Toxicology in Vitro 35 (2016) 24–30

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

# Mechanistic studies of the toxicity of zinc gluconate in the olfactory neuronal cell line Odora

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ARTICLE INFO

*Article history:*
Received 24 February 2016
Received in revised form 6 May 2016
Accepted 9 May 2016
Available online 12 May 2016

*Keywords:*
Olfactory neurons
Toxicity
Odora
Zinc gluconate
Pyroptosis

ABSTRACT

Zinc is both an essential and potentially toxic metal. It is widely believed that oral zinc supplementation can reduce the effects of the common cold; however, there is strong clinical evidence that intranasal (IN) zinc gluconate (ZG) gel treatment for this purpose causes anosmia, or the loss of the sense of smell, in humans. Using the rat olfactory neuron cell line, Odora, we investigated the molecular mechanism by which zinc exposure exerts its toxic effects on olfactory neurons. Following treatment of Odora cells with 100 and 200 μM ZG for 0–24 h, RNA-seq and in silico analyses revealed up-regulation of pathways associated with zinc metal response, oxidative stress, and ATP production. We observed that Odora cells recovered from zinc-induced oxidative stress, but ATP depletion persisted with longer exposure to ZG. ZG exposure increased levels of NLRP3 and IL-1β protein levels in a time-dependent manner, suggesting that zinc exposure may cause an inflammasome-mediated cell death, pyroptosis, in olfactory neurons.



## 1. Introduction

Zinc is an essential element for many organisms, and zinc deficiency has been associated with multiple adverse conditions, including impaired immune function (Bonaventura et al., 2015; Brooks et al., 2004; Muller et al., 2001). In response to this observation, over-the-counter zinc lozenges and nasal sprays were introduced for the treatment of the common cold in the United States during the late 1990s. Unfortunately, the scientific and medical community began to report incidences of anosmia, or the loss of the sense of smell, among people who had used intranasal zinc sprays and gels (Alexander and Davidson, 2006; DeCook and Hirsch, 2000; Jafek et al., 2004). Due to potential bias in reporting symptoms and actual use of the zinc gluconate (ZG) nasal products, Davidson and Smith evaluated all available evidence at the time concerning the probability of a causal relationship between zinc nasal products and loss of the sense of smell. They concluded that there was evidence of causality between over the counter ZG nasal sprays and impaired sense of smell (Davidson and Smith, 2010). The large number of incidences of anosmia resulted in the removal of intranasal (IN) zinc sprays from store shelves. However, a recent article has reported on several clinical trials which use IN insulin for the treatment of food cravings, forgetfulness in Alzheimer's patients, and diabetes (Dash et al., 2015; Freiherr et al., 2013; Hallschmid et al., 2012; Hamidovic, 2015). This is a concern due to the use of zinc to stabilize insulin and to prevent protein aggregation in these formulations (Manallack et al., 1985). While there have not yet been any published reports of anosmia in these trials, possibly due to the relatively small samples size and low zinc concentration, there is the potential for more cases of anosmia in these clinical trials which use IN insulin formulations containing zinc.

Unlike most neurons in the body, olfactory neurons are generated throughout one's lifetime. This process is not synchronized among the neurons, which means that there is a heterogeneous distribution of immature, mature, and dying neurons present in the nose at any given time (Cancalon and Elam, 1980; Farbman, 1994). Thus, it is surprising and alarming that the anosmia in some users of IN ZG spray seem to be permanent, suggesting that the basal cell population, which gives rise to olfactory neurons was destroyed; this very phenomenon was reported in mice treated with 2.6-methylsulfonyl-2.6-dichlorobenzene, a toxicant that destroyed the olfactory epithelium, including basal cells, resulting in respiratory metaplasia, but not recovery of the olfactory epithelium (Bahrami et al., 2000).

