INFECÇÃO POR *TRYPANOSOMA CRUZI IN VITRO* COMO MODELO DE ESTUDO DA TOXICOLOGIA CELULAR DE METAIS

LAÍS PESSANHA DE CARVALHO

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE
DARCY RIBEIRO – UENF
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Orientador: Prof. Dr. Edésio José Tenório de Melo

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RESUMO

Trypanosoma cruzi, agente etiológico da doença de Chagas, é um parasito com um complexo ciclo de vida que envolve hospedeiros invertebrados e vertebrados. Nos vertebrados ocorre uma fase intracelular obrigatória, com o estabelecimento da infecção através da multiplicação dos parasitos no citoplasma. Muitos eventos que ocorrem nesta fase são dependentes de íons metálicos, por isso, o controle da homeostase destes metais é de grande importância para o estabelecimento e manutenção da infecção. No entanto, pouco se sabe sobre a influência da adição de metais e seus metalocompostos no sucesso da infecção. O objetivo deste trabalho foi avaliar a adição dos metais $ZnCl_2$, $CdCl_2$ e $HgCl_2$ nas diferentes formas do *Trypanosoma cruzi* e no seu ciclo de vida intracelular. Os parasitos foram incubados com nas concentrações de 1 a 20 μM durante 2, 12, 18 ou 24 horas. Os resultados obtidos pela quantificação destes parasitos e a observação estrutural e ultraestrutural mostraram que os metais não essenciais (Cd e Hg) foram mais tóxicos para os parasitos. No entanto, a susceptibilidade do *T. cruzi* foi dependente do seu estágio morfológico; os epimastigotas foram os mais vulneráveis, seguido pelos amastigotas e pelos tripomastigotas extracelulares. As incubações com os metais não prejudicaram o escape do parasito do vacúolo endocítico e o estabelecimento da infecção. Entretanto, o tratamento causou diminuição na atividade mitocondrial e compactação nuclear nos amastigotas, seguidos de acúmulo de vacúolos acídicos, inclusive lisossomos e marcação positiva para vacúolos autofágicos contendo parasitos estruturalmente alterados. Os resultados mostraram que o *T. cruzi* foi um ótimo modelo de estudo sobre a presença de metais nos processos de interação parasito – hospedeiro e desenvolvimento intracelular. Além disto, os eventos desencadeados pelos metais, principalmente o zinco, são de grande interesse no desenvolvimento de novos compostos antiparasitários, pois se mostraram eficazes em eliminar o amastigota sem prejudicar a viabilidade da célula hospedeira

ABSTRACT

Trypanosoma cruzi, the etiologic agent of Chagas' disease, is a parasite with a complex life cycle involving invertebrate and vertebrate hosts. In vertebrates an obligate intracellular phase occurs, with the establishment of the infection through the multiplication of the parasites in the cytoplasm. Many events that occur at this stage are dependent on metal ions, so the control of the homeostasis of these metals is of great importance for the establishment and maintenance of the infection. However, little is known about the influence of the addition of metals and their metallocompounds on the success of the infection. The objective of this work was to evaluate the addition of ZnCl2, CdCl2 and HgCl2 metals in the different forms of *T. cruzi* and in their intracellular life cycle. The parasites were incubated at concentrations of 1 to 20 μM for 2, 12, 18 or 24 hours. The results obtained by the quantification of these parasites and the structural and ultrastructural observation showed that the nonessential metals (Cd and Hg) were more toxic to the parasites. However, the susceptibility of *T. cruzi* was dependent on its morphological stage; epimastigotes were the most vulnerable, followed by amastigotes and extracellular trypomastigotes. Incubations with the metals did not impair the escape of the parasite from the endocytic vacuole and the establishment of infection. However, the treatment caused a decrease in mitochondrial activity and nuclear compaction in the amastigotes, followed by accumulation of acidic vacuoles, including lysosomes and positive marking for autophagic vacuoles containing structurally altered parasites. The results showed that *T. cruzi* was a good model of study on the presence of metals in the processes of parasite - host interaction and intracellular development. Moreover, the events triggered by metals, especially zinc, are of great interest in the development of new antiparasitic compounds as they have been shown to be effective in eliminating the amastigote without impairing the viability of the host cell.

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1. INTRODUÇÃO

1.1 Aspectos biológicos do *Trypanosoma cruzi*

Trypanosoma cruzi, agente etiológico da doença de Chagas ou tripanossomíase americana, é um protozoário parasito hemoflagelado da ordem Kinetoplastidae e da família Trypanosomatidae (COURA; CASTRO, 2002). Este patógeno, que foi primeiramente descrito e estudado por Carlos Chagas em 1909, tem um ciclo de vida complexo que envolve insetos triatomíneos como hospedeiro invertebrado e mamíferos como hospedeiros vertebrados (TYLER; ENGMAN, 2001). As três principais diferenciações morfológicas encontradas no ciclo de vida do *T. cruzi* são: (a) epimastigotas: formas alongadas (20-40 μm comprimento, 2-5 μm largura) e com flagelo livre, extracelulares e replicativas, encontradas no hospedeiro invertebrado; (b) tripomastigotas: parasitos extracelulares alongados (18 μm comprimento, 2-3 μm largura) e com flagelos livres, mas que não se multiplicam, encontradas no sangue dos vertebrados e na região distal do intestino do inseto vetor e (c) amastigotas: formas esféricas (2-7 μm) sem flagelo livre e que se multiplicam no ambiente intracelular dos hospedeiros vertebrados (Figura 1) (TONELLI *et al*., 2010). O *Trypanosoma cruzi* é capaz de infectar todo tipo de célula nucleada (FERNANDES; ANDREWS, 2012). No entanto, o parasito apresenta um tropismo por células musculares cardíacas e lisas, que pode, em parte, ser explicado pela grande capacidade destas células em reparar suas membranas (BANSAL; CAMPBELL, 2004).

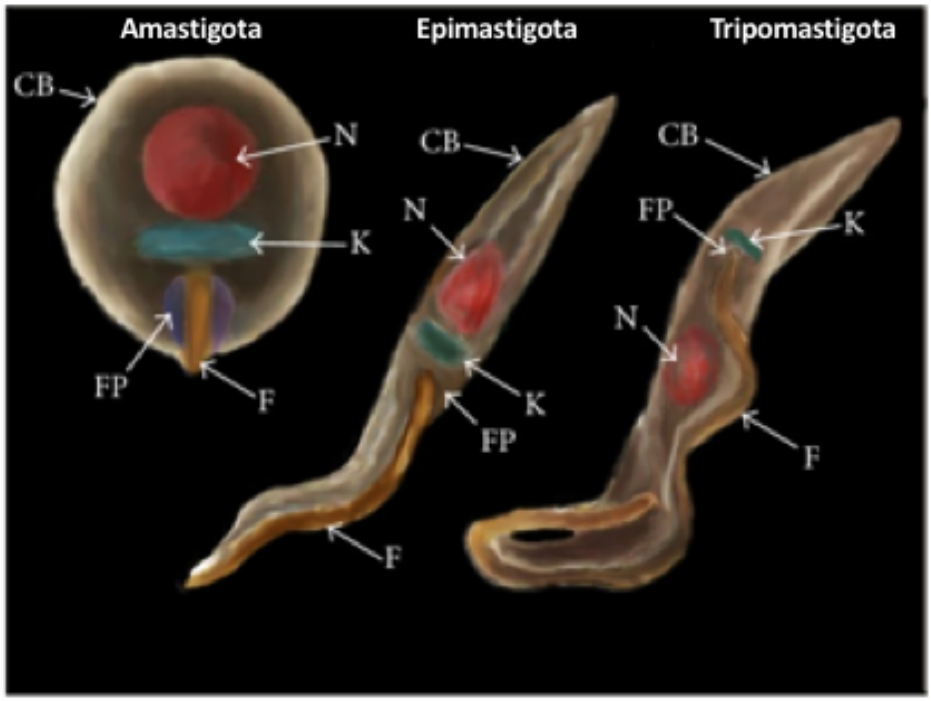


Figura 1 - Diferentes estágios do *Trypanosoma cruzi* incluindo o amastigota, epimastigota e tripomastigota. (N): núcleo, (K): cinetoplasto, (F): flagelo, (FP) bolsa flagelar, (CB): corpo celular. Retirado de: (PECH-CANUL; MONTEÓN; SOLÍS-OVIEDO, 2017).

O *Trypanosoma cruzi* possui as estruturas e organelas características das células eucarióticas, mas também tem especializações estruturais e funcionais próprias para ajudar no processo de invasão e estabelecimento da infecção no hospedeiro. Dentre estas especializações estão:

- **Superfície celular:** composta principalmente por diferentes glicoconjugados que auxiliam na interação parasito / célula hospedeira (revisado em SOUZA, 2009).
- **Membrana plasmática:** não homogênea quanto a sua densidade e distribuição de componentes proteicos e lipídicos, formando domínios estruturais como: corpo celular, bolsa flagelar, flagelo e base do flagelo (revisado em SOUZA, 2009).
- **Citóstomo:** porção especializada da membrana plasmática que se invagina passando pelo núcleo e alcançando a bolsa flagelar. O citóstomo é uma estrutura encontrada nas formas epimastigotas e amastigotas dos tripanosomatídeos e é por onde o parasito

internaliza macromoléculas necessárias para sobrevivência (revisado em SOUZA, 2009).

- **Reservossomos:** organela pré-lisossomal que estoca as macromoléculas internalizadas pelo citóstomo. Estas moléculas estocadas são utilizadas a medida que os nutrientes se tornam escassos ou nos processos de diferenciação como da forma epimastigota para a tripomastigota (SOUZA *et al.*, 2000).
- **Flagelo**: tem a função de locomoção e adesão inicial do parasito às células hospedeiras. O flagelo origina-se na bolsa flagelar e é formado por um conjunto de 9 pares de microtúbulos periféricos e 2 pares de microtúbulos centrais, com seu comprimento variando de acordo com o estágio morfológico do parasito (revisado em SOUZA, 2009).
- **Citoesqueleto:** composto pelos microtúbulos subpeliculares, assim chamados por se localizarem abaixo da membrana plasmática do parasito, dando a esta uma grande rigidez. Os microtúbulos têm arranjos helicoidais e são capazes de manter uma distância específica entre estes e a porção interna da membrana plasmática tendo, contudo, pontos de conexões (SOARES; SOUZA, 1979; revisado em SOUZA, 2009).
- **Acidocalcissoma:** é uma organela vacuolar ácida com uma alta concentração de fósforo na forma de pirofosfato ou polifosfato e cálcio (DOCAMPO; MORENO, 2011)**.** Estudos citoquímicos mostraram a presença de H^+ ATP-ase, Ca^{2+} ATPase e pirofosfatase na membrana no ácidocalcisoma. A quantidade destas organelas varia de acordo com o estágio do parasito, estando mais presentes nas amastigotas em comparação com epimastigotas e tripomastigotas (BENCHIMOL *et al.*, 1998). No interior dos acidocalcisomas podem ser encontrados outros íons como sódio, potássio, zinco e ferro (DOCAMPO; MORENO, 2011). Entre as funções descritas para esta organela estão: estocagem de íons e compostos fosfatados tais como pirofosfatos e polifosfatos, manutenção do pH e osmorregulação (DOCAMPO *et al.*, 2005).

- **Complexo mitocôndria – cinetoplasto:** os tripanosomatídeos possuem apenas uma mitocôndria que se distribui por todo o citoplasma do parasito. O cinetoplasto, estrutura que caracteriza a ordem dos Cinetoplastídeos, consiste na porção do DNA mitocondrial cujos anéis encontram-se concatenados em maxicírculos e minicírculos. A morfologia ultraestrutural do cinetoplasto e sua localização no citoplasma são dependentes do estágio morfológico do parasito (RIOU; YOT, 1977). Nas formas epimastigotas e amastigotas, o cinetoplasto é bastante compactado na forma de bastão e se encontra na porção posterior ao núcleo e anterior ao flagelo, enquanto nos tripomastigotas, o cinetoplasto está localizado na porção anterior do flagelo e núcleo, é arredondado e o DNA encontra-se mais disperso (RIOU; YOT, 1977) (Figura 1).
- **Glicossomos:** são organelas esféricas presentes no citoplasma do *T. cruzi* onde ocorrem as vias glicolíticas e metabólicas no parasito (OPPERDOES; BORST, 1977). Como exemplo, o metabolismo de peróxidos, a β- oxidação de ácidos graxos, a síntese de fosfolipídios, a fixação de dióxido de carbono, a reciclagem de purina e biossíntese *de novo* de pirimidina, o alongamento de ácido graxo e a biossíntese de isoprenóides e esteróis (OPPERDOES; COTTON, 1982; OPPERDOES, 1987), revisado em SOUZA, 2009.

1.2 O ciclo de vida

O ciclo de vida do *T. cruzi* é bastante complexo e envolve um hospedeiro invertebrado (insetos da família Reduviidae - Triatomíneos e Hemiptera) e diversas espécies de mamíferos, incluindo humanos como hospedeiros vertebrados (SÁNCHEZ VALDÉZ *et al.*, 2013) (Figura 2). Uma vez que um vetor se alimenta do sangue de um indivíduo infectado, o inseto pode adquirir o parasito, majoritariamente, na forma tripomastigota. Ao atingir o intestino médio do inseto, o *T. cruzi* adquire a forma de epimastigota, multiplica-se e, ao chegar à porção posterior do intestino, passa para a forma tripomastigota metacíclica, já próximo de ser excretado pelo vetor (OSÓRIO *et al.*, 2012). No próximo repasto, estes parasitos serão excretados junto com as fezes para infectar outros mamíferos. Normalmente, os tripomastigotas penetram no hospedeiro vertebrado através de mucosas ou ferimentos. Uma vez na corrente sanguínea, estes invadem diferentes tipos de células hospedeiras (OSÓRIO *et al.*, 2012). No ambiente intracelular, o parasito permanecerá por um curto período dentro do vacúolo endocítico que será reconhecido pelo sistema fagocítico da célula. A fusão com lisossomos diminui o pH do vacúolo e induz os parasitos a secretarem enzimas que criam poros na membrana deste vacúolo, desestabilizando-a, permitindo o escape do *T. cruzi* para o citoplasma (LEY *et al.,* 1990). Neste momento, os tripomastigotas já estão se diferenciando em amastigotas, processo que termina no citoplasma. Após um período de adaptação ao ambiente citoplasmático, os amastigotas começam a se multiplicar por fissão binária até o momento em que retornam a forma tripomastigota e rompem a célula hospedeira, podendo infectar novas células (OSÓRIO *et al.*, 2012) (Figura 2).

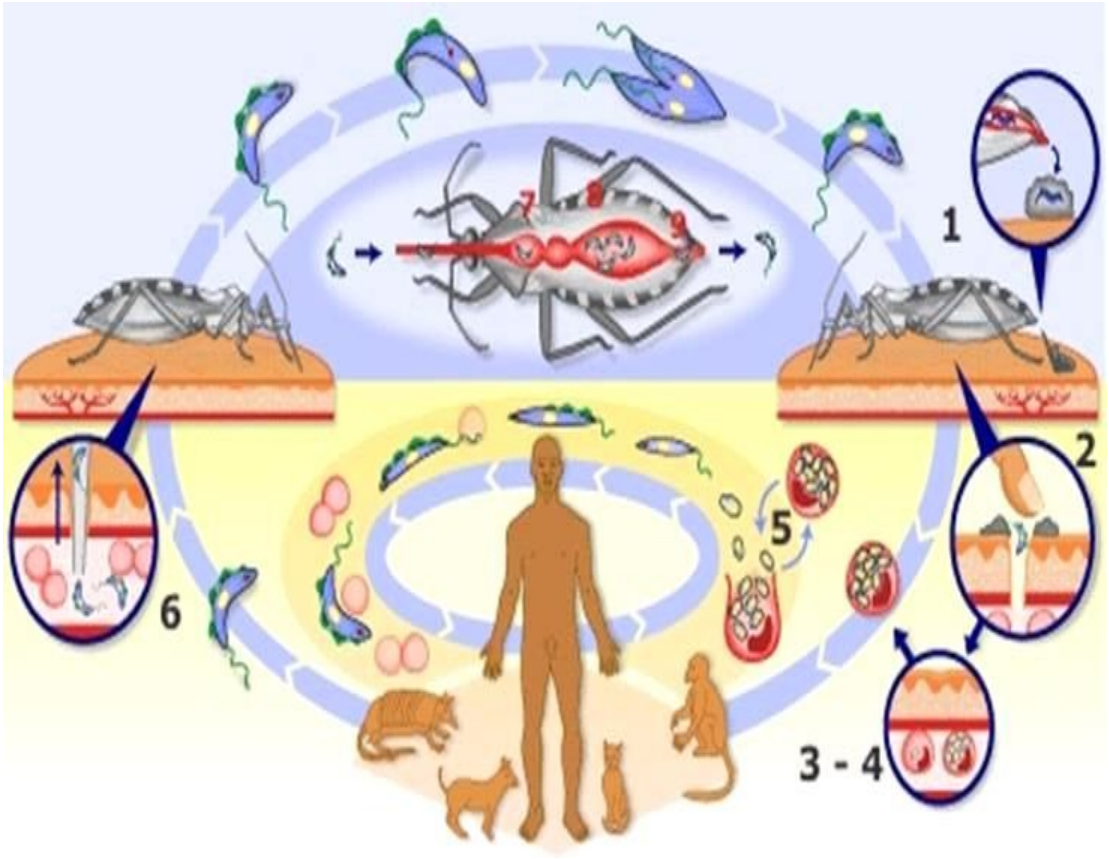


Figura 2 - Esquema representando o ciclo de vida do *Trypanosoma cruzi*. (1) O tripomastigota metacíclico é transmitido para o hospedeiro vertebrado através das fezes ou urina do inseto vetor. (2) O hospedeiro vertebrado, ao coçar o local da picada do mosquito causa lesões possibilitando a entrada do parasito na sua corrente sanguínea. (3-4) No hospedeiro vertebrado, os parasitos invadem qualquer tipo de célula nucleada, (5) multiplicam-se, rompem a célula e propagam a infecção através da invasão de novas células. (6) Durante o período em que os tripomastigotas estão na corrente sanguínea para infectar novas células e propagar a infecção, ele pode ser capturado por um novo vetor, que poderá transmitir o parasito para outros vertebrados. Retirado de (OSÓRIO et al., 2012).

1.3 Ciclo intracelular do *T. cruzi*

1.3.1 Interação parasito - célula hospedeira

Para o sucesso da infecção, os tripomastigotas metacíclicos (as formas especializadas na infecção) utilizam diversos mecanismos para invadir as células hospedeiras (TYLER; ENGMAN, 2001). Dentre estes mecanismos estão incluídos etapas de adesão pela porção

flagelar, desencadeamento de cascatas de sinalizações tanto no parasito quanto na célula e invasão, como revisado em SOUZA; CARVALHO; BARRIAS, 2010.

O início do processo de infecção é dependente de moléculas de superfície encontrados em ambos parasito e célula hospedeira que possibilitam a adesão entre estes dois. A quantidade e o tipo destas moléculas podem variar de acordo com a cepa e estágio morfológico do parasito e o tipo de célula hospedeira, e agem como receptores - ligantes desencadeando as cascatas de sinalizações de vários eventos celulares (SOUZA; CARVALHO; BARRIAS, 2010). A superfície do *T. cruzi* é majoritariamente recoberta por uma camada de glicocálix; as glicoproteínas mais presentes são as mucinas, enquanto os glicoinositolfosfolipídios são os glicolipídios mais abundantes (GIORGI; LEDERKREMER, 2011). No entanto, outros três grandes grupos de moléculas também podem ser encontrados: as gp85/transialidases (TONELLI *et al.*, 2010), as proteínas tipo mucinas (ACOSTA-SERRANO; ALMEIDA; FREITAS-JUNIOR, 2001) e as proteases gp63 (KULKARNI *et al.*, 2009). A transialidase é responsável por transferir ácido siálico dos sialoconjugados das células hospedeiras para as mucinas do parasito, visto que este é incapaz de sintetizá-lo (NARDY *et al.*, 2016). As mucinas e moléculas tipo mucinas estão distribuídas na região da bolsa flagelar e flagelo do *T. cruzi* (BUSCAGLIA, et al., 2006). As principais funções destas moléculas incluem o auxílio no processo de invasão da célula hospedeira e modulação da resposta imune do hospedeiro (CÁNEPA *et al.*, 2012), embora não se saiba ainda o mecanismo exato de funcionamento. Alguns exemplos destas moléculas são as gp82 e gp35/50, sendo a gp82 mais eficaz em disparar cascata de liberação de cálcio e é mais encontrada em cepas virulentas (YOSHIDA, 2006). O terceiro conjunto de moléculas engloba a família Gp63 (metaloproteases com zinco na estrutura) que podem ser divididas em dois grandes grupos: TcGP63-I e TcGP63-II (CUEVAS; CAZZULO; SÁNCHEZ, 2003). A expressão das Gp63 é varia entre os estágios dos parasitos, com maior quantidade nos amastigotas do que nos epimastigotas ou tripomastigotas (GRANDGENETT *et al.*, 2000). O primeiro grupo está presente nos três principais estágios

morfológicos do *T. cruzi* (CUEVAS; CAZZULO; SÁNCHEZ, 2003). Outras moléculas presentes em menor quantidade também são importantes para o processo de adesão e reconhecimento parasito – célula hospedeira e invasão tais como: a penetrina, cruzipaína, oligopeptidase B, entre outros, revisado em SOUZA; CARVALHO; BARRIAS, 2010.

A adesão da forma infectiva na superfície da célula hospedeira dispara sequências de sinalizações (Fig. 3). Durante a etapa de sinalização, ocorre o aumento da concentração do cálcio no citoplasma do parasito e da célula hospedeira não fagocítica (cascatas bidirecionais) induzido por monofosfatos de adenosina cíclicos (AMPs cíclicos) ou mediada por inositol-1,4,5-trifosfato (IP3). Este aumento de cálcio induzirá uma reorganização do citoesqueleto da célula, facilitando a invasão do parasito. Quanto às células fagocíticas, a sinalização induzirá a ativação de quinases para auxiliar na invasão celular (SOUZA; CARVALHO; BARRIAS, 2010).

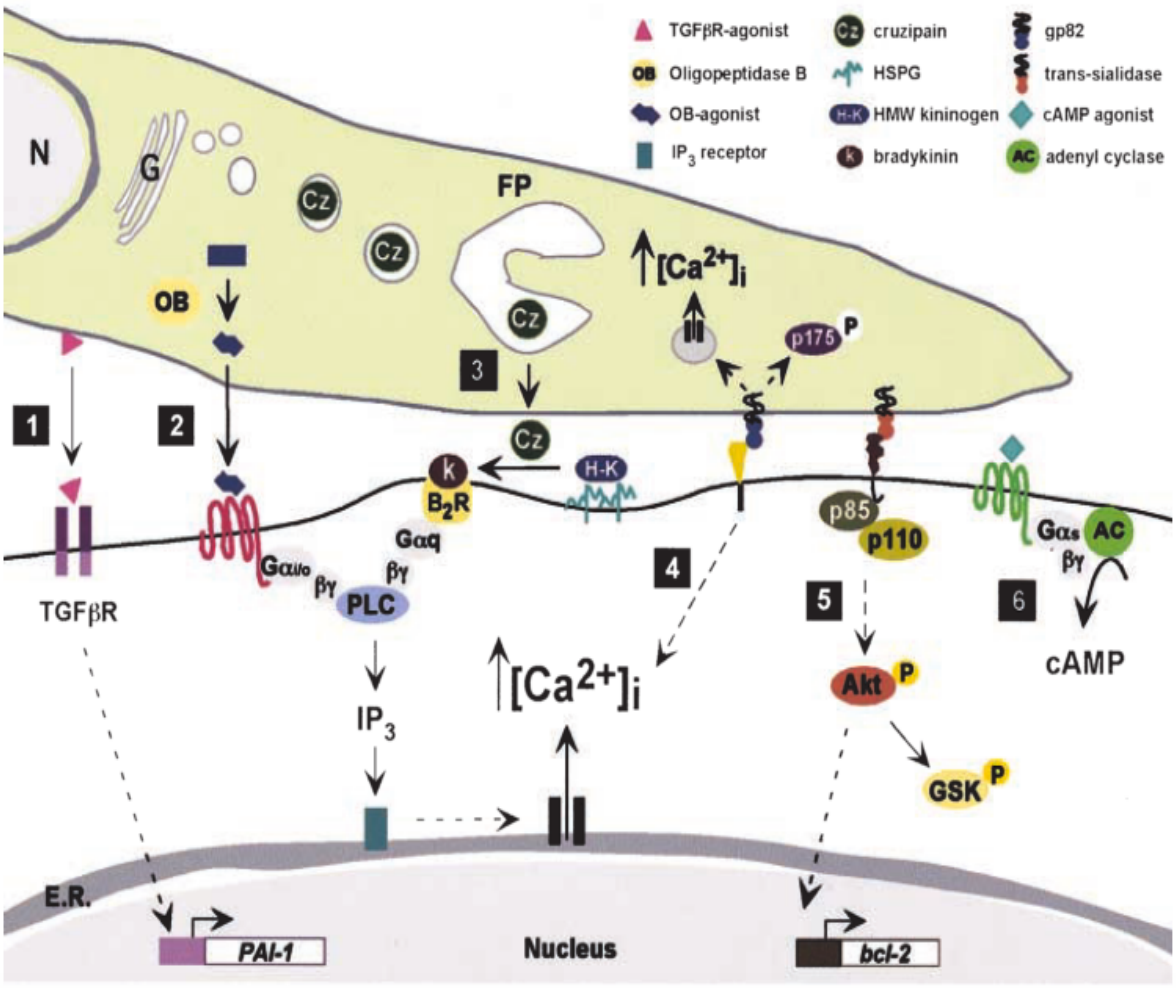


Figura 3 - Esquema representando os componentes moleculares tanto do tripomastigota quanto da célula hospedeira não fagocítica envolvidos da sinalização bidirecional do cálcio e na garantia da sobrevivência desta célula hospedeira. O agonista do TGF-β, ao ser reconhecido pelo seu receptor produz genes (PAI-1) que inibem a ativação do plasminogênio. As Oligopeptidase B, cruzipaína e bradiquinina ativam a fosfolipase C ao serem reconhecidas por seus receptores e induzirão a liberação de cálcio mediada pelo IP3. A gp82 assim como os agonistas do AMPc também causam a liberação de cálcio no citoplasma; enquanto a gp85 promove a expressão do gene bcl-2, conhecido por ser antiapoptótico (BURLEIGH; WOOLSEY, 2002).

Os mecanismos envolvidos na entrada do parasito na célula estão relacionados com a dependência ou não de lisossomos no momento da invasão, e variam de acordo com o tipo de célula hospedeira (ALVES; COLLI, 2007). Em células não fagocíticas, como as epiteliais, os processos de invasão são dependentes de lisossomos. Nesta etapa, o cálcio é liberado no citoplasma pelas cascatas de sinalização, induzindo a migração de lisossomos para o local da

invasão. O recrutamento destes lisossomos é dependente da presença de cinesinas e da reorganização dos microtúbulos, mas independentes dos filamentos de actina (ANDRADE; ANDREWS, 2005). Como consequência, ocorre à liberação do ácido esfingomielinase, uma enzima lisossomal envolvida no processo de reparação da membrana plasmática (TAM *et al.*, 2010). As membranas lisossomais reconstituirá parcialmente a membrana plasmática da célula hospedeira e também ajudará na formação do vacúolo parasitóforo (SOUZA; CARVALHO; BARRIAS, 2010). Estes eventos mostram que o *T. cruzi* explora os mecanismos de reparação da membrana plasmática da célula para infectá-la (IDONE *et al.*, 2008; TAM *et al.*, 2010).

Os processos de invasão independentes de lisossomos podem ocorrer em células fagocíticas profissionais (fagocitose) e não profissionais (endocitose) (BARRIAS; CARVALHO; SOUZA, 2013). Os eventos são desencadeados com a liberação do cálcio no citoplasma do parasito e da célula hospedeira e a acumulação de PIP3 na membrana plasmática da célula hospedeira. A presença do PIP3 leva a reorganização dos filamentos de actina que induzirão a formação de pseudópodes que internalizam o parasito (no caso da fagocitose) ou que levarão a invaginação da membrana plasmática da célula (endocitose) (BURLEIGH; WOOLSEY, 2002). Ambos os casos vão dar origem a um vacúolo intracelular endocítico que se funde com lisossomos (BARRIAS *et al.*, 2013). No entanto, os processos de invasão parasitária independentes de lisossomos são reversíveis e não culminam no estabelecimento da infecção (ANDRADE; ANDREWS, 2004).

1.3.2 Estabelecimento da infecção

Uma vez o parasito internalizado, este ficará no interior do vacúolo parasitóforo por um período entre 10 – 16 h, onde começará seu processo de transformação para amastigota e a ativação de enzimas que permitirão o escape deste vacúolo (CARVALHO; SOUZA, 1989). A membrana do vacúolo parasitóforo é majoritariamente constituída pela membrana da célula hospedeira, contudo há marcações de proteínas de endossomos primários (EEA1 – early

endosome antigen 1), endossomos secundários (Rab 7), vacúolo autofágico (LC3) e lisossomos (LAMP1 e LAMP 2). Estas marcações indicam que o vacúolo parasitóforo contendo o parasito pertence às vias endocíticas tradicionais. Nesta via endocítica acontece à digestão intracelular do material endocitado via enzimas lisossomais. Estas enzimas são liberadas após a fusão vacúolo-lisossomos e leva a acidificação deste ambiente. No entanto, o parasito utiliza-se do baixo pH para ativar enzimas próprias que o auxiliam no escape para o citoplasma do hospedeiro (ANDREWS; WHITLOW, 1989; CARVALHO, SOUZA, 1989).

Esta etapa do ciclo intracelular do *T. cruzi* acontece em torno de 10 horas (cepa DM28) após a invasão do parasito e é o resultado da desorganização da membrana do vacúolo parasitóforo no qual o parasito está. Esta desorganização envolve a ação combinada das enzimas transialidase e neuraminidase que retiram o ácido siálico da membrana do vacúolo, tornando-o mais vulnerável. Em seguida, as enzimas Tc-Tox e perforina causam a ruptura dos poros na membrana do vacúolo, facilitando a saída dos parasitos para o citoplasma do hospedeiro (ANDREWS; WHITLOW, 1989; LEY *et al.*, 1990).

