EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 5
23-32
Year: 2016

NON-ESSENTIAL AND ESSENTIAL METAL EFFECTS ON INTRACELLULAR TOXOPLASMA GONDII

Laís Pessanha de Carvalho* and Edésio José Tenório de Melo

Laboratory of Cellular and Tecidual Biology, Norte Fluminense Darcy Ribeiro State University, Campos dos Goytacazes 28013-602, RJ, Brazil.

***Corresponding Author: Laís Pessanha de Carvalho**
Laboratory of Cellular and Tecidual Biology, Norte Fluminense Darcy Ribeiro State University, Campos dos Goytacazes 28013-602, RJ, Brazil.

Article Received on 26/02/2016 **Article Revised on 17/03/2016** **Article Accepted on 07/04/2016**

ABSTRACT
Toxoplasma gondii is an obligatory protozoan parasite that uses sophisticated mechanisms to guarantee its survival at the intracellular environment of all eukaryotic host cells. However, metals are very important for survival and development of several parasites at host intracellular environment. The parasites use essential metals as co-factors or structural components of metalloproteins. These proteins are involved in host cell invasion, establishment on cytoplasm, vacuole formation, parasite multiplication and host cell egress. Nonetheless, the understanding of toxicological and biochemical impact of metallic ions on biological systems of intracellular replicative form of T. gondii - tachyzoite is poorly known. The aim of this study was utilize the relationship of host cell - Toxoplasma gondii to verify the effects of non-essential (CdCl2 and HgCl2) and essential (ZnCl2) metals on the maintenance of intracellular infection. We used different concentrations (1, 3, 5, 8, 10 and 20 µM) and incubation times (2, 12 and 24 h) to observe the progressive toxic effect to parasite. Ultrastructural features were analyzed under light and transmission electron microscopy. The results indicated that uninfected culture were more susceptible to toxicity of non-essential metals than infected ones. However, the infection decreased during the incubation with these metals while tachyzoites underwent elimination at low time and concentrations of the non-essential metals. These results suggest that parasites were very susceptible to these metals. Therefore, the essential metal ($ZnCl_2$) protected the host cells and parasites against toxic effects of non-essential metals ($HgCl_2$ and $CdCl_2$).

KEYWORDS: cadmium, infected cells, mercury, T. gondii and zinc.

INTRODUCTION
Toxoplasma gondii, the protozoan parasite causative of Toxoplasmosis disease, is an intracellular obligatory parasite able to infect all nucleated cells of the vertebrate host.[1] The tachyzoite, invasive and fast replicative form of T. gondii, penetrate all eukaryotic cells and forms a parasitophorous vacuole where it can replicate or transforms into bradyzoite, the slow replicative form, perpetuating the infection in vertebrate host.[2] The parasite mechanism to survive in host cell environment involves two concomitant steps: (1) secretion of parasite products during the formation and development of the parasitophorous vacuole, and (2) structural and functional reorganization of host cell organelles around the parasitophorous vacuole membrane. These reorganizations involve organelles as mitochondria and endoplasmic reticulum elements[3], microtubules[4] and vesicle traffic from Golgi complex.[5] The efficiency of these processes allows the tachyzoite multiplication and avoids the recognition by the host cell microbicide mechanisms, mainly the endocytic fusion. Although many parasite molecules are involved in all these events, little is known about the importance of metal ions on survival and development of the intracellular tachyzoite.

Essential metals are found in all forms of organisms and they are co-factors of metabolic reactions or of a wide range of physiological processes[6] and are present as structural or catalytic sites of metalloenzymes (as Fe (II), Zn (II), Cu (II), Mg (II) or Ca (II)).[7] Many metalloenzymes regulate invasion processes of several parasites, survival at the intracellular environment, intravacuolar development, replication and egress from the host cell.[8][9][10] Nonetheless, the cells are also in contact with non-essential metallic ions which mimicking the essential ones, can enter the cells through ions pumps, transporters and channels.[11] Inside the cell, metals such as cadmium and mercury can produce free radicals and/or mimic and substitute essential metals in molecular targets, including metalloenzymes, blocking their functionality, leading to organelle dysfunction and cell death.[12][13] Mechanisms to select, distribute and detoxify metal ions, including sequestering or chaperones and chelating molecules, metal-responsive

gene regulators and transmembrane transporters, are important to guarantee the cell homeostasis.[14]

Non-essential metals have been largely discussed in the literature, mainly their high toxic effects even in low concentrations in different cell types[15], and mechanisms that host cell utilizes them to eliminate intracellular microorganisms and how the pathogens can subvert this defense mechanism.[16] However, the effect of these metals in infected cells and the influence of metallic ions or related molecules on the development of T. gondii is unclear.

For the first time, the effects of cadmium, mercury (both non-essentials) and zinc (essential) on infected host cells were studied. The study of metallic ions is necessary to understand their essential, toxicological and biochemical impact on biological systems in the relationship of parasite - host cell.

MATERIALS AND METHODS

Host cell culture

Vero cells (kidney fibroblasts of the African monkey) were grown in plastic Falcon flasks (25 cm^2) containing Dulbecco's Modified Eagle's Medium (DMEM-1152, SIGMA, ST. LOUIS, USA) supplemented with 5% fetal bovine serum (FBS, NUTRICELL, CAMPINAS, BRAZIL). The culture was trypsinized when the cell density approached to monolayer. For experimental proposals, the cells were placed on Linbro 24-well plates with a sterile coverslip at a density of 3 x 10^4 cells per well or on a medium flasks (3 x 10^6 cells) and allowed to attach for 24 h at 37°C in a 5% CO_2 atmosphere.[17]

Parasite maintenance

Tachyzoites of T. gondii (RH strain) were maintained in Vero cell culture. The culture was infected at a ratio of 10 parasites per cell, before reaching the monolayer stage. This culture was kept at 37 °C for 48 h to allow cell lysis and liberation of the tachyzoites into the medium. The medium was centrifuged at 500 × g for 10 min, and the pellet was resuspended in 1 mL of medium. A 0.01 mL aliquot of the suspension was transferred to a Neubauer chamber and the parasites were counted using an optical microscope Zeiss Axioinvert 135, 20x objective (ZEISS, OBERKOCHEN, GERMANY). Then, the tachyzoites were transferred to a new Vero cell culture. For experimental purpose, the released parasites were transferred to the cells previously seeded on the plates in a rate of 5:1 parasite - cell, during 24 h, when the infection is established.[18]

