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Research Article

Manganese-Induced Disruption of Cross-Talking Pathways in *Danio rerio* (Zebrafish) is Potentially Linked to Toxicity and Neurodegeneration

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Abstract

Manganese (Mn) is an essential element; but in humans, chronic and/or acute exposure to this metal can lead to neurotoxicity and neurodegenerative disorders including Parkinsonism and Parkinson´s Disease by unclear mechanisms. To better understand the mode of action of Mn^{2+} on the biology of a cell, we exposed embryos of *Danio rerio* (zebrafish) to $MnCl_2$ and Citrate of Mn(II), which followed by functional analyses. Our results of gene expression revealed a genetic relationship between Mn and the process of protein biosynthesis as a possible target for Mn-induced toxicity. Additionally, using Comparative Toxicogenomic Database we revealed that Mn shared certain similarities in toxicological mechanism with neurodegenerative disorders including Alzheimer´s disease (AD), Amyotrophic Lateral Sclerosis (ALS), Parkinson´s disease (PD) and Huntington´s disease (HD). Altogether, these findings contribute to our current understanding of the mechanisms that governs cytotoxicity that is triggered by Mn^{2+}.

Keywords: *Manganese; Toxicity; Neurodegeneration; Danio rerio; Gene Expression; Protein Biosynthesis*

Introduction

Trace elements play an important role in the life's balance on our planet. Manganese (Mn) is an example of these crucial elements, which is the twelfth most abundant element in the earth's crust. Natural levels of Mn range from 1 to 200 µg/L in fresh water and 410 to 6700 mg/kg (dry weight) in sediment [1]. In aquatic environments, Mn^{2+} is the most dominant and stable water-soluble species at a low redox and pH. The Mn^{3+} is soluble only in complex form and the Mn^{4+} has very limited solubility [2]. Because of geogenic reasons and/or anthropogenic activities such as mining, local levels of Mn can be significantly higher in certain regions [3-6]. According to recent epidemiological and toxicological studies, certain levels of Mn can be beneficial to specific biological processes and detrimental to others, which may lead principally to neurotoxicity [7] and mental disabilities. These alterations can be influenced by chemical speciation and fractionation [7,8], developmental stage [7,8], disease state [9,10], cell type [7] and/or the organism itself. Despite the fact that several hypotheses have been proposed to explain the toxicity of the manganese; including oxidative stress, energy-mitochondrial impairment, apoptosis, metal dyshomestasis and others, the mechanisms behind these effects is unclear by far [11]. However; experimental data with different biological models have suggested that Mn can disturb the vital flow of genetic information from DNA to RNA to protein [12-15] and dynamics of the endoplasmic reticulum (ER) [15]. Protein synthesis is controlled by the ribosome in connection with the ER [16]; which is an organelle in contact with the plasma membrane, Golgi, mitochondria, and other cellular components [17]. Due to its central importance to cell survival and its energy requirements, protein synthesis is firmly regulated and strongly connected to other cellular processes such as cell cycle and metabolic pathways [18,19].

Nowadays it is well accepted that many human diseases are directly linked to the environmental toxin exposures which have stimulated studying the toxicity of environmental contaminants in different model organisms [20,21]. In this sense; the zebrafish embryos, larvae, and adults are valuable models to investigate and address pressing issues in environmental health such as contaminant detection, environmental monitoring, toxicity/teratogenicity testing and investigations into mechanisms of action and disease phenotypes associated with exposure to toxic chemicals. Our recent studies suggested that chemical speciation and fractionation are important for Mn-induced metal dyshomoestasis in zebrafish, including calcium disruption [8], which can lead to endoplasmic reticulum stress. In greater details, we studied multiple genes associated with the metabolism of proteins, using a systems biology approach, which allows to examine interactions on a global scale such as protein-protein interaction (PPI) networks. Since, it has been revealed that over 80% of proteins are not individually functional and their role is defined based on the complex network that they partake [22,23]. Thus, we provided evidences that connect the toxicity of divalent chemical species of manganese to impairment of protein biosynthesis and toxicity.

Materials and Methods

Preparation and characterization of manganese complexes for toxicity assays

$MnCl_2$ stock solution was prepared by dissolving manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in sterile ultra pure water to obtain a final concentration of 1M. Exposure solutions were prepared in test medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.75 mM $NaHCO_3$, 0.08 mM KCl) in the concentration range of 0.75 - 400 mM. Divalent manganese complexes were prepared and characterized, according to Hernández., *et al* [8]. Briefly, manganese(II) citrate [(Mn(II)Cit] were obtained by mixing $MnCl_2$ stock (6 mM) with 6 mM sodium citrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Exposure concentrations of Mn(II)Cit were 0.19 - 6 mM.

