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Original Research Article

Zinc Toxicity Causes Necrotic Death of C6 Glioma Cells and Affects Chemosensory Ability of Caenorhabditis elegans

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Abstract :

Aims and Objectives: Zinc is an essential micronutrient responsible for cellular homeostasis and functioning. Exposure to elevated zinc levels leads to neurological disorders such as brain ischemia, trauma and epileptic seizures. The purpose of this study was to investigate the cellular mechanisms affected by high zinc exposure both *in vitro* (using C6 glioma cells) and *in vivo* (*Caenorhabditis elegans*) systems.

Materials and methods: Survival and mitochondrial integrity of C6 cells were measured by MTT assay with control and Zinc-exposed cells. Microscopic analysis following Giemsa staining was performed to assess the nature of cell death. Chemotaxis assay was performed with age-synchronized adult *C. elegans* using 2-Nonanone.

Results: Our results from the *in vitro* studies indicate that progressively increasing levels of zinc exposure affects cell survival and proliferation of C6 cells, with changes in cellular morphology, mitochondrial dysfunction and induction of reactive oxygen species (ROS)at 150 μM $ZnCl_2$. At 200 μM of $ZnCl_2$ necrotic cell death was observed. In vivo behavioural study with *C. elegans* showed that zinc increases the chemo-repulsive effect caused by AWB neuron-specific agent 2-Nonanone possibly due to higher levels of cholinergic signalling leading to increased repulsion.

Conclusion: High zinc exposure to glioma cells leads to necrotic cell death. Moreover, it leads to changes in chemotactic behaviour in nematode *Caenorhabditis elegans* suggesting that zinc toxicity adversely affects neuronal functions.

Keywords: zinc toxicity; reactive oxygen species; necrosis; glioma cell; *C. elegans*; chemotaxis

Introduction

Zinc is a micronutrient, and 2-3 g of zinc is present in adult humans, of which 90% is found in the bone and muscles.[1] In a cell, the nucleus contains 30–40% of total zinc, the cytosol 50%, and the remainder is membrane associated. [1] Zinc plays an essential role in cell division (mitosis), immune system function, protein and nucleic acids synthesis, and acts as a co-factor for more than 300 enzymes or metalloproteins.[2]In the central nervous system, high concentrations of zinc are found in brain regions like the hippocampus, amygdala, cerebral cortex, thalamus, and olfactory cortex.[2] Zinc is second only to iron in abundance in the central nervous system.[3] During embryonic development, zinc is essential for neural tube formation, neurogenesis and stem cell proliferation.[3] In the brain, zinc is required for synaptic and axonal transmission, tubulin growth & phosphorylation, & nucleic acid metabolism. [4] Besides nervous system, zinc plays an important catalytic (as

metalloenzymes), structural (eg, zinc-fingers domains of proteins) and gene expression regulation (Zn-finger transcription factors).[5] Therefore, maintenance of Zn homeostasis is crucial for many physiological functions.

Zinc deficiency adversely affects the gastrointestinal, central nervous system, immune, skeletal, and reproductive systems.[5] In the brain, Zn deficiency leads to impaired cognitive function, behavioural abnormalities, impaired memory, learning disability, and neuronal atrophy.[6] Moreover, high zinc level in the brain primarily acts as a neurotoxin than a gliotoxin.[7] C6 glioma cells have been routinely used for studying cellular oxidative stress. Low intracellular zinc concentrations showed decreased proliferation, increased oxidative stress, and increased single-strand DNA breaks in these cells.[8] On the other hand, high Zn concentration above 200 µM has been shown to cause DNA fragmentation[9]as well as necrosis (above 300 µM).[10]

The effects of zinc toxicity on the functioning of nervous system is limited. Therefore, the effect of zinc toxicity on neuromodulation was the focus of this study**.** Our *in vitro* studies suggest that high zinc level (in micromolar concentration) affects various cellular processes, which lead to necrotic cell death. Moreover, *in vivo* findings suggest that high zinc affects chemotactic behaviour of *C. elegans*.