Metal treatments

Uninfected and infected Vero cells were incubated with 5, 8, 10 or 20 µM of $HgCl_2$ and $CdCl_2$ through 2, 12 or 24 h. In addition, the $ZnCl_2$ (20 µM) and $HgCl_2$ and $CdCl_2$ (1 and 3µM) were incubated only through 24 h, due to no significant effect at this time. For the co-treatments with the metals, the infected or uninfected cells were incubated with 20 µM of $ZnCl_2$ for 24 h, after, the medium was replaced by a refresh one containing $HgCl_2$ or $CdCl_2$ at 8 µM for more 24 h. Dilutions of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ salts originated 0.1 M stock solutions in an ultra-pure quality water. Dilutions with the culture medium prepared the final concentrations. The metals were obtained from SIGMA

Quantification and morphological analyses

After treatments, the coverslips containing cells were rinsed in PBS, fixed in Bouin's solution for 5 minutes and stained with Giemsa's solution (diluted in PBS, pH 7.2, 10%, v/v) during 6 hours at room temperature. The mounting of the coverslips occurred on glass slides with Entellan (MERCK, DARMSTADT, GERMANY) for observation by light microscopy. The examination of all preparations used a Zeiss Axioplan microscope, equipped with 20x and 40x objectives. Analysis System software obtained the images. The observation of three random fields of each of six samples (individual treatments) gave the quantification of the parameters. The observation of morphological alterations and reduction of cells and parasites indicated the $HgCl_2$ and $CdCl_2$ cytotoxicity.[19]
The estimative of LD 50 considered the dose that reduced 50% of the cultures.

Ultrastructural studies

For ultrastructural analyses, uninfected and infected Vero cells were treated with 5 µM of $HgCl_2$ and $CdCl_2$. After 24 h, the cultures were washed with PBS at 37 °C and fixed at room temperature in a solution containing 1% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$, and 5% saccharose in cacodylate buffer 0.1 M, pH 7.2. The cells were postfixed for 1 h in a solution containing 2% OsO_4, 0.8% potassium ferrocyanide. The samples were rinsed with 0.1 M cacodylate buffer, pH 7.2, dehydrated in graded acetone and embedded in PolyBed812 (Polyscience Inc., Germany) and then polymerized for 2 days in 60° C. Ultra-thin sections obtained with a LEICA (NUSSLOCH, GERMANY) ultramicrotome were stained with uranyl acetate and lead citrate, and observed with a Zeiss 900 Transmission Electron Microscope at 80 kV acceleration.[20]

Biochemical Studies

Medium flasks containing infected or uninfected cells were incubated with 5 or 20µM of $HgCl_2$, $CdCl_2$ and $ZnCl_2$, during 24 h. Untreated T. gondii and Vero cells were used as control.

The cells were harvested from the flasks and centrifuged at 3.000g, 20min, 4°C. After, the pellets were washed with Tris-HCl buffer, 5mM, pH 8 containing 5% of saccharose and then the pellets were centrifuged at the same conditions. So, the pellet were ressuspended in a solution of 100 µL of TRIS-HCl buffer, 5mM, pH 8 in the presence of a 1% of inhibitor cocktail (Protease inhibitor cocktail for general use, SIGMA) and 0.1% of TRITON X-100 (SIGMA). Afterward the samples were centrifuged at 10.000 g, 20min, 4°C and the supernatant

protein contents were quantified by the bicinchoninic acid method (SIGMA) with albumin from chicken egg whites (SIGMA) used as the protein standard. For Gel electrophoresis, protein analyses were performed on a tricine-SDS-PAGE gel.[21] Before the electrophoresis, 40 µg of the samples were reduced by boiling in a sample buffer containing 125 mM Tris-HCl pH 8.0, 2.5 % SDS, 15 % saccharose and 0.25 % bromophenol blue in the presence of 5 % β-mercaptoethanol. The gel was stained with Coomasie Brilliant Blue R-250 (BIO-RAD, CALIFÓRNIA, EUA) and protein markers from 200 to 6.5 KDa were used as standards (SIGMA).[22]

RESULTS AND DISCUSSION

Toxicity of metals to host cells and intracellular parasite

Number of host cell and morphological features during all treatments were determined observing the progression of toxic effects (drastic cellular disorganization) until cell elimination (toxic effect).

The 1 and 3 µM concentrations did not lead toxic effect until 24 h of time incubation. The progressive toxic effects (elimination) to uninfected cells occurred after the incubation at concentration 5 µM, 2 h hours with both $HgCl_2$ and $CdCl_2$, when approximately 10% of cell number reduction was observed. The increase of time incubation and concentration, led to a greater cell elimination, but only the $HgCl_2$ eliminated all culture after 12 hours of incubation at 20 µM. Differently, $ZnCl_2$, only caused a minimum toxic effect after incubation through 24 hours and in the high dose of 20 µM (18% of cells elimination) (Table 1).

Table: 1 The mean number Vero cells in the presence of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ at different times and concentrations of incubations. ± Standard deviation of six different samples. – NE :no effect Tox: no remaining cells .

Vero toxicity							
	HgCl2			CdCl2			ZnCl2
(µM)	2 hours	12 hours	24 hours	2 hours	12 hours	24 hours	24 hours
Untreated	NE	NE	149.5±19.0	NE	NE	157.8±17.5	NE
1	NE	NE	163.5±24.4	NE	NE	164.4±24.4	NE
Untreated	NE	NE	149.5±19.	NE	NE	157.8±17.5	NE
3	NE	NE	146.1±22.9	NE	NE	157.2±19.1	NE
Untreated	229.7±5.3	140.6±6.7	135.4±5.9	335.5±5.3	267±3.8	319.7±4.7	NE
5	203.1±5.8	80.4±7.0	48.0±9.1	312.7±5.3	164.1±3.8	153.1±7	NE
Untreated	153.4±5.3	79.0±7.0	139.3±4.8	170.1±20.8	88±28.6	311.5±6.3	NE
8	119.5±4.9	28.7±14.0	28.4±3.9	141.1±30.7	48.4±20.9	58.4±4.9	NE
Untreated	229.6±5.3	176.8±6.1	166.5±21.1	341.2±5.6	287.7±13.2	166.5±3.9	NE
10	170.1±5.4	50.7±7.7	12.6±10.9	212.8±5.8	108±4.7	19.5±4.7	NE
Untreated	123.9±6.1	176.8±6.1	139.3±4.8	341.2±5.6	287.7±13.2	311.5±6.3	156.2±25
20	71.2±5.4	TOX	TOX	171.6±6.1	52.7±4.4	TOX	128±21

Next, we evaluated the effects of metals only on the infected culture, to differentiate from the Vero cell toxicity. After 24 h, Infected Vero cells showed intracellular proliferative parasites, confirming the establishment of infection. In this phase the metals were incubated. At a time of 2 h of incubation with $HgCl_2$ no cytotoxic effect was observed, neither 5 µM 12 h nor 1 and 3 µM 24 h. Nonetheless, considerable cytotoxicity was seen from 8 µM with 12 h and 5 µM 24 h. The concentration of 20 µM of $HgCl_2$ eliminated all uninfected and infected cells since 12 h of treatment. In the case of $CdCl_2$, again the time of 2 h was not toxic neither 12 h up to 5 µM or 24 h up to 3 µM, but the cells were totally eliminated at 20 µM, 24 h. In addition, $ZnCl_2$ has no toxic effect at all concentration and time tested (Table 2). These results showed that $HgCl_2$ is more toxic than $CdCl_2$, mainly when the cell became not infected, while $ZnCl_2$ did not affect in both situations. These results suggest that the host cells and parasites are able to deal with high extra concentration of this essential metal.