Exposure of zebrafish embryos for chemical species of manganese

The zebrafish wildtype strain was established from a stock originally purchased from a local supplier (São Paulo - SP, Brazil) and had been kept for two generations in the laboratory. Fish were cultured at 26 ± 1°C and 14:10h light:dark cycle. They were fed daily, once with *Artemia salina* and twice with commercial flake food (Tetra, Melle, Germany). Collection of eggs and exposure of embryos were performed with minor modifications according to Hernandez., *et al* [8]. Thirty embryos were exposed for 0, 1 and 3 mM of chemical species of manganese, in each dish plates. Static exposure was performed from 48 - 120 hpf (hours post fertilisation), followed of gene expression analysis. Controls were exposed to exposure medium without manganese supplementation but including sublethal concentrations of 6 mM citrate (least of 5% of lethality and without significant change in gene expression), in case of experiments with the manganese-complexed with these molecule. All exposure experiments were performed at least in triplicates using batches of embryos from different breeding tanks. All work was undertaken under the ethical regulations stablished at the Univ. Federal of São Paulo (CEUA 5022030214).

Gene expression analysis through transcriptomics experiment, using RT-PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was used to assess mRNA expression of zebrafish larvae, after treatment with chemical species of manganese. Total mRNA from this zone was extracted using trizol (Thermo Fisher), according to manufacturer's protocols. Extracted mRNA from all samples was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific) and reverse transcribed into cDNA using SuperScript Vilo Master Mix (Applied Biosystems). Real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems) with the primers for the following genes: Carboxypeptidase A (CPA1, Dr03116112_m1), ubiquilin 4 (UBQLN4, Dr03095311_m1), eukaryotic translation initiation factor 2, subunit 1 alpha a (EIF2S1a, Dr03080657_m1), matrix metalloproteinase 2 (MMP2, Dr03106215_m1), branched chain aminotransferase 2, mitochondrial (BCAT2, Dr03108413_m1), sarcoglycan, epsilon (SGCE, Dr03114960_m1) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Dr03436842_m1), which used as reference. 40 ng of cDNA template was prepared per 20 μL reaction for the genes of interest and the endogenous gene. Real-time PCR analysis was carried out using a TaqMan Fast Advanced Master Mix and StepOnePlus™ Real-Time PCR System (Applied Biosystems), for 2 minutes at 50°C, for 2 minutes at 95°C, followed by 40 cycles of 95°C for 1s and 60°C for 20s. Data was analyzed using

the comparative CT method, where average CT values were first normalized to GAPDH gene expression followed by relative quantification to the positive control (normal condition or without treatment). All samples were measured in triplicate.

Protein-Protein interaction (PPI) prediction and gene ontology (GO) analysis

Considering that the majority of proteins or genes working in complexes instead of alone, we inferred the potential Protein-Protein interaction (PPI) of the genes studied by PCR. A PPI network can be described as a heterogeneous network of proteins joined by interactions as edges. Studies about protein network and GO enrichment analysis were based on the data from the current project and analyzed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (http://string-db.org) [24]. Additional GO analysis was conducted at the Comparative Toxicogenomic Database - CTD (http://ctdbase.org/) [25] to verify the hypothesis about conserved mode of action of the manganese between yeast and human. Both STRING and CTD database were accessed at January 31st, 2018.

Data analysis

The results were expressed as mean ± sem of at least three independent experiments. To detect statistically significant differences ANOVA (analysis of variance) followed by Bonferroni's tests was be used; additionally, in some case performed t-test analysis between pairs of treatments. Fitting and statistical analyses were performed with GraphPad Prism (GraphPad 4.0 Software Inc, San Diego, CA, USA).

Results

Previous works from our group with yeast model and mammalian neuronal models (manuscripts in elaboration) suggests that the manganese can disrupt different pathways linked to protein biosynthesis. In the same context, we conducted a preliminary transcriptomics array assay with zebrafish embryos exposed for $MnCl_2$ and Citrate of manganese(II), (supplementary material: figure SM-1 and table SM-1). There we identified six genes (BCAT2, SGCE, CPA1, eIF2S1a*,* UBQLN4 and MMP2) potentially involved in protein digestion and absorption and protein processing in ER pathways (supplementary material: table SM-2). We investigated these genes by using RT-PCR and eventually verifying that they are up-regulated with exposure to 1 mM Mn and quite contrary, they are down-regulated in the presence of 3 mM Mn (Figure 1).

