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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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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

cellular lines are a good model for studying also this homeopathic remedy. We tested *Zinc met* low- (2c, 3c and 4c) and high-dilutions (15c) on THP-1 and primary human macrophages observing cell vitality and cytokine production. The same dilutions were tested on rat astrocytes (C6 cell line) that are able of releasing NO upon cell activation with interferon gamma (IFN-γ) and LPS or tumour necrosis factor alpha (TNF-α). In the latter cellular model we could not evaluate the cytokine production due to budget limitations since this research was done without external sponsors.

Materials and methods

Materials

The human monocytic leukaemia cell line THP-1 was purchased from DSMZ (Germany) and C6 glioma cells were kindly provided by Dott. Vittorina della Bianca (Department of Medicine, University of Verona). Growth media RPMI 1640, Ultraglutamine 20 mM solution, Trypsin−Versene Mixture (1×), Penicillin 10.000 UI/ml Streptomycin 10.000 UI/ml were purchased from Lonza (Verviers, Belgium) and DMEM F12 from Aurogene (Rome, Italy). Foetal bovine serum (FBS), phorbol 12-myristate 13-acetate (PMA), pure ethanol and ultra-pure water (W3500) were purchased from Sigma−Aldrich Co. (St. Louis, MO, USA). Human macrophage colony stimulating factor (MCSF) was purchased from Macs-Miltenyi Biotec (Bergisch Gladbach, Germany). Ficoll-Hypaque and Percoll were purchased from GE Healthcare Life Science (Uppsala, Sweden). The intracellular zinc chelator N,N,N′,N′-Tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), zinc chloride/$ZnCl_2$ and zinc acetate/$Zn(CH_3COOH)_2$ were purchased by Sigma−Aldrich Co. Sulfanilamide, N-1-napthylethylenediamine dihydrochloride (NED), sodium nitrite standard and lipopolysaccharide from *Escherichia coli* O26:B6 were purchased by Sigma−Aldrich Co., cell proliferation reagent WST-1 assay by Roche Life Science (Monza, Italy) and Bio-Rad protein assay by Bio-Rad (Milan, Italy). Recombinant rat IFN-γ was purchased by R&D Systems (Minneapolis, MN, USA) while recombinant rat TNF-α by PeproTech (Rocky Hill, NJ, USA). The DMEM F12 contains zinc sulphate heptahydrate at the concentration of 1.5 μM and RPMI 10% FBS 0.3 μM.[25]

Test solutions

Soluble Zn^{2+} solutions were prepared by serial dilutions (from 3 mM to 10^{-4} mM) of stock solution of Zn-acetate or Zn-chloride as indicated. The Zn-acetate solutions were prepared in bidistilled ultrapure water, sterile filtered (0.22 μm) and succussed in sterile glass tubes after each dilution step. The neutrality of pH was controlled. Zn-chloride serial dilutions from 3 mM to 10^{-4} mM were prepared in DMEM F12 plus 2% FBS and 100 U/ml penicillin/streptomycin (complete cell culture medium) from a 200 mM stock in sterile filtered distilled water. All procedures for drug preparation and cell treatments were done in sterile conditions.

We used two different salts of soluble zinc but throughout the manuscript the effects are referred to the actual concentration of zinc ions (Zn^{2+}) since acetate or chloride are dissolved in culture buffers and those compounds cannot affect the cell responses. The following final concentrations of Zn^{2+} were applied for 24 h to THP-1 and C6 cells: 300, 100, 30, 10, 3, 1, 0.3, 0.1, 0.01 and 0 μM. Zn^{2+} dilutions or control were added to cell cultures at the final volume of 10% v/v.

Zinc met preparation (1c) was obtained by fine triturating the metallic zinc filings in lactose powder (1 g in 100 g). *Zinc met* 2c and 3c were prepared by centesimal dilution and trituration in lactose, while higher dilutions were prepared in water. Lactose control samples (LAC) were made with identical lactose batch, with the same handling and storage procedures without the *Zinc met* supplementation. All the medicines were prepared following the Brazilian Homeopathic pharmacopoeia and provided by the Pharmacy Faculty of Rio de Janeiro UFRJ (BR) to our laboratory and to all the other laboratories involved in the *Zinc met* multicenter study. The medicines were dissolved in water before the addition in cell culture as follows. *Zinc met* 1c, 2c, 3c or their control LAC powders 1c, 2c, 3c were dissolved in ultrapure water (1% w/v), transferred into clean sterile soda-lime glass tubes and succussed with a DynaA mechanical shaker for 7.5 s (150 strokes) to obtain the centesimal dilutions 2c, 3c and 4c, respectively. *Zinc met* 15c and the corresponding control solution were prepared starting from the 4c dilution, by serial centesimal (1% v/v) dilutions/succussions in ultrapure water. After filtering through 0.22 μm Millipore filters, *Zinc met* 2c, 3c, 4c and 15c and the corresponding LAC dilutions were stored in metallic box at room temperature, wrapped with aluminium foil.

Cells cultures and treatments

The THP-1 cell line was cultured in RPMI medium added with 10% FBS and 2 mM ultraglutamine, at 37°C in controlled atmosphere (5% CO_2, 95% air) and complete humidity. Cells were seeded in microplates (4×10^4 cells/well) in RMPI with 2% FBS and exposed to PMA 20 ng/ml for 48 h, to induce differentiation towards a macrophage phenotype. After 48 h the medium was removed, the cells were washed and maintained in new fresh RPMI with 2% FBS 2 mM ultraglutamine. THP-1 cells were exposed for a short time (2.5 h) to different doses of zinc acetate (0, 10 and 100 μM), then the medium was replaced with fresh one and the cell cultures were supplemented with the agonist LPS (10 ng/ml) and the increasing Zn^{2+} doses for 24 h. The cells were treated with *Zinc met* or LAC controls at the final volume of 10% v/v 1 h before the LPS treatment (10 ng/ml) and then incubated for further 24 h.