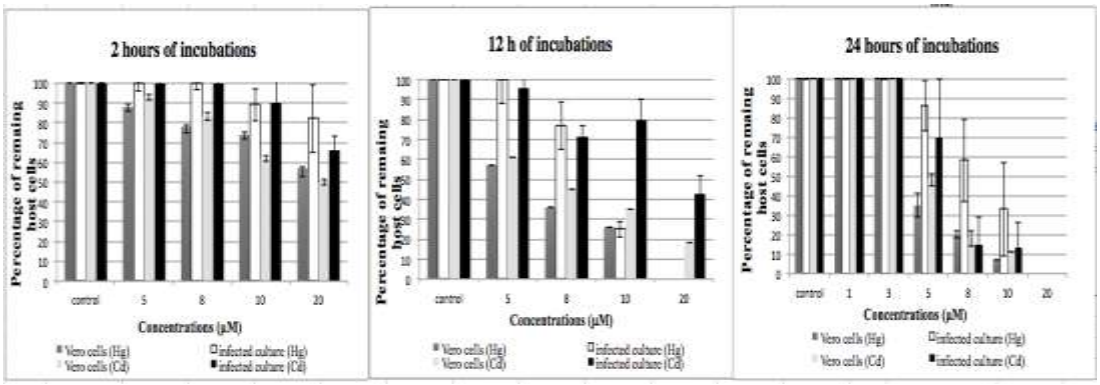
Table: 2 The mean number of Vero cells in the infected culture in the presence of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ at different times and concentrations. ± Standard deviation of six different samples. – Not determined. TOX: no more cells.

Infected Vero toxicity							
	HgCl2			CdCl2			ZnCl2
(µM)	2 hours	12 hours	24 hours	2 hours	12 hours	24 hours	24 hours
Untreated	-	-	159.1±22.4	-	-	172.6±22.4	-
1	-	-	196.8±28.4	-	-	172.8±22.6	-
3	-	-	177.2±31.3	-	-	178.1±18.8	-

Untreated	112.2±18.7	98.1±11	152.5±29.2	182.5±26.4	195.6±18.9	204.7±51.3	-
5	115.2±24.5	103.2±12.6	133.1±96.9	185.6±32.5	187.5±17.7	123.6±89.3	-
8	109.3±19.3	76.1±12.8	89.6±64.4	174.1±21.6	140.1±13.7	28.2±39.9	-
Untreated	124.8±8.3	123.8±9.6	148.8±18	174.7±13.7	172.3±16.8	172.6±22.4	140.15.2
10	111±10.1	50.7±71.7	57.7±7.1	157.6±21.8	140.1±13.7	26.6±36.8	-
20	102.8±22.7	TOX	TOX	116±13.6	75.5±16.3	TOX	139.2±4.1

The CdCl2 toxicity is consequence of factors including the bioaccumulation of the metal as a result of very slow elimination. The interaction of CdCl2 with cellular components has been described as causing several dysfunctions such as the blockage of oxidative phosphorylation, glutathione depletion and inhibition of antioxidant enzymatic activity, DNA damage, production of ROS and decrease of protein synthesis. [cited in 23] In the case of $HgCl_2$, the toxic effects caused can alter a wide range of cellular and biochemical processes. Mercury has a high and general attraction for thiol groups that constitutes proteins, nucleic acids and transcription factors[24], and can block antioxidant enzymes, impair mitochondria metabolism and induce oxidative stresses involving O_2 and N_2.[25] In addition, HgCl2 can also disturb functionally and structurally organelles as endoplasmic reticulum, microfilaments, mitochondria, acidic organelles.[13] Our results suggest that due to these wide ranges of effects on metabolism, $CdCl_2$ and $HgCl_2$ had more drastic toxic effects on intracellular parasites.

As demonstrated at table 1 and 2, the elimination of Vero cell occurs in lower concentration and in a fast manner than in infected Vero cell, suggesting that the infection can give resistance to non-essential metals. To quantify the difference of cell resistance, the percentage of cell survival after each treatment was plotted and analyzed in Fig 1. As showed, the infected culture was more resistant to metals than the uninfected ones. As example, the incubations of 2 h of $HgCl_2$ and $CdCl_2$ did not lead significant cell death to infected cells (except to $CdCl_2$ 20 µM). Comparatively, these same conditions cause among 10 to 50 % of cell death in uninfected culture (Fig 1 A). Toxic effect is also seen to others treatments, but 12 h of $HgCl_2$ incubation is high enough to eliminate both the infected and uninfected cells (Fig 1 B), the same happened with $CdCl_2$ 24 h (Fig 1 C). These results showed that $HgCl_2$ is more toxic to the infected cells than $CdCl_2$, and also the differences in cell survival showed at table 1 and 2 and Figure 1 suggests that intracellular tachyzoites protect the host cell against non-essential metals toxic effects.



A **B** **C**

Fig 1. Graphical representations of the comparative metal toxic effects in uninfected and Infected Vero cell culture . A,B,C: Comparative percentage of uninfected and infected cell survival after the treatment with $HgCl_2$ and $CdCl_2$ during different times and concentrations. The high standard value is due to the irregular elimination of the culture, resulting in cell spots.

Several studies showed that intracellular parasites such as C. albicans,[26] C. neoformans[27] and Plasmodium falciparum[28] must acquire all essential nutrients as well as transition metals from the host cell and many of them need elevated metal quotas to achieve pathogenesis.[29] For these purposes, they use a variety of mechanisms to sense, acquire, store and export the metal ions, as example the siderophores and the expression of high-affinity transporters to metal ions.[30] Although the need of metals to establish and develop an infection and to achieve pathogenesis is relatively well established regarding many intracellular pathogens, nothing is known to T. gondii. Nonetheless, the metallic ions should have important roles for maintenance of the tachyzoite infection since many enzymes responsible for development and replication of the parasite have metallic ions as structural or catalytic sites.[31] Whereas the non-essential metals can substitute the essential ones in the enzymes, the parasite's enzymes that have faster activity to support their replicative cycle, can be damaged before those of the host, making the tachyzoite the primary target of the metals.

