6.25 to 100 µg/ml. Specifically, no differences were observed in the levels of Beclin-1 in any of the groups when compared to the control group ($p > 0.05$) (Figure 4a). The only significant increase in active LC3B levels was observed in the Zn^0 + 12.5 µg/ml IgG group ($p = 0.046$) (Figure 4b). Comparisons between the Zn^0 and Zn^0 + IgG groups also revealed no significant difference in terms of Beclin-1 and active LC3B protein levels ($p > 0.05$, Supplementary Table 5). Similar to Beclin-1, the differences between Zn^0 and Zn^0 + IgG groups in MFN-2 levels were also not significant ($p > 0.05$) (Figure 4c, Supplementary Table 5). Conversely, higher doses of IgG (50 and 100 µg/ml) upregulated Parkin expression significantly when compared to both the control ($p = 0.016$ and $p = 0.006$, respectively) and Zn^0 groups ($p = 0.0005$ and $p = 0.0002$, respectively) (Figure 4d, Supplementary Table 5), while the PINK1 levels were found to be significantly decreased in these groups compared to Zn^0 alone ($p < 0.0001$) (Figure 4e). Lastly, IgG supplementation upregulated TRIM21

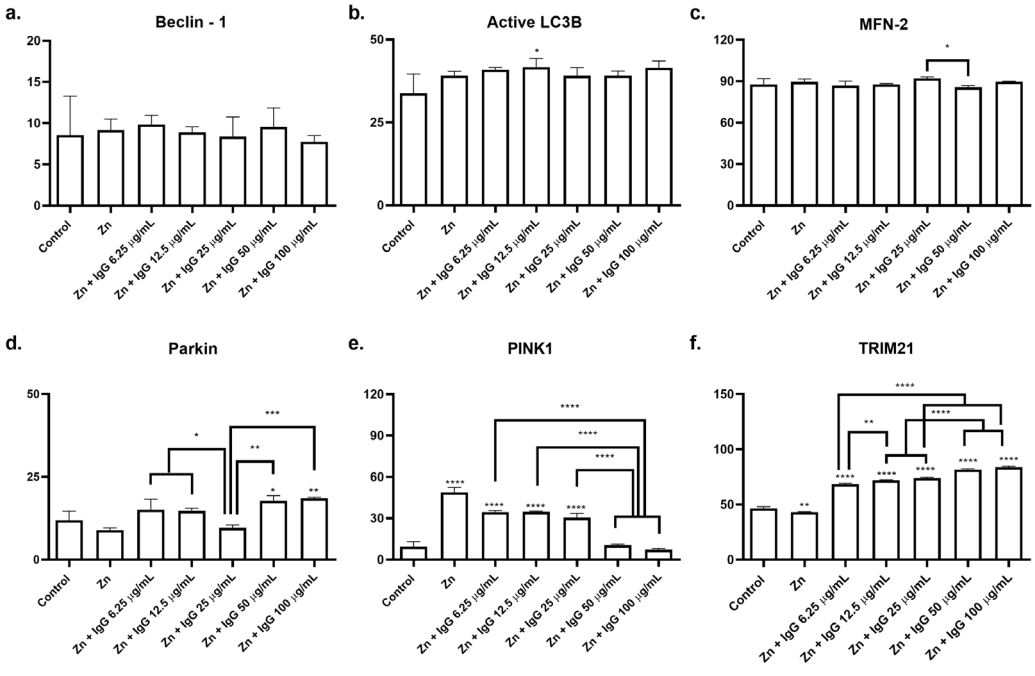

FIG. 4. Zn^0 exposure didn't promote macro-mitophagy. However, upregulated PINK1 levels suggested that Zn^0 may enhance Parkin-independent mitophagy while IgG led to a dose-dependent decrease in PINK1 levels along with the upregulation of Parkin. Bar graphics indicating (a) Beclin-1 (b) Active LC3B (c) MFN2 (d) PINK1 (e) Parkin and (f) TRIM21 protein levels. *Indicates differences between the groups. One-Way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Data are presented as mean ± standard deviation.

levels compared to both the control and Zn^0 groups ($p < 0.0001$, Supplementary Table 5); comparisons between the Zn^0 + IgG groups also suggested that IgG increases TRIM21 in a dose-dependent manner (Figure 4f, Supplementary Table 5).

