Homeopathy (2017) 106, 103–113

http://dx.doi.org/10.1016/j.homp.2017.03.003, available online at http://www.sciencedirect.com

In vitro effects of Zinc in soluble and homeopathic formulations on macrophages and astrocytes

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Zinc is an important metal in body homeostasis. Zinc in soluble form (Zn^{2+}) and homeopathic *Zincum metallicum* were tested in macrophages and astrocytes in order to investigate its potential toxic or therapeutic effects. We evaluated cell viability (WST assay), cytokine production such as tumour necrosis factor alpha (TNF-α) and interleukin 10 (IL-10) by enzyme-linked immunosorbent assay (ELISA) and nitric oxide release by Griess reaction. The effect of zinc-depletion and high zinc pre-treatments on the cell adaptation capability was also investigated. In THP-1 macrophage cell line and in human primary macrophages, Zn^{2+} at sub-toxic doses (30 μM) caused stimulation of TNF-α and IL-10 with different dynamics reaching the maximum peak at the zinc concentration 100 μM, before the cell death. Highest doses (300 μM) impaired dramatically cell vitality. Similar effects on cell viability were obtained also in C6 astrocytes, where Zn^{2+} slightly increased the nitric oxide release only in cells activated by one of the pro-inflammatory stimuli used in our cellular model (interferon gamma plus TNF-α). Zinc depletion markedly reduced IL-10 production and cell viability. *Zincum metallicum* did not cause toxicity in any cell type and showed some small stimulation in WST assay that was statistically significant in a few experimental conditions. *Homeopathy* (2017) **106**, 103–113.

Keywords: *Zincum metallicum*; Zn^{2+}; Macrophage; Astrocyte; Cytokine production; Inflammation; Nitric oxide; Hormesis

Introduction

Zinc is an essential micronutrient and plays a key role for many functions at the cellular level, because of its occurrence in over one thousand enzymes as a catalytic ion and in at least the same number of proteins as a structural metal (metalloproteins). Moreover, zinc is involved in the activation of DNA expression regulatory proteins, the 'zinc finger' proteins, that act as a component of transcription factors in eukaryotes.

Being the second most abundant trace element in the body, zinc affects both innate and adaptive immune cells but also the central nervous system (CNS) behaviour, since the brain contains the highest cation concentration of any other organ. A disrupted zinc homeostasis causes impaired immune and brain function, affecting the expression of genes and the activity of enzymes related to the inflammation and oxidative stress.[1] At the cellular level, the zinc balance is highly regulated and Zn^{2+} excess, as well as its deprivation, are detrimental to cells. Zinc deficiency is closely linked to insufficient dietary intake, impaired resorption, or to some chronic diseases; controlled zinc supplementation can restore normal states of health.[2]

During *in vitro* zinc deficiency, phagocytosis and cytotoxicity increase and monocyte oxidative burst is enhanced.[1] Additionally, increased maturation into monocytes was observed after zinc depletion, suggesting that low zinc status promotes differentiation of myeloid precursors into monocytes.[3] The zinc-induced release of proinflammatory cytokines *in vitro* has already been reported.[4] In monocytes, zinc influences the secretion of proinflammatory cytokines in a concentration-dependent manner both enhancing and inhibiting cytokine release. In monocytes *in vitro* low zinc concentrations increase lipopolysaccharide (LPS)-induced secretion of proinflammatory cytokines, whereas higher concentrations negatively affect cytokine production.[5] Nevertheless, the

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Received 29 July 2016; revised 27 February 2017; accepted 8 March 2017

specific amount of zinc, which influences cytokine release depends on the cell model and experimental conditions.

Zinc signals are required for NF-κB activation and subsequent cytokine production.[6] At the molecular level, zinc regulates the NF-κB activation, modulating the degradation of the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate and the activity of protein kinase A. In the presence of critically decreased or elevated intracellular zinc concentrations, cell signaling induced e.g. by LPS, like TLR-4-mediated cytokine release via cyclic nucleotide, is affected. The result of this altered signaling is that the NF-κB-activating pathways are inhibited, resulting in impaired cytokine release.[7]

Besides macrophages, another cell type affected by the zinc effects is the astrocyte, a key cell for neurodevelopment and neuroinflammation and involved in several pathological conditions including Alzheimer's disease and aging.[8,9] Astrocytes are the major glial cells present in central nervous system (CNS). In counting, they greatly outnumber neurons, microglia or oligodendrocytes. Inducible nitric oxide synthase (iNOS) is expressed in astrocytes in a signal-dependent fashion. Nitric oxide (NO) in excess of physiological thresholds is produced and this excess NO then plays a role in the pathogenesis of stroke, demyelination and other neurodegenerative diseases. Astroglial iNOS is upregulated in response to a wide range of inducers like bacterial and viral products and proinflammatory cytokines. Changes in Zn^{2+} free concentration ($[Zn^{2+}]_i$) makes an important contribution to signal transduction by NO.[10]