Both $CdCl_2$ and $HgCl_2$ effects led to an increase on the number of uninfected cells as consequence of intracellular parasite elimination with no cytotoxicity

effects. However, when the concentration used was high enough (from 8 µM) to induce a toxic activity (cell death), the uninfected cells were eliminated first, and after the infected ones. These results indicate that the parasites have some protective effect to cells (Table 3). So, to better understand this protective effect, the progression of parasite elimination was determined.

Comparative effects of $HgCl_2$ and $CdCl_2$ on the infection

We considered only infected cells to show the progressive reduction of the infection during the different treatments with metals.

With the elimination of intracellular tachyzoites, the percentages of infection decreased. However, 1 µM until 24 h and 3 µM until 12 h of $HgCl_2$ and $CdCl_2$ was not enough to reduce the infection. While 2 h of incubation of $HgCl_2$ at 5 µM reduced 22 % of the infection, $CdCl_2$ did not show activity until 10 µM, when decreased 22 %. After 12 h of treatment, $HgCl_2$ led to a greater reduction of the infection (18, 63, 93 and 100%) than the $CdCl_2$ (33, 39, 35 and 77) at concentrations of 5, 7, 10 and 20 µM, respectively. Nonetheless, after 24h, $CdCl_2$ decreased the infection (15, 73, 97, 95 and 100) more than the $HgCl_2$ (17, 63, 77, 92 and 100%) at concentrations of 3, 5, 8, 10 and 20 µM. For $ZnCl_2$, there is no reduction on infection.

Table 3. Mean number of infected Vero cells after the treatment with $HgCl_2$, $CdCl_2$ and $ZnCl_2$ in different times and concentrations. NE: no effect. TOX. No more remaining cells.

Mean number of infected cells									
$HgCl_2$ (µM)									
	Untreated	1	3	Untreated	5	8	Untreated	10	20
2 h	NE	NE	NE	79±13	61±12	60±10	95±5	55±12	52±5
12 h	NE	NE	NE	67±4	55±6	24±3	92±5	6±1	TOX
24 h	110±14	119±16	92±15	96±17	36±10	22±16	104±11	8±7	TOX
$CdCl_2$ (µM)									
2 h	NE	NE	NE	106±16	118±22	105±13	124±6	96±15	62±9
12 h	NE	NE	NE	110±7	63±4	68±7	102±8	67±6	23±5
24 h	127±13	129±15	108±8	114±38	39±28	4±6	127±13	7±9	TOX
$ZnCl_2$ (µM)									
24 h	139±14	NE	NE	NE	NE	NE	NE	NE	139±15

Progressive parasite elimination

For quantify the number of intracellular tachyzoites during metal incubations, just the parasites with normal morphological features (half-moon, as shown at figure 4 B) were considered.

Infected cells incubated with $HgCl_2$ showed percentage of parasite survival ranging from 65 to 40 % (5 and 20 µM, respectively) after 2 h of incubation. Considering $CdCl_2$, the percentage of survival varied from 84 to 60 % with 2 h and 52 to 16 % (5 and 20 µM, respectively for both) to 12 h. Until 24 h of treatment, the concentrations of 1 for $HgCl_2$ and 3 µM for $CdCl_2$ did not affect the tachyzoites. But from these concentrations, 86 % of parasites survived after 1 µM of $HgCl_2$ and 67% after 3 µM of $CdCl_2$. All tachyzoites stayed alive after $ZnCl_2$ treatments.

Table 4. Mean number of intracellular parasites in the presence of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ in different times and concentrations. ± Standard deviation of three different samples. NE: no effect. TOX. no more remaining cells.

Mean umber of intracellular tachyzoites							
	$HgCl_2$			**$CdCl_2$**			**$ZnCl_2$**
(µM)	**2 hours**	**12 hours**	**24 hours**	**2 hours**	**12 hours**	**24 hours**	**24 hours**
Untreated	NE	NE	711.3±44.6	NE	NE	892.6±85.4	NE
1	NE	NE	615.3±64.1	NE	NE	900.1±54.1	NE
3	NE	NE	536.6±57.3	NE	NE	600.5±57.7	NE
Untreated	343.2±52.1	353±26.1	635.5±47.3	300.7±33.8	423±89.9	692.6±65.5	NE
5	226.1±45.6	243.4±34.4	314.3±226.2	251.1±45.3	264.2±27.8	140.4±100.2	NE
8	212.8±43.2	112.5±15.4	143.8±106.1	228.5±5	216.5±18.7	22.7±32.2	NE
Untreated	392.2±26.3	393.8±19.9	489.3±42.5	302±26.4	540±61.2	892.6±85.4	NE
10	164±34.3	49.7±70.3	22±7.3	204.8±31.3	241.2±26.8	132.3±99.3	718±59
20	157.3±17.2	TOX	TOX	180.1±14.2	88.9±20.9	TOX	691.9±37.5

In this study, we found that at low concentrations (1 and 3 µM) of incubation of toxic metals just the tachyzoites of T. gondii underwent progressive drastic morphological alterations that led to its elimination, while the host cells were not eliminated until 5 µM, 12 h. These results indicate that the parasites are the main

targets of non-essential metals and their presence protects the host cells from toxicity. The parasite susceptibility was also found in studies utilizing drugs able to arrest the enzyme Ribonucleotide Reductase, which is responsible for replicative cycle. These studies also suggest that the replicative cycle of 6 hours of the parasite become them less resistant than the host cells.[20][32][19][33]

Lethal dose values (LD50)

After the screening of the treatments with the metals, the lethal dose to 50 % of each population (LD 50) was determined. The time of 2 h in the presence of the metals did not lead effects to the cultures, except to T. gondii (HgCl2) whose LD 50 was 9 µM. After 12 h, a toxic effect could be seen for both uninfected and infected cultures, nonetheless, these values can confirm that the parasites are more susceptible to metals than the cells, protecting them from the toxic effect. Meanwhile the treatment with ZnCl2 was not enough to determine its LD 50.

Table 5: Lethal dose values to uninfected, infected and parasites after different treatments.