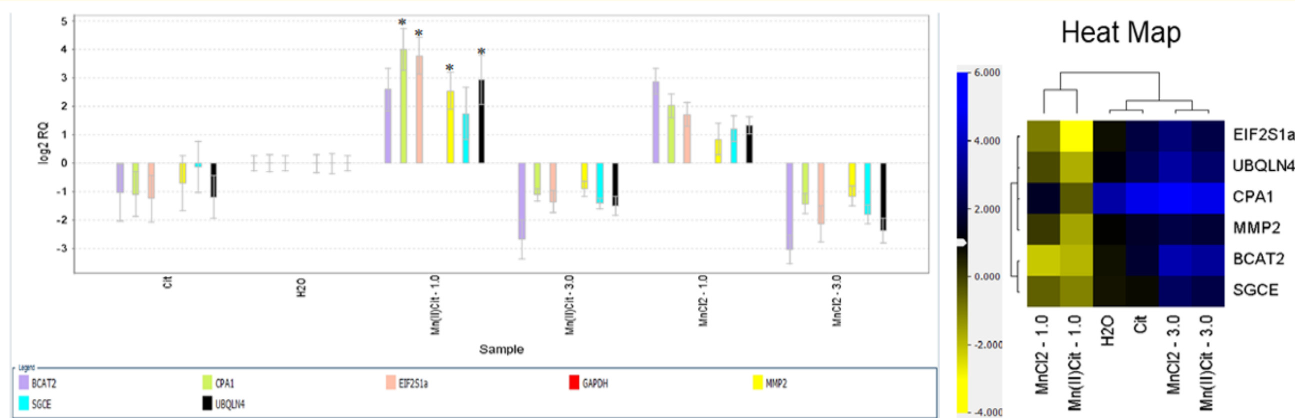

Figure 1: *Relative gene expression of preselected genes associated with the metabolism of proteins. The statistical difference between each treatment and the control was verified by two-way Anova, followed of Bonferroni posttest, *(p < 0.05). Carboxypeptidase A (CPA1), ubiquilin 4 (UBQLN4), eukaryotic translation initiation factor 2, subunit 1 alpha a (EIF2S1a), matrix metalloproteinase 2 (MMP2), branched chain aminotransferase 2, mitochondrial (BCAT2), sarcoglycan, epsilon (SGCE) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).*

Manganese perturbed multiple pathways in zebrafish, which mimic mechanisms of neurodegeneration

Environmental toxicants, including heavy metals for lower concentrations can cause significant neurological deficits. These problems can manifest quite rapidly and severely or delayed and more gradual. For instance, neuropsychiatric disorders can appear decades after exposure. Recently, the number of neuroepidemiological reports associated with human exposure to manganese has been elevated, which can be studied in embryos of zebrafish. Since, this model organism conserves key cellular processes and genes found to be associated with human neurodegenative diseases [26].

For a major understanding of our experimental data, we conducted a functional proteomic and Gene Ontology at the String-database based on protein-protein interaction (PPI) [24]. String uses physical interactions and functional associations to study a defined set of proteins and expands it by including associated proteins to different biological processes. In this way, the network of functional interactors for Mn was increased to approximately 1702 edges (p-value < 1.0e-16); where, approximately 85% of the added interactions have been experimentally verified. A schematic representation of these interactors is shown in figure 2.

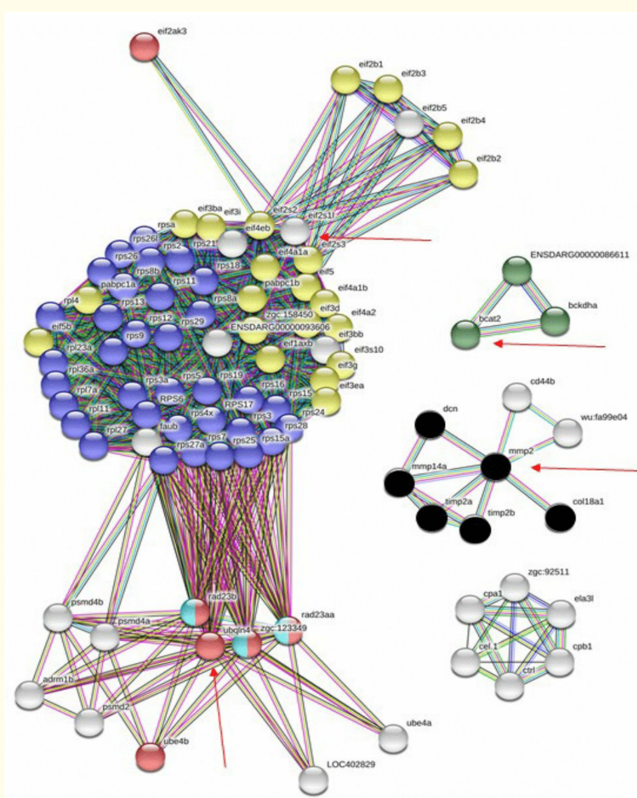

Figure 2: *Inferred protein-protein interactions (PPI) network, based in genes differentially expressed in zebrafish (indicated by red cross in the map), after exposure 72 hours for 1 mM and 3 mM of MnCl2 and Mn(II)Cit respectively. This PPI map suggest that the Mn induced alteration of cellular signaling processes (black: TGF-beta signaling pathway, GnRH signaling pathway, AGE-RAGE signaling pathway, signal transduction); Valine, leucine and isoleucine degradation (dark green); Ribosome (blue); RNA transport (yellow); Protein processing in endoplasmic reticulum (red); Nucleotide excision repair (cyan). Parameter of the PPI: p-value < 1.0e-16; number of nodes (91); expected number of edges (269); clustering coefficient of 0.892; number of edges (1702); average node degree (37.4); avg. local clustering coefficient (0.929). The predicted PPI was calculated with a high confidence score (0.7), at the String data-base and proteins not connected were removed of this picture. Interactions are represented by: Red line - indicates the presence of fusion evidence; Green line - neighborhood evidence; Blue line - cooccurrence evidence; Purple line - experimental evidence; Yellow line - text-mining evidence; Light blue line - database evidence; Black line - co-expression evidence.*