When indicated, the cell cultures were treated supplemented with 2.5 μM TPEN in order to chelate residual Zn^{2+} ions present in the medium and to test the effect of Zn^{2+} deprivation and subsequent supplementation.

Human monocytes were isolated from blood of healthy volunteers, provided at the Blood Bank of the University of Verona. Monocytes were isolated from buffy coats by Ficoll-Hypaque and Percoll density gradients. Monocytes were purified from remaining contaminants lymphocytes by culturing at 37°C in 5% CO_2 for 1 h in RPMI 1640 without FBS; non-adherent cells were carefully removed and the medium was replaced by RPMI 1640 supplemented with heat-inactivated, low-endotoxin FBS (2% final concentration), 2 mM glutamine and 100 U/ml penicillin/streptomycin. Monocytes were differentiated with h-MCSF for 5 days. The cells were treated with *Zinc met* or LAC controls as indicated for THP-1 cells.

C6 rat glial cell line was cultured in DMEM F12 10% FBS plus 100 U/ml penicillin/streptomycin until the passage 10, then the cells were detached with trypsin treatment and seeded at day one in 96 well plates (with black walls) in complete medium at the density of 5×10^4 cells/well. After 24 h cells were exposed for 1 h to test solutions (zinc chloride or *Zinc met* dilutions) or control dilutions as indicated, then treated with the administration of the inflammatory treatment (LPS 1 ng/ml + IFN-γ 10 ng/ml or LPS 10 ng/ml + IFN-γ 10 ng/ml or TNF-α 10 ng/ml + IFN-γ 10 ng/ml) and incubated for further 24 h.

Cell function assays

Nitric oxide was measured in C6 supernatants by Griess reaction. Briefly, 50 μl of cultured media were collected and mixed with 25 μl of 1% sulfanilamide and 25 μl of 0.1% NED under acidic (phosphoric acid) conditions for the nitrite concentration determination. Following 10 min incubation at room temperature, the absorbance was measured at 550 nm using a microplate reader Victor3 multilabel reader (PerkinElmer, Shelton, CT, USA). A standard curve was generated with sodium nitrite in concentrations from 0.39 to 100 μmol/l.

The release of pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines was monitored by immune detection with ELISA assays starting from the culture supernatants.

Cell viability was checked on cells in the microplates at the end of the experiment by WST-1 assay, by measuring the 450 nm absorbance (OD) of the samples using a Victor3 multilabel reader (PerkinElmer, Shelton, CT, USA). WST is a dye that is reduced by intracellular enzymes and its colour changes are index of metabolic activity of living cells. Total proteins of cell extracts were quantified by Bio-Rad protein assay according to the manufacturer's instructions.

Statistics

Statistical analysis of effects of various doses of zinc was performed by ANOVA followed by Least Significance Difference or Dunnet post hoc test where indicated. The Friedman test was used to compare the group of zinc samples (irrespective of dilution) with the corresponding control samples group.

Results

Effects of soluble zinc (Zn^{2+})

We started the investigation by using different doses of soluble zinc to test our cell systems with a formulation whose concentration in the medium can be precisely determined. Moreover, Zn^{2+} in molecular form is presumably more suitable for direct interaction with biochemical regulatory systems of cultured cells. In order to evaluate the effects of zinc in soluble form on cells involved in immune defence we tested increasing doses of zinc acetate or zinc chloride on THP-1 macrophages and C6 astrocytes respectively.

Zn^{2+} supplementation in THP-1 cells: The potential effect of increasing doses of Zn^{2+} was assessed in THP-1 cells in resting condition and subjected to a mild inflammatory activation (treatment with LPS 10 ng/ml). The viability of LPS-activated THP-1 macrophages was measured after 24 h incubation (Figure 1A white bars). No viability loss was observed in samples treated with Zn^{2+} from 0.01 μM to 30 μM, but rather the WST signal slightly increased at 30 μM Zn^{2+} supplementation. Higher Zn^{2+} doses (100 μM and 300 μM) were cytotoxic and induced a significant decrease of viability to 63% and 19%, respectively. Similar results were obtained in resting THP-1 macrophages (data not shown).

Pre-conditioning of the cells with a short-time exposure (2.5 h) with Zn^{2+} (10 and 100 μM) did not change significantly the behaviour of cells subsequently exposed to increasing doses (Figure 1A grey and black bars). In particular, pre-treatments did not vary significantly the survival of the cells to longer exposure at the toxic concentrations 100 μM and 300 μM zinc. These short-term pre-treatments per-se did not impair the viability (data not shown).

Production of cytokines in THP-1 cells upon exposure to increasing doses of Zn^{2+}: The release of the cytokines TNF-α and IL-10 by THP-1 macrophages was measured after 24 h incubation with increasing doses of Zn^{2+}, in resting condition or in cells mildly activated with LPS (10 ng/ml). TNF-α production was not observed in resting cells. As shown in Figure 1B, in LPS-activated cells, TNF-α production followed a hormetic behaviour, with increment starting at 10 μM Zn^{2+}, the maximum peak at 100 μM and a sharp drop at higher zinc concentration. Notably, maximum TNF-α production corresponded to the first toxic Zn^{2+} dose (100 μM), where the cells lose 40% of viability (Figure 1A). TNF-α release was induced by Zn^{2+} treatment at medium doses. At higher Zn^{2+} doses the marked stimulatory effect on TNF-α was associated with cellular stress and death. The short pre-treatment with non toxic Zn^{2+} dose (10 μM) resulted in a sensitization of the cells, expressed as a significant TNF-α increase at 10 μM Zn^{2+} and an earlier peak at 30 μM instead of 100 μM (dark grey bars Figure 1B), compared to control cells without pre-treatment. This sensitizing effect of low zinc doses did not appear after pre-treatment with a higher Zn^{2+} concentration (100 μM), that did not change the dose–response of TNF-α release compared to control (Figure 1B black bars).