LD 50 (µM)									
		HgCl2				CdCl2			ZnCl2
		2 h	12 h	24 h		2 h	12 h	24 h	24 h
Uninfected		> 20	6	3.5		20	8.2	4.8	>20
Infected	Cells	> 20	9	8.5		>20	16	5.5	>20
	T. gondii	9	6.5	5		>20	8	4	>20

Once it was determined the effect scale of two non-essential metals and the essential $ZnCl_2$, the next question was if the $ZnCl_2$ is able to protect the host cells and parasites against the toxic effects of $CdCl_2$ and $HgCl_2$. Zinc is known for protecting the cells against oxidative stress and intracellular pathogens.[34][35][36] Because of that, the host cells were previously treated with $ZnCl_2$ and after with $CdCl_2$ or $HgCl_2$.

The pre-treatment with $ZnCl_2$ protects the cultures from $CdCl_2$ and $HgCl_2$ cellular toxic effects

The toxic effects and the cell elimination after treatment with CdCl2 and HgCl2 were reduced significantly when both the cell cultures were pre-treated with ZnCl2 (20 µM/ 24 h) and, after that, incubated with CdCl2 (8 µM/ 24 h) or HgCl2 (8 µM/ 24 h) (Fig 2 A). We also observed a similar decrease on parasite elimination, explaining the high number of remaining parasites (55 and 95 % for $CdCl_2$ and $HgCl_2$, respectively) and percentage of infection (60 and 80% for $CdCl_2$ and $HgCl_2$, respectively). Nonetheless, as shown at figure 2 B, the protective ZnCl2 effects were higher to parasites treated with HgCl2. Considering these results, it was showed that ZnCl2 avoids host cell and parasite elimination, suggesting a protective action against cellular destruction (Fig 2 B and C).

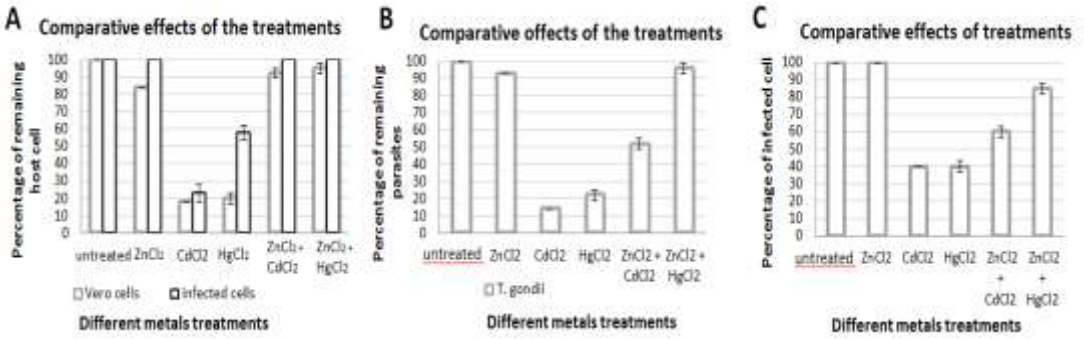


Fig 2. Comparison among the effects of the isolated or sequential treatments with $ZnCl_2$ (20 µM/ 24 h), $CdCl_2$ (8 µM/ 24 h) and $HgCl_2$ (8 µM/ 24 h) in (A) uninfected or infected cultures, (B) parasites and (C) percentage of infected cells.

As our results showed, the zinc pre-treatments protected mainly the cells, but also the parasites against the effects of cadmium and mercury. Zinc is fundamental for cell membrane integrity and is a component of more than 300 enzymes with variable functions of cellular metabolism, involving metabolism of proteins, lipids and carbohydrates.[37] The Zinc is also known for protecting biological structures from free radical damage through sustaining an adequate level of MTs, as a protective agent for thiols and other chemical groups and as an essential component of Cu, Zn-SOD.[34][38]

Although our zinc treatment did not reduce the infection, some studies have demonstrated that the importing of zinc is an important component of phagolysosomal killing of bacteria within immune efector cells.[39][40] However, it has been describe some pathogens' Adaptations have reduced the metals uptake or

increased efflux, chemical modifications and cellular repair.[16]

Maybe the protein adaptive expressions inductions in the host cell, as observed through electrophoretic profiles, are responsible for this protection and maybe the metallothioneins are in the key roles.[41] However it remains unclear the mechanisms of metal detoxifying of protozoans, including T. gondii, and nothing is known about its capability to express metallothioneins.

Gel eletrophoresis

Protein analyses of uninfected untreated or treated Vero cells and the T. gondii samples were performed by electrophorese. The protein profile of the untreated control (Vero) and cells treated with 5 µM of $HgCl_2$ or CdCl2 did not show many differences, but increasing the concentration of both the metals to 10 µM, the cells expressed new proteins (~ 21 kDa). The parasite protein profile was also observed, and consistent difference were showed, as expected. Less proteins were traced, mainly from 31 KDa below (Fig 3).

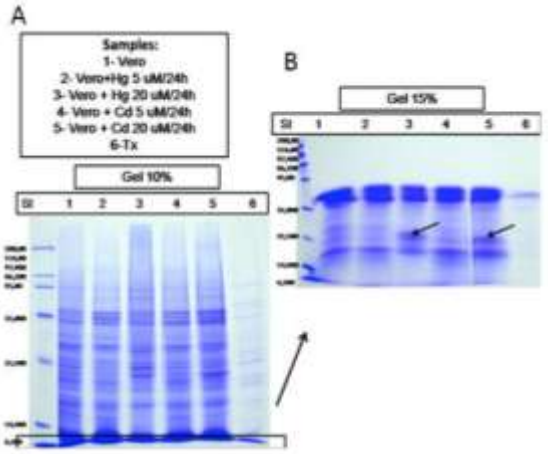


Fig 3. Electrophoretic visualization of the samples after different treatments with metals. The concentrations of metals influence the expression of different proteins in Vero cells.

Eukaryotes and prokaryotes developed several mechanisms to avoid metal accumulation or oxidative stresses caused by them. Among these mechanisms are mainly enzymes such as glutathiones, catalases, superoxide dismutases and metallothioneins that play roles to chelate intracellular oxidants or metals.[34] Highlighting the metallothioneins, they are included in a specific group of metalloenzymes found in many cell structures.[42] Metallothioneins are characterized by the high content of cystein residues (approximately one third) and thiol ligands, low molecular weight (6-14 kDa), and have high affinity by transition metals as Cadmium, Lead, Cobalt, Mercury e Zinc.[43] However is stated that the metallothioneins are present in vertebrates, invertebrates and microorganism, but they were isolated for the first time in parasites with medical importance, epimastigotes of T. cruzi, only recently.[44] The quantity of metallothioneins is adaptive to cell environment and it can increase or decrease according to metal availability.[45] In this respect, zinc plays an essential role, mainly in induction of the expression of metallothioneins, but although zinc be commonly bound to them, cadmium can also displaces and releases it in the cytoplasm blocking the enzyme activity.