Metallothioneins (MTs) are critical to intracellular Zn^{2+} homeostasis, as it has the ability to bind up to seven zinc atoms per MT molecule. MTs act as sensor of cellular redox such that a shift to more oxidizing conditions leads to release of zinc, whereas a shift to a more-reducing environment leads to binding of zinc.[11] NO-dependent increase of $[Zn^{2+}]_i$ critically depends on the S-nitrosation of cystein residues of MTs, promoting Zn^{2+} release both *in vitro* and *in vivo*.[12,13] NO is capable of increasing the amount of labile Zn^{2+} in living cells from the hippocampus.[14] An elevated $[Zn^{2+}]_i$ inhibits iNOS activity, and it could be a regulatory mechanism through zinc released by MTs controls acute inflammatory reactions.[15] The resultant changes in labile Zn^{2+} could then have a modulating influence on a number of intracellular events including effects on enzyme activity and/or the regulation of gene expression.

Low zinc levels are associated with decreased cell survival, altered neuronal differentiation, and, in particular, synaptic function.[16] The lower zinc concentration is related to aging and to some pathological events like amyotrophic lateral sclerosis,[17] aluminium-induced neurodegeneration,[18] epilepsy,[19] and the beneficial effects of zinc supplementation is documented in these cases. A low zinc bioavailability during aging has been linked to abnormal increments of zinc-bound MTs in hippocampus of old rats.[20] Furthermore, in aged astrocytes and microglia, genes implicated in 'zinc binding' showed increased transcript levels compared with young cells.[21] The molecular mechanisms underlying the beneficial effects of zinc restored physiological concentrations are still in many cases to be clarified although in certain cases it seems clear that zinc plays an important role in antioxidant defence.[22] However, controversies remain regarding the effect of zinc supplementation in the improvement of oxidative stress.

The physicochemical nature of zinc — namely a soluble, ionic, formulation or a condensed state as in form of nanoparticles (NPs) — is of paramount importance for mediating its biological and pharmacological effects. The presence of zinc in its soluble ionic form in the CNS has been implicated in the regulation of many channels and receptors, exerting important modulatory effects on neurotransmission and synaptic function. Zinc is released upon synaptic signaling and astrocytes are capable of binding and taking up extracellular zinc.[23] Others have found that astrocytes are less sensitive to the toxic action of $[Zn^{2+}]_i$ elevation compared with neurons for their high antioxidative capacity. A recent study[24] focuses on the interaction of zinc oxide (ZnO) NPs with rat C6 glial cells revealing that the NPs are taken up by the astrocytes and induce a toxicological reaction, when delivered at a concentration above 10 μg/ml. ZnO NPs can reach the brain through the olfactory neuronal pathway and can interfere with the brain zinc homeostasis.

Given the uncertainties on the role of zinc in human health further research is needed both at clinical and laboratory level to ascertain if zinc supplementation may be beneficial or detrimental, especially with regard to the doses (e.g. homeopathic or oligotherapic) and formulations (soluble or nanoparticulate) employed. In this context, we selected two possible cellular targets of zinc action, namely macrophages and astrocytes, to evaluate the effects of this metal on some parameters of inflammation like proinflammatory cytokines and NO production, important factors of their function.

The THP-1 cell line resembles primary monocytes and when differentiated with phorbol esters it develops macrophage morphology and functional properties. C6 cell line is one of the most used glial cell lines and is characterized by functional properties similar to astrocytes and by high release of NO upon cell activation. The advantage of NO detection is also linked to the extreme sensitivity and precision of this method and its low cost.

In the first part of the work here reported, we tested the soluble ionic form (Zn^{2+}), in a wide range of doses, from 0.001 to 300 μM confirming that these factors are good zinc targets in our cellular models. We also investigated the ability of the cells to pre-adapt to zinc toxicity during short pre-incubation with sub-cytotoxic doses of Zn^{2+}, and to zinc depletion. Then, we studied the effects of treatment with *Zincum metallicum* (*Zinc met*) that is a homeopathic medicine obtained by fine triturating the metallic zinc filings in lactose powder and by subsequent serial dilutions in water.

This research was done using water dilutions from the original preparations manufactured at the Federal University of Rio de Janeiro for the Italo-Brazilian multicenter study. *Zinc met* is defined in *Materia medica* by several behavioural and inflammatory symptoms, suggesting that our