No ambiente citoplasmático, o *T. cruzi* termina sua transformação para a forma amastigota (TOMLINSON *et al.*, 1995). Após um período de latência, os amastigotas começam a se multiplicar e depois de vários ciclos de divisão, estes voltam a se diferenciar em tripomastigotas (ALVES; COLLI, 2007). Ainda não é completamente entendido os eventos que desencadeiam multiplicação e diferenciação dos parasitos, mas acredita-se que fatores genéticos dos parasitos estão envolvidos (TEIXEIRA, 1998). Após, aproximadamente, 6 dias da infecção celular (cepa DM28), os movimentos dos tripomastigotas no citoplasma levam a ruptura da célula hospedeira, liberando os tripomastigotas no ambiente extracelular de onde podem infectar novas células adjacentes (OSÓRIO *et al.*, 2012).

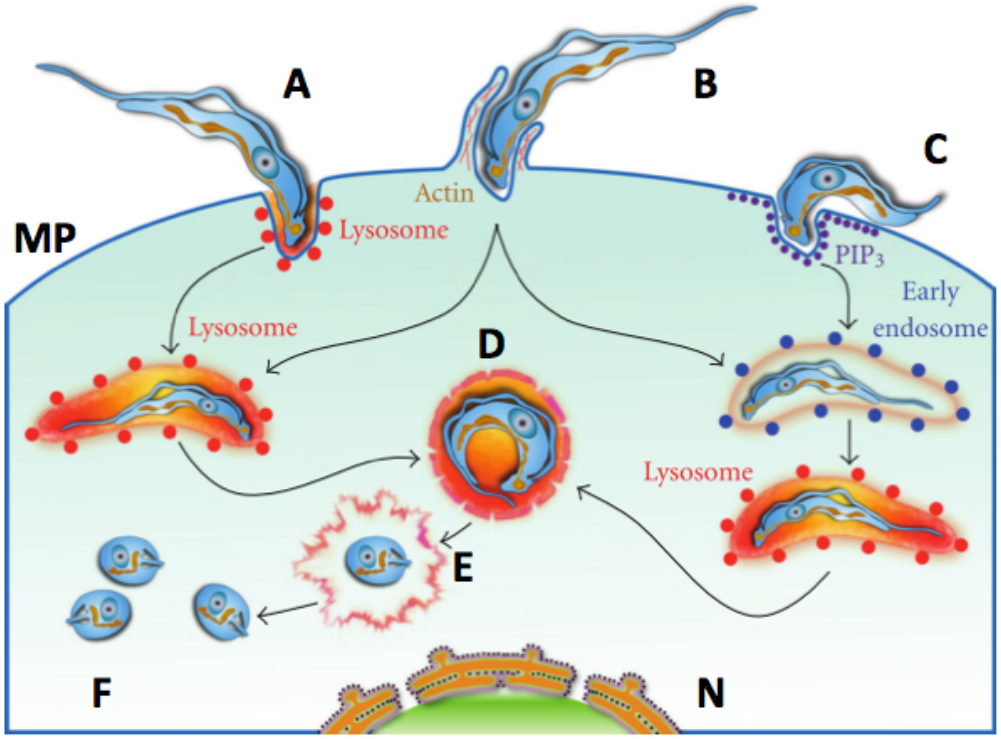


Figura 4 - Representação do processo de invasão da célula hospedeira e estabelecimento da infecção pelo *Trypanosoma cruzi*. (A) Processo de invasão dependente de lisossomos, (B) invasão através da fagocitose e (C) e mediada por endocitose. (D) Através dos mecanismos mostrados, o parasito vai direta ou indiretamente para um vacúolo que se funde com lisossomos. (E) A membrana do vacúolo se rompe com a acidificação deste e (F) os parasitos vão para o citoplasma onde ocorre a multiplicação. MP: membrana plasmática da célula hospedeira e N: núcleo da célula hospedeira. Retirado de: (SOUZA; CARVALHO; BARRIAS, 2010)

1.4 Mecanismos celulares envolvidos na eliminação patógenos intracelulares

Patógenos que se desenvolvem no ambiente intracelular como o *T. cruzi* evitam as respostas imunes extracelulares humorais dos seus hospedeiros. Contudo, estes também precisam evadir as respostas microbicidas intracelulares para estabelecer a infecção (LEVINE; DERETIC, 2007). As células hospedeiras utilizam reações físico-químicas do seu metabolismo como respostas microbicidas. Neste caso, o estresse oxidativo causado pela produção exacerbada de espécies reativas de oxigênio e nitrogênio são importantes na proteção inicial a invasores. A endocitose e os tipos de morte programadas I (apoptose) e II (autofagia) também

são muito importantes porque induzem a digestão de patógenos presentes em vacúolos endocíticos e autofágicos (RADTKE *et al*., 2006).

Endocitose, um processo que ocorre nas células eucarióticas, é fundamental na captura de nutrientes para o metabolismo celular com a formação de um vacúolo endocítico através da invaginação da membrana plasmática. Neste processo, patógenos também podem ser internalizados em vacúolos. Nesta via, os vacúolos internalizados são reconhecidos por organelas acídicas (endossomos e lisossomos) levando seu conteúdo à digestão intravacuolar (ELKIN; LAKODUK; SCHMID, 2016).

A fagocitose é uma endocitose que ocorre por meio de reconhecimento por receptores e internalização de grandes partículas contidas em vacúolos chamados fagossomos (GORDON, 2016). Este também é um processo essencial tanto para adquirir nutrientes quanto um mecanismo de resposta microbicida. Neste sentido, os animais superiores possuem células fagocíticas especializadas, como macrófagos, que atuam na defesa contra patógenos, sendo muito importantes na imunologia celular destes organismos (ARANDJELOVIC; RAVICHANDRAN, 2015).

Apoptose é a morte celular programada que acontece em condições fisiológicas para o controle populacional de células. Este processo também é usado contra danos externos e nas respostas imunológicas como mecanismo de defesa celular, como revisado em ELMORE, 2007). As células especializadas em eliminar micro-organismos como neutrófilos, monócitos e macrófagos utilizam a apoptose para disparar a resposta imune molecular (LIM; GRINSTEIN; ROTH, 2017). O processo de apoptose é bastante complexo e resulta de uma cascata de eventos moleculares dependentes de energia. Estas cascatas são desencadeadas por fatores extrínsecos (receptores que sinalizam a morte celular) e intrínsecos (via mitocondrial), e podem ser dividido em 3 grupos: (a) I (dependente de caspase), (b) II (independente de caspase) e (c) III (independente de caspase e mediada por células T) (NAGATA, 2018).

A apoptose tipo I utiliza proteínas (capase-8, caspase-9, caspase-12, caspase-7, caspase-3) e uma série de receptores específicos (TNF-alpha, FasL) para ativar a proteína Bcl-2 que induz mudanças na membrana da mitocôndria e a liberação da citocromo c, uma molécula pró-apoptótica (ELMORE, 2007). No tipo II, os eventos morfológicos como o aparecimento de um vacúolo de dupla membrana são bem característicos. Estes eventos ocorrem depois de uma alteração no potencial de membrana mitocondrial e um aumento na produção de espécies reativas de oxigênio (ROS), induzindo a apoptose independente de caspase. Neste caso, o fator de indução de apoptose (AIF) pode ser liberado da mitocôndria e causar danos no DNA que se não reparados, levarão a apoptose celular. Outros fatores como a permeabilização da membrana lisossomal, supressão do fator tumoral p53 e algumas proteínas virais também podem induzir apoptose tipo II (JOHANSSON *et al.,* 2010). O terceiro tipo de apoptose é desencadeada pelas células T citotóxicas e consiste na liberação de grânulos líticos mediada por cálcio após o reconhecimento de antígenos na superfície da célula alvo. Estes grânulos são lisossomos modificados contendo perforina e granzimas. As perforinas são enzimas que induzem a formação de poros transmembranares nas células alvo, enquanto as granzimas desencadeiam uma cascata de enzimas que culminará na degradação do DNA (ELMORE, 2007). Como descrito, os três tipos de apoptose apresentam características morfológicas podem ser observadas nas células tais como fragmentação nuclear, disfunção mitocondrial e desorganização celular, podendo formar estruturas conhecidas como corpos apoptóticos (HENGARTNER, 2000).

A autofagia é um processo celular catabólico importante na reciclagem de moléculas e na homeostase celular. Dentre as funções da autofagia estão: a geração de energia quando a célula está com baixo nível de nutrientes, degradação de agentes tóxicos (DIKIC; ELAZAR, 2018), reciclagem de proteínas, reciclagem de organelas danificadas e eliminação de patógenos intracelulares (GLICK; BARTH; MACLEOD, 2010). Basicamente, na autofagia ocorre formação de um fagóforo e, posteriormente, do autofagossomo, organelas circundadas por uma

membrana específica com características presentes na membrana do retículo endoplasmático (SEGLEN; BOHLEY, 1992). Uma vez que este autofagossomo se funde com outros componentes da via endocítica e com lisossomos forma-se o autofagolisossomo, uma organela de dupla membrana onde a partícula fagocitada é digerida (GLICK; BARTH; MACLEOD, 2010).

Embora a autofagia também seja uma resposta microbicida do hospedeiro contra patógenos, alguns deles modulam este processo para estabelecer uma infecção (ROMANO *et al.*, 2007). No caso do *T. cruzi,* este parasito se aproveita da autofagia no processo de escape para o citoplasma do hospedeiro (BRENNAND; RICO; MICHELS, 2012).

1.5 Mecanismos de sobrevivência intracelular do *T. cruzi*

Os patógenos intracelulares como o *T. cruzi* lidam com vários desafios para ter sucesso na sobrevivência e proliferação no ambiente intracelular do hospedeiro. Os eventos relacionados com o estabelecimento da infecção incluem evadir dos mecanismos microbicidas, sobrevivência em diferentes microambientes e obtenção de nutrientes (SIBLEY, 2011). Neste contexto, o *T. cruzi* sofre diversas modificações morfológicas, fisiológicas e bioquímicas durante o seu ciclo de vida que permitem o sucesso da infecção (OSÓRIO *et al.*, 2012). Estas mudanças, inclusive na expressão de proteínas e moléculas de superfície, auxiliam o parasito a se adaptar e garantir sua sobrevivência em diferentes ambientes como o organismo do seu vetor, a corrente sanguínea e o ambiente intracelular do hospedeiro vertebrado (CUERVO; DOMONT; DE JESUS, 2010).

Um grupo de moléculas bastante importante para a sobrevivência parasitária é o de fatores de virulência, cuja presença é dependente do hospedeiro e do estágio morfológico do parasito (EPTING; COATES; ENGMAN, 2010). As principais funções dos fatores de

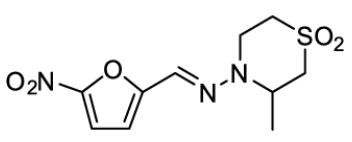
virulência incluem: a evasão ou resistência ao sistema imune, a adesão e invasão da célula hospedeira, a diferenciação e proliferação celular e a detoxificação de espécies reativas de oxigênio e nitrogênio, como revisado em Osorio *et al.* (2012). Tal grau de desenvolvimento do *T. cruzi,* o torna um modelo de estudo biológico para outros patógenos intracelulares que têm um modelo de infecção semelhante como o escape das vias microbicidas intracelulares e a multiplicação livre no citoplasma.

1.6 A doença de Chagas e seu tratamento

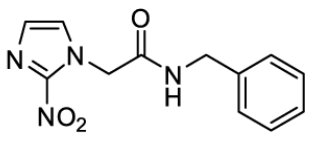
Quando os mecanismos microbicidas das células falham, a infecção pelo *T. cruzi* pode causar a doença de Chagas ou tripanossomíase americana. De acordo com a WHO (2018), em torno de 8 milhões de pessoas estão infectadas com o *T. cruzi* na América latina, com a ocorrência de dez mil pessoas por ano (WHO, 2018). A doença de Chagas é transmitida majoritariamente pelo inseto vetor (90%), mas a infecção também pode acontecer por acidentes laboratoriais, transmissão congênita e sanguínea (HERWALDT, 2001), transplante de órgãos (PIERROTTI *et al.*, 2018) e ingestão de bebidas e comidas contaminadas (STEINDEL *et al.*, 2008). São observadas duas fases distintas da doença: a fase aguda ou inicial, com duração de 4 a 8 semanas, e a fase crônica, que pode durar por toda a vida do indivíduo (RASSI; RASSI; MARIN-NETO, 2010). A fase aguda é caracterizada pela grande quantidade de parasitos circulantes no sangue, uma alta infecção nos músculos cardíacos e esquelético (SOARES; PONTES-DE-CARVALHO; RIBEIRO-DOS-SANTOS, 2001), nervos entéricos (DA SILVEIRA *et al.*, 2008) e adipócitos (COMBS *et al.*, 2005) e uma resposta inflamatória exacerbada no miocárdio (ZHANG; TARLETON, 1999). Na maioria dos casos os sintomas são oligossintomáticos, e em torno de 10 % das vezes pode desenvolver em uma intensa miocardite (BILATE; CUNHA-NETO, 2008). Com o desenvolvimento da imunidade adquirida, o número de parasitos diminui drasticamente e os sintomas da doença tendem a desaparecer espontaneamente em torno de 90 % dos casos, mesmo sem o uso de medicamentos

(FERNANDES; ANDREWS, 2012). A fase crônica da doença de Chagas só se desenvolve em, aproximadamente, 10 % dos indivíduos infectados e os motivos não são muito bem determinados, mas pode envolver a susceptibilidade genética do paciente e a cepa e linhagem filogenética do parasito (MACEDO, et al., 2004; FERNANDES; ANDREWS, 2012;). Esta fase pode durar anos ou décadas e dentre suas manifestações clínicas estão sérias complicações cardíacas, digestivas ou cárdiodigestivas (JUNQUEIRA *et al.*, 2010).

O tratamento da doença de Chagas é feito pelo uso do Nifurtimox [(3-methil-4-(5-nitrofurfurilideneamina) tetrahidro-4H-1,4-tiazina-1,1-dióxido)] e Benzonidazol (N-benzil-2-nitroimidazol acetamida), medicamentos desenvolvidos há mais de quatro décadas. No entanto, estes medicamentos apresentam alta toxicidade para o paciente e sua eficiência varia de acordo com a fase da doença, idade do paciente, região demográfica e resistência por parte dos parasitos (COURA; CASTRO, 2002; SOEIRO *et al.*, 2009). Entre os efeitos colaterais descritos para o benzonidazol e o nifurtimox estão: náusea, anorexia, perda de peso, depressão na medula óssea, dermopatia alérgica, polineurite dos nervos periféricos, trombocitopenia púrpura e agranulose, que podem interromper o tratamento (RASSI; RASSI; MARIN-NETO, 2010). Brevemente, o modo de ação das duas drogas está ligado a biorredução de grupamentos nitro (DOCAMPO, 1990); o benzonidazol leva a formação de interações de intermediários da nitroredução que alteram covalentemente macromoléculas como DNA, lipídios e proteínas (CASTRO; DEMECCA; BARTEL, 2006; POLAK; RICHLE, 1978), enquanto o nifurtimox causa alta produção de ROS, levando ao estresse oxidativo no parasito (BOIANI *et al.*, 2010). Devido aos vários fatores que limitam o uso do nifurtimox e do benzonidazol, o desenvolvimento de novos compostos com ação contra o *T. cruzi* é de grande importância.



Nifurtimox



Benznidazole

Figura 5 – Estruturas químicas dos compostos Nifurtimox e Benzonidazol.

Baseados nos conhecimentos adquiridos sobre a biologia da interação parasito – hospedeiro, uma nova droga ideal deveria ter algumas características incluindo: (a) efeito sobre as formas intracelulares e extracelulares presentes nos mamíferos; (b) alta atividade contra diversas cepas; (c) ser eficaz em ambas as formas da doença; (d) ter formulação oral e em poucas doses; (e) baixa toxicidade; (f) baixo custo; (g) capacidade de acumular nos tecidos e ter meia vida longa, (h) baixo risco de toxicidade cardíaca e (i) e baixa possibilidade de induzir resistência (NWAKA; HUDSON, 2006). Neste sentido, os íons metálicos têm sido bastante utilizados na síntese de novos compostos devido as suas características farmacológicas. Contudo, pouco se sabe sobre a influência destes metais na interação parasito-hospedeiro, no desenvolvimento e estabelecimento da infecção.

1.7 Os metais essenciais e não essenciais

Metais são elementos encontrados naturalmente na crosta terrestre e são classificados pelas suas propriedades físicas quando encontrado no estado sólido, como por exemplo: condutividade elétrica e térmica, brilho, força mecânica e maleabilidade (EL-MOSELHY *et al.*, 2014). Os metais podem ser divididos em dois grandes grupos dependendo das funções nos organismos: essenciais e não essenciais.

Os metais essenciais, que incluem o cálcio, ferro, zinco, cobre, magnésio, sódio, molibdênio e potássio, vanádio, cromo, e cobalto são assim chamados porque são componentes fundamentais de fenômenos biológicos cruciais e da homeostase. Estes metais podem funcionar como receptores, íons cofatores de reações enzimáticas (enzimas ligantes de metais) ou componentes estruturais de proteínas e enzimas (metaloproteínas ou metaloenzimas)

(ANDREINI *et al.*, 2008). Estes dois tipos de proteínas e enzimas dependentes de metais constituem grande parte do total do organismo humano (KENNEDY, 2001).

Os metais essenciais como o cobre, ferro, manganês e molibdênio ligam-se as metaloproteínas com grande afinidade por causa das características físico-químicas como pequeno raio atômico, densidade e interações por forças eletroestáticas e eletromagnéticas. Já o sódio e potássio são mais comuns nas proteínas ligantes de metais e se ligam com baixa afinidade (GARCIA; MAGALHÃES; ARRUDA, 2006). Para adquirir o ambiente intracelular, os metais utilizam transportadores não específicos ou específicos como transportadores de metais divalentes, ATPases tipo P, canais iônicos e ligação a proteínas (MARTINEZ-FINLEY *et al*., 2011).

Zinco, o metal essencial investigado neste estudo, é proveniente da alimentação e absorvidos como íons livres pelo intestino por mecanismo mediado por carreadores durante a ingestão de comida (KREBS, 2000). Existem pelo menos 25 tipos de transportadores de zinco, divididos em 2 grupos, cuja função é manter a homeostase do metal nas células (COUSINS; LIUZZI; LICHTEN, 2006). O grupo dos transportadores ZnTs promovem o efluxo do zinco para o ambiente extracelular em vesículas, enquanto o influxo é feito por transporte passivo ou também por vesículas, através dos transportadores Zip (SEKLER *et al.*, 2007). O zinco é o segundo metal mais abundante no corpo humano e o metal mais encontrado no ambiente intracelular e está presente em mais de três mil proteínas ou enzimas (10 % do total em humanos) tanto quanto elemento estrutural quanto como cofator catalítico (ANDREINI *et al.*, 2006). Estas proteínas e enzimas, incluindo proteases, fosfatases, esterases e deacetilases, estão envolvidas em diversos processos celulares como proteção celular contra radicais livres ou outros grupamentos químicos, reparo do DNA, replicação e translação, transporte e estocagem (ANDREINI *et al.*, 2006; AULD, 2001).

A importância dos metais na interação do parasito com o hospedeiro e no estabelecimento da infecção já foi descrita há algumas décadas (WEINBERG, 1966). Estudos mais recentes também confirmaram que alguns parasitos intracelulares necessitam adquirir os metais essenciais da célula hospedeira para se replicar e ser patogênico (PORCHERON *et al.*, 2013). No caso do *T. cruzi*, metaloproteínas estão envolvidas nos processos de internalização, diferenciação morfológica e estabelecimento da infecção (ALVAREZ; NIEMIROWICZ; CAZZULO, 2012).

No segundo grupo de metais estão elementos como alumínio, cádmio, chumbo, mercúrio, entre outros, que não possuem funções vitais ou efeitos benéficos nos organismos (FRAGA, 2005). Estes metais estão disponíveis por fontes naturais como erosão do solo e pelas atividades humanas como mineração, efluentes industriais e muitos outros (ZHAIR *et al.*, 2005). A acumulação destes metais nos organismos mesmo em concentrações muito baixas pode causar sérios danos por diferentes razões, incluindo: (a) habilidade de reagir diretamente com proteínas, inativando-as, (b) substituição de elementos necessários ao metabolismo e (c) produção de espécies reativas de oxigênio e mudanças no sistema antioxidante (TAMÁS *et al.*, 2014). O primeiro caso é resultado da afinidade entre os metais com os resíduos tiol, histidina e carboxil, causando a ligação dos íons metálicos com elementos estruturais das células e proteínas do transporte celular e nos sítios ativos de enzimas (TAMÁS *et al.*, 2014). No segundo caso, outros elementos como o cálcio dos ossos e ferro nos eritrócitos são substituídos, causando danos e alterações na estrutura e metabolismo (CAILLIATTE *et al.*, 2009; MOULIS, 2010).

O cádmio (Cd), um dos metais não essenciais usados neste estudo, é um metal pesado do grupo IIB da tabela periódica dos elementos (coluna 12 assim como o zinco). A exposição de humanos ao cádmio ocorre pela inalação e ingestão de produtos contaminados e, devido à dificuldade de excreção e meia vida longa, o metal se acumula nos organismos causando danos (MOULIS, 2010). O cádmio ganha o ambiente intracelular através dos transportadores de

metais divalentes, principalmente cálcio e zinco ou por endocitose ligados a proteínas como glutationa e cisteína (ZALUPS; AHMAD, 2003). Uma vez no meio intracelular, o cádmio pode causar, dentre outros efeitos, a inibição de enzimas antioxidativas, produção de espécies reativas de oxigênio, bloqueio da fosforilação oxidativa e transdução de sinais (WAISBERG *et al.*, 2003; ZALUPS; AHMAD, 2003).

O segundo metal não essencial utilizado neste estudo foi o mercúrio (Hg), outro metal pesado que torna-se disponível no ambiente devido aos resíduos de mineração, queima de carvão, incinerações, entre outros (ZAHIR *et al.*, 2005). O mercúrio pode ter diferentes formas químicas como o Hg0 (ou mercúrio elementar), Hg^{2+} and Hg^{+} (mercúrio inorgânico mono ou divalente) e o MeHg (mercúrio orgânico ou metil mercúrio) e seus efeitos tóxicos são dependentes delas (FARINA *et al.*, 2013). As fontes majoritárias deste metal são o consumo de comidas e bebidas contaminadas e seu transporte através da membrana plasmática se dá através de mecanismos ativos e passivos que envolvem transportadores de outros metais essenciais ou de moléculas como aminoácidos e polipeptídeos (BRIDGES; ZALUPS, 2017). No interior das células, o mercúrio pode desestabilizar vários processos biológicos devido a sua grande afinidade por grupos tiol e sulfidrilas encontrados nos ácidos nucléicos, fatores de transcrição e proteínas (SYVERSEN; KAUR, 2012). Outros efeitos diretos também observados são a disfunção de outras estruturas celulares como mitocôndria, citoesqueleto e retículo endoplasmático, que podem induzir um estresse oxidativo e peroxidação de lipídios, podendo causar morte celular (SYVERSEN; KAUR, 2012; VERGILIO; CARVALHO; MELO, 2014).

Os metais não essenciais utilizam os mesmos mecanismos dos metais essenciais porque são capazes de mimetiza-los ou porque conseguem passar livremente pela membrana plasmática (MARTINEZ-FINLEY *et al*., 2011). Desde modo, as células precisam discriminar e regular os dois grupos de metais para usar os essenciais em seus processos vitais, enquanto se detoxifica ou bombeia para fora os metais não essenciais (JAISHANKAR *et al.*, 2014). As

metalochaperonas, importadores e transportadores de metais são os responsáveis por manter as concentrações dos íons metálicos no citoplasma. A expressão gênica destes fatores é controlada por proteínas metaloregulatórias que identificam concentrações altas ou baixas dos metais no citoplasma e levam a regulação de genes de expressão de proteínas de detoxificação e de transporte de metais, afim de manter a homeostase (REYES-CABALLERO; CAMPANELLO; GIEDROC, 2011).

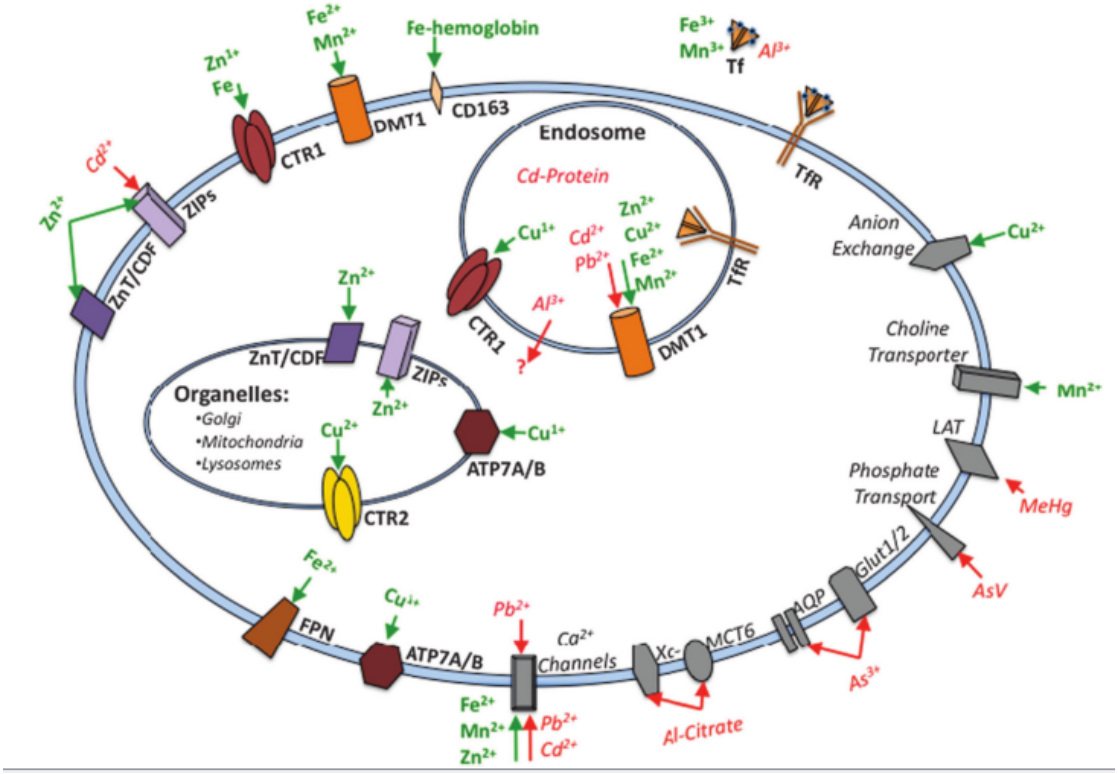


Figura 6 - Transporte celular de metais. Os principais mecanismos envolvidos no transporte de metais essenciais e não essenciais. Os transportadores cuja principal função é o transporte de metais estão em negrito, enquanto nos que o transporte é uma função secundária estão em itálico. Em verde estão os metais essenciais e em vermelho, os não essenciais (MARTINEZ-FINLEY et al., 2012).

1.8 As defesas celulares contra excesso de metais

As células de mamíferos possuem vários mecanismos para manter a homeostase dos metais como absorção e excreção (transportadores transmembranares e os reguladores de genes responsivos aos metais, como citados acima) (GÜNTHER; LINDERT; SCHAFFNER, 2012; MARTINEZ-FINLEY *et al.*, 2012), estocagem e estabilização (metalotioneinas e glutationas) (BABULA *et al.*, 2012; JOZEFCZAK *et al.*, 2012). Quando a concentração de íons metálicos sai do controle, o principal efeito tóxico é o estresse oxidativo, e contra isso, as células de mamíferos possuem proteções dependentes ou não de enzimas. As defesas enzimáticas incluem as superóxidos dismutases, catalases, glutationas peroxidases e glutationas-S-transferases, enquanto as defesas não enzimáticas são o ascorbato, α-tocoferol, β-caroteno e a glutationa (TURRENS, 2004).

As metalotioneinas constituem um grupo de metaloproteínas de baixo peso molecular que contém entre 61-68 aminoácidos ricas em cisteínas que se ligam a diferentes metais (cobre, zinco, cádmio e mercúrio) (SABOLIĆ *et al*., 2010). Estas proteínas têm várias funções celulares como proteção contra danos causados por excesso de metais ou radicais livres por exemplo (CHEN *et al.*, 2004), mas a detoxificação de metais pesados e a manutenção da homeostase de metais essenciais são as funções mais estudadas (DAVIS; COUSINS, 2000). Nos mamíferos, as metalotioneinas se ligam ao zinco, que pode ser substituído por outros metais como o cobre ou cádmio se estes estiverem em excesso (SUTHERLAND, STILLMAN, 2011). A expressão das metalotioneínas pode ser induzida por alguns fatores como o elemento responsivo ao metal (MRE), o fator de ligação da transcrição (MTF-1) (RADTKE, FREDDY *et al.*, 1993) e o fator nuclear 1 (NF1) (ANDREWS, 2000) e sua ativação depende de vários estímulos como íons metálicos, citocinas e fatores de crescimento (HAQ; MAHONEY; KOROPATNICK, 2003). Existem pelo menos dez isoformas funcionais de metalotioneínas que são divididas em 4 diferentes grupos (MT 1-4) de acordo com pequenas diferenças nas características, sequência e expressão (BABULA *et al.*, 2012). As metalotioneínas dos grupos MT 1 e 2 são as mais

abundantes e expressas em diferentes tipos celulares de vários órgãos (BABULA *et al.*, 2012). Em comparação, o subtipo MT-3 inclui um único fator de inibição de crescimento neuronal expresso em células neuronais com características biológicas, estruturais e químicas bastante diferentes do MT-1 e 2 (BABULA *et al.*, 2012), enquanto grupo MT-4 são proteínas restritas ao epitélio escamoso (SABOLIĆ *et al.*, 2010). As metalotioneinas estão majoritariamente no citoplasma, mas também em outras organelas como mitocôndrias, núcleo e lisossomos (BABULA *et al.*, 2012; YE; MARET; VALLEE, 2001).