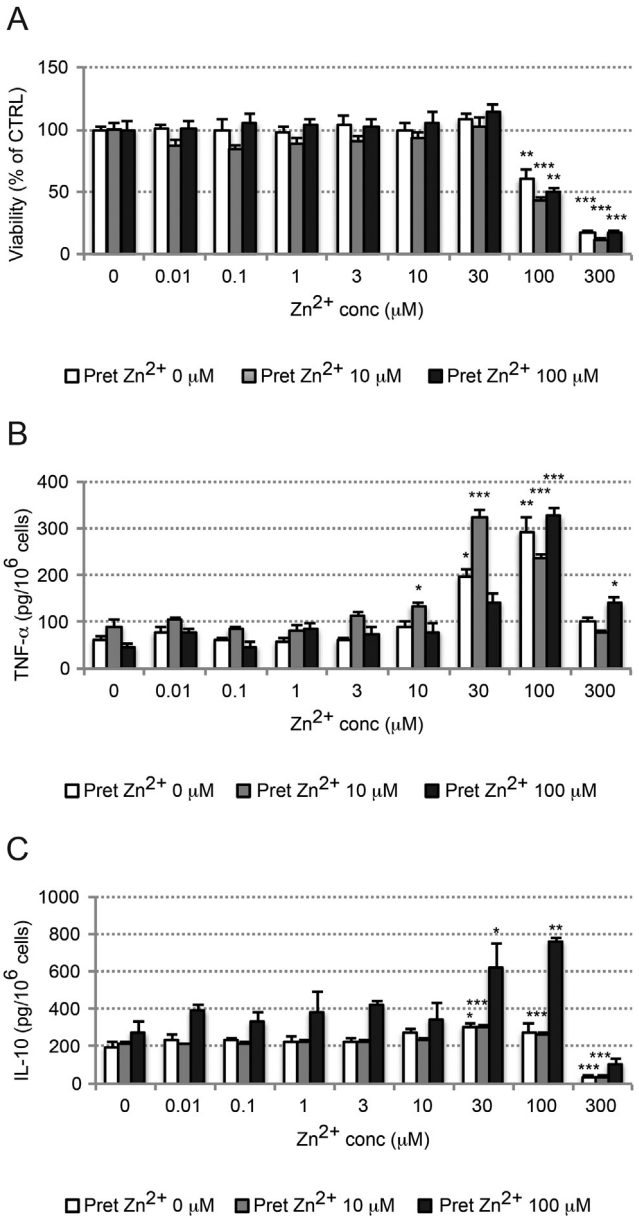

Figure 1 Effect of increasing doses of Zn^{2+} on THP-1 macrophages activities after 24 h incubation in presence of mild activation with LPS 10 ng/ml. Values are measured without zinc pre-treatment (Pret Zn^{2+} 0 μM) and after 2.5 h of pre-treatment with two doses of Zn^{2+} (10 or 100 μM) that for this time of treatment are not toxic. (**A**) Viability of cells after 24 h of treatment, measured as % of control (CTRL, no added Zn^{2+}). Values are means ± SE (n = 6). (**B**) Release of TNF-α and (**C**) release of IL-10. Values are pg/10^6 cells ± SE (n = 3). *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$), Dunnet post hoc *p* values calculated vs CTRL.

IL-10 showed a different behaviour (Figure 1C), since the treatment with increasing doses of Zn^{2+} induced a very little increase of this anti-inflammatory cytokine. The small peak of production was followed by a dramatic drop at 300 μM associated with cell toxicity and death. Pre-treatment with low zinc dose (10 μM) did not change this trend, while 100 μM Zn^{2+} promoted the release of IL-10. In general, the zinc pre-treatments increased the cellular sensitivity in terms of cytokines production without any change of cell viability.

In conclusion, we observed the typical hormetic curves of a substance that is toxic at high doses and with potentiating effects at lower doses. However, under these conditions we did not observe the effect of ultra-low doses.

Effect of Zn^{2+} depletion on cell viability and cytokine release: Since a certain amount of Zn^{2+} is present in the culture medium (about 0.3 μM in RPMI with 10% FBS) we analysed the cell behaviour under conditions in which Zn^{2+} has been totally depleted. Zinc depletion in the cell cultures was obtained by adding the intracellular chelator TPEN, which in those conditions was able to sequester free zinc ions up to a concentration of 2.5 μM. Comparing the grey areas of the two panels of Figure 2, panel A shows that the presence of TPEN caused a significant decrease of cell viability. The supplementation of zinc at doses up to 1 μM did not offset TPEN chelating action, as expected, while higher zinc amounts (10 and 30 μM), that overcome the chelating capacity of TPEN, partially restored the cell viability. However, Zn^{2+} 100 μM and 300 μM was still toxic to THP-1 macrophages in both conditions.

Concerning TNF-α (Figure 2A black circles), zinc depletion, as observed in the first part of the dose—response curve, increased the release of this cytokine respect to the non depleted control (Figure 2B) (from 130 to 103 pg/10^6 cells as average values) in correspondence of the high cytotoxic effects on the cells. Between 3 μM and 10 μM, the cell viability was partially recovered and the production of TNF-α was reduced. Upon addition of excess doses of the metal (100 μM), a peak of release was noted again in association with the decrease of viability. The TNF-α values at 3, 10 and 100 μM were lower in cells pre-treated with TPEN compared to no TPEN control, suggesting that this pre-treatment impaired subsequent effect of zinc addition on this cytokine release, possibly because the cell viability was not fully recovered.