DISCUSSION

The neurotoxic effects of Zn target the mitochondria, reducing ATP production and increasing ROS levels. In cultured mouse cortical neurons, Zn reduced cellular nicotinamide adenine dinucleotide, leading to a progressive decrease in ATP levels and subsequent cell death, suggesting a potential inhibition of mitochondrial respiratory enzymes.[28-30] Studies on hepatic mitochondria have shown that Zn inhibits several mitochondrial enzymes, including α-ketoglutarate dehydrogenase, NAD+ -dependent isocitrate dehydrogenase, succinate dehydrogenase, and cytochrome c oxidase.[31,32] In rat neurons, Zn upregulates the Ras/MEK/ERK pathway (mitogen-activated protein kinase/extracellular-signal-regulated kinase pathway), leading to mitochondrial dysfunction and subsequent cell death.[33] Numerous studies demonstrate the interconnection between oxidative stress and neuroinflammation in the pathology of chronic neurodegeneration in various neurological diseases.[34] Moreover, mitochondria play a role in coordinating innate and adaptive immune responses, as well as managing oxidative stress. Therefore, they form a link between neuroinflammatory and neurodegenerative processes.[35,36]

Dysregulation of apoptosis has been documented in many neurodegenerative disorders.[23] The in silico evaluations presented in our study also confirmed that the cytotoxic effect of Zn^0 is linked with apoptosis. A multifaceted role in the initiation and inhibition of apoptosis has been demonstrated for both intracellular and extracellular Zn homeostasis.[23] Zn acts as an inhibitor of the procaspases, including proenzymes of caspase-3, caspase-8, and caspase-9.[37] Moreover, at low concentrations, Zn^0 promotes neuroglial cell proliferation,[38] which is consistent with our findings indicating microglial proliferation occurred after 24 hours of incubation at low doses, whereas higher doses led to decreased viability. Overall, our findings suggested that Zn^0 exerted cytotoxicity in microglia in vitro in a dose- and time-dependent manner.

Oxidative stress is highlighted as one of the key mechanisms that affect the progression of neurodegenerative diseases.[39] In our study, the in silico analysis revealed that oxidative stress is involved in Zn neurotoxicity, which was also confirmed by the DHR123 assay, where Zn significantly increased mitochondrial ROS accumulation after 24 hours, although this effect was not observed at further time points. These data may indicate that Zn^0 exposure leads to acute mitochondrial stress in vitro, and that the cells may compensate for Zn^0 induced stress through unknown cellular mechanisms.

In our study, the in silico analysis of the molecular functions implicated in neuroinflammatory diseases revealed that the most significant factors were oxidoreductase activity, as well as IgE and IgG receptor activity and binding. The neuroprotective effect of intravenous immunoglobulin (IVIg) administration is well documented. In a murine model of experimental stroke, IVIg treatment promoted neuroprotective responses in microglia, resulting in a decrease in lesion size.[12] In a murine in vivo epilepsy model, the administration of IVIg pre- and post-treatment attenuated acute inflammation by reducing local glial activation and complement system activation. Moreover, the authors revealed that IVIg administration altered the expression and function of IgG-specific receptors (FcγR, Fc-gamma receptors) and blocked the complement receptors on mononuclear phagocytic cells.[40] However, none of these studies were performed using Zn^0. In this study, the direct chemical-protein interaction analysis demonstrated that Zn can interact with the constant region of the Ig heavy chain, supporting the hypothesis that IgG can alleviate Zn^0-induced cytotoxicity. However, additional studies are required to clarify the binding between Zn ions and the F(c) fragments of human IgGs, in addition to elucidating the mechanism by which Zn ions are taken up into cells through the Zn-IgG complex.[26] Some of the factors that should be considered in a more thorough examination of the IgG binding capacity are the various binding sites on the different antibody fragments, the degree of antibody glycosylation, the structural change in F(c) upon binding, and the flexibility of the loop structures.[41] Consistent with the in silico findings, IgG administration ranging in concentrations between 6.25 and 100 µg/ml significantly decreased Zn^0-induced mitochondrial stress. Given that the exact mechanism of Zn binding via human IgG is not yet fully understood,[26] this study's significant finding is that mitochondrial ROS accumulation may not depend on IgG concentration.