Materials and Methods

Culture and Maintenance of C6 Glioma Cells and *Caenorhabditis elegans*

C6 glioma cell line was obtained from the Cell Repository at National Centre for Cell Sciences, Pune, India (Sophia College), and *Caenorhabditis elegans*(wild type N2 strain) stocks were obtained from Dr Yasmin Khan from Sophia College for Women, Mumbai. Cells were cultured using standard cell culture techniques using DMEM (Himedia), 5% FBS (Sigma Aldrich) and antibiotic mixture (Himedia). *Caenorhabditis elegans* were cultured on standard NGM (Nematode growth media) plates as described in the wormbook website (www.wormbook.org/toc/_wormmethods.html)

Zinc Exposure

C6 cells were exposed to various concentrations of $ZnCl_2$ (from 50µM to 200 µM) for 24 h. Age-synchronized adult *C. elegans* were exposed to 55µM to 220µM concentration of $ZnCl_2$ for 24-h.

Giemsa Staining

Giemsa staining was carried out using standard protocol. In brief, C6 cells were grown on circular glass coverslips in a 12-well culture plate. Following, coverslips were washed with 1X PBS and cells were fixed using ice-cold 100% methanol for 4 min, incubated with Giemsa solution for 30 min, washed with distilled water, mounted on slides using DPX and observed under the Olympus microscope.

MTT Assay

The mitochondrial enzymes [NADP(H)-dependent oxidoreductases] produced by viable cells are capable of reducing the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble form purple formazan. C6 Cells were cultured in a 96-well plate. Upon various concentrations of $ZnCl_2$ exposure for 24 hrs, MTT reagent (5 mg/ml, prepared in DMSO) was added to control and Zn-exposed cells for 4 hr. Formazan crystals formed were solubilized using DMSO to give a coloured solution and absorbance was measured at 570 nm.

DCFDA Staining

2',7'–dichlorofluorescin diacetate (DCFDA, also known as H2DCFDA) measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell. Asolution of 10 µg/mL DCFDA was prepared. After 24-h zinc exposure, C6 cells were washed with PBS and incubated in DCFDA solution for 45 min. The coverslips containing the cells were then mounted on a slide and viewed under fluorescence microscope (Olympus) at 480 nm and images were acquired using a cool CCD camera.

Neutral Red Uptake Assay

Lysosomal integrity and its ability to bind to neutral redis an indicator of cell viabilityand cell's capacity to maintain pH gradient via ATP production.[11] After 24 h of $ZnCl_2$ exposure, 100

µL of neutral red medium (40 µg/mL in DMEM) was added to C6 cells. After three hours, neutral red containing medium was removed and cells were washed with 1X PBS. Bound stain was dissolved with a de-staining solution (5.0 mL ethanol + 4.9 mL water + 0.1 mL glacial acetic acid) by keeping the plate on shaker for 10 min. The colour formed was measured using *Epoch 2 plate reader* at 540 nm.

Chemotaxis Assay of *C. elegans*

Chemo-repulsive behaviour of *C. elegans*, mediated by AWB neuron in response to 2-Nonanone was tested and measured using the chemotaxis index. The L3 stage worms were subjected to $ZnCl_2$ concentrations of 55 µM, 110 µM and 220 µM for a 24-hour exposure and the chemotaxis assay was performed as described by Margie et al.[12] In short, control and Zn-treated animals were placed on separate 60-mm assay plates and either control (vehicle solution) or test (2-nonanone) solutions were added in diagonally opposite quadrants on the plate. After one hour, the number of animals present in control or test quadrants were counted and chemotaxis index was calculated using the following formula.

$$CI = \frac{(\text{No. of Worms in Test Quadrant} - \text{No. of Worms in Control Quadrants})}{(\text{Total no. of Scored Worms})}$$

An index of +1.0 score indicates maximal attraction towards the target and represents 100% of the worms arriving in the quadrants containing the chemical target. An index of -1.0 indicates maximal repulsion.