Pathways	# of observed genes	FDR
Ribosome	34	1.86E-54
RNA transport	21	2.53E-26
Protein processing in endoplasmic reticulum	6	0.00174
Nucleotide excision repair	3	0.0212
Valine leucine and isoleucine degradation	3	0.0218

***Table 1**: Enriched cellular pathways influenced by Mn in zebrafish embryos.*

In fact, we inferred that the Mn-induced toxicity in zebrafish shares certain characteristics with the pathways that are involved in neurodegenerative diseases (Figure 3). For example, the Mn-induced perturbation of the gene MMP2 can lead to Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease (HD) and Parkinson's disease (PD), which are associated with disruption of cell signaling pathways, such as AGE-RAGE signaling pathway, GnRH signaling pathway and TGF-beta signaling pathway. These signals are often amplified before evoking a response, which can regulate the translational apparatus. Defection or inhibition of protein synthesis can cause many severe health problems. In some cases, protein synthesis can be considered a mechanism of tolerance or adaptation as well as it can be neurotherapeutic [27], whereas in other cases, inhibition of protein synthesis provokes the onset of certain neurodegenerative disorders [27] such as Alzheimer´s, Parkinson´s and Huntington´s diseases. For example; over expression of PRKN (parkin RBR E3 ubiquitin protein ligase) protects against neurotoxicity in the 6-hydroxydopamine rat model for Parkinson's disease [28]. However, studies with *in vitro* and *in vivo* models have revealed that exposure for manganese chloride can results in increased methylation of the gene PRKN, with consequent decreased of this mRNA expression [29] as well as other authors have verified increased expression of PRKN protein [30], which is associated with alteration of mitochondrial function, Parkinsonism and Parkinson´s disease [29,30].

It has been previously suggested that ER has various domains and membrane contact sites that are required for multiple cellular processes including synthesis of proteins and lipids, regulation of calcium levels and exchange of macromolecules [17]. In the same context, we verified disruption of the gene UBQLN4 directly linked to protein processing in endoplasmic reticulum (PPER) and member of the ubiquitin domain. The PPER pathway have been linked to development of AD, ALS and PD (Figure 3). Additionally; we verified disruption of the gene BCAT2, potentially linked to perturbation of valine, leucine and isoleucine degradation pathway, which is involved in protein synthesis [31]. Finally, we inferred disruption of nucleotide excision repair (NER) pathway, which is used by eukaryotes to remove bulky DNA lesions [32].

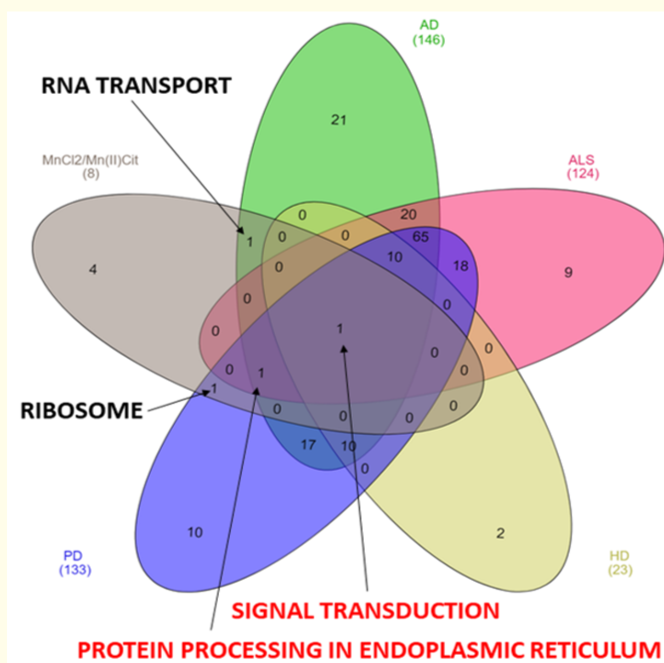

***Figure 3:** According to analysis performed with the Comparative Toxicogenomic Database, the Mn-induced toxicity in Danio rerio share similarities with certain pathways linked to neurotoxicity and neurodegeneration. The results these analyses indicate overlapping pathway among Mn and [Alzheimer's Disease (AD)] and [Amyotrophic Lateral Sclerosis (ALS)] and [Huntington's Disease (HD)] and [Parkinson's Disease (PD)] due to disruption of the gene MMP2, associated with signal transduction as well as among Mn, AD, ALS and PD due to disruption of the gene UBQLN4, associated with protein processing in endoplasmic reticulum. Other putative associations imply Mn and AD because alteration of RNA transport as well as Mn and PD because perturbation of the Ribosome.*

Discussion and Conclusion

The toxic role of Mn, especially in neurotoxicity and neurodegeneration yet remains unclear with several proposed hypotheses [11]. The Comparative Toxicogenomic Database (CTD) introduces Mn as an essential trace element, with possible connections to approximately 570 biological processes and/or pathways [25]. Precisely; in this work, using a systems biology approach, we inferred the involvement of the Mn in various interconnected pathways, including signal transduction, protein processing in ER (PPER), Ribosome; RNA transport; Valine, Leucine and Isoleucine degradation and Nucleotide Excision Repair.