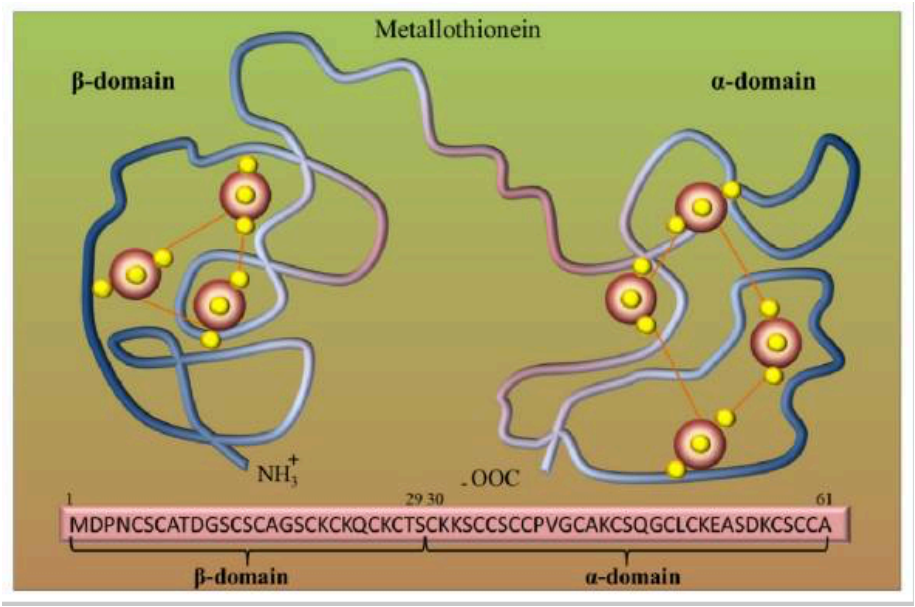


Figura 7 Estrutura da metalotioneína. Modelo de dois sítios ligantes da metalotioneína. Os grandes círculos vermelhos sãos os átomos de metais e os em que os círculos amarelos são átomos de enxofre. Retirado de: (RUTTKAY-NEDECKY et al., 2013)

A glutationa (GSH) é um tripeptídeo (L-Glu-L-Cis-Gli) presente em todos os tecidos dos mamíferos principalmente no citoplasma, mas pequenas quantidades também são encontradas no retículo endoplasmático e nas mitocôndrias (MEREDITH; REED, 1982). As funções da glutationa incluem defender as células contra estresse oxidativo, detoxificação de xenobióticos, manutenção da homeostase de metais, apoptose, ciclo celular, entre outros (LU,

2013). A presença do grupo tiol (-SH) na glutationa que é o responsável pelas características bioquímicas que possibilitam diferentes funções, como a alta afinidade aos metais, possibilitando sua imobilização e excreção das células através de transportadores (RUBINO, 2015).

As superóxidos dismutases (SODs) são enzimas presentes em todos os organismos aeróbicos localizadas em diferentes organelas. Estas enzimas são as principais linhas de defesa celulares contra os radicais livres de oxigênio, visto que são responsáveis pela transformação do superóxido ($O_2^{\bullet-}$) em oxigênio ($2O_2^{\bullet-}+2H^+ \rightarrow H_2O_2+O_2$) e peróxido de oxigênio (H_2O_2) (YONUS, 2018). Os ânions superóxidos são produtos de muitos processos fisiológicos, incluindo a respiração mitocondrial e sua reatividade pode produzir espécies reativas de oxigênio e nitrogênio (WINTERBOURN, 2008). Existem três classes de superóxido dismutase com diferentes íons metálicos como fatores catalíticos: Cu/Zn SODs, Mn SOD/Fe SODs, and Ni SODs (WANG *et al.*, 2018), mas os eucariotos só possuem Cu/Zn SODs no citoplasma e ambiente extracelular e Mn SODs nas mitocôndrias (ANNE-FRANCES, 2012).

As catalases são enzimas com um átomo de ferro em seu sítio catalítico presentes em organismos de plantas e de mamíferos capazes de converter H_2O_2 em água e oxigênio (ZAMOCKY; FURTMÜLLER; OBINGER, 2008). A principal localização das catalases são os peroxissomos, visto que estas têm uma sequência sinal para serem internalizadas nesta organela (LAZAROW; DE DUVE, 1973), mas elas também já foram descritas no citoplasma (MIDDELKOOP *et al.*, 1993). Existem três grupos de catalases que são divididos de acordo com sua estrutura e função. Os grupos 1 e 2 são as catalases verdadeiras ou catalase-peroxidase porque englobam enzimas contendo heme, enquanto o terceiro grupo possuem manganês (ZAMOCKY; FURTMÜLLER; OBINGER, 2008). As catalases humanas estão no primeiro grupo e são conhecidas por suas quatro pequenas subunidades (62 kDa), com o heme como seu grupo prostético e o NADPH como cofator. As catalase-peroxidases são encontradas em

fungos, arqueobactérias e bactérias, enquanto as catalases com manganês são exclusivas de bactérias (ZAMOCKY; FURTMÜLLER; OBINGER, 2008).

As glutationas peroxidases são uma família de enzimas homólogas as glutationas peroxidase-1 de mamíferos que contém uma selenocisteína no sítio catalítico e são responsáveis pela redução de H_2O_2 a água ou hidroperóxidos a álcoois, usando a glutationa como agente redutor (LUBOS; LOSCALZO; HANDY, 2011). Outros subtipos de glutationa peroxidases não usam a glutationa e não apresentam a selenocisteína no sítio catalítico. Ao invés disto, estas são caracterizadas funcionalmente como peroxidases dependentes de tioredoxina que possuem uma cisteína no lugar da selenocisteína (LUBOS; LOSCALZO; HANDY, 2011). A glutationa peroxidase 1 é o subtipo mais comum desta família de enzimas e está presente em todas as células no citoplasma e na mitocôndria (LUBOS; LOSCALZO; HANDY, 2011), e nos peroxissomos em certos tipos celulares (SINGH et al., 1994).

As glutationas transferases (GSTs) são uma superfamília de proteínas encontradas nos procariotos e eucariotos. Nos eucariotos, as glutationas transferases são divididas nos grupos GSTs citoplasmáticas, mitocondriais e microssomais (RAZA, 2012). A grande variedade de funções deste grupo de enzimas inclui: o controle dos produtos tóxicos da oxidação dos lipídeos e do estresse oxidativo, entre outros (LISTOWSKY, 2005).

Como descrito acima, os mamíferos possuem um arsenal de mecanismos para manter a homeostase ou controlar os efeitos deletérios que podem ser causados pelo desequilíbrio nas concentrações dos metais. Por outro lado, o *Trypanosoma cruzi* possui mecanismos de controle de homeostase dos metais e de defesa contra seus efeitos tóxicos bastante simples. As metalotioneínas são expressas principalmente no estágio epimastigota e suas concentrações são dependentes da fase de crescimento, estando em maior concentração durante a fase exponencial (MAYA *et al.*, 2004). A enzima superóxido dismutase possui uma atividade bem baixa (TURRENS, 2004), enquanto o sequenciamento do genoma do *T.*

cruzi mostrou que estes não possuem os genes para glutationa redutases, tioredoxina redutases, catalases e glutationa peroxidases contendo selenocisteína (EL-SAYED *et al.*, 2005; KRAUTH-SIEGEL; COMINI, 2008). O principal mecanismo de defesa do *T. cruzi* contra os radicais livres são as tripanotionas e tripanotionas redutases, que consistem de duas moléculas de glutationa ligadas covalentemente a uma espermidina (OZA *et al.*, 2002). Estes fatos sugerem uma maior susceptibilidade destes parasitos aos danos provocados por excesso de metais em comparação com células de mamíferos devido aos mecanismos de defesa contra estresse oxidativo serem mais precários.

Com o crescente número de compostos usando metais para aumentar a eficácia e prevenir a resistência dos micro-organismos, o estudo da influência destes metais nos processos biofísicos e bioquímicos nas células hospedeiras e nos parasitos intracelulares são de grande importância para o design de novas metalodrogas. Estudos anteriores utilizando os mesmos metais zinco, cádmio e mercúrio foram feitos com células hospedeiras Vero e LLC-MK2 (ambas linhagens epiteliais) com o *Toxoplasma gondii.* A escolha específica destes três metais ocorreu porque estes estão presentes na mesma coluna da tabela periódica, compartilhando características físico-químicas. No entanto o zinco é um metal essencial e o cádmio e mercúrio, não essenciais. Os parâmetros usados no presente estudo como concentrações e tempos de incubação com os metais em células não infectadas e infectadas foram baseados nestes estudos prévios (CARVALHO; MELO, 2016; CARVALHO; MELO, 2018).

1.9 O estudo da interação de metais com outros modelos parasitários

Os estudos sobre as interações de metais com parasitos ressurgiram recentemente. Diferentes efeitos celulares foram mostrados em promastigotas de *Leishmania sp.* Após a

utilização de zinco e cobre (PALETTA-SILVA *et al.*, 2012; KUMARI *et al.*, 2017; SAINI *et al.*, 2017)

O primeiro modelo de parasito intracelular usado no estudo dos metais foi o *Toxoplasma gondii.* Carvalho; Melo, 2016 e 2017 investigaram os efeitos do zinco, cádmio e mercúrio no ciclo intracelular deste parasito. Neste estudo também foi estabelecido parâmetros importantes como a escala de toxicidade dos metais (concentrações e tempos) viáveis para uso em células hospedeiras não infectadas e infectadas.

Os resultados mostrados nestes estudos anteriores são bastante promissores e sugerem que esta linha de estudo deve ser empregada em outros modelos parasitários. Neste contexto, o presente estudo baseou-se nos parâmetros já estabelecidos anteriormente para sua realização.

2. OBJETIVO GERAL:

Investigar o efeito do zinco (metal essencial), cádmio e mercúrio (metais não essenciais) nos três principais estágios morfológicos do *T. cruzi* e seus papéis na invasão, estabelecimento da infecção e replicação do parasito no ambiente intracelular.

Objetivos específicos:

- Analisar a cinética do desenvolvimento da infecção pelo *T. cruzi;*
- Quantificar a toxicidade do zinco, cádmio e mercúrio nas formas epimastigotas, tripomastigotas, amastigotas e nas células hospedeiras;
- Estabelecer o valor do LD 50 para as três formas do parasito e para as células hospedeiras;
- Descrever a estrutura e ultraestrutura dos epimastigotas, tripomastigotas, amastigotas e células hospedeiras após a incubação com os metais;
- Investigar a capacidade de reversibilidade dos efeitos tóxicos nos amastigotas e células hospedeiras;
- Investigar se a incubação prévia com zinco nos epimastigotas, tripomastigotas, amastigotas e células hospedeiras os protegem contra os efeitos tóxicos do cádmio e do mercúrio;
- Analisar se os tripomastigotas tratados com zinco, cádmio e mercúrio são capazes de infectar as células hospedeiras e estabelecer uma infecção;
- Investigar se os parasitos tratados são capazes de sair do vacúolo parasitóforo e estabelecer uma infecção no citoplasma;
- Análisar os principais eventos celulares envolvidos na morte e eliminação dos amastigotas do ambiente intracelular.

3. MATERIAL E MÉTODOS E RESULTADOS

Os materiais e métodos e resultados serão apresentados em forma de artigos científicos.

3.1 Life and death of *Trypanosoma cruzi* in the presence of metals

(Publicado na Biometals / 2017)

3.2 Intracellular development of *Trypanosoma cruzi* in the presence of metals

(Publicado na Journal of Parasitic Diseases / 2018)

3.3 Cellular events related to *Trypanosoma cruzi* elimination in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$

(Submetido na Journal of Microbiology 09/01/2019)

3.1 Life and death of *Trypanosoma cruzi* in the presence of metals

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Life and death of *Trypanosoma cruzi* in presence of metals

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Abstract *Trypanosoma cruzi* has many molecules that need metallic elements to work, allowing cell invasion and the establishment of infection, causing Chagas disease. Nonetheless, knowledge regarding how the parasites address metals and maintain homeostasis is lacking. To study this relationship, zinc, cadmium and mercury were chosen. Epimastigote, trypomastigote and intracellular forms of *T. cruzi* were incubated with these metals for different times and at different concentrations. In general, epimastigotes were the most sensitive and trypomastigotes the most resistant to metals. $ZnCl_2$ induced low toxic effects to all parasite forms. Although the parasites were very sensitive to the toxic effects of $CdCl_2$ and $HgCl_2$, pretreatment with $ZnCl_2$ decreased the death rate. The trypomastigotes pretreated with $CdCl_2$ were unable to infect the host cells, and the treated intracellular forms were damaged after 2 h of incubation, when the toxic effects were poorly reverted. New insights on metal toxicity mechanisms are provided, helping to understand how metallic ions influence the parasite's biochemical and physiological processes.

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Keywords Cadmium · Essential metals · Mercury · Non-essential metals · Trypanosoma cruzi and zinc

Introduction

Trypanosoma cruzi is a hemoflagellated protozoan parasite of the Kinetoplastidae order and Trypanosomatidae family. This parasite has an heteroxenic life cycle involving the epimastigote and metacyclic trypomastigote forms found in the vector/definitive hosts (hematophagous insects, triatomids) and trypomastigotes and amastigotes, found in the intermediary hosts (mammals, including humans), as reviewed in (Souza 2002). In humans, *T. cruzi* invades phagocytic and non-phagocytic cells through progressive steps to establish an infection. The first step of invasion is the attachment of the parasite to the host cell, which involves different surface molecules working as receptors that are present in the membranes of both parasite and host cells (Rosestolato et al. 2002; Eptin et al. 2010). Thereafter, the invasion is triggered by calcium bidirectional signaling cascades and different enzymes and results in endocytic vacuoles containing parasites in the host cell (Florencio-Martínez et al. 2010). The fusion of endocytic vacuoles with lysosomes induces the escape of parasites to the cytoplasm, where they transform into the proliferative form—amastigotes. After several divisions, the parasites return to the trypomastigote form and rupture the

host cell, reaching the extracellular milieu and infecting new cells (Ley et al. 1990; Alves and Colli 2007). The set of molecules that *T. cruzi* utilizes to survive and replicate in the extra- and intracellular environments encompasses protein classes that contain metals as structural components or reaction cofactors (Alvarez et al. 2012). Nonetheless, little is known about the role of inorganic metals in the *T. cruzi* cell cycle.

All kinds of organisms use essential metals as structural components of proteins and enzymes or cofactors of enzymatic reactions (Andreini et al. 2008). These metallic molecules can convert energy during respiration and are involved in genic regulation and expression (Waldron et al. 2009). For the parasite, all the events related to host cell invasion cited above depend on these metal-binding proteins/enzymes or metalloenzymes/metalloprotein homeostasis, which are differentially expressed at specific stages (Kulkarni et al. 2009; Alvares et al. 2012). In addition, essential metallic ion balances are well known to considerably influence the establishment and maintenance of host-parasite relationships, and a disruption of this equilibrium can lead to severe damage for both (Weinberg 1966). Nonetheless, the typical presence of non-essential metals in the organism environment and consequently the contact of these elements with cells can results in serious cytotoxic effects (Templeton and Liu 2010).

To enter the intracellular milieu, non-essential metals use the same entrance and distribution pathways as essential metals and replace them in structures and reactions. As a result of these interactions, metals can cause physiological and biochemical damage to parasites and cells (Rana 2008). Although the host cells present a variety of defense mechanisms against excessive essential or non- essential metals (Vašák 2005; Reyes-Caballero et al. 2011), the importance of metallic ions in the development and maintenance of parasite infection is poorly described. Currently, this subject has become very important since many drugs have been complexed to metallic ions to improve their efficiency in eliminating parasites (Beraldo and Gambino 2004; Demoro et al. 2010, 2013; Fernández et al. 2013).

Therefore, for the first time, the main developmental forms of *T. cruzi* were incubated with essential (zinc) and non-essential (cadmium and mercury) metals to study their toxicological impacts on these biological models.

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) supplemented with 5% fetal bovine serum (FBS). LLC-MK2 (kidney fibroblasts of *Macaca mulatta*) were grown in plastic Falcon flasks (25 cm^2) containing RPMI 1640 medium supplemented with 5% fetal bovine serum. The cultures were treated with trypsin when the cell densities approached to monolayer. For experimental proposals, the cells were placed on Linbro 24-well plates with a sterile coverslip at a density of 3×10^4 cells per well or on a medium flasks (3×10^6 cells) and allowed to attach for 24 h at 37 °C in a 5% CO_2 atmosphere (Gomes et al. 2012).

Parasite maintenance

Epimastigotes of *T. cruzi* (DM28 strain) were cultivated in Liver Infusion Triptose (LIT) medium supplemented with 0.4% of hemin and 10% FBS at 28 °C. Every 5 days, a 1 mL aliquot of parasite-containing medium was transferred to a new tube and the volume completed to 5 mL with fresh culture medium.

Trypomastigotes of *T. cruzi* were obtained from transformation of epimastigotes. The epimastigotes were centrifuged at 500×*g* during 10 min and the pellet homogenized in DMEM-1152 supplemented with 10% FBS and incubated at 37 °C during 48 h. After this time, around 95% of the parasites were in the form of trypomastigote. For experimental purpose, the parasites were centrifuged at the same condition and homogenized in 1 mL of DMEM-1152. An aliquot of 0.1 mL was scored at Neubauer chamber and a rate of 20:1 parasite: cell was used to infect the culture.

After 5–6 days of infection the trypomastigotes were released on the supernatant. Then, the supernatant was collected, centrifuged as described above and new cultures were infected.

Metal treatments

Toxicological assays: infected host cells and extracellular parasites were incubated with 1, 3, 5, 8, 10 or 20

μM of $HgCl_2$ (mercury chloride), $CdCl_2$ (cadmium chloride) and $ZnCl_2$ (zinc chloride) during 2 and 24 h.

Co-treatments with the metals: the infected cells were incubated with 20 μM of $ZnCl_2$ during 24 h, then the medium was replaced by a refresh one containing $HgCl_2$ or $CdCl_2$ at 8 μM during 24 h.

Invasion assays: the parasites or the host cells or both of them were treated with 5 μM of $CdCl_2$, $HgCl_2$ or 20 μM of $ZnCl_2$ during 2 h, washed and cultured 48 h more. Untreated host cells and parasite were used as control.

Reversibility assays: infected host cells were incubated with $CdCl_2$, $HgCl_2$ or $ZnCl_2$ at 3, 5 and 8 μM during 24 h. Then, the cells were washed and the medium was replaced by a fresh one and the cells were cultured during 24 h more.

Dilutions of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ salts originated 0.1 M stock solutions in an ultra-pure quality water. The final concentrations were prepared diluting the stock solution with medium.

These times and concentrations were based on the paper published by (Carvalho and Melo 2016) where the same uninfected cells were treated at the same conditions, establishing the parameters for the next studies.

Quantification and morphological analyses

After treatments, the coverslips containing cells were rinsed in PBS, fixed in Bouin's solution for 5 min and stained with Giemsa's solution (diluted in PBS, pH 7.2, 10%, v/v) during 6 h at room temperature. The mounting of the coverslips occurred on glass slides with Entellan for observation by light microscopy. The extracellular parasites were centrifuged and the pellets were suspended in formaldehyde 4% for 30 min and rinsed with PBS, pH 7.2. An aliquot of parasites was put on glass slides. To examine all preparations was used a Zeiss Axioplan microscope, equipped with 20× and 40× objectives and the Analysis System software to obtain the images. Three random fields of each of six samples (individual treatments) of infected cells were scored to the following parameters: (1) uninfected host cells; (2) infected host cells and (3) number of intracellular parasites. The observation of morphological alterations and reduction of cell and parasite numbers indicated the metal cytotoxicity.

The estimative of LD 50 considered the dose that reduced 50% of the cultures.

Ultrastructural analyses

For this purpose, infected LLC-MK2 cells were incubated with $CdCl_2$ at 5 μM, $HgCl_2$ at 10 μM or with $ZnCl_2$ at 20 μM during 24 h. Meanwhile, the extracellular parasites (epimastigotes and trypomastigotes) were incubated with $CdCl_2$ or $HgCl_2$ at 8 μM or $ZnCl_2$ at 20 μM, during 12 h. These concentrations showed an intermediary effect, indicated to visualize the ultrastructural aspects. After the treatments, the samples were washed with PBS pH 7.2 at 37 °C and fixed at room temperature in a Karnovsky's solution containing 1% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$ and 5% saccharose in cacodylate buffer 0.1 M, pH 7.2. The samples were post fixed 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 (Fluka®) and then polymerized for 2 days in 60° C. Ultra-thin sections obtained with a ultramicrotome (LEICA®) were stained with uranyl acetate and lead citrate, and observed with a JEOL 1400 Transmission Electron Microscope at 60 kV acceleration (Carvalho et al. 2010).

Statistical analysis

For analysis of cell viability, the mean and standard error were calculated from the raw data and then subjected to the One Way ANOVA (analysis of variance). Significant difference was taken as $p < 0.05$, < 0.01.

Results

Toxicity quantification of essential and non-essential metals

On the epimastigote form

Epimastigotes in the exponential phase were incubated with $ZnCl_2$, $CdCl_2$ or $HgCl_2$ for 2, 12 or 24 h at progressive concentrations of 1, 3, 5, 8, 10 and 20 μM.

Epimastigotes treated with $ZnCl_2$ for 2 h showed an early toxic effect (parasite elimination) ranging from 6 to 20% at 1 to 20 μM, respectively. After 12 h, the treatments eliminated 11 (1 μM), 10 (3 μM), 20

(5 μM), 20 (8 μM), 22 (10 μM) and 28% (20 μM) of the parasites. After 24 h of treatment, the reductions were 0% (1 and 3 μM), 7% (5 μM), 14% (8 μM), 18% (10 μM) and 24% (20 μM) (Fig. 1a).

The $CdCl_2$ treatment had no toxic effect at 1 and 3 μM and 2 h of incubation. However, $CdCl_2$ became toxic to parasites when incubated at 5 μM, and progressive eliminations of 17 (5 μM), 25 (8 μM), 44 (10 μM) and 55% (20 μM) were observed. Metal incubation for 12 h caused no toxicity at 1 or 3 μM but eliminated 16 (5 μM), 29 (8 μM), 40 (10 μM), and 77% (20 μM) at higher concentrations. The incubation period of 24 h was toxic to parasites from 1 μM, with 42% elimination, followed by 43 (3 μM), 43 (5 μM), 50 (8 μM), 54 (10 μM) and 83% (20 μM) (Fig. 1b).

Unlike $CdCl_2$, $HgCl_2$ was toxic at 3 μM after 2 h of incubation and decreased the parasites by 33, 41, 52, 40 and 40% at 3, 5, 8, 10 and 20 μM, respectively. After 12 h of treatment, the $HgCl_2$ eliminated 11, 44, 44, 61, 80 and 98% of the epimastigotes at 1, 3, 5, 8, 10 and 20 μM, respectively. After 24 h of incubation, the parasite reductions were 32, 28, 37, 47, 67 and 95% (Fig. 1c), and a low growth rate was observed for all concentrations in comparison to 12 h of treatment.

$HgCl_2$ showed a greater progressive toxicity than $CdCl_2$ for the first 2 and 12 h. In addition, after 24 h of treatment, the $CdCl_2$ concentrations of 1-8 μM were more toxic than the $HgCl_2$. However, a low toxic effect was also observed for $ZnCl_2$ under all the treatment conditions.

On the trypomastigote form

Toxic effects on extracellular trypomastigotes were not observed for 2 h of incubation under any of the conditions tested (data not shown). The treatment with $ZnCl_2$ was also not toxic up to 12 h, but it showed toxic effects of approximately 20% for all concentrations tested after 24 h of treatment (Fig. 2a). After 12 h of $CdCl_2$ incubation, the most toxic activity on the parasites was approximately 9% (20 μM). After 24 h of treatment, $CdCl_2$ reduced 20% of the trypomastigotes at 1 μM, reaching 30% at 5 μM and 45% at 20 μM (Fig. 2b). Incubation with $HgCl_2$ during 12 h of treatment showed toxic effects at 5–10 μM (7%), and at 20 μM, the toxic effect was approximately 5%. After 24 h of incubation, the toxic effect of $HgCl_2$ increased to 10 (1 μM), 20 (3 μM), 30 (5 μM), 45 (8 μM), 50 (10 μM) and 65% (20 μM) (Fig. 2c).

The trypomastigote form was more resistant to non-essential metal treatments than the epimastigotes, suggesting that molecular and biochemical adaptations to the environment are extremely important for resistance against metals. Again, in general, $HgCl_2$ was more toxic to the parasites than $CdCl_2$ under the tested conditions.

On infected cultures

The number and morphological features of untreated and treated infected cell cultures and parasites were determined during the treatments with metals. The untreated infected culture showed proliferative cytoplasmic parasites, indicating an established infection (Table 1).

The incubation of infected cultures with $ZnCl_2$ induced a low toxic effect (cell elimination), and this occurred only at 20 μM for 24 h (15%). The treatment with $CdCl_2$ for 2 h induced a reduction of 5–16% (1–20 μM) of the cells. After 24 h of treatment, the

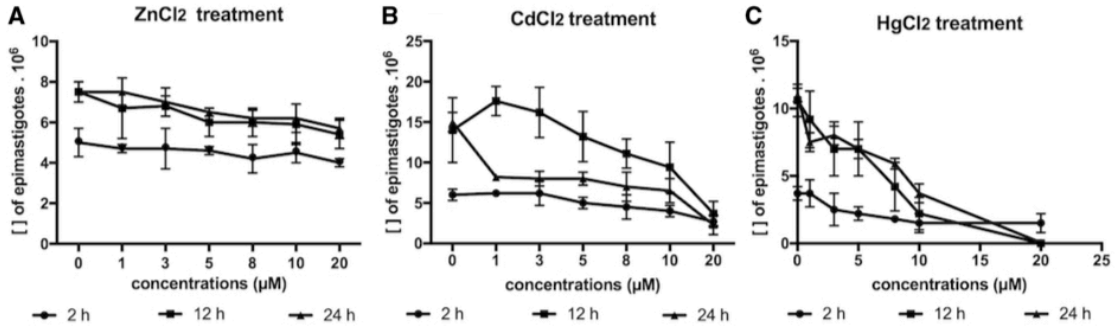

Fig. 1 Graphical representations of the mean number of epimastigotes (triplicates) after incubation for different times (2, 12 and 24 h) and at different concentrations (1, 3, 5, 8, 10 and 20 μM) **a** $ZnCl_2$ **b** $CdCl_2$ and **c** $HgCl_2$

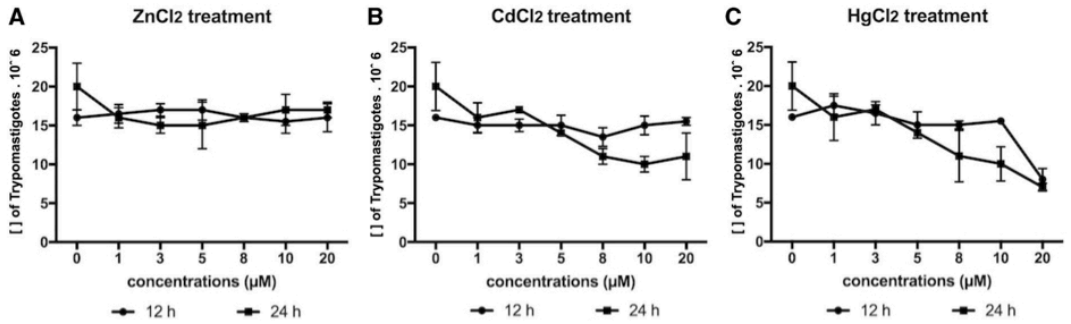

Fig. 2 Graphical representations of the mean number of trypomastigotes (triplicates) after incubation for different times (12 and 24 h) and at different concentrations (1, 3, 5, 8, 10 and 20 μM) **a** $ZnCl_2$ **b** $CdCl_2$ and **c** $HgCl_2$

Table 1 The mean number of host cells in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$ at different times and concentrations

Infected culture toxicity					
	$ZnCl_2$	$CdCl_2$		$HgCl_2$	
(μM)	24 h	2 h	24 h	2 h	24 h
Untreated	NE	259.8 ± 27.1	248.8 ± 32.5	130.6 ± 41.1	248.8 ± 32.5
1	NE	228.1 ± 55.2	200.6 ± 43.1	141.2 ± 16.1	193.6 ± 22.8
Untreated	NE	194.3 ± 38.2	229.7 ± 65.6	130.6 ± 41.1	214.7 ± 38.8
3	NE	173.3 ± 26.1	151.3 ± 42.5	128.9 ± 38.3	157.8 ± 26.1
Untreated	NE	194.3 ± 38.2	229.7 ± 65.6	130.6 ± 41.1	214.7 ± 38.8
5	NE	176 ± 36.6	158.3 ± 34.7	127.4 ± 16	195.8 ± 26.4
Untreated	NE	194.3 ± 38.2	229.7 ± 65.6	129.5 ± 28.1	214.7 ± 38.8
8	NE	186.6 ± 39.8	50.3 ± 15.9	145.3 ± 21.9	156.9 ± 43.1
Untreated	NE	259.8 ± 27.1	198.9 ± 54.9	129.5 ± 28.1	221.3 ± 45.9
10	NE	246.6 ± 39.2	41.3 ± 20.9	143.9 ± 17	41.6 ± 6.1
Untreated	248.8 ± 32.5	259.8 ± 27.1	198.9 ± 54.9	129.5 ± 28.1	221.3 ± 45.9
20	213.4 ± 34.6	219.7 ± 37.8	22.3 ± 10.5	161.9 ± 40	TOX

± Standard deviation of six different samples

NE no effect. *TOX* no more cells

eliminations were 20% (1 μM), 32% (5 μM), 78% (8 μM) and 89% (20 μM). $HgCl_2$ did not reduce the number of cells at any concentration for up to 2 h of incubation. Nonetheless, a toxic effect occurred after 24 h of treatment, when the elimination ranged from 23 to 28% at 1 and 8 μM and 82 to 100% at 10 and 20 μM.

These results show that $HgCl_2$ has no toxic effect until 2 h of incubation is reached, unlike $CdCl_2$. However, $HgCl_2$ at 10 and 20 μM for a 24 h period was more toxic than $CdCl_2$, indicating that the effects of mercury are not as immediate as those of cadmium (Table 1).

Effects of metals on intracellular parasites

The intracellular parasites were quantified after the metal treatments (Table 2). Incubation with $ZnCl_2$ at 20 μM for 24 h was able to eliminate 25% of the parasites. The $CdCl_2$ treatment for 2 h induced parasite eliminations of 17 (1 μM), 22 (3 μM), 35 (5 μM), 45 (8 μM), 42 (10 μM) and 48% (20 μM). After 24 h, the percentages of elimination were 50 (1 μM), 93 (8 μM) and 98% (20 μM). The $HgCl_2$ incubation for 2 h led to the elimination of 5% (1 μM), 62% (8 μM) and 49% (20 μM) of the parasites. After 24 h, the elimination rates were 21 (1 μM), 57 (3 μM), 60 (5 μM), 73 (8 μM), 88 (10 μM) and 100% (20 μM).