Statistical analysis:

Student's t-test ($P < 0.05$) was carried out to study any significant difference in the outcome between control and treated groups.

Results

Zinc toxicity induces cellular disintegration & chromosomal condensation

The C6 cells were grown on coverslips and exposed to different concentrations of Zn for 24 h. Upon microscopic examination, no distinct morphological changes was observed in the control cells as well as 50 and 100 µM $ZnCl_2$ exposed cells (Fig. 1 a-c). However, at150 $ZnCl_2$, cells became circular and completely lost their processes (Fig. 1d, arrows)and nuclear condensation started (Fig. 1i). At 200 µM $ZnCl_2$, cell membrane ruptured completely and the nucleus condensed, as seen by Giemsa staining of the nuclei (Fig. 1e, arrows& 1j). At 100 µM $ZnCl_2$ cells have started retracting their processes (asterisks in Fig. 1h) confirming the initiation of cell vulnerability to high dosage of zinc.

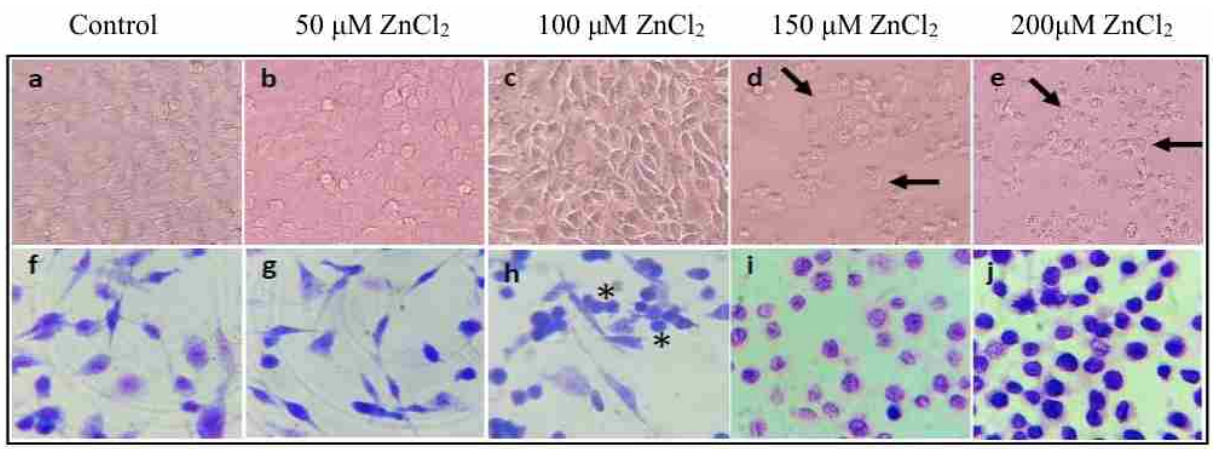

Figure 1 : *Morphological analysis of C6 glioma cells following exposure to different concentrations of $ZnCl_2$. Upper panel shows cellular morphology under bright field (a-e), lower panel (f-j) shows cellular morphology and changes in nuclear appearance following Giemsa staining.*

High zinc dosage alters cellular metabolic function

Both control and $ZnCl_2$-exposed cells were processed for MTT assay, in which viable and metabolically active cells reduce yellow tetrazolium salt into a purple formazan product, and the absorbance was measured at 570 nm. Percent survival of cells were calculated using the following formula:

$$\frac{(OD_T - OD_B) \times 100}{(OD_C - OD_B)}$$

Where OD_T = absorbance of the treated cells, OD_C = absorbance of control (no treatment) cells and OD_B = absorbance of blank.