According to our experiments, appear be that Mn affects translation. Coincidently, previous studies have suggested the possibility that Mn may disturb the flow of genetic information that could influence protein synthesis [13-15], including ER stress [16,33-36]. Indeed, in this study, we verified disturbance of PPER pathway due to dysregulation of the gene UBQLN4 for 1 mM and 3 mM of Mn respectively. Other authors have suggested that a persistent ER stress condition can compromise the protein synthesis, leading to apoptosis [37,38]. Recent studies in *Caenorhabditis elegans* using RNA-Seq approaches revealed that Mn induced regulation of ER-related protein families (FKB and ABU) implicated in ER stress [33]. This can be exacerbated by metal dyshomeostasis. For example; iron depletion increases phosphorylation of the eukaryotic translation initiation factor 2α (phospho-eIF2α) [37], activation of PERK and IRE1 signaling pathways [34,35] and ER swelling [38]. Recently, Hernández., *et al.* [8] verified that the manganese induced calcium disruption in zebrafish embryos, after exposure from 48 hour post fertilization (hpf) to 120 hpf for similar chemical species used in this study, $MnCl_2$ and Mn(II) Cit. Previous studies implicated that imbalance calcium homeostasis has been associated with ER stress, potential protein folding stress and neurodegenerative diseases [39,40]. Studies in Human SH-SY5Y cells have been also identified Mn-induced ER stress associated with increased phosphorylation of translation initiation factor eIF2α [37]. In line with this, we verified regulation of the gene eIF2S1*a* (eukaryotic translation initiation factor 2, subunit 1 alpha a), suggesting that increased ER-stress, after exposure to Mn could lead to translation arrest. Additionally; in our analysis, String database added other complementary proteins linked to eIF2S1*a* (Figure 2), suggesting perturbation of the ribosome and RNA transport at the same time. Recent unpublished studies from our group with yeast and cerebellar granule neurons identified significant alterations of genes associated with the ribosome and translation, including RPS15, RPL14, eIF4*b* and eIF5*a* (manuscripts in elaboration). All these findings, observations and inferences reinforce the hypothesis of manganese-induced impairment of PPER pathway and protein synthesis as well as their potential linking for neurodegenerative disorders such as AD, ALS and PD [25], as showed in figure 3.

We discussed above that Mn-induced perturbation of PPER pathway can lead to ER stress, and some studies have speculated that it can stimulate AGE-RAGE signaling pathway, exacerbating oxidative stress and inflammation, which is involved in neurodegeneration as well [41,42]. Despite; we not verified the potential induction of oxidative stress, indeed we identified perturbation of the gene MMP2*,* which is directly linked to AGE-RAGE signaling pathway. Recent studies suggested that manganese as well as other heavy metals and metalloids including methylmercury, selenium, zinc, and arsenic have been associated with disturbance of AGE-RAGE signaling pathway in bovine aortic endothelial cells, *Caenorhabditis elegans*, *Mus musculus* and *Homo sapiens* [42]. It is known that alteration in AGE-RAGE signaling pathway can activate TGF-beta signaling pathway [43,44], which directly regulate the secretion of GnRH, the neurohormone that controls sexual maturation, reproduction [45] as well as other functions, including learning, memory and feeding behavior with implications for neurodegeneration development [46]. Altogether; these alterations can be potentially involved in Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease (HD) and Parkinson's disease (PD). Since; its are associated with alteration of cell signaling pathways [25], figure 3.

Furthermore; we verified the regulation of the gene BCTA2*,* in the presence of Mn, which could lead to alteration of valine, leucine and isoleucine degradation pathway (VLiL). It is well known that these amino acids are among the most hydrophobic, with a crucial role in globular proteins [31]. Specially, disruption of VLiL pathway, as mentioned above, could trigger the inhibition of protein biosynthesis. Since; other works have suggested that leucine can modulate the rate of protein synthesis, particularly by stimulating the activity of

translation initiation factors such as eIF4E, RPS6, and eIF4G and elongation factor eEF2, mediated by mTOR pathway [47,48]. Although; we did not identify alteration of mTOR pathway, we discussed that Mn induced disruption of the gene *e*IF2S1*a*, which is associated with translation initiation. In addition, leucine is the amino acid most effective in reducing proteolysis [48] therefore perturbation of leucine metabolism could eventually lead to protein accumulation and apoptosis as well [49]. This may also be due to proteolysis disturbance, which have been observed in neurodegenerative disorders such as AD, AlS, HD and PD [49,50].