In cells where zinc was chelated without further addition (0 Zn^{2+}), the production of IL-10 decreased from about 228 pg/10^6 cells (control samples, Figure 2B) to about 54 pg/10^6 cells (TPEN treated samples, Figure 2A), suggesting that a 'physiological' dose of zinc in the cell medium is necessary for IL-10 production by macrophages. This is confirmed also by the net increase of IL-10 production upon addition of 3—30 μM zinc (Figure 2A). At higher zinc doses, the decrease of IL-10 was correlated with cell stress observed both in depletion and excess of zinc.

In conclusion, we observed hormetic effects of zinc on pro-inflammatory cytokines and we have confirmed the physiological effect of zinc in the range of low doses. It is interesting to observe a certain degree of dissociation between IL-10 (anti-inflammatory cytokine) and TNF-α (pro-inflammatory cytokine). These results suggest that the optimal zinc concentration has a narrow range, due to subtle equilibrium between zinc uptake and release.

Metabolic response of C6 astrocytes to Zn^{2+} increasing doses: In Figure 3A, the effect of Zn^{2+} on astrocyte cell viability in the presence of different types of cellular activation (resting, IFN-γ with two doses of LPS, and IFN-γ with TNF-α) is shown. In all conditions, increasing doses

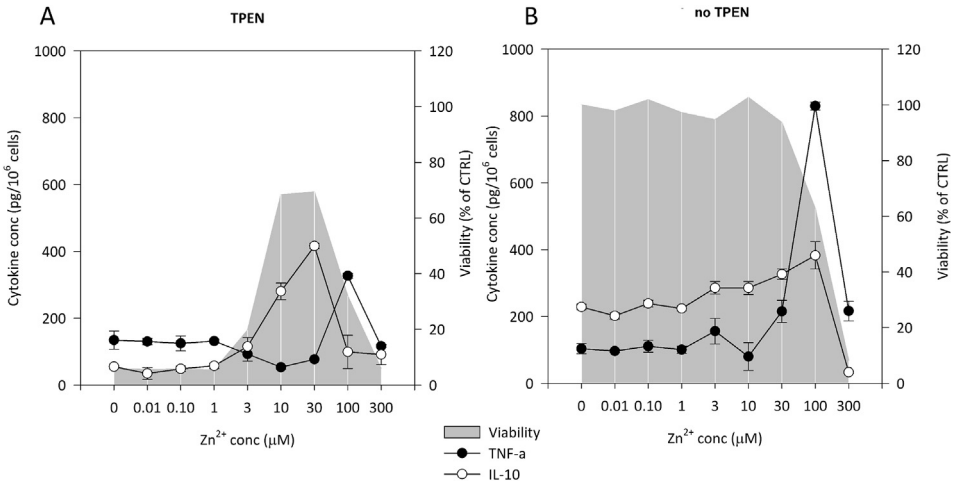

Figure 2 Effect of zinc depletion and increasing doses of Zn^{2+} on the production of cytokines (TNF-α, IL-10) and cell viability in LPS-activated THP-1 cells. (**A**). Cells with zinc depletion by TPEN chelating agent (2.5 μM). (**B**) Normal THP-1 cells (No TPEN). Cytokine values are Mean $\pm$ SE (n = 3); viability is expressed as % of control (CTRL = 0 Zn^{2+}).

of Zn^{2+} caused a decline in vitality only above 30 μM. Lower Zn^{2+} concentration did not impair cell viability, and this behaviour was similar for each type of cell activation, with the exception of a slight decrease in viability in the presence of LPS10 + IFN-γ and TNF-α + IFN-γ at 30 μM that, however, is not statistically significant.

The metabolic response in terms of the NO production is shown in Figure 3B. The cells in the absence of the stimuli (Resting) did not produce NO. The release was triggered by all the three different stimuli, among which LPS10 + IFN-γ appeared to be the strongest. On this parameter, Zn^{2+} addition had no effect at very low doses, while at higher doses (above 10 μM) had different effects according to the stimulant used. In the presence of LPS, the NO secreted ranged around the control value (0 μM Zn^{2+}) until a marked reduction at doses of 100 μM. In the presence of TNF-α, Zn^{2+}