From the graph (Fig. 2) it is evident that the cells remain metabolically active up to 100 µM $ZnCl_2$. However, at 150 µM, the viability reduced to 57% and further to 26% at 200 µM $ZnCl_2$ indicating metabolic breakdown of the cells and thus affecting cell survival, proliferation as well as mitochondrial activity. Our result suggests that $ZnCl_2$ at 150 µM and higher concentration is increasingly toxic to cells.

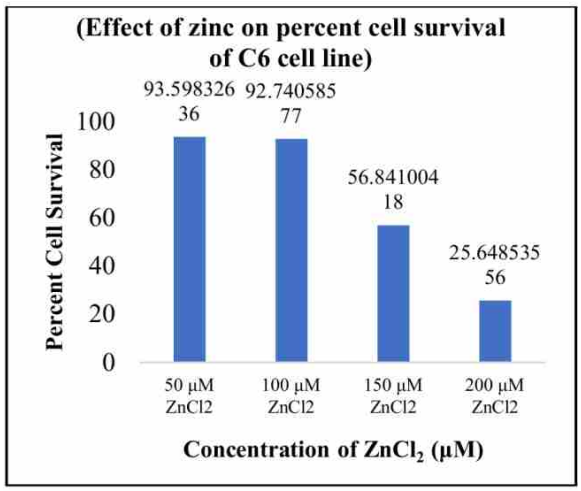

Figure 2*. Graphical representation of percent cell survival following MTT assay of $ZnCl_2$-treatedC6 glioma cells, suggesting that metabolic activity of C6 cells reduces significantly at 150 µM and more of $ZnCl_2$.*

Zinc induces ROS production by interfering mitochondrial respiratory chain function

In order to assess if ROS generation is responsible for cellular toxicity, DCFDA staining were carried out. DCFDA measures hydroxyl, peroxyl and other reactive oxygen species activity in a cell. In healthy cells the dye gets deacteylated to a non-fluorogenic product via cellular esterase. Under oxidative stress, ROS oxidises non-fluorogenic compound to a fluorogenic product DCF (2,7 dichlorofluorescin), which is visualized at 495/529 nm using a florescent microscope. No detectable fluorescence was observed in untreated (Fig. 3a and f), 50 µM (Fig. 3b and g) and 100 µM $ZnCl_2$ treated (Fig. 3c and h) cells. However, cells treated with 150 µM and 200 µM $ZnCl_2$showed intense fluorescence (Fig 3d and 3i ; 3e and 3j, respectively) suggesting that high zinc dose leads to high ROS generation by interfering with the functioning of mitochondrial respiratory chain.

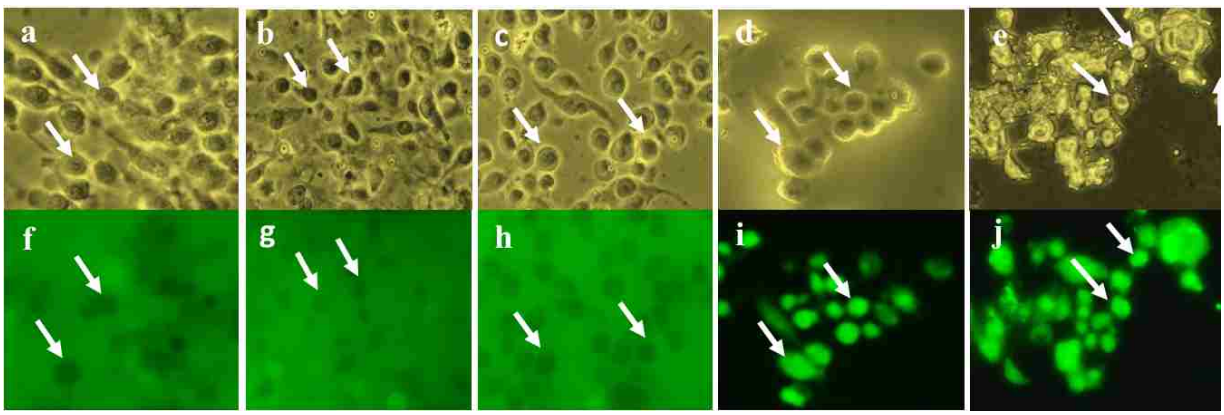

Figure 3. *DCFDA Fluorescencewas not observed in untreated (a and f), 50 µM (b and g) and 100 µM (c and h) ZnCl2-treated cells, but observed in 150 µM (d and i) and 200 µM (e and j) ZnCl2 treated cells.*