Additionally; impairment of cell survival, under Mn stress can be exacerbate due to alteration the nucleotide excision repair (NER) pathway, which is employed by the cells to remove bulky DNA adducts and restore the canonic nucleotide sequence. Although, we did not conduct experiments to verify DNA alteration, previous works have verified that Mn^{2+} causes oxidative DNA damage, which requires base excision repair [51]. At the same time; we identified alteration of the gene UBQLN4*,* involved in PPER and ubiquitination as well. Various authors have suggested that protein synthesis [52] and ubiquitination [53] are critical processes for NER regulation. Thus, up-regulation of UBQLN4, for lower concentration of Mn^{2+} could lead to induction of NER and cell survival increase. Contrary effect must be expected to increased Mn concentration and down-regulation of UBQLN4*.* For example, for *in vitro* assays, the NER activity is inhibited by ubiquitin-mediated proteolysis system, including inhibition of proteasomal ATPases, which causes an increased sensitivity to UV [54] Decreasing of NER activity have observed under apoptosis condition [55]. However; additional experiments must be conducted to verify that mechanisms may be involved in NER pathway, under Mn stress. Since it is well known that early steps in NER can be divided into two subpathways: global genomic repair (GGR) that recognizes and removes lesions throughout the genome, and transcription-coupled repair (TCR) that preferentially repairs the transcribed strand of active genes, most likely by using translocating RNA polymerase as a lesion sensor [53]. Furthermore; clinical observations suggest that defective NER/TCR might also play a critical role in neurodegenerative disorders, such as AD and PD [56].

Final Considerations

At today we have consolidated robust hypothesis about Mn induced-toxicity that include mitochondrial dysfunction, energy impairment, oxidative stress, disruption of neurotransmitter, ER stress, neuroinflammation, DNA damage and epigenetic alterations, apoptosis, autophagy, among others [9,10,29,57]. All these processes occur after the process of protein biosynthesis is completed and/or are directly linked to it. The preliminary results discussed in this work, referent to zebrafish embryos exposed for divalent species of manganese suggest that Mn-toxicity can occur with disruption of orchestrated essential pathways, including protein biosynthesis. Disruption of protein synthesis appears to connect Mn-induced toxicity to Mn lead to neurotoxicity and neurodegeneration. Altogether, our findings and inferences can add new evidences to our current understanding about the mode of toxicity of the Mn. Additional experiments in course with mammalian models can to validate these findings, specially our inferences of Mn-induced neurodisorders.

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Author Contributions Statements

The professors Hernández is responsible of the principal idea of this study, co-executor of all designed experiments and data analysis as well as coordinator of the grants that supported this work. Sr. Moteshareie participated directly in the experiments performed and data analysis. The professor Golshani participated in data analysis. All authors wrote and reviewed the manuscript.

Competing Interests

The authors declare no competing interests.

Supplementary Material

Micro-Array Gene Expression

Microarray approaches were conducted to identify the mode of action of the manganese. In this way, thirty embryos of each treatment in biological replicate were analyzed, after exposure from 48 - 120 hour post fertilization- hpf, for chemical species of manganese (manganese chloride, $MnCl_2$; citrate of manganese (II), Mn(II)Cit and citrate of manganese (III), Mn(III)Cit). The array assays were performed by using available and commercial "GeneChip™ Zebrafish Gene 1.1 ST Array Plate", according to the Affymetrix protocols available in: (https://www.thermofisher.com/order/catalog/product/902004?SID=srch-srp-902004). The experiments were developed in the platform of the Epidemiology Genetic Laboratory of Institute of Biomedical Sciences at the University of São Paulo. Finally, the results were verified with the software "Expression Console and Transcriptome Analysis Console" from Affymetrix. The principal results are showed below (Figure SM-1 and table SM-1, including analysis at the Comparative Toxicogenomic Database - CTD, table SM-2.

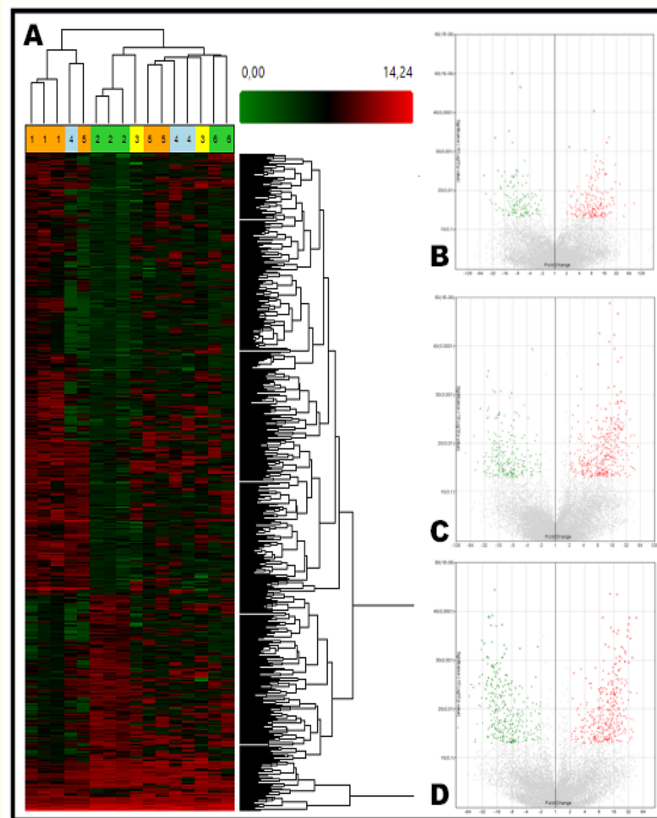

Figure SM-1: *Hierarchical clustering of the full data analysis (A) and volcano plot showing differential expression between H2O-iso and MnCl2, 6 mM (B); Citrate and Mn(II)Cit, 3 mM (C); and finally MnCl2, 6 mM vs Mn(II)Cit, 3 mM (D).*