Morphological cell features during metal treatments

As showed at Fig 4, uninfected cells treated with with $CdCl_2$ (Fig 4 C) and $HgCl_2$ (Fig 4 E) at 8 µM through 24 h underwent more drastic morphological effects than the infected ones (Fig 4 D and F, respectively). As observed, greater cell condensation or elimination occurred. In the case of ZnCl2, until 24 h of incubation at 20 µM no significant toxic effects were observed, and many tachyzoites containing parasitophorous vacuole could be seen (Fig 4 G and H). It was also be observed through light microscopy that the toxic effect to cells and parasites is asynchronous, since they are in different stages of destruction.

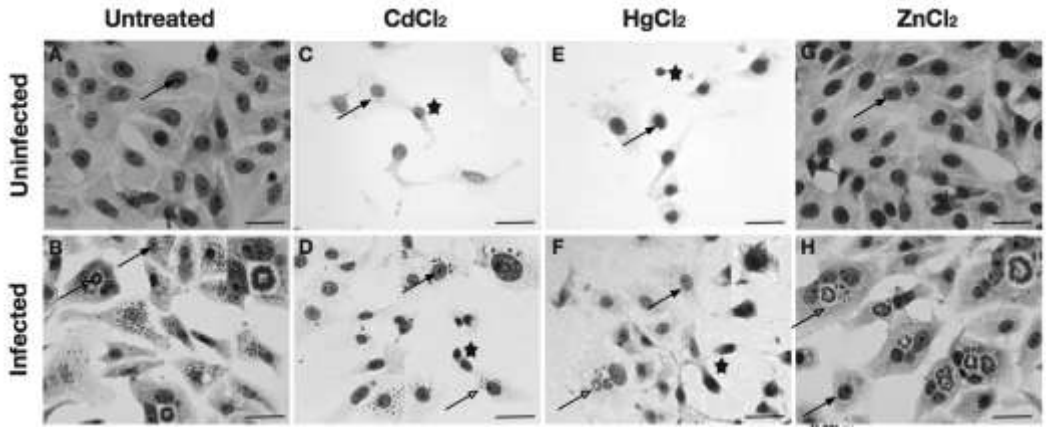


Fig 4. Representative micrographies of the effects of the incubation with metals. Black arrow: cell nucleus. White arrowhead: tachyzoite rosettes. Star: morphological altered host cells. Scale bars: 50 µm.

Following, the protective effects of ZnCl2, for parasites and cells are showed at (Fig 5). The Vero cells still presented their normal morphological feature and dense population while half-moon tachyzoites were remained.

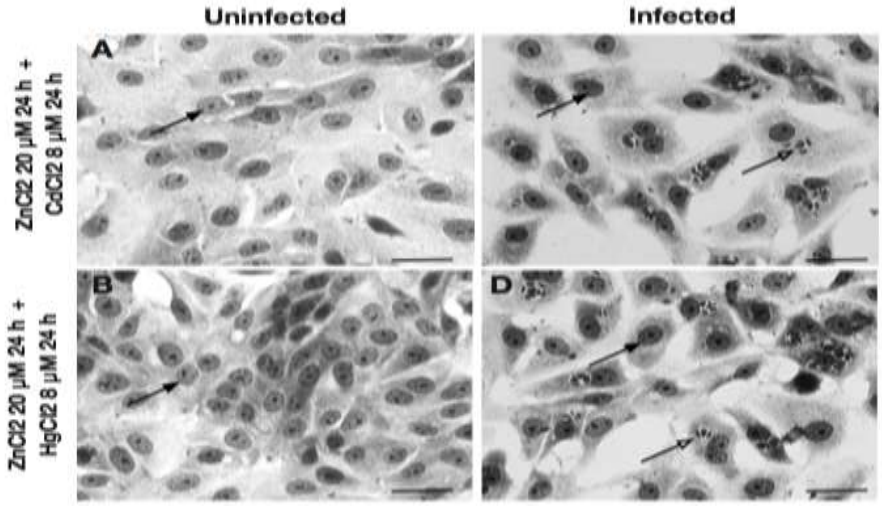


Fig 5. Micrographies representing the sequential treatments with $ZnCl_2$, $CdCl_2$ and $HgCl_2$. Black arrows: cell nucleus. White arrows: parasites. Scale bars: 50 µm.

Transmission electron microscopy analysis showed the control cells presented usual morphological features and proliferative tachyzoites within the parasitophorous vacuoles (Fig 6 A and B). Both the host cells and parasites presented nuclear disorganization; many vacuoles can be seen inside the parasite after treatment with CdCl2 (Fig 6 C and D) and in addition, the tachyzoite was amorphous, mainly with HgCl2 treatment (Fig 6 E and F).

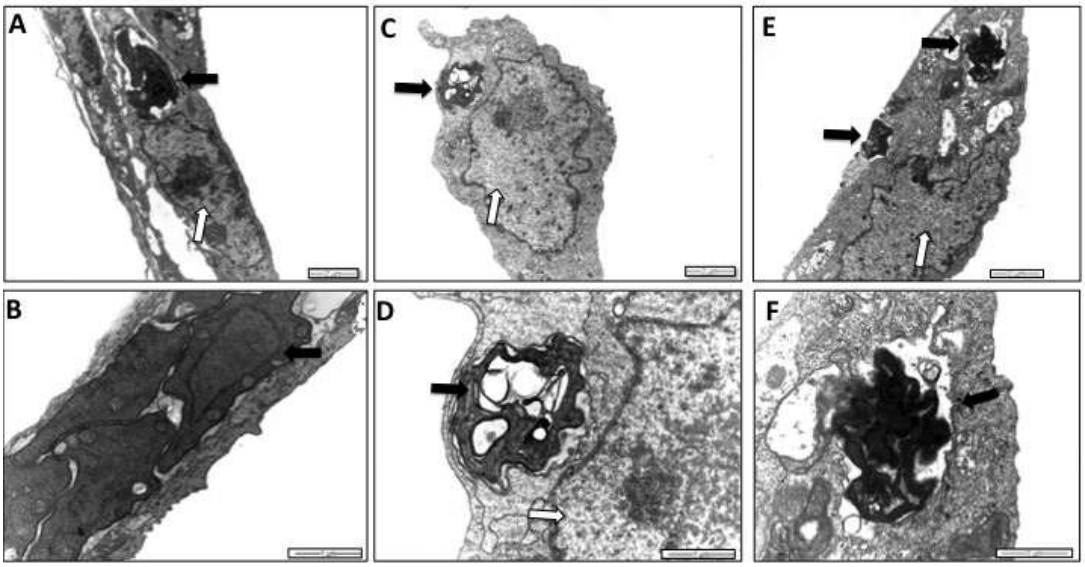


Fig 6. The different metal treatments of infected Vero cells. Black arrows: parasites. White arrows: host cell nucleus. Scale bars: A, C and E: 2 µm. B, D and F: 1 µm.