There are many proposed mechanisms for zinc-induced toxicity ranging from oxidative stress to impaired ATP production to metal dyshomeostasis. Thus, the elucidation of the mechanism of zinc toxicity is more complicated than it would appear, and is affected by many factors, such as concentration of zinc tested, the length of exposure, the cell

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http://dx.doi.org/10.1016/j.tiv.2016.05.003
0887-2333/

type, and the presence of other toxic chemistries. These mechanisms are summarized below in Table 1.

In this study, we used the rat olfactory neuronal cell line, Odora. These cells are an excellent model for olfactory neurons in whole animals because they can properly target odorant receptors to the cell membrane and respond to odorants (Murrell and Hunter, 1999). In addition, as with the intact olfactory mucosa in vivo, Odora cells can be grown as immature undifferentiated neurons or as mature differentiated neurons. In order to elucidate the mechanism underlying zinc-induced anosmia, we performed a series of experiments, beginning with RNA-seq to identify genes and pathways that were up- or down-regulated in response to zinc exposure. After analysis of significantly regulated pathways, zinc-induced changes were confirmed using biochemical assays to measure changes in oxidative stress and ATP production as well as determining the critical amount of time for zinc exposure to irreversibly lead to cell death. Upon finding negative evidence for zinc-induced apoptotic cell death, based on the absence of caspase 3 and 9 upregulation, as well as negative results in an in vitro apoptosis assay, we pursued pyroptosis as a potential mechanism of zinc-induced cell death. Pyroptosis is a caspase-1 mediated mechanism of cell death involving the formation of the inflammsome via recruitment of Nod-like receptor protein 3 (NLRP3) and caspase-1. The activation of caspase-1 results in the activation and release of interleukin-1β, −6, and −18 from the cell into the extracellular space. Characteristics of pyroptosis include loss of plasma membrane integrity, cell swelling, and release of pro-inflammatory intracellular contents (Fink and Cookson, 2005). In vitro studies also identified the important role of caspase-1, the cytokine IL-1β and inflammasome activation, suggesting pyroptosis as a mechanism of cell death.

The goals of this study were to (i) determine what genes and pathways are up- or down-regulated by exposure to ZG in the olfactory neuronal cell line Odora, (ii) determine whether apoptosis occurs in zinc-induced cytotoxicity in Odora cells and (iii) determine whether zinc-induced toxicity in Odora cells can be prevented using anti-oxidants and enzyme inhibitors.

## 2. Materials & methods

### 2.1. Chemicals and reagents

Odora cells were plated in 96-well plates (Falcon, Tewksbury, MA) and 145 × 20 mm tissue culture dishes (Greiner Bio One, Monroe, NC). DMEM, HBSS, and trypsin/EDTA from Hyclone (GE Healthcare Life Sciences, Logan, UT) were used for the cell culture work. Zinc gluconate (MP Biomedicals LLC, Santa Ana, CA) and crystal violet (Allied Chem. Corp., NY, NY) solutions were prepared using distilled water. For the biochemical assays, nitric oxide, and apoptosis work, *o*-phthalaldehyde (OPA, Alfa Aesar, Ward Hill, MA); luminol, horseradish peroxidase, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), apocynin and sodium arsenite (Sigma-Aldrich, St. Louis, MO); perchloric acid (Thermo Fisher Scientific, Waltham, MA), *N*-acetylcysteine (NAC, Acros, NJ) were used. The caspase-1 inhibitor, Ac-YVAD-cmk was obtained from Calbiochem (Billerica, MA). All assays utilizing a plate reader were performed with the Spectramax M2e multi-mode plate reader (Molecular Devices, Sunnyvale, CA). Fluorescence measures were performed with the F-4500 Fluorescence Spectrophotometer (Hitachi, Schaumburg, IL), while luminescence measurements were performed with either the Autolumat Plus luminometer (Berthold, Oak Ridge, TN) or Luminometer Model TD-20/20 (Turner BioSystems, Sunnyvale, CA).