Lysosomal integrity is maintained at 150 µM $ZnCl_2$despite disruption of cellular metabolic function

As the DCFDA assay suggested a decreased mitochondrial function leading to less ATP production, an assessment of the ATP production was carried out using Neutral Red Uptake assay, which also indicates lysosomal integrity. Loss of lysosomal integrity is one of the hallmarks of cellular degeneration.

Neutral Red Uptake assay is based on the ability of the lysosomes to incorporate the weakly cationic dye neutral red. The dye diffuses into into lysosomes where it binds by electrostatic interactions with negatively charged phosphate or other anionic groups present in the lysosomal matrix. In a living cell, a proton gradient maintained by ATP inside the lysosomes makesthe dye more acidic. So, the dye becomes positively charged and is retained in the lysosomes. However, if ATP production is decreased, these pH gradients are disturbed thus leading to the leakage of the dye into the surrounding environment.

C6 cells exposed with various concentrations of zinc for 24 hrs and then stained for neutral red dye showed that there was high uptake of the dye up to 150 µM of $ZnCl_2$. However, at 200 µM concentration, the uptake was drastically reduced (Fig. 4a and b). At this concentration, only 17.48% cells survived.

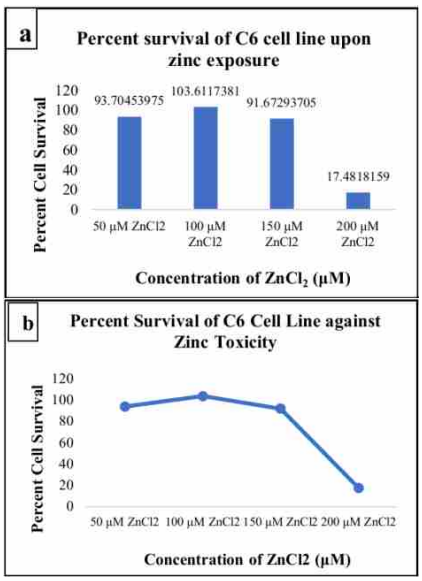

Figure 4*. Graphical representation of percent cell survival following ZnCl2 treatment as measured through Neutral Red uptake assay. This suggests that lysosomal activity of C6 cells remain intact up to 150 µM of ZnCl2.*

Zinc enhanced repulsion to 2-Nonanone mediated by AWB neurons via acetylcholine signalling

Chemosensory ability of *C. elegans* is responsible for detecting food (attraction) and toxic substances (repulsion). The worms were synchronized, and the L3 stage larvae were exposed to 55 µM, 110 µM, 220 µM of zinc for 24 hrs. The L4/adult worms were then used to perform a chemotaxis assay and chemotaxis index was calculated as mentioned in Material and Methods.

Chemotactic ability of the worms was tested using 2-Nonanone, a compound is sensed by the olfactory AWB sensory neurons in *C. elegans*.[13] These neurons are responsible for avoidance to 2-nonanone, which is mediated via acetylcholine signalling.[14] The worms were treated with various zinc concentrations for 24-h. With increasing zinc concentration, the worms exhibited progressively increasing ability to avoid 2-Nonanone (Fig. 5). This indicates that exposure to higher concentrations of zinc increases sensitivity to the compound leading to a greater avoidance.