Structural damages of these non-essential metals are poorly described. Nonetheless, it was shown that cadmium can lead to the condensation of the fiber bundles at adhesive foci, the separation of stress fibers into their individual filament and tubular components, the loss of microfilaments, disorganizing the cytoskeleton (cadmium induced changes in cell organelles) and also led to nuclear condensation.[46] In the case of mercury, it was described that the reticular network became punctuate instead of spread through the cell, the microfilament projections into the cytoplasm were lost, leading to a cell condensation.[13]

Despite the developed mechanisms to avoid intracellular metal accumulation, sometimes it happens and a wide range of effects can occur. Firstly, although the majority of non- essential metals enter the cells through essential metals transporters or channels in a process namely ionic mimicry, it has also been described domains involved in non- essential metals transport.[47] Once non-essential

metals are within the cells, an imperfect selection of metal ions by metal-binding proteins – mismetallation – can occur, leading to intracellular oxidative stress, the impairment of protein function, genotoxicity and/ or membrane perturbation.[16] Since the metalloproteins are involved in many events related to cell survival, including the regulation of replicative cycle, their mismetallation tends to affect preferentially the cell with the faster replicative cycle–T. gondii.

CONCLUSION

Concluding, tachyzoites of T. gondii are high-priory to metal effects probably due to their fast-replicating cycle or absence of metal detoxifying mechanisms. As consequence, infected culture are more resistant than uninfected ones. However, at low concentrations (1 and 3 µg/ 24 h) no morphological changes are seen in host cell even after a significant percentage of tachyzoites were eliminated. But high concentrations (20 µm) were able to eliminate both the parasites and cells.

ACKNOWLEDGEMENTS

For technical support: Dra. Cristiane dos Santos Vergílio and Arthur Rodrigues.

For financial support: FAPERJ and CNPq.

REFERENCES

1. Dubey, JP. The history of Toxoplasma gondii - The first 100 years. J Eukaryot Microbiol, 2008; 55(6): 467–475.
2. Carruthers, V, Boothroyd, JC. Pulling together : an integrated model of Toxoplasma cell invasion. Curr Opin Microbiol, 2007; 10: 83–89.
3. De Melo, EJT, De Carvalho, TU, De Souza, W. Penetration of Toxoplasma gondii into host cells induces changes in the distribution of the mitochondria and the endoplasmic reticulum. Cell Struct and Funct, 1992; 17(5): 311–317.
4. De Melo, EJT, De Carvalho, TU, De Souza, W. Behaviour of microtubules in cells infected with Toxoplasma gondii. Biocell, 2001; 25(1): 53-9.
5. De Melo, EJT, De Souza, W. Pathway of C6-NBD-Ceramide on the host cell infected with Toxoplasma gondii. Cell Struct and Funct, 1996; 21(1): 47–52.
6. Suresh, V, Singh, R. Metals and apoptosis: Recent developments. J Trace Elem Med Biol, 2008; 22: 262–284.
7. Garcia, JS, De Magalhães, CS, Arruda, MAZ. Trends in metal-binding and metalloprotein analysis. Talanta, 2006; 69(1): 1–15.
8. Adjogble, KDZ, Mercierb, C, Dubremetz, JF, Huckea, Cron-DelaR, Ces, MacKenzie, MF, Daubener, W. GRA9, a new Toxoplasma gondii dense granule protein associated with the intravacuolar network of tubular membranes. Int J Parasitol, 2004; 34(11): 1255–64.
9. Eidell, KP, Burke, T, Gubbels, MJ. Development of a screen to dissect Toxoplasma gondii egress. Mol Biochem Parasitol, 2010; 171(2): 97–103.
10. Hajj, HE, Lebrun, M, Fourmaux, MN, Vial, H, Dubremetz, JF. Characterization, biosynthesis and fate of ROP7, a ROP2 related rhoptry protein of Toxoplasma gondii. Mol Biochem Parasitol, 2006; 146(1): 98–100.
11. Williams, RJP. Chemical selection of elements by cells. Coord Chem Rev, 2001; 216: 583–595.
12. Martinez-Finley, EJ, Chakraborty, S, Fretham, SJB, Aschner, M. Cellular transport and homeostasis of essential and nonessential metals. Metallomics, 2012; 4(7): 593–605.
13. Vergílio, CS, Carvalho, CEV, Melo, EJT. Mercury-induced dysfunctions in multiple organelles leading to cell death. Toxicol In Vitro, 2014; 29(1): 63–71.
14. Reyes-Caballero, H, Campanello, GC, Giedroc, DP. Metalloregulatory proteins: Metal selectivity and allosteric switching. Biophys Chem, 2011; 156: 103-114.
15. Klaassen, CD, Liu, J, Diwan, BA. Metallothionein protection of cadmium toxicity. Toxicol Appl Pharmacol, 2009; 238(3): 215–220.
16. Lemeire, JA, Harrisson, JJ, Turner, RJ. Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nature, 2013; (11): 371-384.
17. Gomes, MAGB, Carvalho, LP, Melo, EJT, Oliveira, RR, Maria, EJ. Evaluating anti- Toxoplasma gondii activity of new serie of phenylsemicarbazone and phenylthiosemicarbazones in vitro. Med Chem Res, 2012.
18. Soares, AMS, Carvalho, LP, Melo, EJT, Costa, HPS, Vasconcenlos, IM, Oliveira, JTA. A protein extract and a cysteine protease inhibitor enriched fraction from Jatropha curcas seed cake have in vitro anti-Toxoplasma gondii activity. Exp Parasitol, 2015; 153: 111–117.
19. Carvalho, LP, Gomes, MAGB, Rocha, BS, Oliveira, RR, Maria, EJ, Melo, EJT. Anti-parasite effects of new Thiosemicarbazones and their Products Thiazolidinone including cellular aspects of intracellular elimination of Trypanosoma cruzi in vitro. J Develop Drugs, 2014; 03(02).
20. Carvalho, CS, Melo, EJT, Tenório RP, Góes, AJS. Anti-parasitic action and elimination of intracellular Toxoplasma gondii in the presence of novel thiosemicarbazone and its 4-thiazolidinone derivatives. Braz J Med Biol Res, 2010; 43: 139–149.
21. Shagger, H, Von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem, 1987;166(2): 368-379.
22. Souza, GS, Nascimento, VV, Carvalho LP, Melo, EJT, Fernandes, KV, Machado, OLT, Retamal, CA, Gomes, VM, Carvalho, AO. Activity of recombinant and natural defensins from Vigna unguiculata seeds against Leishmania amazonensis. Exp Parasitol, 2013; 135: 116–25.
23. Chiarelli, R, Roccheri, MC. Heavy metals and metalloids as autophagy inducing agents: focus on cadmium and arsenic. Cells, 2012; 1(3); 597–616.