Table 2 Mean number of intracellular parasites in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$ at different times and concentrations

Infected culture toxicity					
	$ZnCl_2$	$CdCl_2$		$HgCl_2$	
(μM)	24 h	2 h	24 h	2 h	24 h
Untreated	NE	710.1 ± 40.5	790.5 ± 66.6	243.4 ± 41.4	790.5 ± 66.6
1	NE	593.8 ± 95.6	400.2 ± 76	231.8 ± 18	627.1 ± 47.3
Untreated	NE	240.2 ± 44.3	398.3 ± 63.4	243.4 ± 41.4	1091.9 ± 97.6
3	NE	188.2 ± 33.6	156.6 ± 50.9	216.9 ± 58.9	475.3 ± 44.8
Untreated	NE	240.2 ± 44.3	398.3 ± 63.4	243.4 ± 41.4	1091.9 ± 97.6
5	NE	157.9 ± 38.8	111.9 ± 23.2	194.3 ± 26.1	447.1 ± 85.2
Untreated	NE	240.2 ± 44.3	520.3 ± 62.4	286 ± 23	1091.9 ± 97.6
8	NE	132.1 ± 51.9	30 ± 8.1	178.3 ± 45	299.9 ± 67.6
Untreated	NE	710.1 ± 40.5	520.3 ± 62.4	286 ± 23	717.7 ± 164.6
10	NE	415.8 ± 95.3	12.5 ± 6	154 ± 23.4	88.926.9
Untreated	790.5 ± 66.6	710.1 ± 40.5	520.3 ± 62.4	286 ± 23	717.7 ± 164.6
20	595 ± 69.2	376.6 ± 44.3	8.6 ± 6.7	141.1 ± 30	TOX

± Standard deviation of three different samples

NE no effect; *TOX* no more remaining cells

Incubation with both $CdCl_2$ and $HgCl_2$ for 2 h led to significant parasite elimination (toxic effect) with no toxic effect to host cells, as seen above (Table 1). The results below show that at concentrations lower than 8 μM, the intracellular parasites were more resistant to $HgCl_2$; however, at 10 and 20 μM, both metals were very toxic (Table 2). Consequently, all intracellular parasites were eliminated after treatment with $CdCl_2$ and $HgCl_2$ at 20 μM for 24 h.

Here, we considered only infected host cells to demonstrate the progressive reduction of infection during the different treatments with metals. The percentages of infection decreased after the elimination of intracellular parasites. The results showed a reduction of infected cells but not of host cell number after treatment with $CdCl_2$ at 8 μM or $HgCl_2$ at 10 μM, indicating that the parasites were the main target of the metals. However, after metal treatments at high concentrations (10 and 20 μM), the percentage of infected cells increased, while that of uninfected cells decreased, suggesting that infected cells were more resistant. These results indicate that the intracellular parasites protect the host cell from the toxic effects of $HgCl_2$ and $CdCl_2$.

Structural and ultrastructural analyses involving metal incubations

On epimastigotes

The analyses by light microscopy showed that the untreated epimastigotes presented their typical morphological features such as an elongated body, flagellum and well-marked kinetoplast (Fig. 3a insert).

The parasites were treated with $CdCl_2$ or $HgCl_2$ at 8 μM (representing the intermediate elimination of 50% of non-essential metals) or with $ZnCl_2$ at 20 μM for 12 h. After incubation with $ZnCl_2$, the parasites presented their normal features as seen in the control (Fig. 3b). However, when the parasites were treated with $CdCl_2$ (Fig. 3c) or $HgCl_2$ (Fig. 3d), the epimastigotes began to present a rounded and condensed cell body.

The ultrastructural analyses showed the untreated epimastigotes with their usual morphology (Fig. 3a), while few changes were observed in the parasites treated with $ZnCl_2$ (Fig. 3b). However, after the $CdCl_2$ (Fig. 3c) and $HgCl_2$ (Fig. 3d) treatments, some intracellular vesiculation, alteration to structures of metabolic importance, such as glycosomes and acidocalcisomes, as well as drastic nuclear disorganization were observed.

Table 2 Mean number of intracellular parasites in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$ at different times and concentrations

Infected culture toxicity					
	$ZnCl_2$	$CdCl_2$		$HgCl_2$	
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NE no effect; *TOX* no more remaining cells

Incubation with both $CdCl_2$ and $HgCl_2$ for 2 h led to significant parasite elimination (toxic effect) with no toxic effect to host cells, as seen above (Table 1). The results below show that at concentrations lower than 8 μM, the intracellular parasites were more resistant to $HgCl_2$; however, at 10 and 20 μM, both metals were very toxic (Table 2). Consequently, all intracellular parasites were eliminated after treatment with $CdCl_2$ and $HgCl_2$ at 20 μM for 24 h.

Here, we considered only infected host cells to demonstrate the progressive reduction of infection during the different treatments with metals. The percentages of infection decreased after the elimination of intracellular parasites. The results showed a reduction of infected cells but not of host cell number after treatment with $CdCl_2$ at 8 μM or $HgCl_2$ at 10 μM, indicating that the parasites were the main target of the metals. However, after metal treatments at high concentrations (10 and 20 μM), the percentage of infected cells increased, while that of uninfected cells decreased, suggesting that infected cells were more resistant. These results indicate that the intracellular parasites protect the host cell from the toxic effects of $HgCl_2$ and $CdCl_2$.

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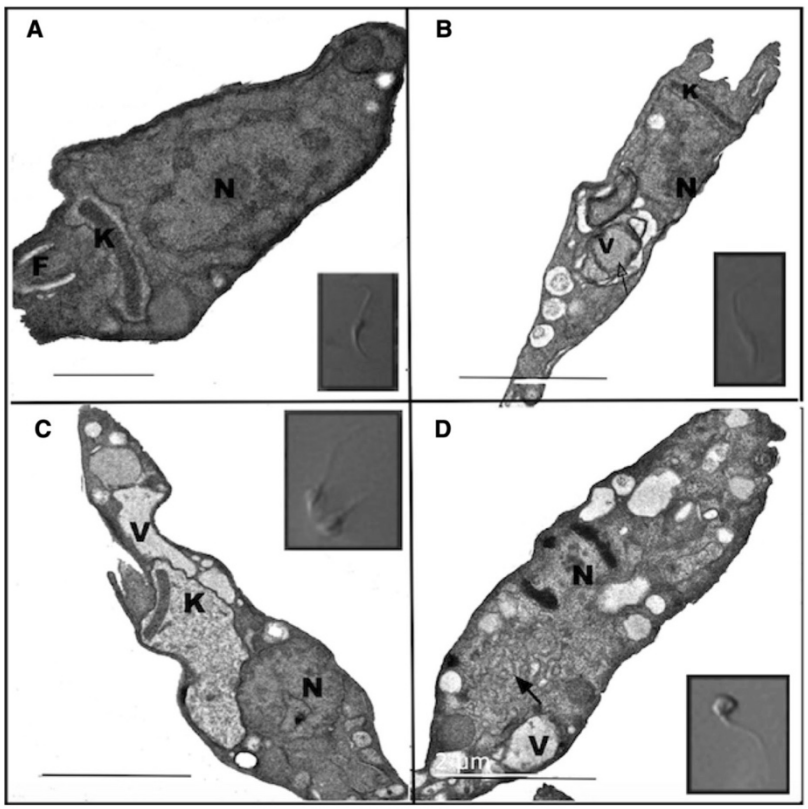

Fig. 4 Light (insert) and transmission electron microscopy showed ultrastructural and morphological features of trypomastigotes treated with $ZnCl_2$ (20 µM), $CdCl_2$, and $HgCl_2$ at 8 µM for 12 h **a** untreated trypomastigotes **b** trypomastigotes treated with $ZnCl_2$ **c** trypomastigotes treated with $CdCl_2$ **d** trypomastigotes treated with $HgCl_2$, *N* nucleus, *K* kinetoplast, *V* vesicular bodies. arrow: endomembrane system disorganization, scale bars: 2 µm

Reversibility assays

To verify whether low doses of $CdCl_2$ or $HgCl_2$ (3, 5 and 8 µM for 24 h) can cause an irreversible or reversible effect, the infected host cells were treated, washed and then incubated for an additional 24 h in a fresh medium.

The zinc treatment led to the progressive elimination of intracellular parasites (15% at 5 µM and 25% at 8 µM) and a reduction in infected cells (17% for 3 µM, 42% for 5 µM and 48% for 8 µM). In comparison, the cadmium reversibility treatment also clearly targeted the parasite at 3 µM, since its number dropped by 50%, while the host cell population remained stable, thereby resulting in an increase in uninfected cells after the elimination of parasites. After $CdCl_2$ at 5 µM, the intracellular parasite elimination was 72%, and at 8 µM, it was 78%. However, a reduction in host cell number (42 and 63%, respectively) occurred. In the case of mercury, the elimination of parasites was 21% at 3 µM, 42% at 5 µM and 55% at 8 µM. Although there was no cellular elimination at 5 µM, a reduction of 23% at 8 µM was seen (Table 4).

Furthermore, the reversibility and direct toxicity assays were compared. No toxic effect was observed after the zinc treatments under any of the conditions tested (Fig. 8a) (Fig. 9a, d, g, j). After the cadmium reversibility assay at 3 µM, the host cells were able to recover their viability, resulting in no toxic effect, unlike in the direct toxicity assay (Fig. 8b) (Fig. 9e). However, the direct and reversibility assays at 5 µM led to similar effects (Figs. 8b, 9h). Nonetheless, the reversibility assay at 8 µM increased cell survival from 20 to 40% (Figs. 8b, 9k). Regarding mercury, the reversibility assay was toxic to host cells only at 8 µM (Fig. 9l), unlike the direct toxicity assay, which was toxic from 3 µM. In this case, the reversibility assay at 8 µM was more toxic (60% of cell elimination) than the direct toxicity assay (25% of cell elimination),

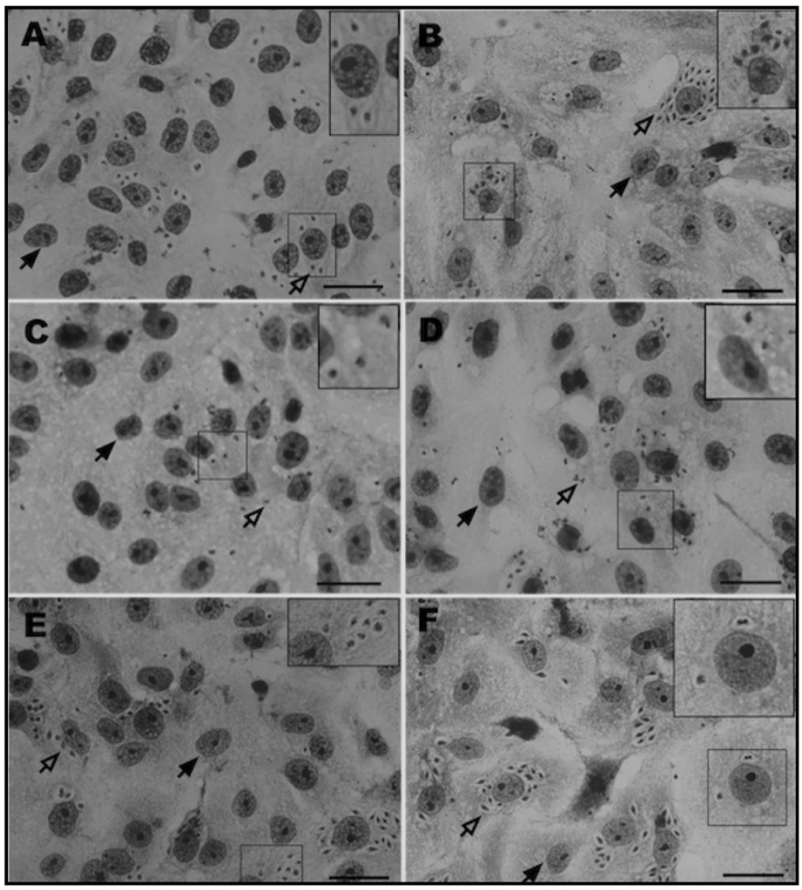

Fig. 5 Morphological aspects of infected host cells treated with metals for 2 h **a** untreated infected cells, **b** infected cells treated with $ZnCl_2$ at 20 μM, **c** infected cells treated with $CdCl_2$ at 3 μM, **d** infected cells treated with $CdCl_2$ at 8 μM, **e** infected cells treated with $HgCl_2$ at 3 μM, **f** infected cells treated with $HgCl_2$ at 8 μM, black arrows: host cell nucleus, white arrow: intracellular amastigotes, scale bars: 50 μm

indicating progressive and irreversible damage (Fig. 8c).

In the case of the parasites, the zinc reversibility assays were able to eliminate them, unlike the direct toxicity assays, indicating that 24 h of treatment was not enough to eliminate the parasite but caused irreversible damage (Fig. 8d). Nonetheless, in the case of cadmium and mercury, the direct toxicity assays were more efficient in eliminating the parasites (Fig. 8e, f).

The influence of $ZnCl_2$ preincubation on $CdCl_2$ and $HgCl_2$ toxicity

To test whether the essential metal ($ZnCl_2$) confers resistance to the host cells or parasites against non-essential metal toxicity ($CdCl_2$ and$HgCl_2$), the cultures were incubated with $ZnCl_2$ at 20 μM for 24 h, washed and then incubated with $CdCl_2$ or $HgCl_2$ at 8 μM for another 24 h.

After the $CdCl_2$ incubation, 85% of the parasites survived, while after $HgCl_2$, only 60% of the parasites survived, showing that $ZnCl_2$ has a more protective effect against $CdCl_2$ than $HgCl_2$. These results showed that the $ZnCl_2$-treated epimastigotes were more

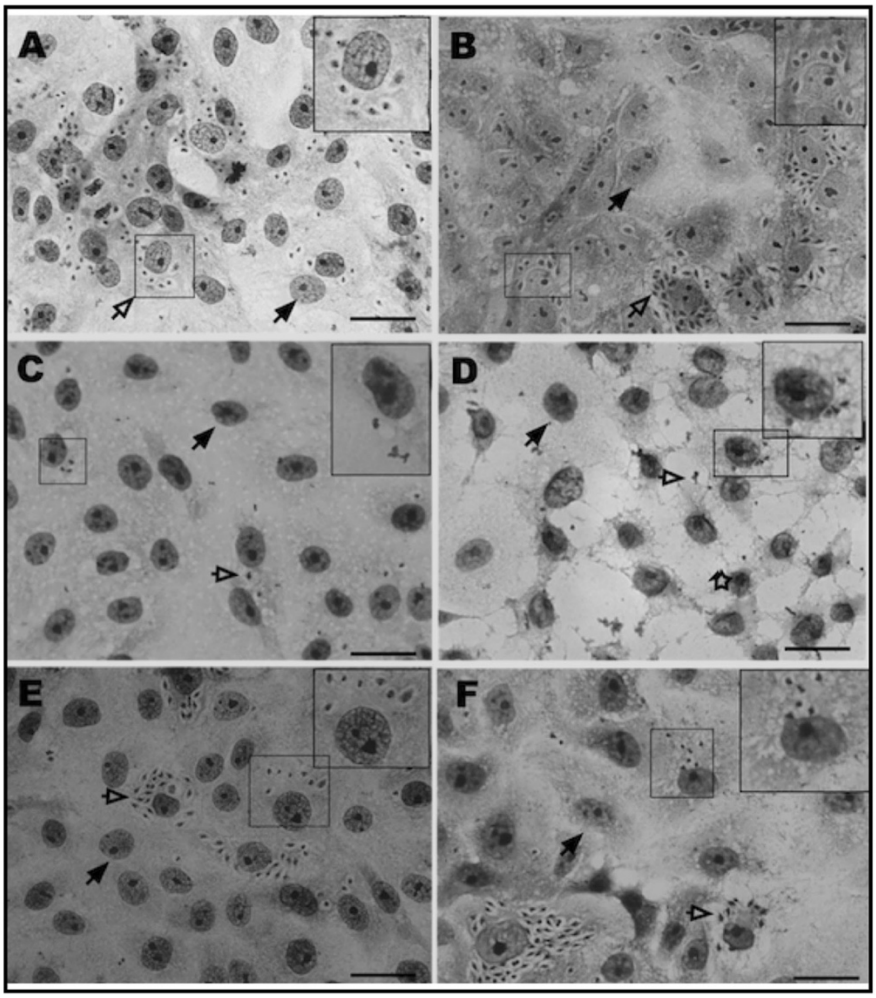

Fig. 6 Morphological aspects of infected host cells treated with metals for 24 h **a** untreated infected cells, **b** infected cells treated with $ZnCl_2$ at 20 μM, **c** infected cells treated with $CdCl_2$ at 3 μM, **d** infected cells treated with $CdCl_2$ at 8 μM, **e** infected cells treated with $HgCl_2$ at 3 μM, **f** infected cells treated with $HgCl_2$ at 8 μM, black arrows: host cell nucleus, white arrow: intracellular amastigotes, star: dead cell, scale bars: 50 μm

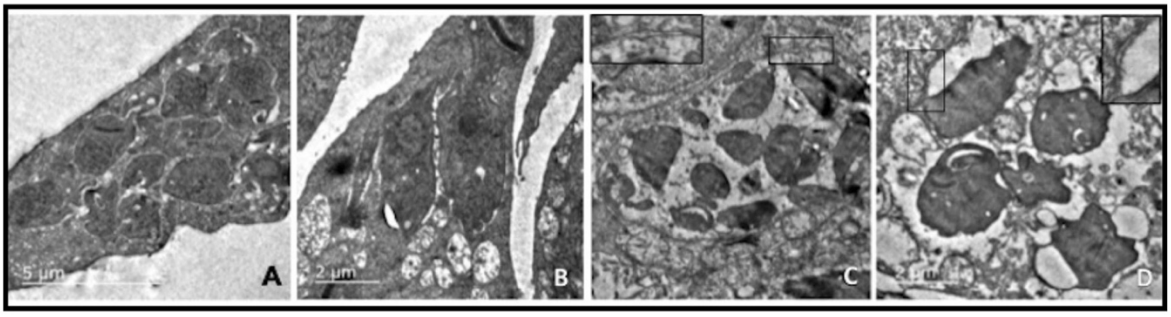

Fig. 7 Transmission electron micrographs of host cells infected with intracellular *Trypanosoma cruzi* and incubated with different metals **a** untreated infected host cells, **b** infected host cells treated with $ZnCl_2$ at 20 μM, **c** infected host cells treated with $CdCl_2$ at 5 μM, **d** infected host cells treated with $HgCl_2$ at 5 μM, all treatments lasted 12 h, scale bars: **a** 5 μm; **b** 4 μm; **c**, **d** 2 μm

Table 3 Lethal dose values to epimastigotes, trypomastigotes, intracellular form and host cells after the different treatments with metals

	LD 50 (μM)						
	$ZnCl_2$	$CdCl_2$			$HgCl_2$		
	24 h	2 h	12 h	24 h	2 h	12 h	24 h
Epimastigotes	> 20	18	15	10	8	6.5	8
Trypomastigotes	> 20	> 20	> 20	20	> 20	15	8
Infected culture							
Cells	> 20	> 20	ND	6.2	>20	ND	9
T. cruzi	> 20	20	ND	4	20	ND	2.5

ND not determined

Table 4 Reversibility assays

	Reversibility assays			
	Concentration (μM)	Host cell		Parasites
		Uninfected	Infected	
$ZnCl_2$	Untreated	89.7 ± 9.8	74.6 ± 3.9	279.2 ± 15.8
	3	109.6 ± 13.3	62.7 ± 6.1	283 ± 13.1
	5	125.9 ± 12.4	43.4 ± 20.6	237.1 ± 26.1
	8	130 ± 11.4	38.9 ± 20.4	211.6 ± 31.1
$CdCl_2$	Untreated	76.5 ± 14.8	72.4 ± 16.1	284.1 ± 59
	3	92.8 ± 18.5	52 ± 14.9	161 ± 42.4
	5	89.7 ± 9.5	45.6 ± 5.1	106.2 ± 41.6
	8	51.1 ± 17.1	23.8 ± 10	85.2 ± 65.5
$HgCl_2$	Untreated	91.7 ± 8.8	60.8 ± 2.8	242.1 ± 5.8
	3	141.9 ± 23.6	58.7 ± 7	191.4 ± 64.7
	5	148.9 ± 47.4	38.3 ± 16.6	140.4 ± 44.8
	8	79.2 ± 26.3	38.9 ± 20.4	110.8 ± 65.5

Infected host cells were treated with different concentrations of $CdCl_2$ and $HgCl_2$ for 24 h, washed and cultivated for an additional 24 h in a metal-free medium

resistant to the toxic effects of non-essential metals (Fig. 10a). The respective micrographs showed the untreated culture with the usual morphology (Fig. 10b), whereas the cultures treated with $CdCl_2$ (Fig. 10c) or $HgCl_2$ (Fig. 10d) had rounded and vacuolated parasites.

Regarding the trypomastigote form, the preincubation with $ZnCl_2$ protected it from the toxic effect of $CdCl_2$ at approximately 95%. Nonetheless, the same did not occur with $HgCl_2$, and the parasites pretreated with $ZnCl_2$ had a survival rate of only 21% (Fig. 11a). As observed by light microscopy, the control (Fig. 11b) trypomastigotes showed the common morphological features, as well as the $CdCl_2$-treated parasites (Fig. 11c), but the $HgCl_2$-treated trypomastigotes presented a rounded and unusual form (Fig. 11d).

In the case of the infected cells, the 24 h treatment with $CdCl_2$ or $HgCl_2$ at 8 μM led to 80 and 30% cell elimination, respectively. Meanwhile, treatment with $ZnCl_2$ at 20 μM had no significant toxic effects, in comparison to control (100% of survival). However, when the infected cultures were incubated with $ZnCl_2$ at 20 μM for a 24 h period, and the culture medium was replaced by another one containing $CdCl_2$ or $HgCl_2$ (8 μM) for an additional 24 h, the toxic effects of the non-essential metals decreased (Fig. 12a). The most obvious protection was against the $CdCl_2$, where the percentage of cell survival was close to 100%. A protective effect of $ZnCl_2$ on $CdCl_2$ incubation was also observed in the intracellular parasites, in which the percentage of survival increased from 7 to 40%. It is unlikely that $ZnCl_2$ protected the host cells against $HgCl_2$ (90% survival) but did not protect the parasites. These results suggest that $ZnCl_2$ has protective activity against cellular destruction (Fig. 12b, c).

The protective effects of $ZnCl_2$ for the intracellular parasites and host cells are shown in Fig. 12d, e. The

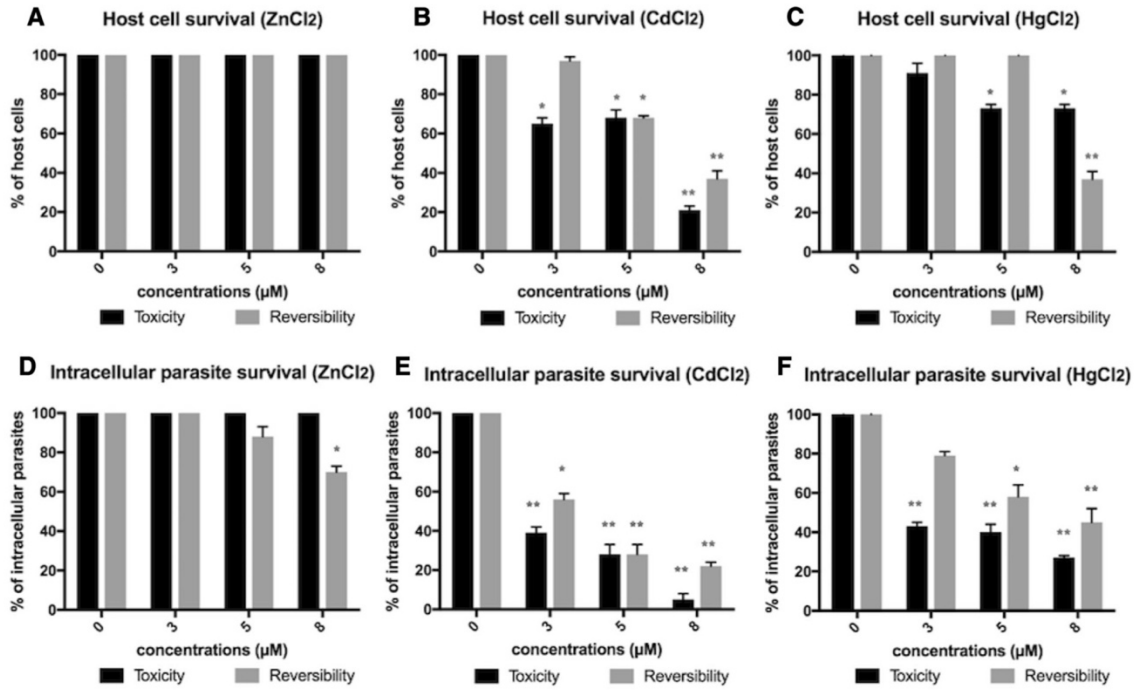

Fig. 8 Graphical representation of comparative effects of toxicity and reversibility (24 h of metal treatment + 24 h growing in metal-free medium) assays of infected host cells and intracellular parasites at different concentrations (3, 5 and 8 μM) of $ZnCl_2$, $CdCl_2$ and $HgCl_2$

cells still presented their usual morphological features, a dense population, and some intracellular parasites after incubation with $CdCl_2$ (Fig. 12d) or $HgCl_2$ (Fig. 12e), but the number of parasites were lower than in the control cultures (Figs. 5, 6a—above).

Influence of metal preincubation on trypomastigote invasion

To investigate whether metal-pretreated trypomastigotes were able to infect the host cells, we used three different tests with metals at 5 μM for 2 h: (1) the host cells were treated, washed and infected with untreated parasites; (2) the parasites were treated, washed and incubated with untreated cells, and (3) both cells and parasites were treated separately, washed and incubated together for 48 h (Fig. 13; Table 5).

The control culture (untreated cells and untreated parasites) had a high number of intracellular parasites (Fig. 13a–c; Table 5), and similar results were observed under all conditions after the pretreatments with $ZnCl_2$ (Fig. 13d, g, j).

When only the trypomastigotes were treated with $CdCl_2$ before infection, low numbers of infected cells (11%) and intracellular parasites (5%) were observed (Fig. 13e). Similar values were observed when the host cells and trypomastigotes were treated simultaneously (Fig. 13k; Table 5). However, when only host cells were treated, no difference was observed in comparison with the untreated ones, indicating that the effects were restricted to the parasites (Fig. 13h).

When only the trypomastigotes were treated with $HgCl_2$, the number of intracellular parasites decreased to 48%, while the number of infected cells dropped to 40% (Fig. 13f). Again, when only the host cells were incubated with $HgCl_2$, no difference was observed (Fig. 13i), and the culture behaved like those that were untreated (Table 5). In all cases, no significant difference in the total number of cells was seen, indicating that the treatments were not toxic to the host cells (Table 5).

These results suggest that $CdCl_2$ significantly impairs parasite invasion and likely disrupts important mechanisms for host cell recognition and adhesion.