The association between exposure to Zn^0 nanoparticles and mitochondrial damage has been well documented. However, the studies investigating its effects on the mitochondrial quality control processes in the central nervous system are rather limited. Recently, Wang et al.[42] revealed that Zn^0 nanoparticles promoted mitochondrial dysfunction and apoptosis in the human neuroblastoma cell line SH-SY5Y. In this study, mitochondrial biogenesis regulator peroxisome PGC-1 (proliferator-activated receptor gamma coactivator 1-alpha) and fission protein dynamin-related protein 1 (Drp1) were found to be upregulated. Zn^0 also promotes autophagic flux, as well as PINK1/Parkin-mediated mitophagy. Similarly, in another study performed on the murine microglia cell line BV-2, Zn^0 was found to promote Parkin-mediated mitophagy, while the genetic ablation of PINK1 resulted in mitophagy failure, eventually leading to cell death.[43] In the context of hypoxia/reoxygenation, Bian et al.[44] examined the impact of ZnCl exposure on the cardiac cell line H9c2. They found that ZnCl induces mitophagy through an interaction with PINK1 and Beclin-1.[44] In this study, Zn^0 significantly upregulated PINK1 levels, which is consistent with these findings. Conversely, we found that the increase in active LC3B and Beclin-1 levels following Zn^0 exposure was not significant, which suggests that higher doses of Zn^0 may be required for the lysosomal degradation of the damaged mitochondria in microglia. Interestingly, Zn^0 exposure led to a substantial decrease in TRIM21, a protein that is involved in the proteasomal degradation of antibodies.[45] Given that TRIM21 is involved in the clearance of the pathogens bound to IgG, this finding suggests that Zn^0 may lead to an impairment of proteasomal degradation of cytosolic pathogens via downregulating TRIM21, which in turn may impair innate immune responses. Furthermore, IgG administration to all groups that were also exposed to Zn^0 resulted in a dose-dependent upregulation of TRIM21 in comparison to both

control and sole Zn^0 groups, suggesting that IgG internalization and subsequent degradation occurred. Concomitant with the increase in TRIM21 levels, IgG administration led to a significant decrease in PINK1 levels, indicating that mitochondrial damage was alleviated. However, IgG administration at high doses has been shown to increase Parkin levels, although Parkin acts as a downstream target of PINK1 and cannot promote mitophagy in the absence/decrease of PINK1.[45]

The results of this study confirmed that Zn^0 induced cytotoxicity in the human microglia cell line HMC3 by promoting oxidative stress. However, this effect was only observed for a brief period, indicating that microglia are capable of tolerating Zn^0-induced mitochondrial stress to a limited extent. Further investigations revealed that Zn^0-induced mitochondrial damage enhanced the mitophagy response by increasing PINK1 expression. However, in the presence of Zn^0, IgG administration protected cells against mitochondrial stress and hampered mitophagy, which may be attributed to its direct interaction via Zn. This conclusion is based on in silico evaluations and requires confirmation through in vitro studies.

A significant limitation of our study is that it is focused on the effects of Zn^0 but not the mechanistic effect of IgG alone, which may be investigated in further studies. In addition, the effect of receptor-mediated mitophagy, which involves FUNDC1, NIX/BNIP3L, and BNIP3, may be investigated along with the PINK1/PARKIN axis when evaluating the protective properties of IgG treatment on Zn^0 toxicity.[46] Moreover, for clarifying the impact of IgG on Zn-induced mitochondrial damage and mitophagy, Zn isotopes or labeled Zn may be used.[47,48]

Although it is well established that oxidative effects are induced by Zn^0 or ZnCl at extremely high and low levels,[49] Zn^0 induced cellular responses remain unclear. Our study is the first to demonstrate that Zn^0 exposure leads to cytotoxicity in microglia in vitro and may induce mitochondrial stress. Moreover, IgG administration along with Zn^0-exposed cells protected them against mitochondrial damage, suggesting that IVIg may be used in cases with high levels of acute Zn exposure. This study provides novel insights into the cellular and molecular mechanisms underlying Zn^0 neurotoxicity and proposes a new approach for therapeutic intervention with IgG in neuroinflammatory conditions associated with Zn dysregulation.

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