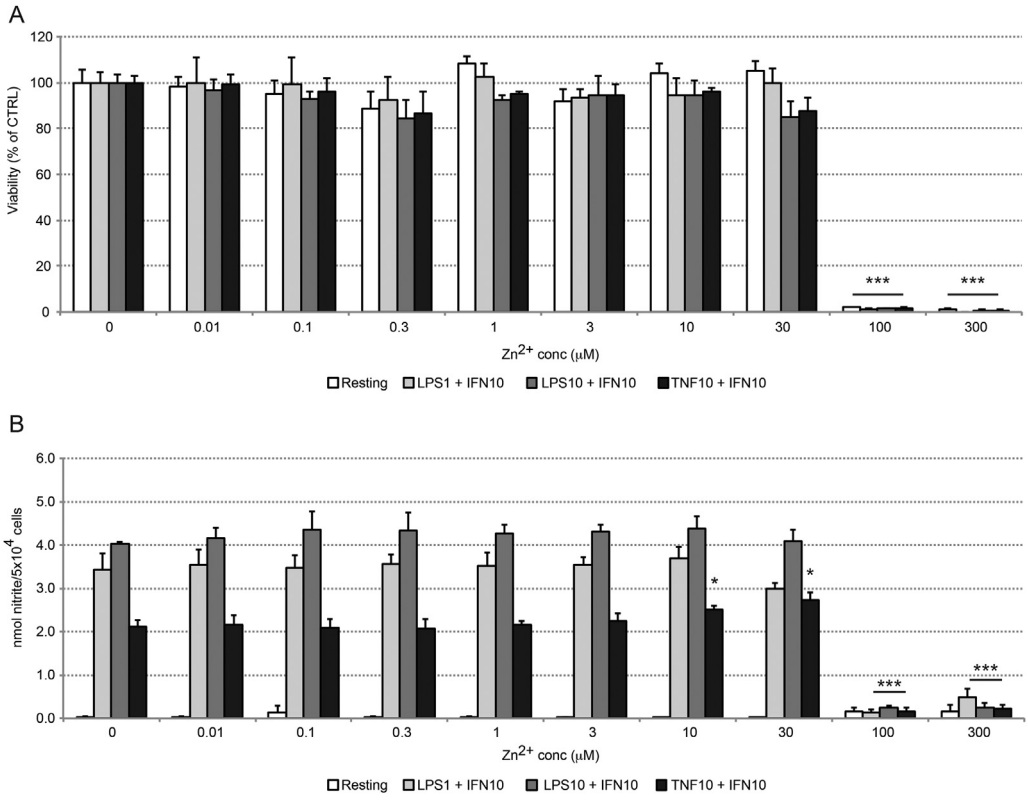

Figure 3 Effect of increasing doses of Zn^{2+} on C6 cells after 24 h incubation with different inflammatory stimuli. (**A**) Cell viability measured as percentage change of treated samples vs control (CTRL = 0 μM Zn^{2+}). Mean $\pm$ SE (n = 3). ***($p < 0.001$), ANOVA followed by Dunnet post hoc p values calculated vs control. (**B**) NO production and secretion in culture media expressed as nmol nitrite/5 $\times$ 10^4 cells. Mean $\pm$ SE (n = 3). LPS1 + IFN10 = LPS 1 ng/ml + IFN-γ 10 ng/ml, LPS10 + IFN10 = LPS 10 ng/ml + IFN-γ 10 ng/ml, TNF10 + IFN10 = TNF-α 10 ng/ml + IFN-γ 10 ng/ml. * ($p < 0.05$) or ***($p < 0.001$) ANOVA followed by Dunnet post hoc p values calculated for treated samples vs CTRL (0 μM Zn^{2+}).

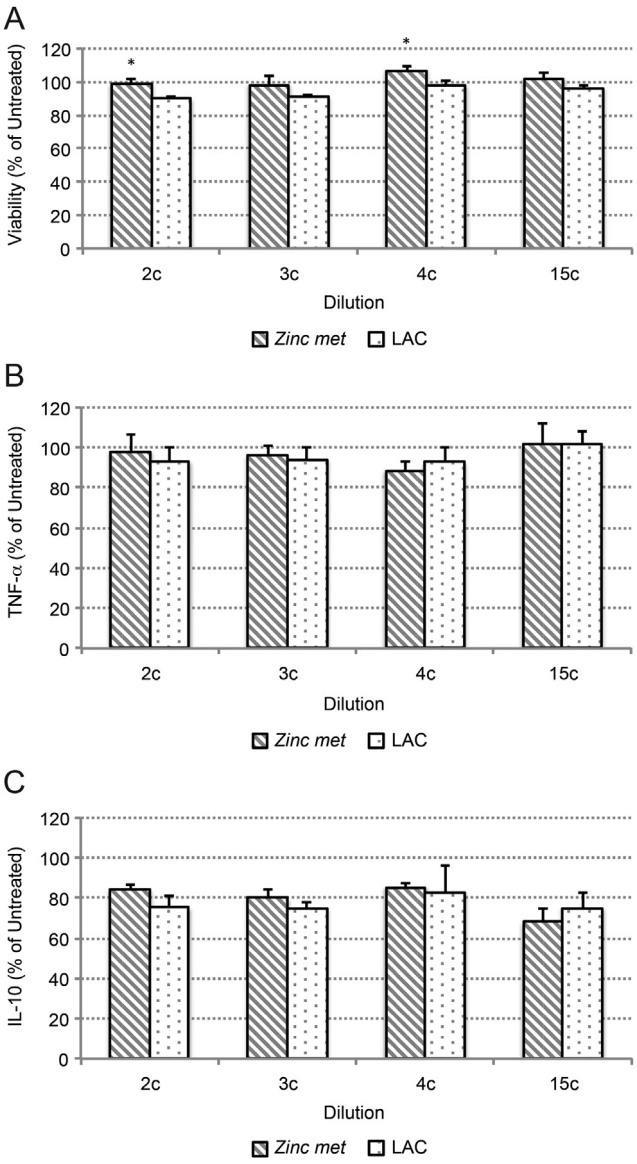

Figure 4 Effects of *Zinc met* treatment in LPS-activated THP-1 cells for 24 h. (**A**) Cell viability level is calculated from the WST absorbance values as % of untreated (cells cultured without addition of *Zinc met* or LAC) (mean ± SE, n = 6); (**B**) Production of TNF-α mean ± SE (n = 12). Values were reported as % of untreated samples (cells cultured without addition of *Zinc met* or LAC); (**C**) Amount of IL-10, mean ± SE (n = 3). Values were reported as % of untreated samples (cells cultured without addition of *Zinc met* or LAC). *($p < 0.05$) LSD post hoc *p* values calculated for *Zinc met* dilution vs corresponding LAC dilutions.

caused a slight but significant increase in the release of NO, with hormetic-like behaviour. These results suggest that Zn^{2+} play a role in the regulation of NO in response to TNF-α and IFN-γ.

Effects of *Zincum metallicum*

The effect of *Zinc met* at low (2c), medium (3c, 4c) and high (15c) dilutions was investigated on THP-1 (Figure 4), on macrophages primary cells (Figure 5) and on C6 astrocytes (Figure 6) and compared with the corresponding dynamized lactose solutions (LAC).