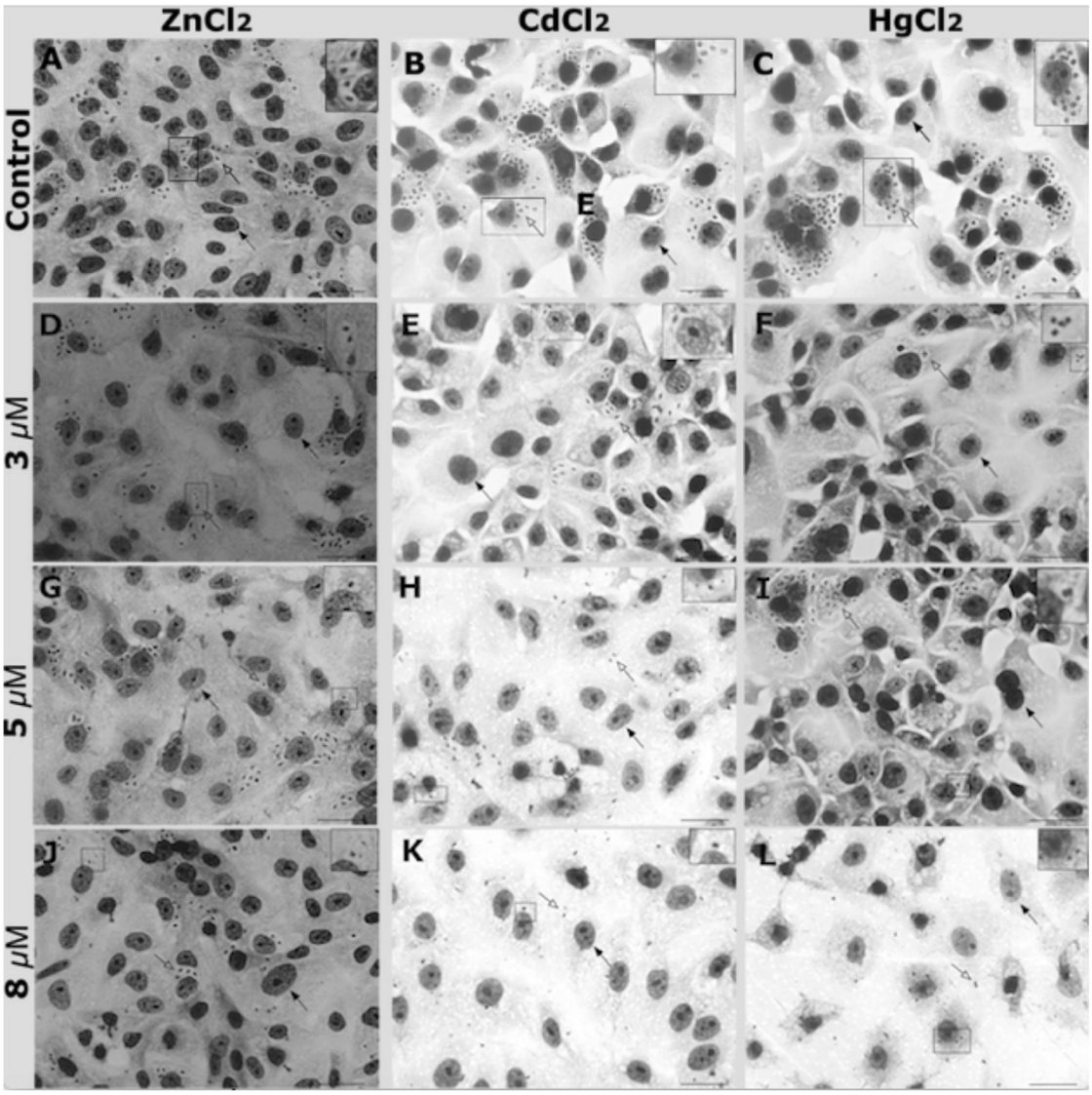

Fig. 9 Light micrograph of the reversibility assays: The infected cultures were incubated with $ZnCl_2$, $CdCl_2$ and $HgCl_2$ at 3, 5 and 8 μM for 24 h, washed and incubated for another 24 h in a metal-free medium, scale bars: 50 μm

Discussion

Intracellular parasites depend on host cell metabolism to survive, and, based on this, the interaction between parasites and host cells is strictly regulated to guarantee the establishment of infection and propagation. Nonetheless, unbalanced *T. cruzi* infection has caused many deaths annually due to the self-limiting effects (Santos et al. 2012). As a result, many research groups have been studying the various advancements in pathogenic disease chemotherapy using metal-complexed drugs, as metals are known to have potential pharmacological properties, to coordinate binding and increase fluidity and redox activity (Sánchez-Delgado and Anzellotti 2004). In addition, metals are essential elements for cell metabolism and replication, and their flux control is fundamental to cell survival. Despite the increase in inorganic compounds as candidates for

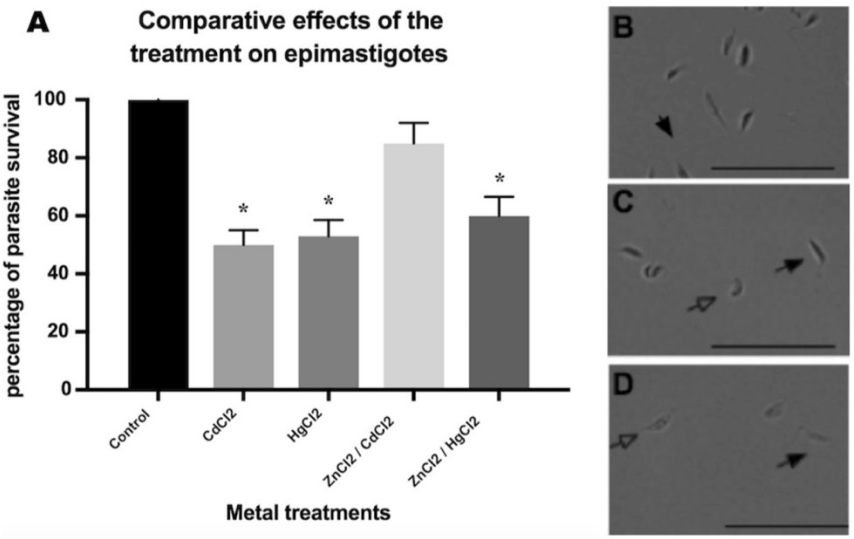

Fig. 10 Graphical representations and light micrographs of metal-treated epimastigotes **a** the percentage of epimastigote survival after treatment with $CdCl_2$ or $HgCl_2$ at 8 µM over 24 h or $ZnCl_2$ at 20 µM over 24 h followed by $CdCl_2$ or $HgCl_2$ at 8 µM within a 24 h period, **b** untreated parasites, **c** $ZnCl_2$ followed by $CdCl_2$ and **d** $ZnCl_2$ followed by HgCl2, black arrow: parasites, white arrows: altered parasites, scale bars: 100 µm

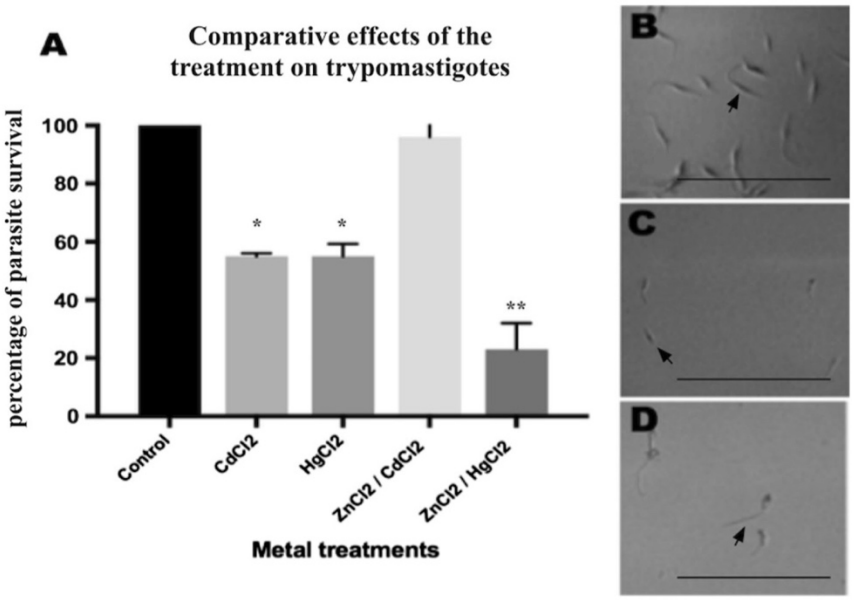

Fig. 11 Graphical representations and light micrographs of metal-treated trypomastigotes **a** the percentage of epimastigote survival after treatment with $CdCl_2$ or $HgCl_2$ at 8 µM over 24 h or $ZnCl_2$ at 20 µM over 24 h followed by $CdCl_2$ or $HgCl_2$ at 8 µM within a 24 h period, **b** untreated parasites, **c** $ZnCl_2$ followed by $CdCl_2$ and **d** $ZnCl_2$ followed by $HgCl_2$, black arrow: parasites, scale bars: 100 µm

drug therapy, there have been few studies about their toxic effects, including the biophysical and biochemical pathways used to intoxicate intracellular protozoan parasites. Therefore, understanding the antiparasitic effects of metals is very important for improving the design of metal-based agents (Lemire et al. 2013).

Zinc is an essential trace element that is found ubiquitously within cells and is present in more than 300 enzymes (Parkin 2004). The biological functions of zinc can be divided into three main groups: catalytic, structural and regulatory (Roohani et al. 2013). The other important roles of zinc are to protect cells against non-essential metals by triggering the up-regulation of cellular elements that have the ability to trap them (Maret and Vallee 1998) or to function as an antioxidant (Bray and Bettger 1990). Although zinc can be indispensable in cell homeostasis, it is toxic in high quantities, and because of this, its concentrations are tightly regulated (Eide 2006). Our results showed

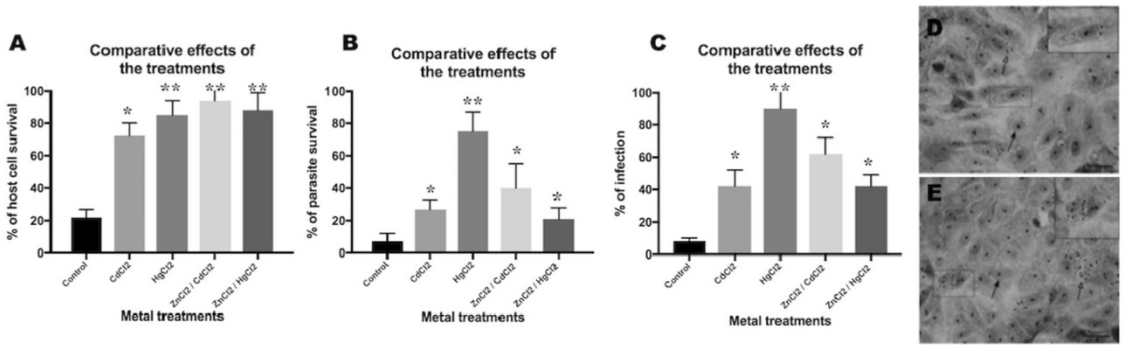

Fig. 12 Comparison of the effects of the isolated or sequential treatments with $ZnCl_2$ (20 μM/24 h), $CdCl_2$ (8 μM/24 h) and $HgCl_2$ (8 μM/24 h) on **a** infected cultures, **b** parasites and **c** percentage of infected cells, **d** $CdCl_2$ at 8 μM for 24 h, **e** $HgCl_2$ at 8 μM for 24 h, black arrows: cell nucleus, white arrows: parasites, scale bars: 50 μm

that the toxic effects of zinc are absent or low for all three main forms of *T. cruzi* and for the host cells (Figs. 1, 2; Tables 1, 2). In addition, zinc led to parasite resistance, mainly in the case of cadmium (Figs. 11, 12, 13).

On one hand, the usual presence of non-essential metals in the environment and, consequently, the contact of cells and parasites with these elements causes serious but little-described toxic effects to cells. In this study, the parasites and host cells were all susceptible to the non-essential metals cadmium and mercury chlorides but in different ways. As shown, the host cells were the most resistant against the non-essential metals, and many of them were able to reverse the effects at low concentrations (μM), Followed by the trypomastigotes, the intracellular forms and the epimastigotes, with the latter being the most vulnerable under the same conditions. The results also showed that cadmium was the most toxic to the host cells, while mercury was the most toxic to the parasites (Figs. 1, 2; Tables 1, 2). On the other hand, trypomastigotes treated with cadmium lost their great capability to infect the host cells. Although there is no specific mechanism for the entrance of non-essential metals, in general, they enter the intracellular milieu through the pathways used by the essential metals and substitute them in structures and reactions (Martinez-Finley et al. 2012). As result of these interactions, metals can cause the oxidative deterioration of biological molecules through the generation of free radicals, the modification of DNA bases and lipid peroxidation, resulting in cell death (Valko et al. 2006; Jomova and Valko 2011). In the case of cadmium, the main mechanism of entry is through divalent metal ion transporters, mainly for calcium, due to its similar ionic radius (Moulis and Thévenod 2010; Moulis 2010), and for zinc, which is located in the same column of the periodic table and has similar physico-chemical properties (Rousselet et al. 2008). Once inside the cells, the cadmium affinity for thiol groups and its chemical mimicry of nutritional divalent metals induce competition for these ion metabolic pathways and binding sites, impairing biochemical pathways such as redox homeostasis, DNA repair and signal transduction (Waisberg et al., 2003). In the case of mercury, its ability to cross lipid membranes (Girault et al. 1997) and its high affinity for the thiol groups present in several biomolecules can cause broad effects in cellular and biochemical processes (Maret and Vallee 1998).

The mammalian cells were more resistant to the toxic effects of the non-essential metals than the parasites. It is known that mammalian cells have evolved highly sophisticated mechanisms of uptake, storage, quelation and secretion to maintain metallic ion concentrations (Andreini et al. 2008). These mechanisms involve chaperones, metal-responsive gene regulators, transmembrane transporters, glutathiones, catalases, metallothioneins, and dismutase superoxide. The latter three are responsible for quelating metals and for oxidative defense (Fairlamb and Cerami 1992; Formigari et al. 2007; Reyes-Caballero et al. 2011). These pathways help the host cell to address the non-essential metal quota, but the knowledge about the metal adaptation mechanisms of *T. cruzi* still remains inadequate. In addition, the

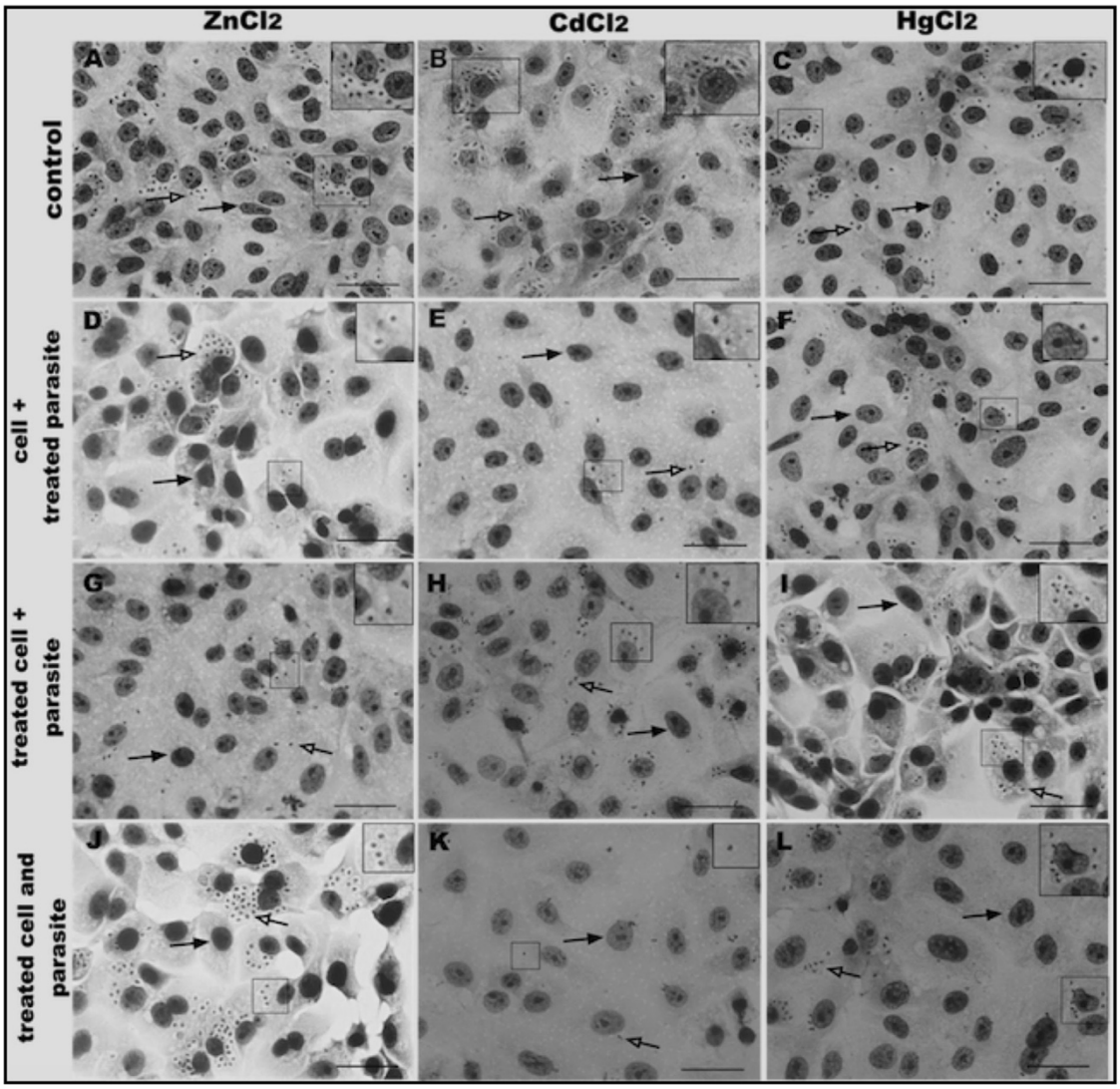

Fig. 13 Micrographs of the invasion assays, host cells, parasites or both were treated with 5 μM for 2 h, washed and incubated together for 48 h

oxidative defense of this parasite is restricted and specific, including only trypanothione instead of catalase and glutathione reductase activity like other eukaryotic cells (Wilkinson et al. 2002). All these adaptive mechanisms interact and increase the capacity of parasites and host cells respectively, to recover their ability to replicate after incubation with low concentrations of non-essential metals.

The results showed that the *T. cruzi* forms are very susceptible to the considered non-essential metals and, to a lesser extent, to zinc (Figs. 1, 2). The parasites responded differently to the metal treatments according to their morphological stages, showing important survival mechanisms as a consequence of the adaptation to environmental change (Nogueira et al. 2015). The epimastigote form, found in the midgut of the insect vector, was the stage that was most susceptible to the metal treatments, followed by the intracellular forms, which are found in niches spread throughout the cytoplasm. These two replicative forms do not

Table 5 Invasion assays

Invasion assays				
	Cell + *T. cruzi*	Cell + Treated *T. cruzi*	Treated Cell and *T. cruzi*	Treated Cell + *T. cruzi*
$ZnCl_2$				
Infected cells	135.7 ± 19	122.4 ± 17.2	129.7 ± 10.7	146.6 ± 11.5
Uninfected cells	51.4 ± 3.7	58 ± 4.7	53.6 ± 6.5	50.6 ± 4.8
Total of cells	187.1 ± 22.7	180.4 ± 21.9	183.2 ± 16.8	196.6 ± 16.3
Parasites	330.6 ± 61.1	283.4 ± 44.7	291.7 ± 31.7	289.8 ± 2.6
$CdCl_2$				
Infected cells	126.8 ± 14.7	13.2 ± 3.1	13.7 ± 2.3	139.1 ± 30.5
Uninfected cells	60.9 ± 17.3	189.9 ± 18.4	173.4 ± 20.3	65.2 ± 12.5
Total of cells	187.7 ± 32	203.1 ± 21.5	187.1 ± 22.7	204.3 ± 43
Parasites	296.3 ± 28.4	12.8 ± 2.1	14.1 ± 3.9	343.4 ± 50
$HgCl_2$				
Infected cells	155.2 ± 9.8	92.3 ± 5.6	105.8 ± 20.7	138.1 ± 3.5
Uninfected cells	69.4 ± 14.1	111.3 ± 10.4	89.3 ± 7.7	81.9 ± 7.2
Total of cells	224.7 ± 24.9	203.6 ± 16	194.2 ± 28.4	220 ± 10.6
Parasites	288.6 ± 15.5	152.2 ± 27.4	224.8 ± 43.7	325.9 ± 14.2

Host cells, parasites or both were treated with 5 μM for 2 h, washed and incubated together for an additional 48 h

endure a harsh environment, nor do they encounter the host microbicidal mechanisms, demonstrating adaptive and protective responses (Tyler and Engman 2001). Furthermore, the trypomastigote form is subjected to substantial physiological changes upon reaching the anterior midgut of the insect vector, including osmolarity changes, lower temperatures and digestive enzymes (Kollien and Schaub 2000), and when it reaches the bloodstream of the mammalian host, it faces against microbicidal defense (Tyler and Engman 2001). The need for rapid adaptations to avoid elimination makes the trypomastigotes more resilient in the harsh environment (Ferreira et al. 2016), mainly through differences in membrane composition and protein expression (Souza et al. 2010), which could make them more resistant to the non-essential metals.

Although the trypomastigote was the most resistant morphological form, its incubation for 2 h with a sublethal cadmium concentration almost completely blocked the parasite's ability to infect the host cells, although a less obvious effect was seen with mercury and zinc (Table 5; Fig. 11). The parasite invasion process is mediated by molecules present at the surface of both the host and parasite cells that function as receptors/ligands, triggering bidirectional cascades and leading to calcium release in both the parasite and cell, resulting in parasite entry (Villalta et al. 2009). The fact that cadmium has a high affinity for surface receptors and perturbs many intracellular signaling pathways, such as cAMP-dependent and calmodulin-dependent pathways (Moulis 2010), can be an explanation for the impairment of parasite entry, since the invasion process is dependent on them.

Zinc is an important trigger of important mechanisms addressing metal quotas and balances, including metallothionein superexpression (Klaassen et al. 1999) and antioxidative defenses (Bray and Bettger 1990). Metallothioneins are metalloenzymes known for their high concentrations of cysteine residues and high affinity for transition metals, including cadmium, copper, cobalt, mercury and zinc, and due to the presence of many thiol ligands (Butcher et al. 2003). They are described in both mammalian cells and *T. cruzi*, although with differences in stage-specific expression (Maya et al. 2004). The activities of metallothioneins include nitrogen and oxygen reactive species sequestration (Yoshida et al. 2005), zinc intracellular trafficking (Feng et al. 2005) and protection against apoptosis (Shimoda et al. 2003; Santon

et al. 2004). The quantity of metallothionein is adaptive to the cellular environment and can increase or decrease according to metal availability (Suhy et al. 1999). To verify whether our host cells and parasites responded to a sublethal dose of zinc and whether this promotes non-essential metal resistance, we preincubated them with zinc before cadmium or mercury. The host cells and all three forms of the parasite became more resistant to cadmium (Figs. 11, 12, 13). The fact that zinc is a metallothionein expression and antioxidative defense inductor can be related to the resistance against non-essential metals. In addition to this, zinc transporters, composing an important mechanism of cadmium entry, can be blocked due to high concentrations of zinc, impairing cadmium entrance. These explanations help to explain why the preincubation with zinc did not significantly alter the toxic effect of mercury, which passes freely through membranes and is not preferably quelated by metallothionein but by glutathiones (Sears 2013).

A previous work using the same metals against uninfected and *Toxoplasma gondii* infected by Vero cells presented results similar to those in this study. For example, the intracellular tachyzoites were also more susceptible to metals than the host Vero cell, with LD 50 values being similar to those of the intracellular form of *T. cruzi* after the cadmium treatments. Nonetheless, in the case of mercury, more tachyzoites were eliminated than the intracellular *T. cruzi* after 2 h of incubation, but at 24 h, the lowest LD was recorded for *T. cruzi*. In the case of infected Vero cells, the LD 50 values were the same under all conditions (Carvalho and Melo 2016).

Once inside the host cell, invading pathogens must acquire all essential nutrients, including transition metals, from the host to maintain their high metabolism during proliferation. Naturally, the host cells use this need as a defense mechanism against the parasites, limiting or replacing the essential metals (Morey et al. 2015). Therefore, understanding the aspects involved in host cell resistance and parasite susceptibility will potentially reveal new targets for novel chemotherapy. This study showed some aspects involved in parasite death and elimination in the presence of different metals. Further studies are necessary to better understand the effects of parasite elimination and the mechanisms or absence of them in parasite resistance or vulnerability.

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3.2 Intracellular development of *Trypanosoma cruzi* in the presence of metals

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ORIGINAL ARTICLE

Intracellular development of *Trypanosoma cruzi* in the presence of metals

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Abstract *Trypanosoma cruzi* is transmitted to vertebrate hosts during the feeding of blood-sucking insects. After the invasion of host cells, the parasite resides within the parasitophorous vacuole until to escape to host cytoplasm and to proliferate, establishing an infection. Studies demonstrated that some intracellular parasites have to acquire all essential nutrients as well as transition metals from the host cell to be pathogenic, to maintain the homeostasis and to replicate. The present study investigated the progressive steps of the intracellular parasite development and establishment of infection in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$. LLC-MK2 cells were infected with trypomastigotes during 6–84 h to investigate the steps of intracellular parasite development. After the host cells were infected during 12 h and treated with metals during 24 or 60 h or they were treated for 24 h and cultured for 72 h more to observe the reversibility. The results showed that the non-synchronous invasion of trypomastigotes resulted in an increasing number of intracellular parasites in intermediary forms (until 24 h post-infection), the appearance (from 36 h) and proliferation (84 h) of the amastigotes. The 24 h-treatments were not enough to impair parasite escape to the host cytoplasm and reproduction. However, 60 h of incubations led to a significant reduction in parasite numbers, as well as the reversibility assays. In conclusion, new insights about the intracellular *T. cruzi* development in the presence of metals were provided, and further studies should be performed to investigate the events involved in parasite death and elimination.

Keywords Cadmium · Mercury · Parasitophorous vacuole · *Trypanosoma cruzi* · Zinc

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Introduction

Trypanosoma cruzi, the causative agent of Chagas' disease, is a protozoan parasite of the Trypanosomatidae family. The parasite life cycle has different stages involving epimastigotes and metacyclic trypomastigotes in the triatomine insect vector, and blood trypomastigotes and intracellular amastigotes in vertebrate hosts, including humans. Trypomastigotes—the infective forms—adhere to the host cell surface via molecules that work as receptor-ligands, invade the cells and reside temporarily within the parasitophorous vacuole (PV) on host cytoplasm, as reviewed in (de Souza et al. 2010). After many hours, the trypomastigotes escape from the PV to cytoplasm where they undergo morphological changes to the replicative forms—amastigotes. After successive divisions, the amastigotes differentiate back to trypomastigotes, rupture the cells and reach the bloodstream from where they can invade new cells, establishing an infection (Tyler and Engman 2001).

The processes of parasite internalization, changes in parasite morphology and establishment of infection are orchestrated by metalloproteins (Alvarez et al. 2012). In addition to this, metal ions play important roles in host-parasite interactions (Weinberg 1966), but they are still poorly described.

Metals are divided into essentials and non-essentials according to their functions to the organisms (Martinez-

Finley et al. 2012). The essential ones, like zinc, are present in many proteins and enzymes as structural component or co-factor, assisting for the cell homeostasis, although their concentrations are tightly regulated to avoid toxicity (Formigari et al. 2007). On the other hand, non-essential metals as cadmium and mercury have any function to the organisms, but are also present on the environment and can get the intracellular milieu through routes destined to the essential ones, causing a range of toxic effects (Martinez-Finley et al. 2012).

Currently, metal ions have been drawn attention due to pharmacological properties, and many metallo-drugs have been synthesized and tested against a wide variety of diseases, including Chagas disease (Vieites et al. 2008; Benítez et al. 2011; Martins et al. 2012). Nonetheless, deeper studies about the role of metals in host cell-parasite interactions lack, as well as about the influence of metals on parasite development and the establishment of infection. Recently, two studies investigating the role of zinc, cadmium and mercury chlorides in host-parasite interactions were published (de Carvalho and de Melo 2017), concerning the *Toxoplasma gondii* and *T. cruzi*, respectively. In the last study, extra- and intracellular proliferative *T. cruzi* were incubated with these metals and the results showed that all parasite forms were susceptible to metal incubations in conditions not toxic to the host cells.

In this context, this study was carried out to investigate whether early metal incubations influence the escape of intracellular parasites from the PV to the host cytoplasm and the parasite proliferation.

Materials and methods

Host cell

LLC-MK2 (kidney fibroblasts of *Macaca mulatta*) (the cells were provided by the Institute of Biophysic Carlos Chagas Filho, Federal University of Rio de Janeiro) were grown in plastic Falcon flasks (25 cm^2) containing RPMI 1640 (Sigma®) medium supplemented with 5% fetal calf serum (FCS) (Sigma®). The cultures were treated with trypsin when the cell densities approached the monolayer. For experimental proposals, the cells were placed on Linbro 24-well plates with a sterile coverslip at a density of 3×10^4 cells per well or in medium flasks (3×10^6 cells). The cells were allowed to attach for 24 h at 37 °C in a 5% CO_2 atmosphere (Gomes et al. 2012). Then, the host cells were infected during different times and submitted to treatments with $ZnCl_2$, $CdCl_2$ and $HgCl_2$.

Parasite maintenance

Epimastigotes of *T. cruzi* (DM28 strain) were cultivated in Liver Infusion Tryptose (LIT) (Fluka®) medium supplemented with 0.4% of hemin and 10% FCS at 28 °C. Every 5 days, a 1 mL aliquot of parasite-containing medium was transferred to a new tube and the volume completed to 5 mL with fresh culture medium (de Carvalho and de Melo 2017).

Trypomastigotes of *T. cruzi* were obtained from transformation of epimastigotes. The epimastigotes were centrifuged at 500 g for 10 min and the pellet homogenized in RPMI 1640 supplemented with 10% FCS and incubated at 37 °C for 48 h. After this time, around 90% of the parasites were in the form of trypomastigote. For experimental purpose, the parasites were centrifuged at the same condition and homogenized in 1 mL of RPMI 1640. An aliquot of 0.1 mL was scored at Neubauer chamber and a rate of 20:1 parasite: cell was used to infected the culture. After 5–6 days of infection, the host cell lysis occurred and the trypomastigotes were released into the supernatant. So, the supernatant was collected, centrifuged as described above and new cultures were infected (de Carvalho and de Melo 2017).

Metal treatments

Dilutions of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ salts originated 0.1 M stock solutions in ultra-pure quality water. The final concentrations were prepared to dilute the stock solution with the medium.

These times and concentrations were based on the paper published by others (de Carvalho and de Melo 2016, 2017) where the same uninfected cells were treated at the same conditions, establishing the parameters for the next studies.

Intracellular parasite development

The host cells were infected with trypomastigotes during times ranging from 6 to 84 h to observe the progressive steps of intracellular development.

Toxicity assays

The host cells were infected during 12 h (before parasite escape) and treated with $ZnCl_2$ at 20 μM or $HgCl_2$ and $CdCl_2$ at 1 μM during 24 or 60 h to investigate whether metals impair the parasite escape from the PV to the host cytoplasm and proliferation.

Reversibility assays

The host cells were infected for 12 h, treated with $ZnCl_2$ at 20 µM or $HgCl_2$ and $CdCl_2$ at 1 µM during 24 h. Thus, the medium was changed to a drug-free medium and the infected cells were cultured for 24 h to observe whether metal incubations induced reversible or irreversible toxic effects to the remaining parasites.

Cell quantification and morphological analyses

After treatments, the coverslips containing cells were rinsed in PBS, fixed in Bouin's solution for 5 min and stained with Giemsa's solution (diluted in PBS, pH 7.2, 10%, v/v) during 6 h at room temperature. The coverslips were mounted on glass slides with Entellan (Merck®) for observation by light microscopy. The extracellular parasites were centrifuged and the pellets were suspended in formaldehyde 4% (w/v) for 30 min and rinsed with PBS, pH 7.2. An aliquot of parasites was put on glass slides. To examine all preparations a Zeiss Axioplan microscope, equipped with 20× and 40× objectives was used.The Analysis System software to obtain the images. Three random fields of each of six samples (individual treatments) of infected cells were scored to the following parameters: (1) uninfected host cells; (2) infected host cells and (3) number of intracellular parasites. The observation of morphological changes and reduction of cell and parasite numbers indicated the metal cytotoxicity (de Carvalho et al. 2013).

Ultrastructural analyses

For this purpose, infected LLC-MK2 cells were incubated with $CdCl_2$ at 1 µM, during 24 h and fixed or cultivated during 24 h more (reversibility assay) to be processed for transmission electron microscopy. After the treatments, the samples were washed with PBS pH 7.2 at 37 °C and fixed at room temperature in a Karnovsky's solution containing 1% (v/v) glutaraldehyde, 4% (v/v) paraformaldehyde, 5 mM $CaCl_2$ and 5% (w/v) saccharose in cacodylate buffer 0.1 M, pH 7.2. The samples were postfixed for 1 h in a solution containing 2% (v/v) OsO_4, 0.8% (v/v) potassium ferrocyanide. The samples were rinsed with 0.1 M cacodylate buffer, pH 7.2, dehydrated in graded acetone, embedded at PolyBed812 (Fluka®). After, the resin was polymerized for 2 days in 60° C. Ultra-thin sections obtained with an ultramicrotome (LEICA) were stained with uranyl acetate and lead citrate, and observed with a JEOL 1400Plus Transmission Electron Microscope at 60 kV acceleration (Carvalho et al. 2010).