24. Syversen, T, Kaur, P. The toxicology of mercury and its compounds. J Trace Elem Med Biol, 2012; 26: 215–226.
25. Fonnum, F, Lock, EA. The contributions of excitotoxicity, glutathione depletion and DNA repair in chemically induced injury to neurones: Exemplified with toxic effects on cerebellar granule cells. J. Neurochem., 2004; 88(3): 513–531.
26. Simm, C, Luan, CH, Weiss, E, O'Halloran, T. High-throughput screen for identifying small molecules that target fungal zinc homeostasis. Plos One, 2011; 6(9): 1–9.
27. Raja, MR, Waterman, S. R, Qui, J, Bleher, R, William, PR, O'Halloran, T. A copper hyperaccumulation phenotype correlates with pathogenesis in Cryptococcus neoformans. Metallomics, 2013; 5(4): 363–371.
28. Marvin, RG, Wolford, JL, Kidd, MJ, Murphy, S, Ward, J, Que, EL, Mayer, ML, Penner-Hahn, JE, Haldar, K, O'Halloran TV. Fluxes in "free" and total zinc are essential for progression of intraerythrocytic stages of Plasmodium falciparum. Chem. Biol., 2012; 19(6): 731–741.
29. Porcheron, G, Garénaux, A, Proulx, J, Sabri, M, Dozois, CM. Iron, copper, zinc, and manganese transport and regulation in pathogenic Enterobacteria: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. Front in Cell and Infect Microbiol, 2013; 3.
30. Hayden, JA, Brophy, MB, Cunden, LS, Nolan, EM. High-affinity Manganese Coordination by Human Calprotectin is Calcium-dependent and Requires the Histidine-rich Site Formed at the Dimer Interface. J Am Chem Soc, 2014; 135(2): 775–787.
31. Odberg-Ferragut, C, Renault, JP, Viscogliosi, E; Toursela, C, Briche, I, Engels EA, Lepage, G, Morgenstern-Badarau, I, Camus, D, Tomavo S, Dive, D. Molecular cloning, expression analysis and iron metal cofactor characterisation of a superoxide dismutase from Toxoplasma gondii. Mol Biochem Parasitol, 2000; 106: 121-129.
32. De Aquino, TM, Liesen, AP, Silva, REA, Lima, VT, Carvalho, CS, Faria, AR, Araújo, JM, Lima, JG, Alves, AJ, Melo, EJT, Góes, AJS. Synthesis, anti-Toxoplasma gondii and antimicrobial activities of benzaldehyde 4-phenyl-3-thiosemicarbazones and 2-[(phenylmethylene)hydrazono]-4-oxo-3-phenyl-5-thiazolidineacetic acids. Bioorg Med Chem, 2008; 16: 446–456.
33. Tenório, RP, Carvalho, CS, Pessanha, CS, Lima, JG, Faria, AR, Alves, AJ, Melo, EJT, Góes, AJS. Synthesis of thiosemicarbazone and 4-thiazolidinone derivatives and their in vitro anti-Toxoplasma gondii activity. Bioorg Med Chem Lett, 2005; 15: 2575–2578.
34. Formigari, A, Irato, P, Santon, A. Zinc, antioxidant systems and metallothionein in metal mediated-apoptosis: Biochemical and cytochemical aspects. Comp Biochem Physiol C Toxicol Pharmacol, 2007; 146(4) 443–459.
35. Mocchegiani, E, Muzzioli, M, Giacconi, R. Zinc and immunoresistance to infection in aging: new biological tools. Trends Pharmacol Sci, 2000; 21(6) 205–208.
36. White, RJ, Margolis, PS, Trias, J, Yuan, Z. Targeting metalloenzymes : a strategy that works. Curr Opin Pharmacol, 2003; 3: 502–507.
37. Parkin, G. Tion as a hydride compound. One metal that was not known to form such view of the unprecedented. Science, 1998; 1: 1–2.
38. Tapiero, H, Tew, KD. Trace elements in human physiology and pathology: zinc and metallothioneins. Biomed Pharmacother, 2003; 57(9): 399–411.
39. White, C, Lee, J, Kambe, T, Fritsche, K, Petris, MJ. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem, 2009; 284(49): 33949–33956.
40. Botella, H, Stadthagen, G, Lugo-Villarino, G, Chastellier, C, Neyrolles, O. Mycobacterial P 1-Type ATPases mediate resistance to Zinc poisoning in human macrophages. Cell Host Microbe, 2011; 10(3) 248–259.
41. Klaassen, CD, Liu, J, Choudhuri, S. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol, 1999; 39: 267–294.
42. Banerjee, D, Onosaka, S Cherian, MG. Immunohistochemical localization of metallothionein in cell nucleus and cytoplasm of rat liver and kidney. Toxicol, 1982; 24(2) 95–105.
43. Butcher, H, Kennettea, W, Collins, O, Demoor, J, Koropatnick, J. A sensitive time-resolved fluorescent immunoassay for metallothionein protein. J Immunol Methods, 2003; 272(2) 247–56.
44. MAYA, JD, Rodríguez, A, Pino, L, Pabón, A, Ferreira, J, Pavani, M, Repetto, Y, Morello, A. Effects of buthionine sulfoximine nifurtimox and benznidazole upon trypanothione and metallothionein proteins in Trypanosoma cruzi. Biol Res, 2004; 37: 61–69.
45. Klaassen, CD, Liu, J. Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. Environ. Health Perspect, 1998; 106; 297–300.
46. Lovric, J, Cho, SJ, Winnik, FM, Maysinger, D. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. Chem. Biol, 2005; 12(11): 1227–1234.
47. Smith, AT, Barupala, D, Stemmler, TM, Rosenzweig, AC. A new metal binding domain involved in cadmium, cobalt and zinc transport. Nature Chem Biol, 2015; 11(9): 678–684.