Probe set	Control	$MnCl_2$ 6mM	Fold Change (linear)	Anova, p<0.05	pFDR	Gene	Gene name
Dr.4244.1.A1_at	5.82	9.24	-10.64	0.00001	0.154887	cpa1	carboxypeptidase A1 (pancreatic)
Dr.21605.1.S1_at	5.98	8.73	-6.74	0.000022	0.175652	wu:fc25e04	wu:fc25e04
Probe set	**Control**	**Citrato**	**Fold Change (linear)**	**Anova, p<0.05**	**pFDR**	**Gene**	**Gene name**
Dr.3664.1.S1_at	9.7	5.74	15.52	0.000007	0.109028	fundc2	fun14 domain containing 2
Dr.9399.2.S1_at	9.09	5.78	9.93	0.000018	0.141985	ppp2r4	protein phosphatase 2A activator. regulatory subunit 4
Dr.15412.2.S1_at	9.5	5.95	11.71	0.000038	0.198802	tssc1	tumor suppressing subtransferable candidate 1
Dr.14203.1.S1_at	5.42	9.24	-14.12	0.000058	0.22639	ppp1r14aa	protein phosphatase 1. regulatory (inhibitor) subunit 14Aa
Probe set	**Citrato**	**Mn(II)Cit 1.5 mM**	**Fold Change (linear)**	**Anova, p<0.05**	**pFDR**	**Gene**	**Gene name**
Dr.14866.1.A1_at	5.16	9.89	-26.55	0.00001	0.159918	gnsa	glucosamine (N-acetyl)-6-sulfatase a
Dr.14481.1.S1_at	9.41	5.87	11.6	0.000072	0.281294	LOC100536058	uncharacterized LOC100536058
Dr.16350.1.A1_at	5.92	10.19	-19.23	0.00005	0.281294	LOC100535214	uncharacterized LOC100535214
Dr.22485.1.A1_at	4.84	9.16	-19.87	0.000067	0.281294	slc14a2	solute carrier family 14 (urea transporter). member 2
Probe set	**Citrato**	**Mn(II)Cit 3 mM**	**Fold Change (linear)**	**Anova, p<0.05**	**pFDR**	**Gene**	**Gene name**
Dr.209.1.S1_at	10.02	6.29	13.3	0.000044	0.23421	pitpnbl	phosphatidylinositol transfer protein. beta. like
Dr.8744.1.A1_at	10.54	6.34	18.43	0.000045	0.23421	actr2a	ARP2 actin-related protein 2a homolog (yeast)
Dr.14481.2.S1_x_at	5.55	9.7	-17.84	0.000037	0.23421	LOC100536018	uncharacterized LOC100536018
Dr.2720.1.A1_at	9.8	4.73	33.7	0.000136	0.236234	sgce	sarcoglycan. epsilon
Dr.18285.1.A1_at	5.8	10.34	-23.25	0.000134	0.236234	ell2	elongation factor. RNA polymerase II. 2
Dr.14849.1.S1_at	10.32	4.79	46.18	0.000136	0.236234	myl6	myosin. light chain 6. alkali. smooth muscle and non-muscle
Dr.12375.1.S1_at	5.61	10.19	-23.99	0.000127	0.236234	si:ch211-285f17.1	si:ch211-285f17.1
Dr.399.1.A1_at	10.2	6.97	9.4	0.000134	0.236234	dda1	DET1 and DDB1 associated 1
DrAffx.2.100.A1_at	4.84	9.16	-19.91	0.000122	0.236234	dpp4	Dipeptidyl-peptidase 4
Dr.20032.1.S1_at	9.21	4.91	19.62	0.000247	0.237002	mc5rb	melanocortin 5b receptor
Dr.9964.1.S1_at	5.55	9.59	-16.52	0.000201	0.237002		
Dr.3966.1.A1_at	8.83	5.04	13.87	0.000215	0.237002	tagln3b	transgelin 3b
Dr.4325.1.A1_at	9.82	4.88	30.79	0.000246	0.237002	bcat2	branched chain aminotransferase 2. mitochondrial
Dr.1429.1.S1_at	10.29	5.29	31.85	0.000184	0.237002	zgc:193541	zgc:193541
Dr.15634.1.S1_at	5.43	9.85	-21.36	0.000192	0.237002	tcea3	transcription elongation factor A (SII). 3
Dr.16312.1.S1_at	5.5	9.16	-12.61	0.00019	0.237002	sb:cb25	sb:cb25
Dr.14752.1.A1_at	10.11	6.13	15.83	0.000258	0.237002	fam100aa . LOC100536798	family with sequence similarity 100. member Aa ; uncharacteriz
Dr.26185.1.A1_at	9.14	4.92	18.53	0.000286	0.248387		