Statistics

The assays were performed in quadruplicate. At least 4 different fields and 500 host cells were counted for each assay. The parameters observed were: the number of (1) uninfected cells; (2) infected cells and (3) intracellular parasites.

The statistical significance was determined using GraphPad Prism v.6 software (GraphPad Software, Inc. CA, USA). Two-way ANOVA followed by a Bonferroni post-test was used to compare the differences in cell viability relative to the control cultures ($p < 0.001$).

Results

Intracellular *Trypanosoma cruzi*

After the establishment of the host cells on the plate, they were infected with trypomastigotes-derived culture. The intracellular parasites were quantified and the parasite morphologies were also considered at the initial steps of development (Figs. 1, 2). A low number of infected host cells were observed in 6 h of infection, but this number increased until 24 h when it was established (Figs. 1a, 2a–c). At the initial periods of infection (6–24 h), the parasites were at an intermediate stage of development (punctuate and condensed morphology) (Figs. 1b—white bars, 2a–c). From 36 h the amastigote forms also appeared (Figs. 1b—grey bars, 2a, b, c).

Metal toxicities on intracellular *Trypanosoma cruzi*

The host cells were infected during 12 h and incubated with $ZnCl_2$ at 20 µM or $CdCl_2$ and $HgCl_2$ at 1 µM during 24 h to verify the effects of a low dose of metals on the initial steps of the intracellular development of *T. cruzi*. The 12 h period is the average time required for parasite escape from the vacuole to host cytoplasm, before the establishment of infection. In these conditions, no reduction in host cell numbers was observed but occurred a decrease of 17, 13 and 4% on parasite number after $ZnCl_2$, $CdCl_2$ and $HgCl_2$ treatments, respectively (Fig. 3).

The analyses of the metal effects confirmed that any treatments cause morphological changes to host cells. Untreated (Fig. 4a) and treated cultures (Fig. 4b–d) showed the same typical features as a spread and non-vacuolated cytoplasm and nucleus. However, the number of punctual parasites decreased.

As previously described, $CdCl_2$ and $HgCl_2$ treatments induced similar morphological effects to parasites (de Carvalho and de Melo 2017). Then, to further investigate the morphology of both host cells and parasites, they were

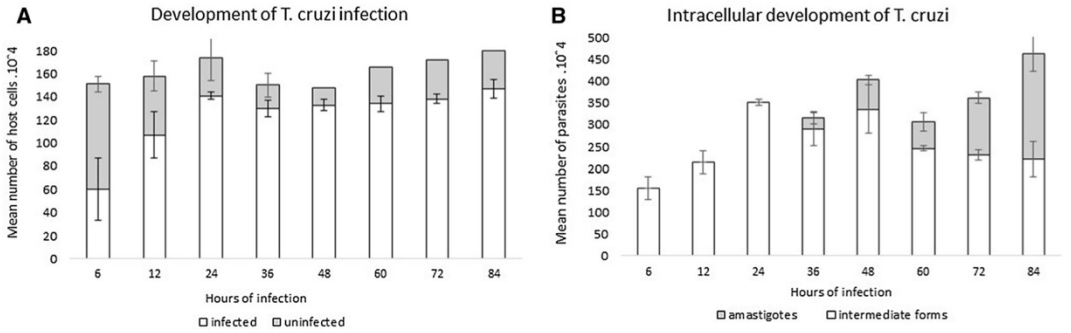

Fig. 1 The establishment of the intracellular *T. cruzi* infection. The intracellular *T. cruzi* was quantified and the morphologies considered to observe the initial steps of the establishment of the infection. The number of infected host cells increased up to 24 h and it was maintained at about 75% until 84 h (**a**—white bars). During the times of 6–24 h, only intermediary stages were observed (**a**—white bars). After 36 h, some parasites started to show the spread portion of cytoplasm—amastigotes, suggesting a more advanced stage of development (**b**—grey bars). From 84 h, the majority of parasites was spread within the cytoplasm and presented two punctuate stains (nucleus and kinetoplast), indicating the establishment of infection and proliferation steps

treated with $CdCl_2$ at 1 μM during 24 h and processed. The untreated host cells showed a homogeneous cytoplasm and a well-established *T. cruzi* with its typical morphology (Fig. 5a). After $CdCl_2$ treatment at 1 μM during 24 h, the host cells remained with is usual features but the intracellular parasites presented highly disorganized morphology (Fig. 5b).

The new metal incubations last 60 h to observe whether longer incubations can eliminate more parasites and also the progressive steps of parasite elimination. The untreated culture had 56% of infected cells, but this percentage decreased after metal incubations. After $ZnCl_2$ treatment, the percentage of infection dropped down to 30% while with $CdCl_2$ to 50%, with no reduction in cell number in comparison to the untreated ones. However, this longer time of incubation with $HgCl_2$ also induced a toxic effect on the host cells and eliminated 52% of them (Fig. 6a). Also, the $ZnCl_2$ and $CdCl_2$ incubations decreased 61% of the number of intracellular parasites, and $HgCl_2$ 72% (Fig. 6b).

In agreement with the quantification above, the optical microscopy showed that untreated cells had typical morphology and a high number of intracellular parasites (Fig. 7a). The $ZnCl_2$ (Fig. 7b) and $CdCl_2$ (Fig. 7c)—treated ones had a lower number of viable parasites and any toxic effect on host cells. Nonetheless, $HgCl_2$ treatment led to cell condensation and elimination in addition to parasite destruction (Fig. 7d). These results showed that $HgCl_2$ has a higher and accumulative toxic effect on both host cells and parasites.

Reversibility assay

A 24 h-treatment induced a low number of parasite elimination, although many morphological changed parasites remained on the culture. Then, the reversibility assays were performed to investigate whether the remained parasites were able to revert the toxic effects caused by the metals or not. For this purpose, 12 h-infected cells were treated with $ZnCl_2$ at 20 μM or $CdCl_2$ and $HgCl_2$ at 1 μM during 24 h, then the medium was replaced by a drug-free medium and the cells were cultivated for additional 72 h. After this time, the untreated culture had 85% of infected cells, after $ZnCl_2$ incubation, this number decreased to 63%, and to 70% after $CdCl_2$ and $HgCl_2$, with no significant reduction on host cell number (Fig. 8a). On the other hand, a greater number of intracellular parasites was eliminated on the reversibility assays in comparison to the direct toxic effect. The $ZnCl_2$, $CdCl_2$ and $HgCl_2$ incubations led to reductions of 54, 69 and 66%, respectively (Fig. 8b). These results suggest that irreversible toxic effects were already triggered during the 24 h of treatment.

The analyses by optical microscopy showed the untreated cells with an established cytoplasmic infection (Fig. 9a). After the reversibility assays, a lower number of intracellular parasites was observed and the remaining parasites had modified and condensed appearance (Fig. 9b).

The ultrastructure analyses showed host cytoplasm with its typical morphology containing proliferative *T. cruzi* with the usual nucleus, kinetoplast and flagellum (Fig. 10a). After $CdCl_2$ reversibility assay, different stages of parasites were observed, including typical and condensed parasites. In addition, many vacuoles appeared on

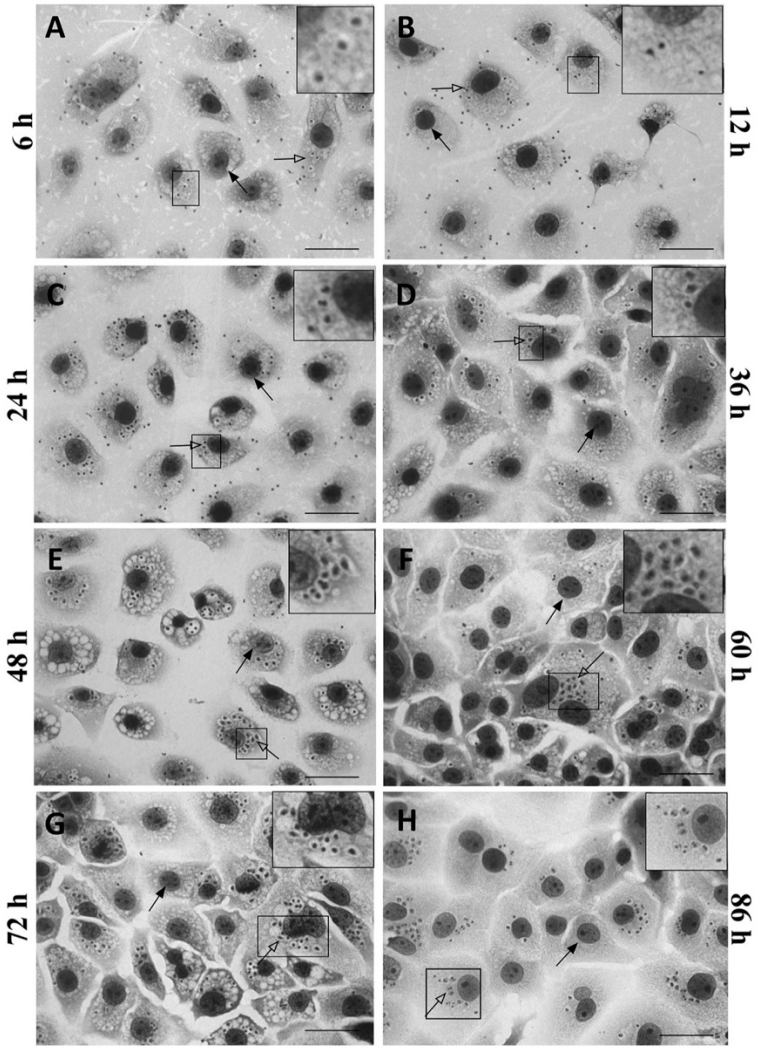

Fig. 2 Optical microscopy of the development of intracellular *T. cruzi* during different times of infection. Intermediary forms were observed in (**a**, **b**, **c**). From 36 h **d** the parasites started to present a spread cytoplasm, a similar amastigote morphology. Black arrows: host cells nuclei. White arrows: parasites. Scale bars: 100 µm. Inserts: amplification of the selected areas

host cytoplasm, suggesting parasite destruction and elimination (Fig. 10a).

Discussion

The establishment of an intracellular infection and parasite propagation is dependent on host cell metabolism (Caradonna et al. 2013). For this reason, the metabolic coupling of intracellular pathogens with host cells is strictly regulated. However, many deaths have been caused annually due to the self-limiting effects of an unbalanced *T. cruzi* infection (Santos et al. 2012). To overcome this problem, many research groups have been using the advances in the rational design of metal-based chemotherapy to synthesize anti-pathogenic therapeutic agents, as metals are known to potentialize pharmacological properties (Vieites et al. 2008; Benítez et al. 2011; Martins et al. 2012).

Many studies demonstrated that some intracellular parasites have to acquire all essential nutrients as well as transition metals from the host cell to be pathogenic, to maintain the homeostasis and to replicate (Porcheron et al.

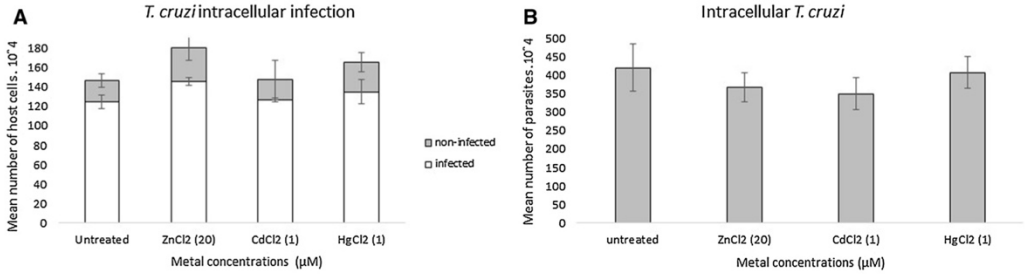

Fig. 3 Mean number of host cells infected with *T. cruzi* during 12 h and incubated with metals at 1 µM during 24 h. **a** The total of host cells separated into uninfected and infected ones. **b** Total of intracellular parasites

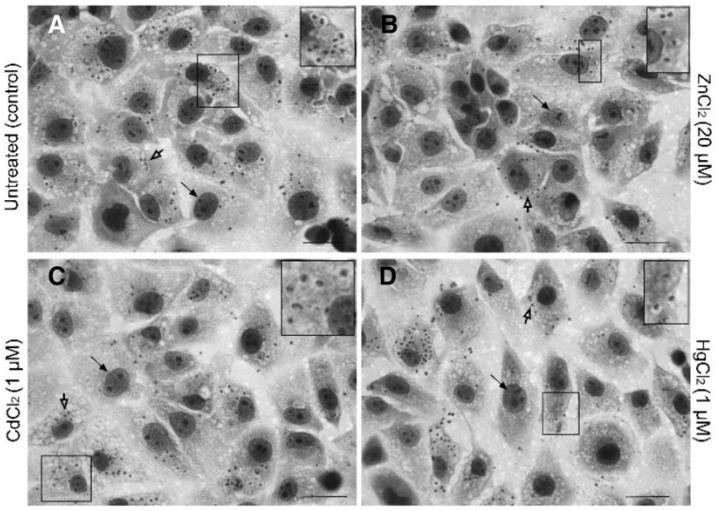

Fig. 4 Host cells infected with *T. cruzi* for 12 h and incubated with metals for 24 h. Black arrows: host cells nuclei. White arrows: intracellular parasites. Scale bars: 100 µm. Inserts: amplification of the selected areas

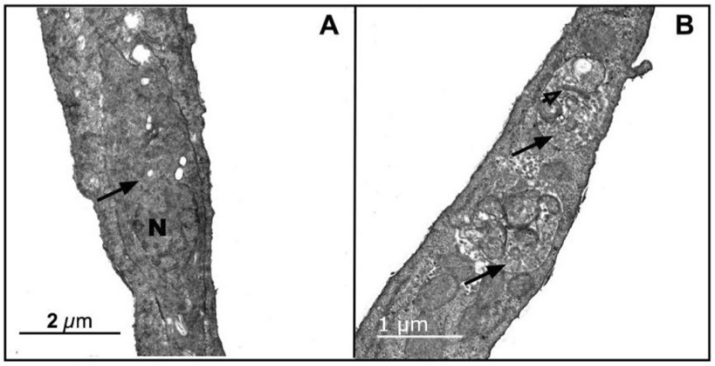

Fig. 5 Ultrastructural analyses of 12 h-infected cells treated with $CdCl_2$ at 1 µM during 24 h. **a** Untreated infected cell. **b** The treated infected cell containing destroyed parasites. N: parasite nucleus. Black arrow: intracellular parasite. Arrowhead: kinetoplast

2013). For these reasons, metal flux control is vital to parasites. However, few studies have been published concerning the toxic effects of metal ions to intracellular protozoan parasites. Therefore, the comprehension of the

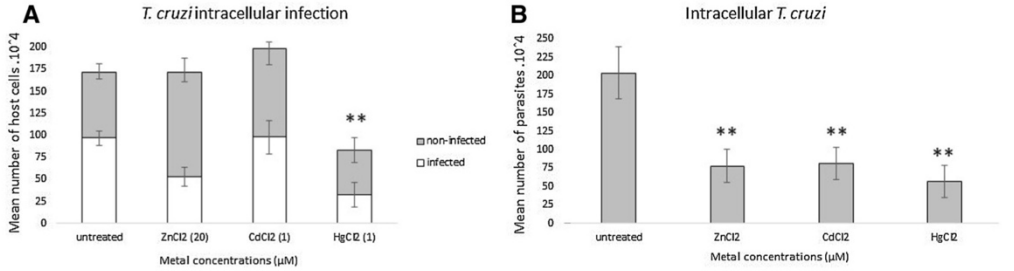

Fig. 6 Mean number of host cells infected with *T. cruzi* during 12 h and incubated with $ZnCl_2$ at 20 μM or $CdCl_2$ and $HgCl_2$ at 1 μM during 60 h. **a** The total of host cells separated into uninfected and infected ones. **b** Total of treated intracellular parasites

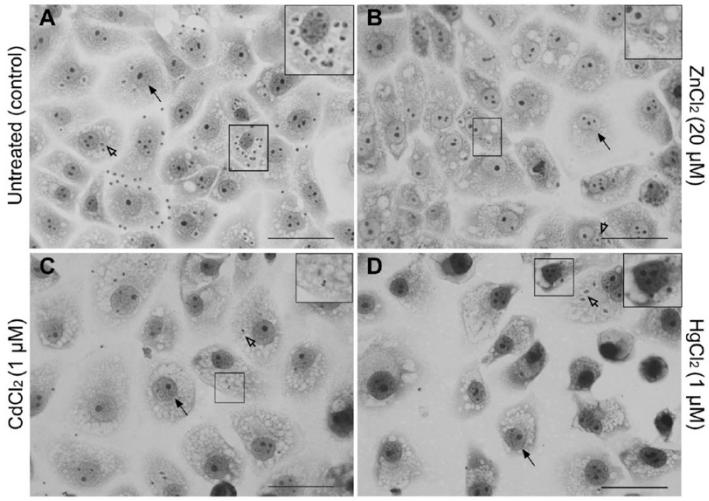

Fig. 7 Host cells infected with *T. cruzi* for 12 h and incubated with metals at 1 μM during 60 h. After this longer time of incubations, a lower number of viable parasites was seen. Black arrows: host cell nuclei. White arrows: intracellular parasites. Scale bars: 100 μm. Inserts: amplification of the selected areas

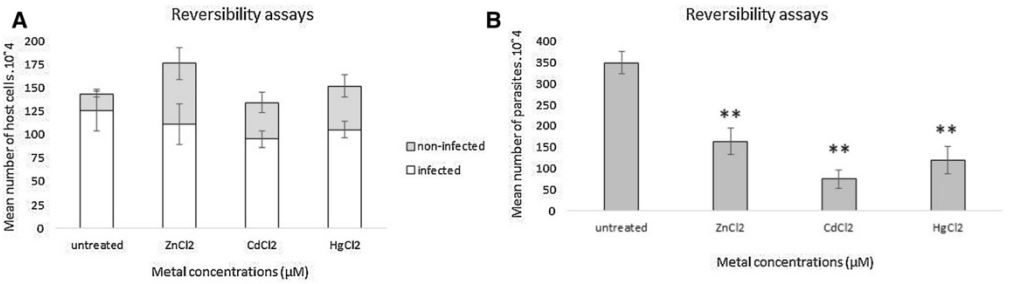

Fig. 8 Mean number of host cells infected with *T. cruzi* during 12 h, incubated with $ZnCl_2$ at 20 μM or $CdCl_2$ and $HgCl_2$ at 1 μM during 24 h, and cultivated during 72 h more on a metal-free medium. **a** The total of host cells separated into uninfected and infected ones. **b** Total of intracellular parasites

antiparasitic impact of metals is crucial for improving the activity of metal-based agents (Lemire et al. 2013).

Trypanosoma cruzi, an obligate intracellular parasite, is transmitted to vertebrate hosts during the feeding of blood-

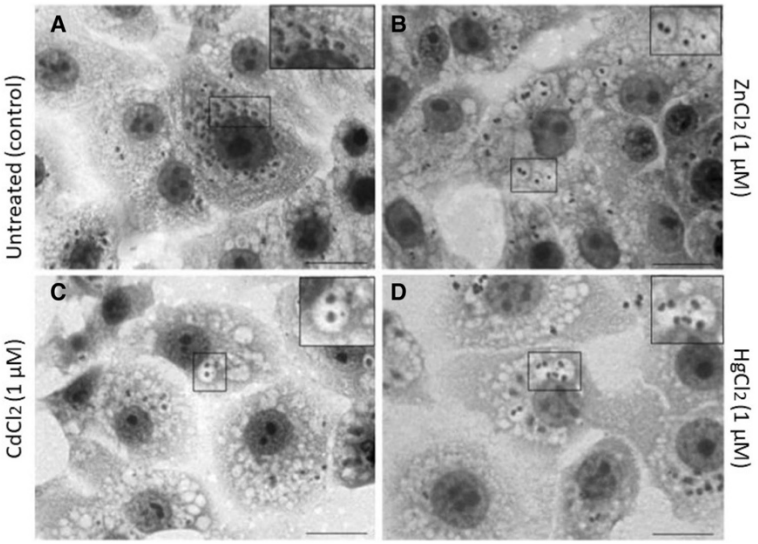

Fig. 9 Reversibility assays of metal treatments in *T. cruzi* infected cells. The 12 h-infected cultures were incubated with metals during 24 h and cultivated for additional 72 h on a metal-free medium. After this time, a large number of inviable parasites was seen. Scale bars: 50 µm. Inserts: amplification of the selected areas

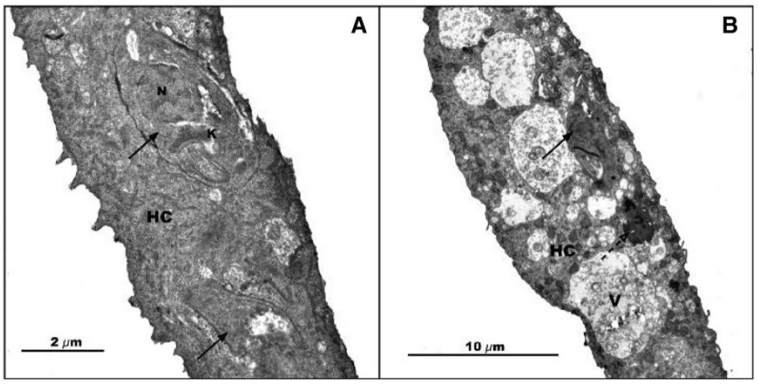

Fig. 10 Reversibility assay of $CdCl_2$ treatment in *T. cruzi* infected cells. The 12 h-infected culture was incubated at 1 µM during 24 h, and cultivated during additional 72 h on a metal-free medium. **a** Untreated culture. **b** $CdCl_2$ treated culture. Different stages of parasite destruction were observed after metal treatment. N: parasite nucleus. K: kinetoplast. HC: host cytoplasm. V: vesicles. Black arrows: intracellular parasites. Dotted black arrow: destroyed parasite

sucking insects. The process of parasite internalization is triggered by molecules present in both parasite and mammalian cells that work as receptor-ligands resulting in the endocytic or phagocytosis pathways in a non-synchronic manner, as reviewed in (de Souza et al. 2010). The host cell plasma membrane and lysosomes contribute to parasitophorous vacuole formation, where the trypomastigotes initiate their intracellular cycle (Woolsey et al. 2003; Tardieux, Nathanson and Andrews 1994; Rodriguez et al. 1996). After 8–16 h post-invasion, the trypomastigotes start the process of transformation to amastigotes, while many events related to the parasitophorous vacuole destruction are activated. The parasitophorous vacuole membrane is marked with early and late endosome proteins, suggesting a process of parasite destruction through endolysosome formation and vacuole acidification (Andrews and Whitlow 1989). However, the parasite uses this host microbicide defense mechanism to escape to the cytoplasm, to replicate and to establish an infection (de Carvalho and de Souza 1989). These steps result from the Tc-Tox activity—a parasite's peptide activated by the pH decrease after lysosome-vacuole fusion—that lead to the disintegration of the parasitophorous vacuole membrane (Andrews and Whitlow 1989; Ley et al. 1990). After *T. cruzi* reach the host cytoplasm, the parasites finish their transformation to amastigotes—proliferative form—and multiply in direct contact with the host cell organelles.

As the present study aimed to investigate the progressive steps of the intracellular parasite development, host cells were infected and the number of intracellular parasites and their morphology were observed. According to expectations, this paper showed that the non-synchronous invasion of trypomastigotes resulted in an increasing number of intracellular parasites in intermediary forms (until 24 h post-infection), the appearance (from 36 h) and proliferation (84 h) of the amastigotes (Fig. 1). From the confirmation that after 12 h of infection, only intermediary forms were observed on host cell indicating that the infection was not established yet, this time was used to further investigate the role of metal ions in the intracellular development. It is well-known that the metal ions play an important role in the establishment and maintenance of host-parasite interactions and an imbalance can cause severe damage to both of them (Weinberg 1966).

In this context, a previous study showed that $ZnCl_2$, $CdCl_2$ and $HgCl_2$ incubations in epimastigotes, trypomastigotes and intracellular proliferative *T. cruzi* were able to eliminate part of the parasites without causing toxic effects to the host cells (de Carvalho and de Melo 2017). Nonetheless, this study showed that early metal incubations ($ZnCl_2$, $CdCl_2$ and $HgCl_2$) at the concentration of 1 μM during 24 h was not enough to impair parasite escape to the host cytoplasm and the infection establishment (Figs. 3, 4). However, it was able to eliminate a high number of proliferative ones mainly with $CdCl_2$ (de Carvalho and de Melo 2017). This higher survival rate of trypomastigote and intermediary forms in comparison to amastigotes can be a result of the different morphological life cycle forms and the changes in gene expression (Tyler and Engman 2001). Trypomastigotes have important survival mechanism to adapt to the environmental changes while the metabolism of the amastigote is directed to proliferation (Nardy et al. 2015). However, longer time of incubation (60 h) led to a significant reduction on parasite number after the treatments with the three metals (Figs. 6, 7), as well as the reversibility assays (Figs. 8, 9, 10).

Metal incubations, mainly non-essential ones, can induce parasite death through many pathways. Mercury, for example, can cross lipid membranes and have a high affinity for thiol groups (Girault et al. 1997), as well as cadmium that can impair intracellular signaling pathways after interacting with surface receptors (Moulis 2010). In case of zinc, an essential metal, it is also toxic in high concentrations and, for this reason, it needs to be tightly regulated (Eide 2006). Then, the misbalance of metal ions can result on the production of free radicals involving in the lipid peroxidation, modifications to DNA bases and disruption of calcium and sulphydryl homeostasis (Valko et al. 2007; Jomova and Valko 2011). Trypanosomatids, including *T. cruzi,* have a peculiar defense mechanism against free radicals, which includes the trypanothione (Ariyanayagam and Fairlamb 2001; Turrens 2004) and low activity of superoxide dismutase (Maya et al. 2007). Due to the lack of defenses against metal effects, the parasites were the main target for metal effects, as observed in our results.

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Authors contribution Lais Carvalho performed all assays while Edésio Melo organized the results and he also wrote the paper.

Compliance with ethical standards

Conflict of interest All authors declares that they have no conflict of interest.

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3.3 Cellular events related to *Trypanosoma cruzi* elimination in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$

Cellular events related to *Trypanosoma cruzi* elimination in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$

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Running title: *Trypanosoma cruzi* infection in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$

Abstract

Trypanosoma cruzi is an obligate intracellular parasite transmitted to vertebrate hosts by blood-sucking insects. Molecules present in parasites and mammalian cells allow the recognition and parasite internalization. Metallic ions play an important role to the establishment and maintenance of host-parasite interaction. However, little is known about how parasites handle with essential and nonessential metal quotas. The aim of this study was to investigate the influence of metal ions on biological processes of *T. cruzi* infected cells. Infected cells were incubated with $ZnCl_2$, $CdCl_2$ and $HgCl_2$ for 12 h and labeled with different specific dyes to investigate the cellular events related to intracellular parasite death and elimination. Infected host cells and parasite's mitochondria underwent functional and structural disorders, in addition to parasite's DNA condensation and pH decrease on host cells, which led to parasite death. Deeper investigations suggested that lysosomes were involved in pH decrease and the double membrane of the endoplasmic reticulum formed vacuoles surrounding damaged parasites, which indicate the occurrence of autophagy for parasite elimination. In conclusion, low concentrations of nonessential and essential metals cause a series of damage to *Trypanosoma cruzi* organelles, leading to its loss of viability, death, and elimination, with no elimination of the host cells.

Keywords: autophagy, cadmium, mercury, nonessential metals, *Trypanosoma cruzi* and zinc

1- *Introduction*

Trypanosoma cruzi is the causative agent of Chagas' disease, also known as American trypanosomiasis, a potentially life-threatening illness. *T. cruzi* has a complex life cycle which involves the developmental stages present in the invertebrate and vertebrate host species [Eptin *et al.* 2010]. During the feeding of the insect vector (Reduviidae family) the metacyclic trypomastigotes infect the vertebrate host. *T. cruzi* uses diverse mechanisms for adhesion and invasion that involve molecules present on both host cells and parasite surfaces [Souza *et al.* 2010]. The early intracellular development includes the formation of an endocytic vacuole (parasitophorous vacuole) where the parasite resides temporarily. However, the acidification of this vacuole allows the parasite to escape to the cytosol, where the differentiation into the amastigote form occurs [Carvalho and Souza 1989]. In the cytoplasm, the amastigotes have several cycles of replication until differentiating into trypomastigotes which, after cell rupture, reach the bloodstream to infect new cells [Romano *et al.* 2012]. The success of the infection of *T. cruzi* depends on the transition among its different morphological stages, and physiological and biochemical modifications, which allows the parasite to survive in different environments [Tyler and Engman 2001]. Many enzymes responsible for these modifications or metabolic pathways use metals as co-factor or structural components [Alvares *et al.* 2012].

In the vertebrate host cells, the metal group can be divided into essential and nonessential. The essential ones, analogous to carbon-based molecules, are important to maintain the homeostasis of the organisms and preservation of life. Essential metal functions include important structural, regulatory and catalytic role in different types of proteins, such as enzymes, receptors and transporters [Phipps 2002]. Because the metals are present in the environment, the cells developed mechanisms to uptake them as the divalent metal transporters or docked with other biomolecules [Nelson 1999]. However, the environment also has

nonessential metals known to be toxic even in very low concentrations. To get the intracellular milieu, the nonessential metals utilize the entry routes as essential ones and substitute them on different structures, triggering a series of toxic effects [Rana 2008; Templeton and Liu 2010].

Zinc, which is an essential metal for eukaryotic cells, works as a structural constituent of many proteins, as catalytic component of more than 300 enzymes, as the regulator of the stability of proteins and also prevents free radical formations [Parkin 2004]. Zinc is involved in several factors that protect biological structures from damage by free radicals such as induction of metallothioneins (MTs), as an essential component of superoxide dismutase (SOD), as protector of the eukaryotic cells for thiols and other chemical groups [Tapiero and Tew 2003]. Zinc also takes part of other biological functions as the immune response, gene expression, cellular proliferation and differentiation and apoptosis [Vallee and Falchuk 1993].