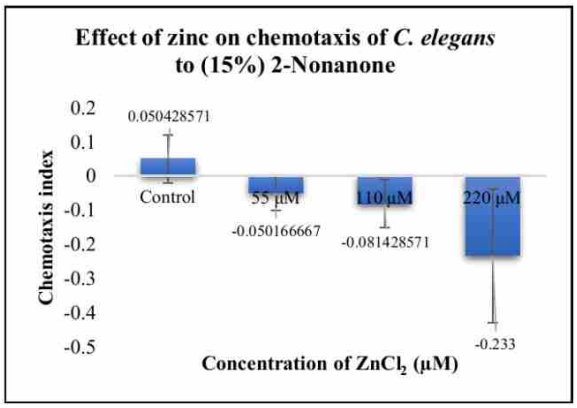

Figure 5. *Bar diagram showing data for chemotaxis of C. elegans to 2-Nonanone specific to the AWB sensory neurons. With increasing concentration of zinc, avoidance to 2-nonanone progressively increased.*

DISCUSSION

Heavy metals are known to be potent neurotoxins. Zinc has deleterious effects in cells through mechanisms of oxidative stress, excitotoxicity or reduced energy metabolism. The project intended to perform an *in vitro* and *in vivo*

analysis to understand the effects of zinc over exposure /toxicity on the nervous system. *In vitro* analyses focused on studying the effect of zinc on C6 glioma cells, while an*in vivo* analysis used *C. elegans* as a model system in order to understand the effect of elevated zinc levels on the behaviour of the organism.

In Vitro Studies: An indication of necrotic cell death

C6 cells have processes extending from the cell body. Morphological analysis post zinc exposure showed that at 100 µM $ZnCl_2$, many cells had retracted cellular processes, which is an indication of microtubular depolymerization/ degradation of the cells. Microtubule depolymerization occurs initially before any other cellular changes, indicating the onset of cell's vulnerability to high zinc doses. At 150 µM $ZnCl_2$, cells turned hypertrophic, became circular, cell surface smoothened and had completely lost their processes. At 200 µM, the cell membrane was completely ruptured, which is a precursor step to cell lysis.

Moreover, Giemsa staining revealed that starting from 150 µM $ZnCl_2$ exposure, the cell bodies were brightly stained, marking the beginning of nuclear condensation. At 200 µM $ZnCl_2$, the intensity of the stain was highest. The hyper-condensed DNA appearance is a characteristic of anucleolytic pyknosis seen in necrosis.[16]

Optimum cellular metabolism is required for cell survival and proliferation. Cellular metabolic function was assessed using MTT assay, which measures the ability of mitochondrial enzymes to reduce the MTT dye to an insoluble formazan product. It was observed that beyond 100 µM zinc concentration, cellular viability has drastically decreased due to degradation of the enzymes in the cell.

One of the contributory factors for sub optimal mitochondrial functioning could be high ROS generation. The DCFDA staining clearly showed that cells treated up to 100 µM $ZnCl_2$ did not generate any significant amount of ROS. However, above 100 µM $ZnCl_2$ green florescent cells were observed as deacetylated DCFDA was oxidised by ROS to DCF. This suggests that toxic doses of zinc lead to generation of high amounts of ROS by interfering with the functioning of mitochondrial respiratory chain.

We lastly assessed lysosomal integrity in the cells as it is known to be one of the hallmarks of cellular degeneration. Our result indicates that the lysosomal integrity is maintained till 150 µM $ZnCl_2$even when cellular metabolic function is disrupted. Moreover, cell membrane lysis was observed at 200 µM $ZnCl_2$. This suggests that lysosomal breakdown causes release of cytosolic hydrolases, which in turn leads to cell membrane digestion. This also suggests that cellular mechanisms that are essential for cell survival, exhibit differential vulnerability to different doses of zinc thus leading to a step-wise breakdown of cellular morphology and metabolic activity and eventually causing cell lysis.A previous study reported a similar observation, wherein zinc treatment led to oxidative stress-induced lysosomal dysfunction through lysosomal membrane permeabilization, which eventually caused cell death in neurons and astrocytes.[17] ROS-mediated lysosomal membrane permeabilization has also been observed in drug-induced death of intervertebral cells.[18] Lysosomal degradation is known to cause release of enzymes of the cathepsin family that cause cytosolic acidification and release of cytosolic hydrolases that lead to cell membrane disruption and eventually cell lysis. This is confirmed by the findings of the morphological studies, wherein, at 200 µM $ZnCl_2$, C6 cells had lost their cell membrane and had begun to undergo cell lysis.