**Table 1**
Mechanisms of zinc toxicity.

| Mechanism of zinc toxicity | Reference |
|---|---|
| Inhibition of glutathione reductase | Bishop et al., (2007) |
| Production of ROS and/or activation of NADPH oxidase & nitric oxide synthase | Bishop et al., (2007), Kim and Koh, (2002), Lopez et al., (2011) |
| Induction of apoptosis via DNA fragmentation and cytochrome C release | Rudolf, (2007), Feng et al., (2002) |
| Induction of necrosis | Iitaka et al., (2001) |
| Impaired ATP production via inhibition of glycolytic and TCA cycle enzymes | Rudolf, (2007), Dineley et al., (2003), Sheline et al., (2000) |
| Impaired function of the electron transport chain | Lorusso et al., (1991), Link and von Jagow, (1995) |
| Inhibition of proton channel (HVCN1), leading to intracellular acidosis | Iovannisci et al., (2010) |
| Dysregulation of copper homeostasis | Willis et al., (2005), Hedera et al., (2009) |
| Dysregulation of calcium homeostasis | Guo et al., (2014) |

### 2.2. Cell Viability

Odora cells were obtained from Dr. Dale Hunter and cultured according to published protocols.(Murrell and Hunter, 1999). Immature Odora cells were seeded in 96-well plates (25,000 cells well$^{-1}$) and allowed to attach and grow for 24 h. Cells were then treated with the ZG at concentrations ranging from 50 μM to 500 μM for times ranging from 1 to 24 h. An equivalent volume of sterile water was added to the media as the vehicle control. After the appropriate length of ZG exposure, crystal violet was used to assess viability as previously described (Kueng et al., 1989) by measuring absorbance at 540 nm. Results were expressed as a percentage of control (water-treated) cells. $LC_{50}$ values were calculated using the SigmaStat software's 4 parameter logistic equation (sigmoidal) for each experiment. The $LC_{50}$ from the three biologically independent replicates were averaged together to yield a mean $LC_{50}$. $LC_{50}$ studies were similarly conducted in differentiated Odora cells; due to the slower rate of growth of the differentiating Odora cells, these were allowed to grow for 72 h prior to ZG treatment.

### 2.3. RNA-seq analysis

Odora cells were seeded in tissue culture dishes (145 × 20 mm dish, $10^6$ cells plate$^{-1}$) and allowed to attach and grow for 48 h. Cells were then dosed with 100 μM ZG for 6, 12, or 24 h or 200 μM ZG for 6 h. An equivalent amount of water was added in the vehicle control dishes. At the specified time points, RNA was isolated with mirVana miRNA Isolation Kit (Lifetech, Carlsbad, CA). All steps of library construction, cluster generation, and HiSeq (Illumina) sequencing were performed with biological triplicate samples by the Genomics, Epigenomics, and Sequencing Core of the Department of Environmental Health, University of Cincinnati. Differential gene expression analyses between the vehicle control and ZG-treated cells was performed separately at each of the three different exposure time points. Significant genes were selected based on a false-discovery rate–adjusted $p$-value <0.01. RNA-seq data was further analyzed using DAVID Bioinformatics Resource 6.7 (Huang da et al., 2009). The gene expression data and results were deposited in GEO (Barrett et al., 2009) and can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79730.

### 2.4. Protein quantification

Protein concentrations were determined by the bicinchoninic acid method using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and following manufacturer's instructions. The plate was incubated at 37 °C for 30 min before the absorbance was read at 562 nm.

### 2.5. Biochemical Assays

Odora cells were seeded in tissue culture dishes (145 × 20 mm, $10^6$ cells plate$^{-1}$) and allowed to attach and grow for 48 h. Cells were treated with 100 μM ZG for 6, 12, or 24 h (or an equivalent volume of sterile water) before the cells were harvested. GSH and GSSG in Odora cell homogenate were fluorometrically measured following