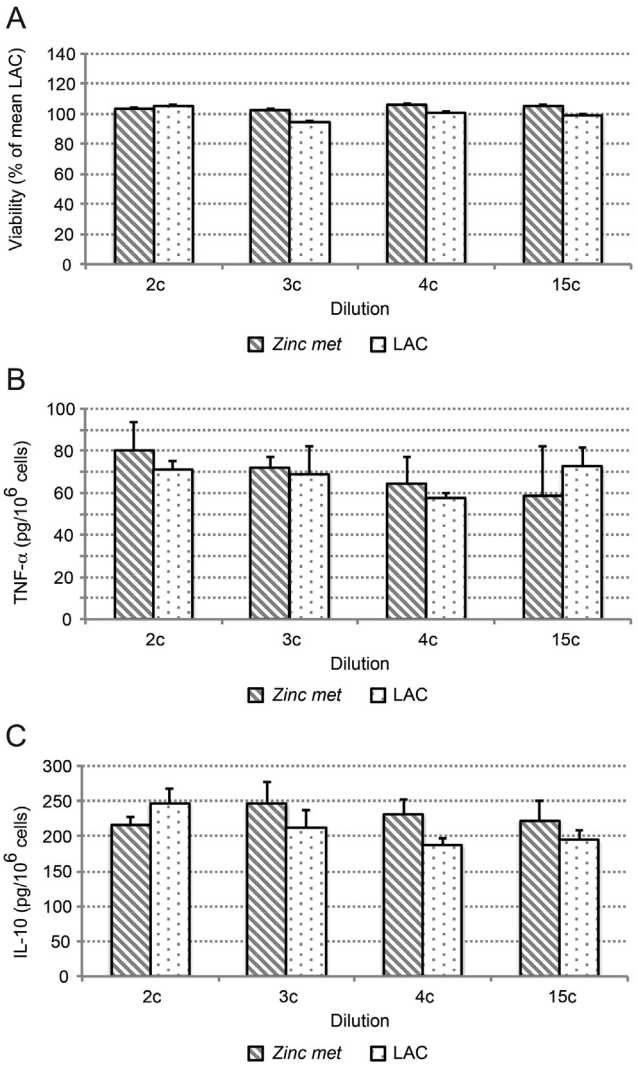

Figure 5 Effects of *Zinc met* treatment in LPS-activated primary macrophages for 24 h. (**A**) Cell viability level is calculated from the WST absorbance values as % of average control (LAC treated samples) (Mean ± SE, n = 6). (**B**) Amount of TNF-α, mean ± SE (n = 3). Values are expressed as pg/10^6 cells. (**C**) Production of IL-10 (pg/10^6 cells), mean ± SE (n = 3). ANOVA test were not significant in all conditions.

Testing Zinc met *on THP-1 macrophages*: Figure 4 A shows that cells treatment with the different dilutions of *Zinc met* determined a slight increase in cell viability compared to the control (LAC), that was statistically significant at dilutions 2c and 4c. These two dilutions were also effective in modulating the amount of TNF-α (Figure 4B) that was slightly increased with *Zinc met* 2c compared to LAC 2c, while decreased in the case of 4c. Both the effects were not statistically significant. The production of IL-10 (Figure 4C) appeared slightly augmented with *Zinc met* low dilution (2c), but no statistically significant effects were reached.

Testing Zinc met *on primary macrophages*: The general metabolic activity of macrophages was not apparently modified by *Zinc met* dilutions compared with the vehicle lactose (LAC) after 24 h incubation in presence of mild LPS activation as reported in Figure 5A. The effect of *Zinc met* treatment on specific immunological activities,