Cadmium is a nonessential transition metal and occurs as divalent cation in biological systems. Cadmium toxicity has historically been closely associated with zinc homeostasis since both share many chemical similarities [Moulis 2010]. These similarities facilitate cellular cadmium traffic via pathways dedicated to transition metals and result in the deregulation of transition metals homeostasis, which lead to metal toxicity or even cell death [Martinez-Finley *et al.* 2012].

Mercury is also a nonessential reactive metal found in different forms: elemental, inorganic and organic and its toxicity is dependent on its form [Farina *et al.* 2013]. In a biological system, mercury is always conjugated to sulfhydryl groups-containing molecules. In many cases, these conjugates may mimic some constitutive molecules [Onsanit and Wang 2011], which inactivates a variety of enzymes present in the cell; leading to several damages [Rana 2008].

Currently, metals-complexed chemical compounds have been synthesized to improve their efficiency and tested against a wide variety of diseases, including cancer and diseases

caused by bacteria and protozoans which include intracellular parasites such as *T. cruzi* [Benítez *et al.* 2013; Beraldo and Gambino 2004; Demoro *et al.* 2012; Fernández *et al.* 2015]. Cisplatin (cis-dichlorodiammineplatinum (II)), is an example of a metallo-drug with high activity in a variety of cancers including ovarian, testicular, bladder, lung, head and neck cancers [Dasari *et al.* 2014]. The use of cisplatin led to interest in other metal-containing compounds as potential anticancer drugs [Frezza *et al.* 2010]. Concerning the protozoan-causing diseases the Ferroquine (FQ; SSR 97193), a new 4-aminoquinoline organometallic chloroquine (CQ) analog, has been tested successfully against resistant *Plasmodium sp.* [Evase *et al.* 2011; Held *et al.* 2015]. In addition, protozoa that cause diseases such as malaria, leishmaniasis and Chagas disease develop in the intracellular environment, limiting the action of chemotherapeutic agents available against these infectious agents. Thus, new compounds need to be studied to also reach the intracellular environment of the host to eliminate the parasites. For this purpose, *T. cruzi* is a great model of study, because the its intracellular cycle depends on a very large set of enzymes that can be susceptible to metallic compounds. Although the effects of metals on uninfected cells are well described, the knowledge of the role of metal ions in parasite infection and subsequent intracellular development lacks. Recently, the first study about the effects of $ZnCl_2$, $CdCl_2$ and $HgCl_2$ on the three main forms of *T. cruzi* was published [Carvalho and Melo 2017]. A detailed study was performed showing that 2 hours of incubations at 3 μM were enough to eliminate a percentage of parasites, including the intracellular forms, with no toxic effects to the host cells. Therefore, the present study aimed to investigate the functionality of parasite organelles and cellular events related to intracellular parasite death and elimination in the presence of zinc, cadmium and mercury chlorides.

2- *Materials and Methods*

2.1 - Host cell culture

LLC-MK2 (kidney fibroblasts of *Macaca mulatta*) were grown in plastic Falcon flasks (25 cm^2) containing RPMI 1640 (Sigma) medium supplemented with 5% fetal calf serum (Sigma). The cultures were treated with trypsin when the cell densities approached to monolayer. For experimental purposes, 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 medium flasks (3 x 10^6 cells) and allowed to attach for 24 h at 37 °C in a 5% CO_2 atmosphere [Carvalho and Melo 2017].

2.2 - Parasite maintenance

Epimastigotes of *T. cruzi* (DM28 strain) were cultured in Liver Infusion Tryptose medium supplemented with 0.4% (v/v) of hemin and 10% (v/v) fetal calf serum at 28°C. Every 5 days, a 1 mL aliquot of parasite-containing medium was transferred to a new tube and the volume completed to 5 mL with fresh culture medium.

Trypomastigotes of *T. cruzi* were obtained from transformation of epimastigotes. The epimastigotes were centrifuged at 500 *g* for 10 minutes and the pellet homogenized in RPMI 1640 supplemented with 10 % (v/v) fetal calf serum and incubated at 37 °C for 48 h. After this time, around 95% of the parasites were in the form of trypomastigote. For experimental purpose, the parasites were centrifuged at the same condition and homogenized in 1 mL of RPMI 1640. An aliquot of 0.1 mL was scored at Neubauer chamber and a rate of 20:1 parasite: cell was used to infect the culture. After 5-6 days of infection the host cell lysis occurred and the trypomastigotes were released into the supernatant. So, the supernatant was collected,

centrifuged as described above and new cultures were infected [Carvalho and Melo 2017, Carvalho and Melo 2018].

2.3 - Metal treatments

For fluorescence assays, the infected cells were treated with $ZnCl_2$ at 20 μM, $CdCl_2$ at 5 μM or $HgCl_2$ at 10 μM during 1, 2 (kinetic assays) or 12 (organelles functionality) hours and processed to microscopy. These times and concentrations were based on the time-concentration effects described in [Carvalho and Melo 2017; Carvalho and Melo 2016; Carvalho and Melo 2018]. These studies showed that part of the intracellular parasites was already eliminated in these conditions, representing a good start point to the further studies. In the published studies, all parameters of times and concentrations were established for the next steps, including to investigate the cellular events related to intracellular parasite elimination.

All assays were visualized using a Zeiss Axioplan fluorescence microscope equipped with different filters. The images were taken using an Olympus DP72 camera and Cell^F Image software.

Three independents experiments were performed and, at least, ten fields of each experiment were considered to confirm the events.

Dilutions of $HgCl_2$, $CdCl_2$ and $ZnCl_2$ salts originated 0.1 M stock solutions in ultra-pure quality water. The final concentrations were prepared diluting the stock solution with medium.

2.4 - Functional and cellular toxicity of metals on intracellular parasites

2.4.1 - Microfilaments

Formaldehyde-fixed treated and untreated infected cultures were incubated with Rhodamine Phalloidin (a high-affinity F-actin probe) (Molecular Probes®) (200 units/mL) for 40 min and observed using a rhodamine filter (546 nm) [Barak *et al.* 1980].

2.4.2 - Acid compartments and lysosomes

The treated and untreated infected cultures were incubated with Acridine Orange (Sigma®) (5 μg/mL) for 40 min at 37 °C to observe acid compartments [Kielian and Cohn 1980]. The lysosomes were stained with LysoTracker Red (high selectivity for acidic cell organelles) (Molecular Probes®) (50 ng) after incubation through 30 min at 37 °C. Both were visualized using a rhodamine excitation filter (546 nm) [Yapici *et al.* 2015].

2.4.3 - Mitochondrial function

Untreated and metal-treated infected cells were incubated with Rhodamine 123 (to stain active mitochondria) (Sigma®) (10 μg/mL) for 30 min at 37 °C and visualized using a rhodamine excitation filter (546 nm) [Johnson *et al.* 1980].

2.4.4 - Autophagy detection

The cultures were incubated with Monodansylcadaverine (accumulates in autophagic vacuoles due to a combination of ion trapping and specific interactions with membrane lipids) (Fluka®) (0.05 mM) for 10 min to stain autophagic vacuole and observed using a UV excitation filter (350 nm) [Biederbick *et* al. 1995].

Especially for the autophagic vacuoles, the percentage of untreated and treated infected cells stained with Monodansylcadaverine was considered in a total of one hundred cells for each situation.

2.4.5 - Nuclei integrity

Ethidium Bromide (an intercalating agent) (Sigma®) was incubated at a concentration of 100 μM during 20 min and observed using rhodamine (546 nm) filter to observed condensed DNA [Baskic *et al.* 2006].

2.4.6 - Cytochemistry analyses

A 48 h infected host cells were treated with $CdCl_2$ at 10 μM during 5 or 12 h and processed for glycose-6-phosphatase cytochemistry assay, an enzyme mainly found in the endoplasmic reticulum. The cells were washed twice with sodium cacodylate buffer at 0.1 M, pH 7.2. After that, the cells were fixed in glutaraldehyde 1% (v/v), paraformaldehyde 2% (v/v) in sodium cacodylate buffer with 5% (w/v) sucrose at 0.05 M, pH 7.2 at 4 °C during 10 min. The cells were washed with sodium cacodylate buffer 0.1 M, pH 7.2 and in TRIS-maleate buffer 0.06 M, pH 6.5 with 5% (w/v) sucrose, both at 4 °C. The cells were incubated with a solution containing $MgCl_2$ at 5 mM, $CeCl_2$ at 4 mM in TRIS-maleate buffer at 0.06 M, pH 6.5 during 30 min at 37 °C. Then, the cells were incubated with a solution containing glycose-6-phosphate 5 mM, $MgCl_2$ at 5 mM, $CeCl_2$ at 4 mM in TRIS-maleate buffer 0.06 M, pH 6.5. The cells were washed with TRIS-maleate 0.06 M, pH 6.5 and sodium cacodylate buffer at 0,1 M, pH 7.2. The cells were shaved from the bottles and post-fixed in osmium tetroxide 1% (w/v) in sodium cacodylate buffer at 0.15 M, pH 7.2, at 4 °C, during 1 h. Posteriorly, the cells were washed twice in sodium cacodylate buffer 0.1 M, pH 7.2, dehydrated in increasing acetone solution and embedded in PolyBed 812 resin. The resins containing the samples were polymerized in a kiln at 60 C for 48 h. Ultrathin sections were obtained using an ultramicrotome in Reichert Ultracuts Leica Instruments® and contrasted with 5% (w/v) aqueous uranyl acetate for 20 min in the dark and lead citrate for 5 minutes. The images were taken using a JEOL 1400 Plus microscope at 60 kV [Carvalho and Melo 2018].

2.5 - Statistics

A *t-test* followed by a Mann-Whitney post-test was used to compare the differences between the control and metal treatments ($p<0.05$) for the incidence of MDC positive staining.

3 - Results

3.1 - Organelle functionality during metal treatments

Selective markers were used to investigate the events related to the effects of metal on organelles and intracellular parasite-host cell interaction. For all assays, the infected cells were incubated with 20 μM $ZnCl_2$ in conjunction with 5 μM $CdCl_2$ and 10 μM $HgCl_2$ for 18 h. The fluorescence assays can be observed on the left side of the figures, while bright fields in the right side.

3.1.1 - Microfilaments

Firstly, the microfilaments (F-actin polymerized form) were visualized on culture with Rhodamine Phalloidin. Differences in distribution of F-actin through the host cells were observed. The untreated cell had the F-actin regularly spread through the cytoplasm and at the border of the cell (Fig 1 a and b). The $ZnCl_2$ treatment caused no severe F-actin rearrangement (Fig 1 c and d). However, after $CdCl_2$ (Fig 1 e and f) or $HgCl_2$ (Fig 1 g and h) incubations, the filaments of F-actin were concentrated in the nucleus and, in addition, they were not visible in cell border.

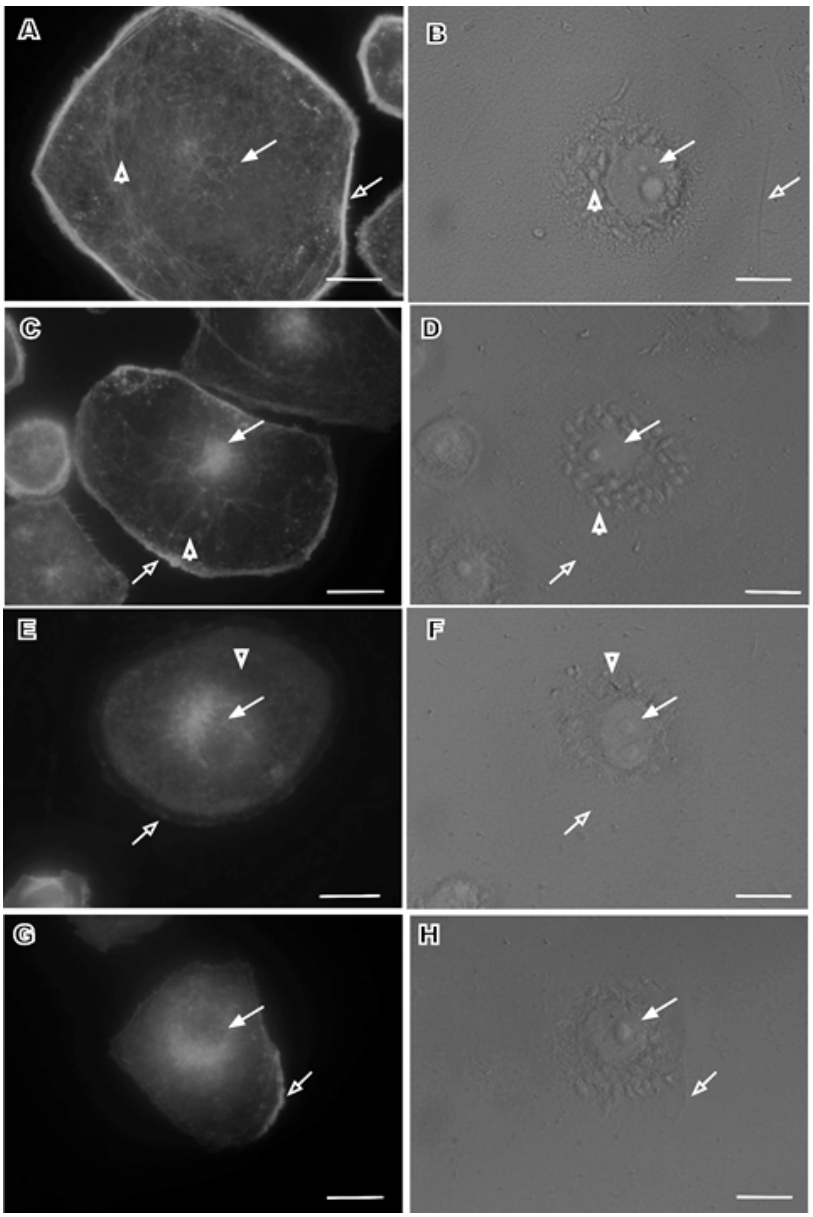

Figure 1 Infected host cell treated with metals for 12 h and stained with rhodamine phalloidin, a selective marker for F-actin. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Scale bars: 20 µm.

3.1.2 – Mitochondrial activity

Rhodamine 123 was then used to detect mitochondrial functionality (Fig 2). Untreated infected host cell showed high markes, which indicate the usual mitochondrial activity (Fig 2 a and b), as well as $ZnCl_2$ treated cultures (Fig 2 c and d). However, after $CdCl_2$ (Fig 2 e and f) and $HgCl_2$ (Fig 2 g and h) incubations, the fluorescence of Rhodamine 123 decreased on host

cells and parasites. This fact suggests that metal treatments led to the loss of infected host cell and parasite mitochondrial membrane potential, which affects their activity.

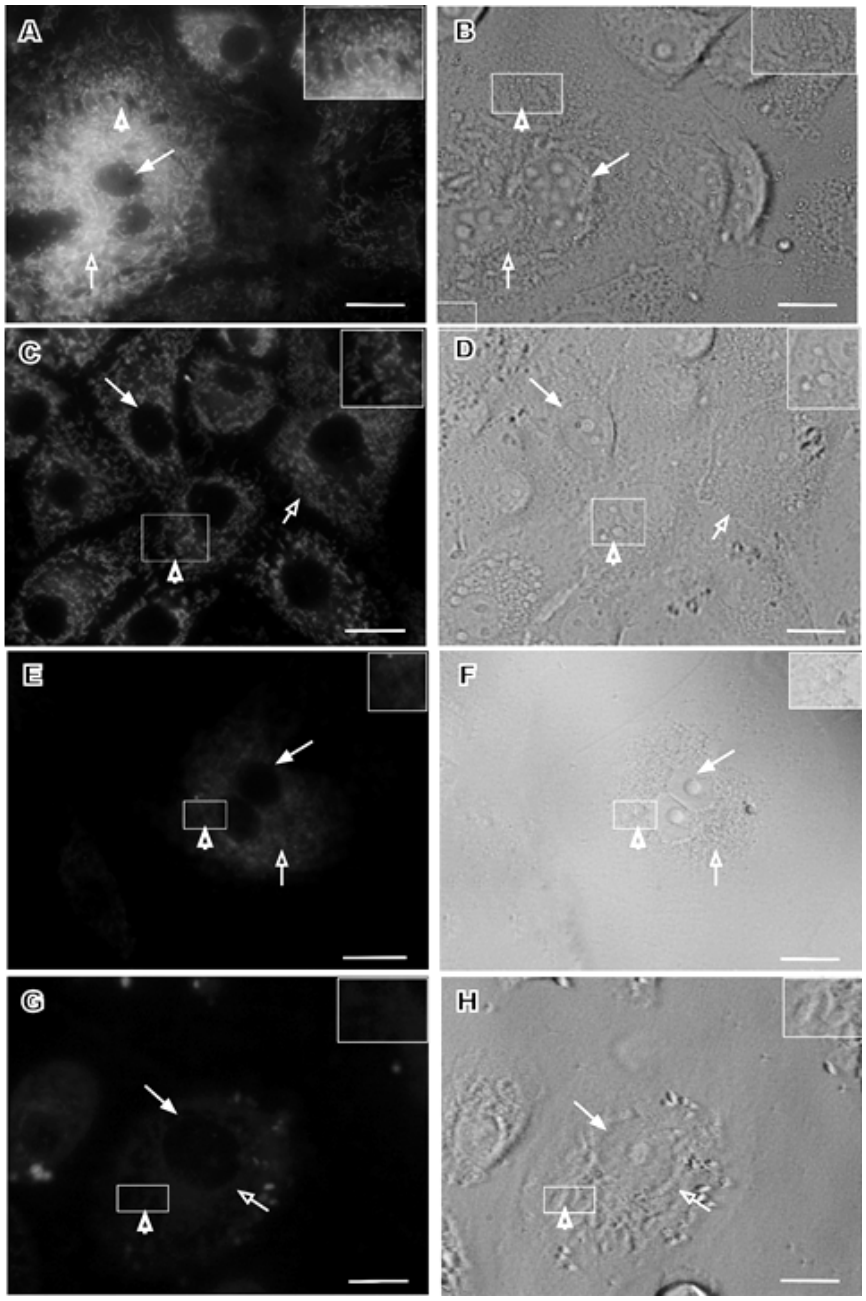

Figure 2 Infected host cell treated with metals for 12 h and stained with the mitochondrial selective marker Rhodamine 123. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 µm

In addition to mitochondria, the proper function of parasite nucleus is vital to parasite survival, because damage in this organelle can trigger DNA fragmentation, one of the main indicators of cell apoptosis. Thus, the next step was to investigate the nucleus structure.

3.1.3 – Nuclei integrity

The cells were stained with ethidium bromide, a DNA intercalating agent, which fluoresce in necrotic cells. The untreated infected cells (Fig 3 a and b) and $HgCl_2$-treated cells (Fig 3 g and h) showed weak markers. However, after $ZnCl_2$ (Fig 3 c and d) and $CdCl_2$ (Fig 3 e and f) treatments, the fluorescence on intracellular parasites and host cells nuclei were stronger, which suggests the occurrence of cell death process.

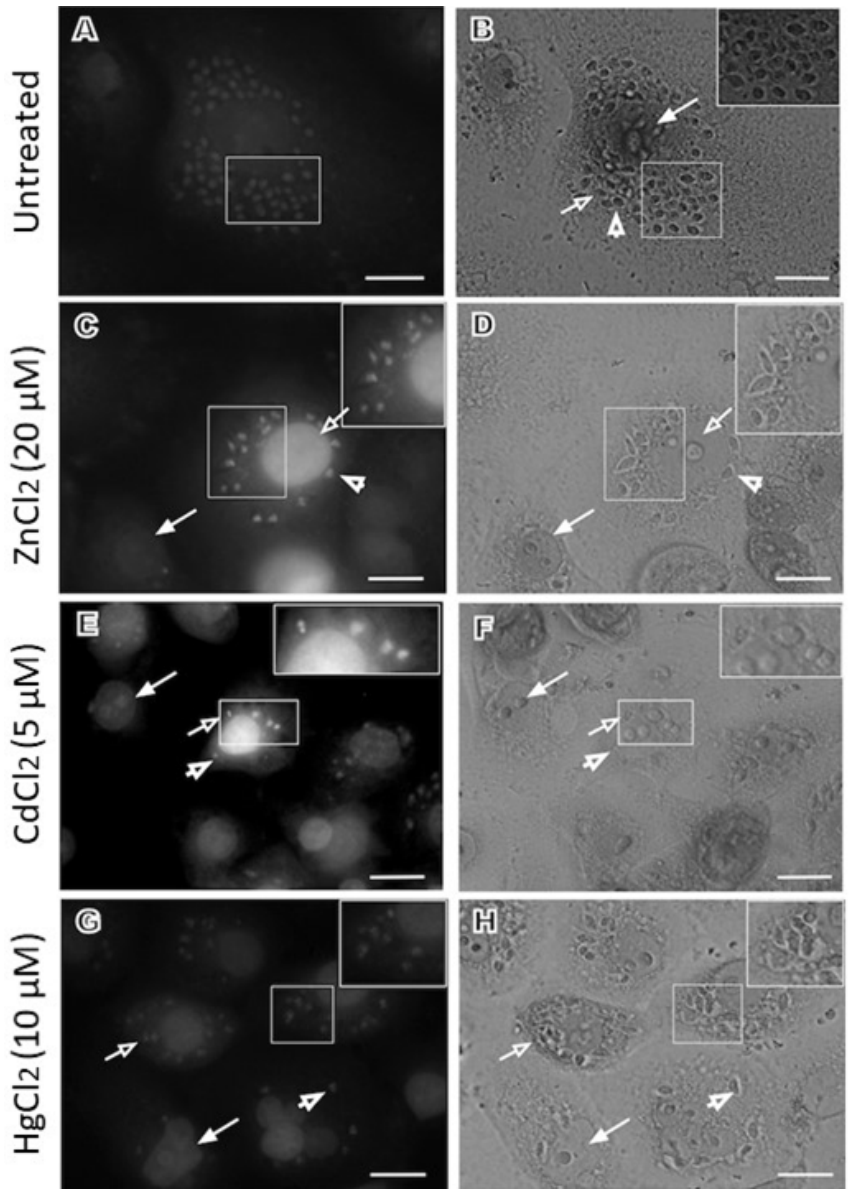

Figure 3 Infected host cell treated with metals for 12 h and stained with Ethidium Bromide. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 µm.

After intracellular parasite death they should be eliminated from the intracellular milieu, but mechanisms to eliminate cytoplasmic parasite is poorly studied and described. Thus, the next steps were to investigate possible cellular mechanisms responsible for intracellular parasite elimination.

3.1.4 – Acid compartments

Therefore, Acridine Orange, a fluorescent dye, was used to verify whether occurrence of compartment acidifications may occur (Fig 4). The untreated infected culture showed weak and diffused fluorescence markers (Fig 4 a and b). After $ZnCl_2$ incubation, the stain seemed as weak as the untreated, but co-localized with morphologically changed parasites (Fig 4 c and d). However, after $CdCl_2$ (Fig 4 e and f) or $HgCl_2$ (Fig 4 g and h) incubations the markers were stronger and more punctual on the host cell and co-localized with the disorganized parasite. These results suggest a general pH decrease on host cytoplasm, especially on altered parasites.

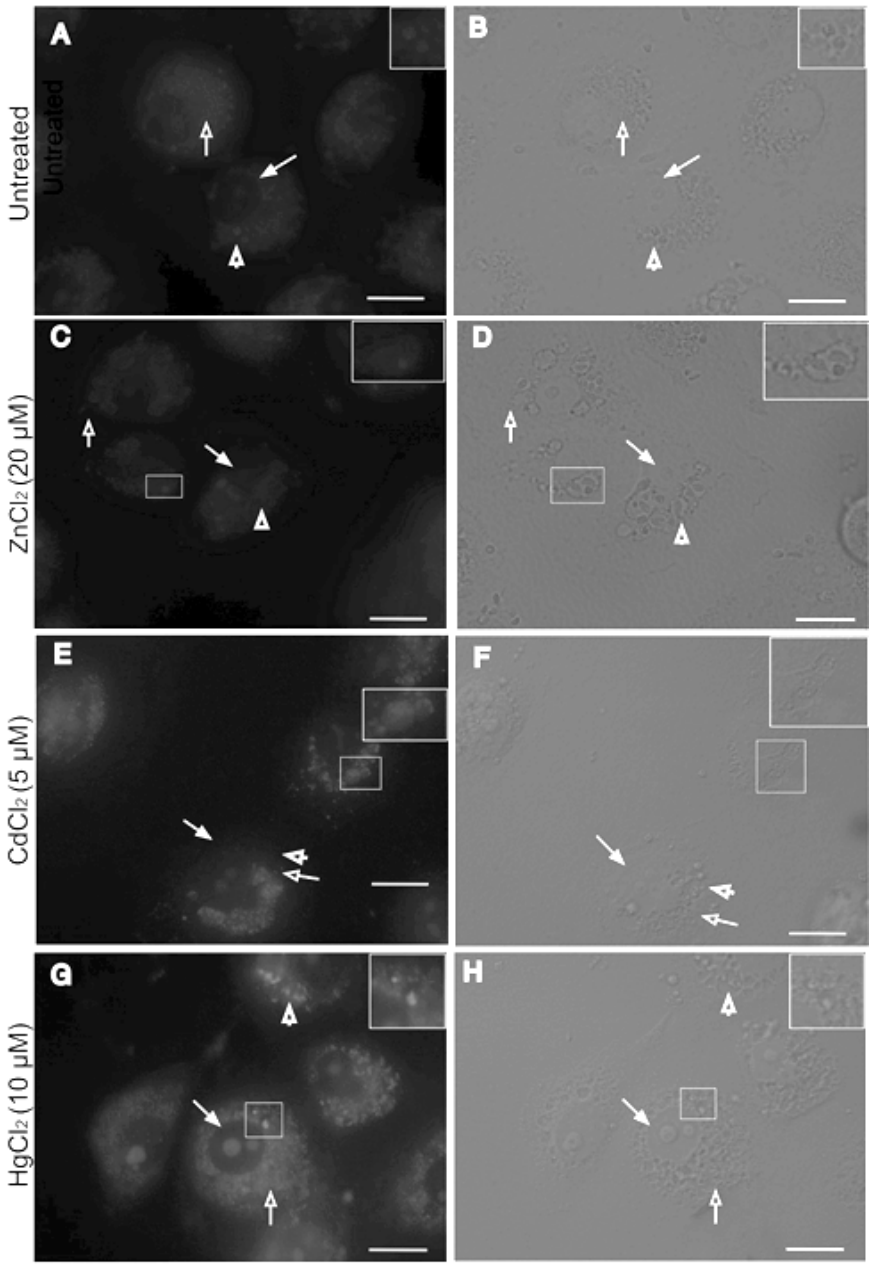

Figure 4 Infected host cell treated with metals for 12 h and stained with the acid compartment selective marker Acridine Orange. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 µm.

3.1.5 - Lysosomes

The cultures were then stained with LysoTracker Red, a selective marker for lysosomes, to investigate whether the lysosomes are involved in host acidification (Fig 5). Untreated cells showed a less intense and spread stain in cytoplasm (Fig 5 a and b). However, intense stains were observed after the three metal treatments in both host cytoplasm and co-localized with morphologically changed intracellular parasites (Fig 5 c-h). This event suggests that host cell lysosomes are involved in parasite destruction through secretion of their hydrolytic enzymes.

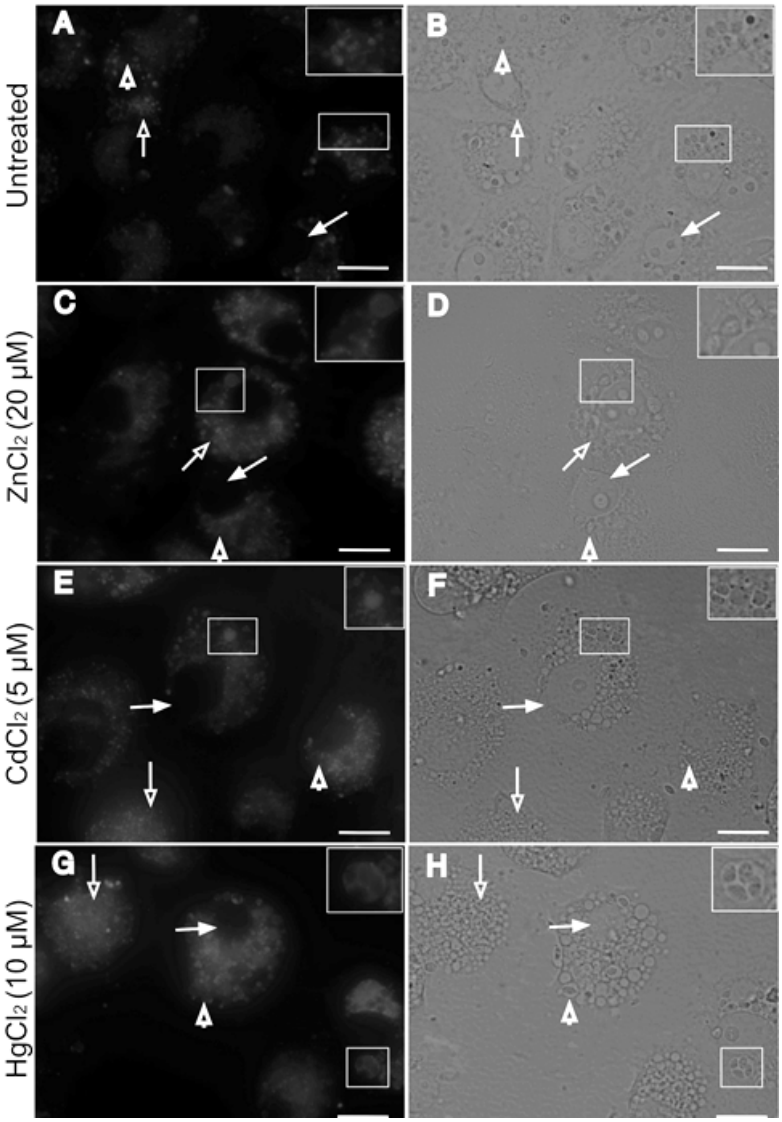

Figure 5 Infected host cell treated with metals for 18 h and stained with the acid compartment selective marker LysoTracker Red. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 µm.