Dr.23818.1.A1_at	10.44	11.27	-1.77	0.000331	0.258301	zfr2	zinc finger RNA binding protein 2
Dr.23024.1.A1_at	10.44	5.54	29.88	0.000325	0.258301	etv5a	ets variant 5a
Dr.14300.1.S1_at	10.18	5.06	34.67	0.000359	0.266758	guca1c	guanylate cyclase activator 1C
Dr.13330.1.A1_at	5.36	7.79	-5.39	0.000574	0.277552	zgc:112153	zgc:112153
Dr.5480.1.A1_at	9.97	5.9	16.8	0.00059	0.277552	snx17	sorting nexin 17
Dr.1139.1.A1_at	5.41	8.93	-11.46	0.000493	0.277552	zgc:154054	zgc:154054
Dr.10109.1.A1_at	5.66	10.73	-33.69	0.000604	0.277552	zgc:92202	zgc:92202
Dr.10393.1.A1_at	10.21	5.69	22.93	0.000532	0.277552	ca5	Carbonic anhydrase V
Dr.15501.1.S1_at	9.93	6.53	10.57	0.000477	0.277552	zgc:85866	zgc:85866
Dr.18241.1.A1_at	5.48	9.82	-20.25	0.000428	0.277552	cenpj	centromere protein J
Dr.2408.2.S1_at	5.55	6.76	-2.32	0.000531	0.277552	mmp2	matrix metalloproteinase 2
Dr.19086.1.S1_at	5.35	8.72	-10.33	0.000589	0.277552	LOC553490	uncharacterized LOC553490
Dr.17250.1.A1_at	9.24	5.6	12.46	0.000535	0.277552	erbb2ip	erbb2 interacting protein
Dr.2028.1.A1_at	10.01	5.37	24.91	0.000572	0.277552	cnot3a	CCR4-NOT transcription complex. subunit 3a
Dr.11831.1.S1_at	5.69	10.56	-29.14	0.000457	0.277552		
Dr.17651.1.A1_at	5.23	10.26	-32.61	0.000399	0.277552		
Dr.5541.1.S1_at	10.35	5.6	26.95	0.00067	0.290737	eif2s1	eukaryotic translation initiation factor 2. subunit 1 alpha
Dr.7485.1.S1_at	9.92	5.14	27.41	0.000659	0.290737	LOC100536256 . pknox1.1	homeobox protein PKNOX1-like ; pbx/knotted 1 homeobox 1.1
Probe set	**$MnCl_2$ 6mM**	**Mn(II)Cit 3 mM**	**Fold Change (linear)**	**Anova, p<0.05**	**pFDR**	**Gene**	**Gene name**
Dr.7503.1.A1_x_at	9.49	5.08	21.26	0.000021	0.166739		
Dr.20274.1.A1_at	9.22	5.39	14.26	0.000013	0.166739	ubqln4	ubiquilin 4
Dr.8516.1.S1_at	9.38	5.22	17.87	0.000059	0.228736	sepp1b	selenoprotein P, plasma, 1b
Dr.6353.1.A1_at	11.25	8.15	8.57	0.000055	0.228736	LOC100536584 , si:ch211-140m22.7	uncharacterized LOC100536584 ; si:ch211-140m22.7
Dr.7313.1.A1_at	8.93	5.14	13.85	0.000082	0.255638	zgc:73340	zgc:73340
Dr.515.1.A1_at	9.54	11.15	-3.05	0.000116	0.258932	gtpbp1	GTP binding protein 1
Dr.15843.1.A1_at	9.34	5.18	17.89	0.000113	0.258932	atrxl	Alpha thalassemia/mental retardation syndrome X-linked, like
Dr.11536.1.A1_at	5.98	5.12	1.81	0.000148	0.289704		
Dr.1712.1.A1_at	10.21	5.57	24.97	0.000172	0.299285	nap1l4b	nucleosome assembly protein 1-like 4b

Table SM-1: *Differential gene expression in Danio rerio exposure for chemical species of manganese.*

Pathway	Pathway ID	P-value	Corrected P-value	Genes Quantity	Annotated Genes	Genome Frequency
Protein digestion and absorption	KEGG:04974	0,00198	0,06350	2	CPA1|DPP4	81/35695 genes: 0,23%
Protein processing in endoplasmic reticulum	KEGG:04141	0,00929	0,29725	2	EIF2S1|UBQLN4	179/35695 genes: 0,50%
Metabolism of proteins	REACT:17015	0,01067	0,34138	3	DPP4|EIF2S1|MMP2	566/35695 genes: 1,59%

***Table SM-2**: Pathways involved in Mn-induced toxicity in Danio rerio larvae, according to CTD-base.*

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