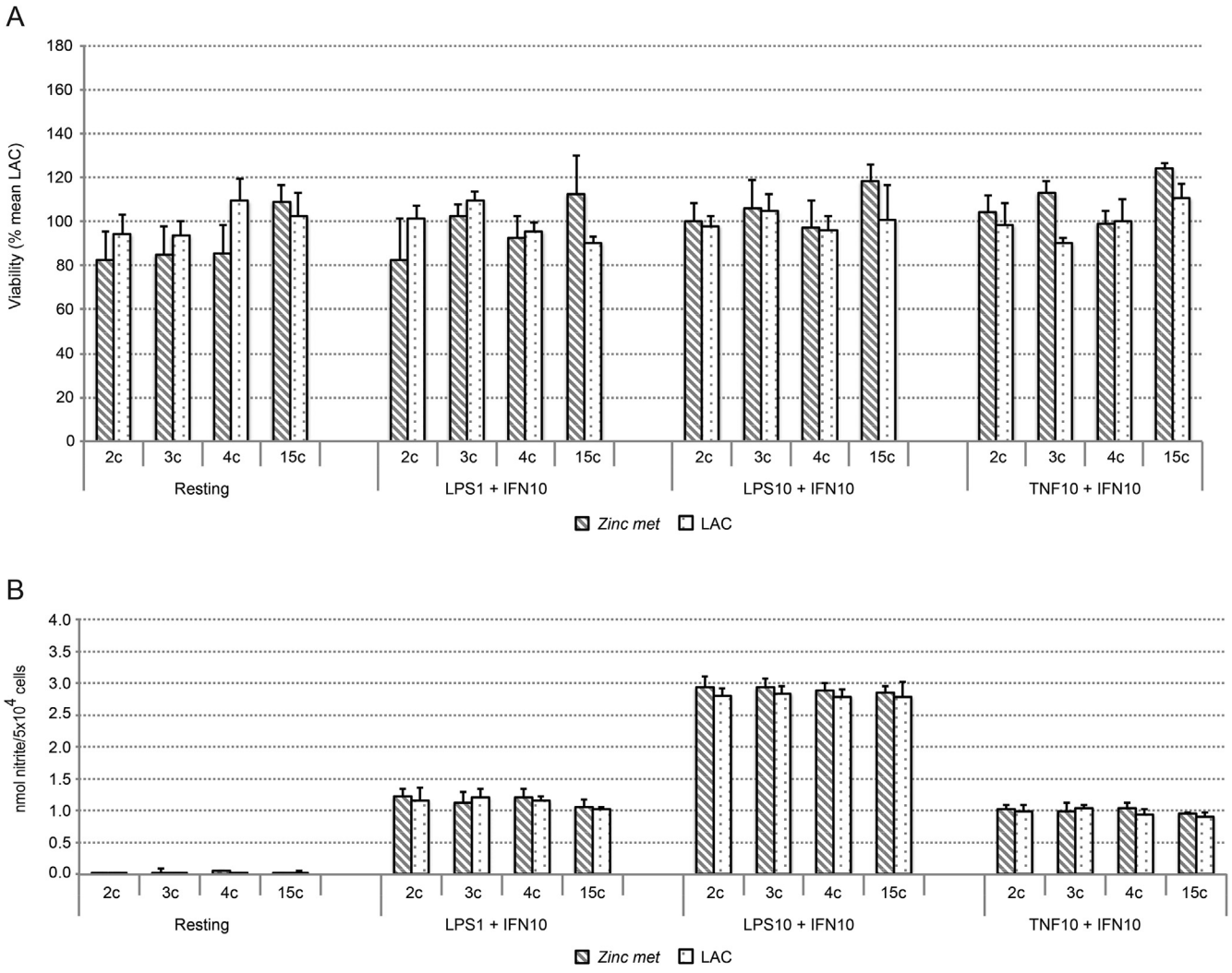

Figure 6 Effect of *Zinc met* on differently activated C6 astrocytes. (**A**) Cell viability level is calculated from the WST absorbance values as % of average control (LAC treated samples) (Mean ± SE, n = 3). (**B**) Production of NO in cell supernatants. Values are mean ± SE (n = 3) and are reported as nmol nitrite/5 × 10^4 cells. LPS1 + IFN10 = LPS 1 ng/ml + IFN-γ 10 ng/ml, LPS10 + IFN10 = LPS 10 ng/ml + IFN-γ 10 ng/ml, TNF10 + IFN10 = TNF-α 10 ng/ml + IFN-γ 10 ng/ml.

expressed as cytokine production was also analysed. The production of TNF-α was variable throughout the treatments and no significant changes were observed (Figure 5B). Large variability and no significant changes were found also for IL-10 (Figure 5C); although no significant changes were found between *Zinc met* and the control, a general trend to upregulation of IL-10 was observed for medium (3c and 4c) and high dilutions (15c) while low dilution (2c) seemed to decrease the production.

Testing Zinc met *on C6 astrocytes*: The effects of *Zinc met* on viability and NO release were analysed in astrocytes after 24 h of incubation with or without activation with three types of stimuli, simulating different inflammatory conditions. The general metabolic activity (Figure 6A) was not significantly affected by the *Zinc met* doses used. We only noted that all the LPS 10 ng/ml + IFN 10 ng/ml samples treated with *Zinc met* were slightly higher than controls. Taken together as a group, those samples showed a small but significant difference with the group of LAC-treated cells (Friedman test $p < 0.05$ *Zinc met* all dilutions vs corresponding LAC controls, not shown in Figure). This could suggest a slightly positive influence of the treatment (irrespective of doses) but due to the small effect size, its meaning remains to be determined.

As reported in Figure 6B, no effect of *Zinc met* were detected on NO release, except a small enhancement in C6 cells activated with LPS 10 ng/ml + IFN-γ 10 ng/ml compared to the respective controls, a finding in keeping with cell viability (Figure 6A). Also in this case, application of Friedman test, comparing the two groups of samples (irrespective of the dose of zinc) with corresponding LAC controls, gave a $p < 0.05$ (not shown in Figure). The same univocal trends were not observed in the other conditions tested.

Discussion

Zinc is both an essential and potentially toxic metal. Damaging effects are reported not only in the case of a

low cellular zinc concentration but also in the case of its excess. Part of the zinc biological effects may be related to its triggering or regulation of inflammatory cells, as it has been reported that cytokine secretion from monocytes is affected by zinc and the intracellular zinc concentration rises in response to stimulation, especially in LPS treated monocytes.[7]

Given the importance of zinc in these mechanisms of physiology and pathology, we investigated its effects on cellular models as part of a multicenter collaborative project that used a homeopathic preparation of zinc in metallic form. Before studying the efficacy of homeopathic doses, we decided to evaluate the effect of soluble zinc on pro-inflammatory factors like TNF-α and NO and the anti-inflammatory cytokine IL-10, which has been poorly studied in relation to zinc. The advantage of the soluble form is that you can control more safely your dose and it is assumed that all the molecules in solution can interact with their cellular targets. On the other hand, in homeopathy the metallic zinc is widely used at 'sub-molecular' doses or in nanoparticulate forms, for which the problem of a precise determination of the concentration is less important.