Cell death is known to occur by two mechanisms: necrosis and apoptosis. Cells respond to toxic agents either by progressing through apoptotic pathway, and in severe condition like hypoxia, immune response, through the necrotic pathway. These pathways have distinct morphological features that help distinguish a cell undergoing apoptosis or necrosis. Although previous studies indicate cells respond viaapoptosis to zinc-induced toxicity, there is evidence of zinc being a protective agent against apoptosis.[19] Moreover, there are other reports indicating zinc's involvement in necrosis. Iguchi and co-workers reported that zinc induces necrosis in prostrate carcinoma cells and increase expression of several molecules involved in necrotic cell death. In the same line, our studies on

C6 glioma cell lines also suggests that toxic amount of zinc causes necrotic cell death.[20]

In-Vivo Studies - Role of zinc toxicity in *C. elegans* sensory neuron activity

Zinc is known to modulate synaptic transmission in the nervous system. In a glutamatergic synapse, zinc is co-released alongside glutamate in the synaptic cleft leading to excitation of the post synaptic neuron. Zinc enters the post synaptic neurons via VGCC, AMPA and NMDA receptors and may cause excitotoxicity. Therefore, understanding the effects of elevated zinc levels on the functional modulation of the postsynaptic neurons was one of the focus of this study. Upon exposure to zinc, we observed an elevated avoidance response of the worms to a known aversive agent 2-nonanone. The AWB olfactory sensory neurons in *C. elegans* are known to exhibit avoidance behaviour mediated by exposure to 2-Nonanone.[21] Such behaviours are important in order to avoid noxious substances, to detect favourable surroundings, for mating and to sense food. A complex integration of these signals is responsible for avoidance or attraction to surrounding cues. AWB sensory neurons detect 2-Nonanone and further executes motor activity (in this case chemorepulsive movement) via postsynaptic cholinergic motor neurons. Zinc is known to modulate neuronal cholinergic receptors in a biphasic manner (leading to potentiation or inhibition) depending on the receptor type it binds to.[22]The observation that with increasing zinc concentration there was an increase in avoidance to 2-Nonanone suggests that increased zinc concentrations cause excessive cholinergic stimulation on the postsynaptic neuron leading to increased avoidance to 2-nonanone.

To the best of our knowledge, this is the first detailed *in vitro* and *in vivo* study analyzing the effect of zinc toxicity on C6 glioma cells and *C. elegans* respectively, wherein we show that exposure to zinc leads to necrotic death of C6 cells by executing a dose-dependent sequential changes in the cellular mechanisms, like generation of high amounts of reactive oxygen species, which is turn leads to permeabilization of lysosomal membrane and eventually cell death by disintegration of the cell membrane. In addition, *in vivo* study of zinc toxicity on *C. elegans* suggests that zinc,even at relatively lower concentrations, alters chemosensory behaviourof the worms.

Limitations of the study

Our study did investigate the molecular pathways regulating the observed cellular and behavioural changes, which could have given a better understanding of the toxic effect of zinc. A more direct approach could be studying the effect of zinc on somatic cell lines rather than a cancerous line.

Acknowledgement

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Ethical statement

Permissions from the Institutional Biosafety Committee (IBSC) was obtained for working with C6 glioma cell lines. For *in vivo* studies, wild-type (N2 strain) *C. elegans* was used, which did not require any ethical clearance.

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