Our results on THP-1 cells show that treatment with Zn^{2+} at sub-lethal concentrations (30 μM) acts synergistically with LPS, inducing the release of TNF-α. This cationic signal is required for the production of pro-inflammatory cytokines because it is necessary for the activation of MAPKs and the TNF-α promoter. TNF-α, in turn, could be responsible for many proposed mechanisms for zinc-induced toxicity, inflammatory processes and cell death. It is hypothesized that the zinc uptake by the mitochondria causes in these organelles an enhanced production of hydrogen peroxide and other generated reactive oxygen species (ROS), leading to increased damage within the mitochondria and nitric oxide production. LPS-driven zinc uptake could in turn improve the ROS production with induction of cellular stress and death. However, these reactive species are not the single cause of zinc cytotoxicity. Another potential mechanism may be adenosine triphosphate (ATP) depletion. In the literature, it has been shown that zinc can affect energy production by inhibiting glycolytic enzymes and interfering with the electron transport chain.[26]

Zinc-induced toxicity is most likely a combination of several processes including, but not limited to, oxidative stress, ATP depletion and inflammasome activation.[27] Moreover, oxidative stress can activate specific transcription factors including NFAT[28] NF-κB and AP-1[29] correlated with the cytokine gene expression involved in the cellular function impairment. The synthesis of immunosuppressive mediators (e.g., IL-10), could be considered as a compensatory mechanism abrogating deleterious TNF-α signals.

Interestingly, we have shown in dose response curves (Figure 2B) for the first time that the IL-10 is not increased by zinc at the same extent as TNF-α, thus the balance between anti and pro-inflammatory mechanisms is shifted towards the latter. The experiments with TPEN have shown that the maximum IL-10 concentration is present when the cell vitality is restored and peaks immediately before the cell death, evidencing the attempt of cells of counteract the toxic effects of $[Zn^{2+}]_i$ enhanced concentration.

The relationship between the enhanced $[Zn^{2+}]_i$ concentration, the production of TNF-α and the cell vitality impairment is confirmed by the TPEN experiment. The Zn^{2+} depletion is related to a high percentage of cell mortality that is correlated with a small enhanced amount of TNF-α respect to control samples, demonstrating that a physiological Zn^{2+} concentration is necessary but also that the cell death induced by the depletion is partially related to a TNF-α production. The minimum TNF-α concentration is reached when the cells are supplemented with Zn^{2+} 3−10 μM and viability is almost completely restored. The TNF-α peaks when the Zn^{2+} concentration raises the toxic concentration (100 μM Zn^{2+} treatment) and is linked to vitality impairment, evidencing that the TNF-α production is induced mostly by an enhanced $[Zn^{2+}]_i$ concentration, promoting the activation of cell death mechanisms. Our results cannot establish if the cell death in these conditions is due to the increased TNF-α or directly to a zinc toxicity.

Pre-treatment of cells with low doses of zinc did not protect the cells from toxicity of higher doses, a result which is in contrast with the current view of the induction of protective mechanisms (pre-conditioning). On the other hand, a reduced TNF-α release was noted in cells pre-treated with Zn^{2+} 100 μM and then treated with 30 μM for 24 h, inducing a retardation in the dose−response of TNF-α production (Figure 1B). Interestingly, the 100 μM pre-treatment enhanced IL-10 production much more than the control (Figure 1C). This enhanced IL-10 production in the pre-adapted cells could more effectively counteract the TNF-α outbreak due to zinc toxicity.

The effects of *Zinc met* on our cell models were extremely small, if any. THP-1 and primary macrophages showed an enhanced metabolic activity induced by the homeopathic remedy (Figures 4A and 5A), without any dose-effect relation. Due to the small effect size the meaning of this observation remains to be determined.

The link between the oxidative stress induction, proinflammatory cytokine production and modification in the $[Zn^{2+}]_i$ balance is well documented also in the case of brain homeostasis. With this background we decided to investigate the zinc relation with the nitric oxide production in C6 glioma cell line activated with three different inflammatory stimuli. All stimuli induced in our cellular model the NO production and secretion, while zinc in soluble form (10−30 μM) increased only the activity of cells stimulated with TNF-α 10 ng/ml + IFN-γ 10 ng/ml samples (Figure 3B). It was reported that the TNF-α treatment increased the $[Zn^{2+}]_i$ in astrocyte[30] and therefore an enhancement in Zn^{2+} intracellular concentration, in turn, may trigger further cell activation and NO release, as noted in our study (Figure 3B) and reported by other in the analogous range of concentration.[15] Given that the nitric oxide production decreased markedly at the Zn^{2+} 300 and 100 μM, we decided to test the cellular toxicity of these

dilutions using the WST assay. As reported in Figure 3A, non significant changes in viability were observed in the Zn^{2+} range 0.01−30 μM while a high percentage of cell death was reported at the 300 and 100 μM Zn^{2+} in all the treated samples confirming the apoptosis induction observed from Haase and collaborators in the same conditions and cellular model.[31]

Also in C6 astrocytes the effects of *Zinc met* were almost undetectable. Also in these experiments, the highest concentration of NO is observed in LPS 10 ng/ml + IFN-γ samples as in the samples treated with Zn^{2+}. In addition, in LPS 10 ng/ml + IFN-γ samples *Zinc met* at all dilutions (Figure 6B) and Zn^{2+} from 0.1 to 10 μM (Figure 3B) seemed able to induce a small increase in NO production, without a clear dose-dependent trend.

Conclusions

Our results, indicating that soluble zinc inhibits NO production at doses higher than 100 μM (Figure 3A), are in keeping with those reported by others in different cell types.[15] In our experiments, low doses of soluble zinc and all dilutions of *Zinc met*, in some experimental conditions, had small positive effects at the level of cell metabolic activity and production of NO. This finding in general confirms the idea of hormetic effects of low doses of a toxic substance. In any case, the fact that *Zinc met* preparations at all dilutions tested did not cause any effect on delicate cell metabolic mechanisms of macrophages and astrocytes suggests that homeopathic drugs are safe and can be used without the harm of inducing toxic or inflammatory reactions.

Further studies are needed to elucidate the effect of low concentrations of zinc in cells subjected to inflammatory stress, also clarifying the possible role in the modulation of cell viability.

Conflict of interest statement

The authors have no conflict of interest.

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