3.1.6 – Autophagy detection

If lysosome enzymes are involved in parasite destruction and elimination, a vacuole surrounding the pathogens might be required for hydrolases activity. The cultures were stained with Monodansylcadaverine, a selective dye for functional autophagic vacuoles to confirm the presence and to investigate the origin of this vacuole (Fig 6). The autophagy pathway is a general mechanism of cells used to get rid of unnecessary organelles and microorganisms

[Glick et al., 2010]. Untreated infected cells showed no stain in the host cytoplasm (Fig 6 a-b). However, the vacuoles that contained destroyed parasites were positive for MDC after incubation with the three different metals (Fig 6 c-h). The counting of this events showed that four in one hundred untreated infected cells were positive to MDC, while twenty-seven, thirty-one and thirty-nine treated infected cells were stained (zinc, cadmium and mercury, respectively) (statistical significance for all ** $p<0.05$). Thus, it suggests that the mechanism of autophagy was involved in recognizing and eliminating the parasite of the host cell, even the parasite residing in the cytoplasm.

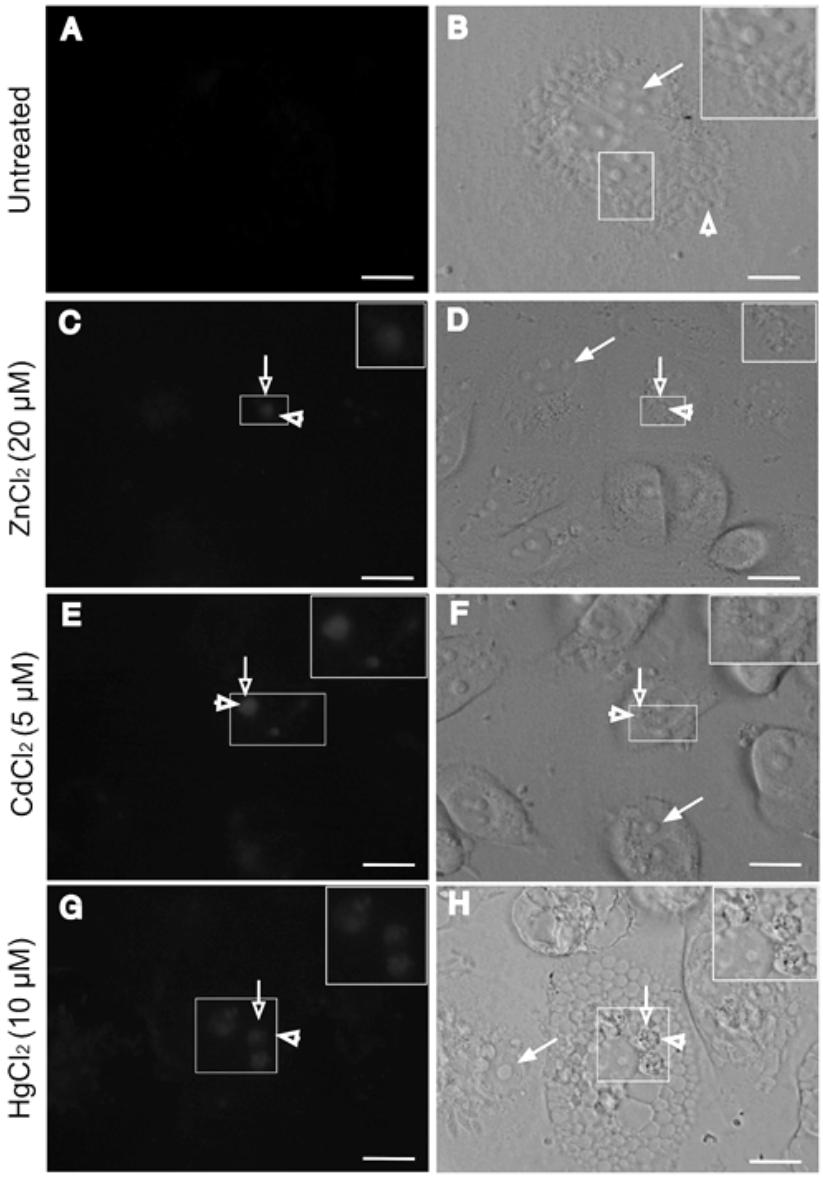

Figure 6 Infected host cell were treated with metals for 12 h and stained with the functional autophagic vacuole selective marker Monodansylcadaverine. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 μm.

3.2 Kinetic studies of metal toxicities on intracellular parasites

It was observed in Figure 5 that only unviable parasites were stained by LysoTracker Red, while the viable ones were not. This feature was explored to observe the time (kinetic) in which cadmium and mercury induce toxic effects on majority of the parasites.

In the fluorescence assays above, the untreated cells had an unstained portion in the cytoplasm co-localized with the parasites (Fig 7 and 8, a and b). However, 5 μM $CdCl_2$ incubations for 1 h induced parasite damage and lysosome markers co-localized with the parasites (Fig 7 c-f). This result was intensified with 10 μM $CdCl_2$ for 1 and 2 h (Fig 7 g-j). Similar structures for vacuoles surrounding one (Fig 7 E and F) or many destroyed parasites (Fig 7 c, d, g-j) were also observed.

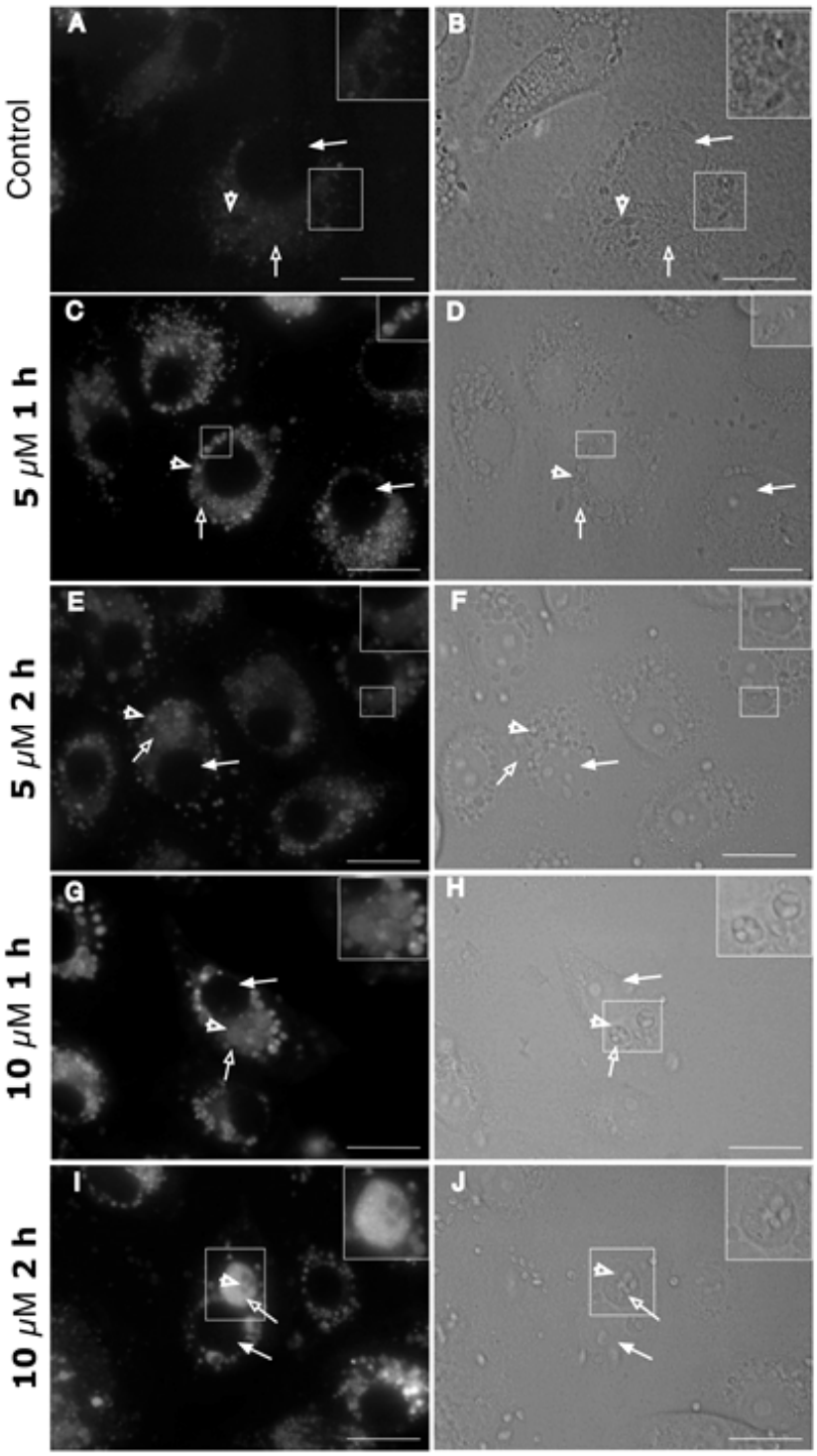

Figure 7 Kinetic assays for $CdCl_2$ incubations and lysosomal staining. The $CdCl_2$ incubations at different times and concentrations showing co-localized staining with vacuole similar structures containing destroyed parasites. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 μm.

Similar results were seen after $HgCl_2$ incubations (Fig 8). Many morphologically altered parasites were surrounded by vacuole-similar structures highly stained for lysosomes (Fig 8 g-j), which suggest that a short period of incubation (2 h, 5 or 10 μM) caused parasite destruction by lysosomal enzymes.

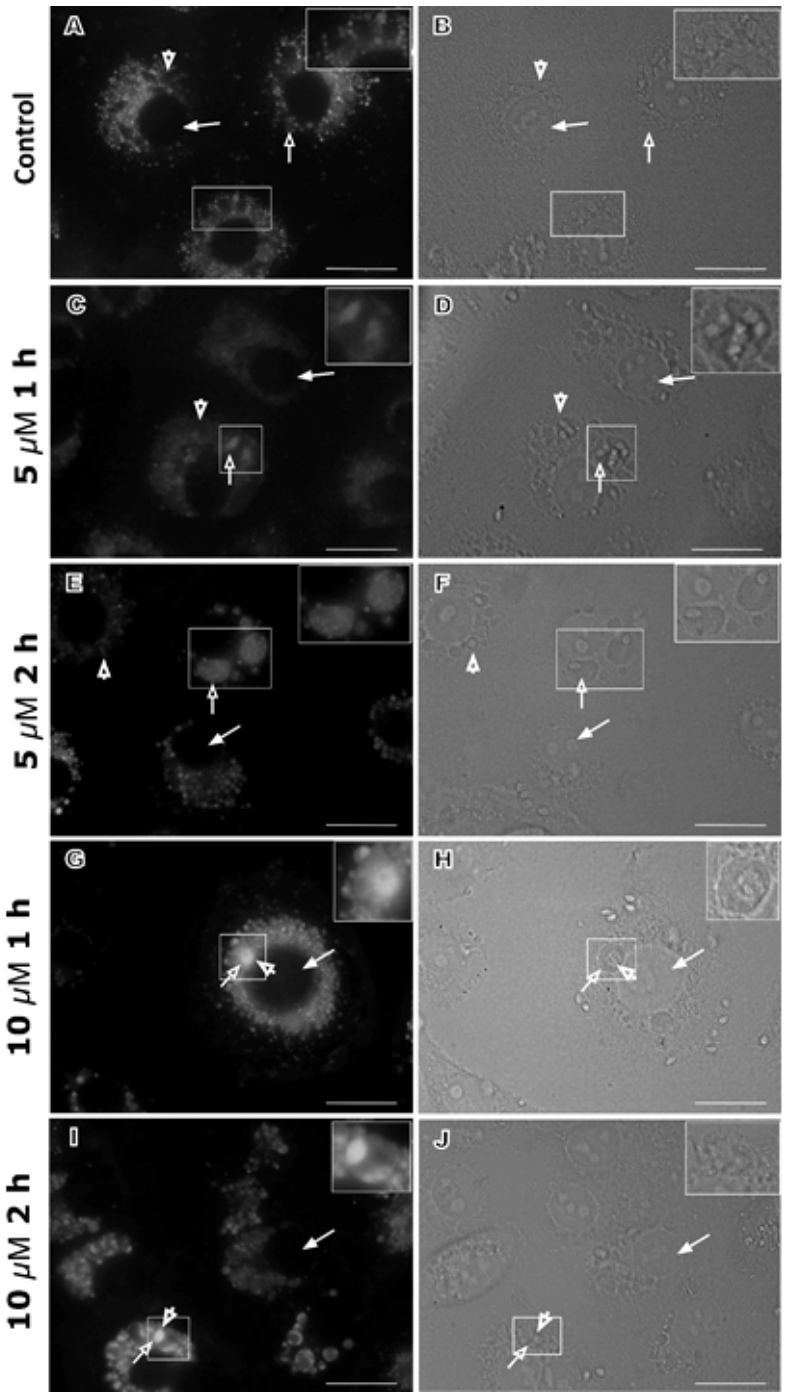

Figure 8 Kinetic assays for $HgCl_2$ incubations and lysosomal staining. The $HgCl_2$ incubations with different times and concentrations showing co-localized staining with vacuole similar structures containing destroyed parasites. Full arrows: host cell nuclei; empty arrows: parasites; arrowhead: the selective marker. Inserts: amplification of the selected areas. Scale bars: 20 μm.

3.3 Cytochemistry analyses

Specific cytochemistry analyses were performed to visualize whether those membranes surrounding the parasites were coming from the endoplasmic reticulum. For this purpose, the product of the reaction of glycose-6-phosphatase, mainly found in the endoplasmic reticulum was marked. In the untreated metal cells, the reaction product of glycose-6-phosphatase was precipitated on endoplasmic reticulum through the cytoplasm and surrounding the nucleus (Fig 9 a), while the parasites were spread on the cytoplasm without marks (Fig 9 b). After 10 μM $CdCl_2$ treatment for 5 h, the precipitations of the products of the reactions of glycose-6-phosphatase were punctuated and discontinued on the reticulum (Fig 9 c), and tightly surrounding the morphologically altered parasites, which indicate that the endoplasmic reticulum was involved in vacuole formation (Fig 9 d). After the 12 h treatment, the effects were exacerbated, and a discontinuous reticulum was observed in the cytoplasm and around the parasite. A double-membrane vacuole originated from the endoplasmic reticulum and isolating a cytoplasmic structure is a specific feature of the autophagic pathway.

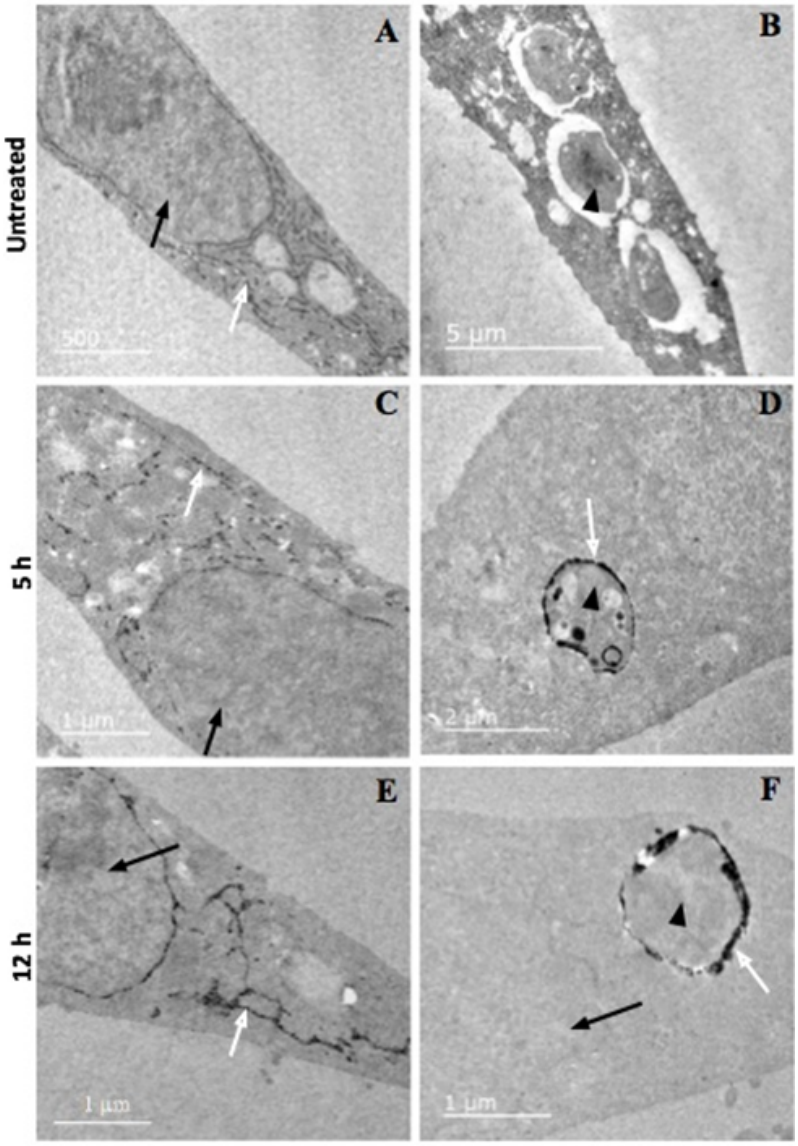

Figure 9 Cytochemistry analysis for endoplasmic reticulum in infected host cells. The host cells were labeled to glycose-6-phosphatase reaction product. Black arrow: host cell nuclei. White arrows: glycose-6-phosphatase reaction product. Arrowhead: intracellular parasites.

4 - *Discussion*

Trypanosoma cruzi, an obligate intracellular parasite, is transmitted to vertebrate hosts by blood-sucking insects. Molecules present in both parasite and mammalian cells are responsible for recognition and triggering of intracellular signaling cascades resulting in parasite internalization via endocytic pathways [Barrias *et al*. 2003]. The intracellular cycle initiates with the formation of an endocytic vacuole also known as the parasitophorous vacuole. This vacuole disrupts hours after its formation, which releases the amastigotes into the cytoplasm where they multiply in direct contact with the host cell organelles, subverting host cell microbicide responses. The pre-replicative and replicative phase of intracellular *T. cruzi* is supported by host cell signaling pathways and cytoskeletal regulators [Caradonna *et al*. 2011]. The metallic ions play an important role in the establishment and maintenance of host-parasite interactions and an imbalance can cause severe damage to both [Weinberg 1966]. As described in Carvalho and Melo [2017], the parasites were more susceptible to metal incubations than the host cells. From 2 hours of treatment with 5 μM of $CdCl_2$ and $HgCl_2$ significant reductions on parasite numbers were observed. However, the significant elimination of the host cells occurred from 5 μM of both metals. In addition, when the reversibility assays were performed with the infected host cells using concentrations until 8 μM of $CdCl_2$ and $HgCl_2$ for 24 h, it was observed that the host cells were able to recover their typical morphology, but the parasites were continuously eliminated. Thus, many specific dyes were used to investigate the cellular events involved in the intracellular parasite elimination in the presence of metal ions

Mammalian cells have many defense mechanisms against nonessential metals including enzymatic and non-enzymatic responses. The enzymatic defense mechanisms involve the use of catalase, glutathione peroxidase, metallothioneins and superoxide dismutase. Non-enzymatic responses include reductive compounds such as ascorbate, β-carotene, α-tocopherol and reduced glutathione or even the rapid metal efflux [Maya *et al*. 2007]. However, the parasite`s

defense mechanisms are poorly described and diverge from the mammalian ones. For instance, no catalase, glutathione peroxidase, β-carotene or α-tocopherol have been described in *T. cruzi* and a low activity of superoxide dismutase is observed, as reviewed in [Morales *et al.* 2016]. Therefore, the trypanothione has been described as the *T. cruzi*'s main response against free radicals and indispensable for glutathione reduction [Ariyanayagam and Fairlamb 2001; Turrens 2004].

Several factors can be involved in parasite death. In general, mercury can cross lipid membranes [Girault *et al.* 1997] and cadmium and mercury have high affinity for thiol groups and its chemical mimicry of essential divalent metals induce competition for these ion metabolic pathways and binding sites, which impairs the biochemical pathways [Maret and Vallee 1998; Waisberg *et al.* 2003]. In addition, the mechanisms of cadmium toxicity are not restricted to the interaction with pathways involved in the homeostasis of other metal cations. The cellular exposure to cadmium also disturbs various intracellular signaling pathway due to its high-affinity interaction with surface receptors [Moulis 2010]. The metal imbalance can lead to the production of free radicals involved in the disruption of calcium and sulfhydryl homeostasis, lipid peroxidation and modifications to DNA bases [Jonova and Valko 2011].

The first target in this study was the mitochondrion that is an important pharmacological target because it is involved in survival and cell death signaling [Szewczyk and Wojtczak 2002]. *Trypanosoma cruzi* has a single and well-developed mitochondrion that spans the entire cell body under the subpellicular microtubules [Lisvane *et al.* 2011]. The mitochondrial membrane can control the uptake of essential metabolic molecules to guarantee the parasite`s survival and allowing an intense flux of inorganic ions [Szewczyk and Wojtczak 2002]. For this purpose, the mitochondria have selective channels to the main inorganic cations (K^+, Na^+, Mg^{2+} and Ca^{2+}) [Fontaine and Bernardi 1999] that are present in various mitochondrial compartments such as ion pumps and chaperones [Atkinson and Winge 2009; Rines and Ardehali 2017]. The

radicals resulting from ATP production can cause oxidative damage to mitochondria, which can cause apoptosis. For this reason, the presence of metals should be tightly regulated [Grubman *et al.* 2014]. The present study showed that $CdCl_2$ and $HgCl_2$ treatments decreased the mitochondrial functionality (Figure 2), suggesting that nonessential metals impair mitochondrial homeostasis and activity, which compromise parasite`s viability. Previous studies showed that the presence of Cd^{2+} in mitochondria produced ROS and led to the opening of the mitochondrial permeability transition pore (MPT) by interacting with calcium- and thiol-dependent domains which culminated in cell death [Belyeva *et al.* 2006]. The imbalance of calcium can also lead to MPT opening, causing exhaustion of the ATP pool and efflux of apoptotic factors from the mitochondria [Fontaine and Bernardi 1999]. As Cd^{2+} is a Ca^{2+} agonist [Marchetii 2013], this effect may also occur. Hg^{2+} the enters mitochondria using the negative charge in the mitochondrial matrix resulted by the respiratory chain [Szewczyk and Wojtczak 2002], which impairs the oxidative phosphorylation [Reyes-Vivas 1996; Belyaeva *et al.* 2006].

'The next studied organelle was the nucleus. In the nucleus occurs the DNA replication and its proper functioning is essential for cell viability and proliferation. For this purpose, the cells have a group of proteins and enzymes to ensure the DNA structure and function or to repair damages on DNA structure [Elmore 2007]. However, previous studies showed that heavy metals may produce ROS and cause cellular modifications, which impair the repair processes of DNA damage [Morales *et al.* 2016]. For example, Cd^{2+} may induces caspase-independent apoptosis after replacement of Zn^{2+} on the tumor suppressor protein (p53), compromising p53-mediated DNA damage repair or cell cycle arrest [Andreini *et al.* 2006]. In agreement, the present study showed that metal imbalance caused by mainly $ZnCl_2$ and $CdCl_2$ incubations induced DNA condensation (Fig 2), which affected parasite`s viability. The nucleus is highly involved in cellular Zn (II) homeostasis since polymerases, transcription factors and DNA remodeling factors need Zn (II) as a cofactor [Andreini *et al.* 2006]. Additionally, the metallothioneins (Zn storage proteins) translocate to the nucleus during the G1-to S phase,

suggesting a tight regulation of nuclear Zn during the cell cycle [Tsujikawa *et al*. 1991]. Thus, the parasites should be the main target for the metals because of their fast-proliferative cycle is more dependent on nuclear proteins and enzymes. The chemical basis for the biological activity of metals is its high capacity for reaction with sulfhydryl groups of cellular components, molecules of low molecular weight and proteins with cysteine residues. In the cells, the metals naturally bind to sites of proteins (amine, imidazole, carboxyl, thiol) and nucleic acids (phosphate and nitrogenous bases and substituent oxygen) and reflect the power of affinity with these. Physical and chemical characteristics of the metals can direct the favorite target because of existing sites in these structures.

The metals damaged vital organelles such as mitochondrion and nucleus, which led to parasite death. Possible mechanisms involved in parasite death are related to the programmed cell death: apoptosis and autophagy. However, the cellular events involved in cytoplasmic parasite elimination are not well known. Our results showed that after metal treatments, the parasites lost mitochondrial membrane potential (Fig 1) and had condensed nucleus (Fig 2), although no blebs on parasite membrane was observed, which indicated intrinsic apoptosis. The dead parasites were eliminated from the host cytoplasm. The autophagy is a cell constituent mechanism involved in the turnover of cytoplasmic molecules and organelles to maintain the cell homeostasis [Glick *et al*. 2010]. In this mechanism, the undesirable or recyclable proteins or organelles are involved in a double-membrane vacuole originated from the endoplasmic reticulum - the autophagosome - which fuses with lysosomes to form the autophagolysosome to degrade the substances, as reviewed in [Broker *et al*. 2005]. Recently, a specific autophagy was described as an innate microbicide mechanism to eliminate different intracellular parasites upon cell invasion – the xenophagy [Glick *et al*. 2010; Songane *et al*. 2012; Yano and Kurata 2011]. The occurrence of autophagy includes the increase of acid compartments on host cytoplasm and parasites, vacuole formation with an endoplasmic reticulum-derived membrane, vacuole-lysosome fusion, and digestion by lysosomal enzymes [Schid and Munz 2007].

Although, after invasion, *T. cruzi* benefits of the lysosome-vacuole fusion to escape from the phagolysosome to cytosol and establishing an infection [Tyler and Engman 2001], this mechanism was further investigated after metal treatments and parasite death. As showed, all these events cited above occurred (Fig 4-9), which suggested that host cells were able to eliminate inviable cytoplasmic parasites after metal treatments, indicating a recovery of the microbicide responses. Taken together, these results delineated some steps involved in parasite death and elimination in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$.

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4. DISCUSSÃO

O *Trypanosoma cruzi* passa parte do seu ciclo de vida no barbeiro (inseto vetor) e outra parte em mamíferos, os hospedeiros vertebrados (TYLER; ENGMAN, 2001). Nestes últimos, ele é capaz de infectar todos os tipos células eucarióticas, embora exista um tropismo por células musculares esqueléticas, cardíacas e lisas (FERNANDES; ANDREWS, 2012). Durante seu ciclo de vida, os parasitos passam por diferenciações morfofisiológicas que os possibilitam sobreviver em diferentes ambientes e estabelecer uma infecção no hospedeiro (OSÓRIO *et al.*, 2012). No entanto, o sucesso do estabelecimento da infecção nos vertebrados e a manifestação da doença de Chagas variam entre diferentes cepas do *T. cruzi,* carga parasitária e condições de saúde do indivíduo. O *T. cruzi* é um parasito dependente do ambiente intracelular nos hospedeiros vertebrados para sua multiplicação, disseminação e estabelecimento da infecção (VILLALTA *et al.*, 2009). No ambiente intracelular, o parasito se protege dos mecanismos imunológicos de defesa do hospedeiro como também dos quimioterápicos tradicionais usados no tratamento da infecção.

Os tratamentos para a doença de Chagas agem prioritariamente na fase aguda da doença (RASSI; RASSI; MARIN-NETO, 2010). No entanto, devido à infecção pelo *T. cruzi* ser assincrônica e o parasito ter um longo ciclo intracelular, os tratamentos tradicionais precisam ser prolongados, causando sérios efeitos colaterais aos pacientes. Por este motivo, diferentes compostos têm sido testados com o objetivo de eliminar os parasitos presentes nas diferentes fases da doença e evitar o desenvolvimento de resistência (FARAL-TELLO *et al*., 2014; OLIVEIRA FILHO *et al*., 2015; Donnici *et al*., 2009). Neste contexto, a adição de metais em diferentes compostos tem sido uma alternativa na química medicinal. Por exemplo, compostos com platina contra câncer (DASARI; TCHOUNWOU, 2014), prata em antimicrobianos (SWATHY *et al.*, 2014) e antimônio para leishmaniose (HALDAR; SEN; ROY, 2011). Várias drogas compostas por rutênio, ouro, titânio e platina estão em fase de teste para diferentes tipos de câncer (NDAGI; MHLONGO; SOLIMAN, 2017), vanádio para diabetes (DOMINGO; GÓMEZ, 2016) e ferro para malária (HELD *et al.*, 2015). No entanto, pouco se sabe sobre a influência destes elementos metálicos no modelo de parasitismo do *T. cruzi*. E vários processos

envolvidos na invasão e estabelecimento da infecção do *T. cruzi* na célula hospedeira são dependentes de metais. Assim, o estudo sobre os efeitos das interações dos elementos metálicos com estruturas importantes no desenvolvimento do parasito abre uma série de possibilidades de alvos celulares e moleculares a serem estudados para o desenvolvimento de novos quimioterápicos.

O estudo da influência dos íons metálicos em parasitos ainda é restrito a poucos grupos de pesquisa, e o uso do *Trypanosoma cruzi* nesta finalidade é muito recente na bibliografia especializada. No entanto, nos últimos anos, alguns grupos passaram a investigar os efeitos de diferentes metais em outros modelos parasitários como *Leishmania* (PALETTA-SILVA *et al.*, 2012; KUMARI *et al.*, 2017; SAINI *et al.*, 2017) e *Toxoplasma gondii* (CARVALHO; MELO, 2016; CARVALHO; MELO, 2018). Um estudo mostrou o efeito tóxico de íons de cobre, um importante metal essencial, sobre promastigotas (forma infectiva encontrada no vetor) de *L. amazonensis* devido a inibição da atividade da enzima 3'-nucleotidase, envolvida hidrólise de ácidos nucléicos para obtenção de nutrientes (PALETTA-SILVA *et al.*, 2012). Recentemente, foi descrita a importância do zinco para a sobrevivência da *Leishmania* e para o estabelecimento da infecção na célula hospedeira (KUMARI *et al.*, 2017; SAINI *et al.*, 2017). Como mostrado nestes dois estudos, o zinco foi essencial para a virulência dos promastigotas e o processo de invasão dos macrófagos e a diminuição da viabilidade deste metal causou a morte destes parasitos por apoptose.

Nossos estudos mostraram que os íons metálicos adicionados à cultura do parasito em diferentes estágios causaram alterações nos processos de invasão, de estabelecimento e disseminação intracelular da infecção, levando a morte e a eliminação dos parasitos em concentrações que não foram tóxicas às células hospedeiras. O processo de invasão da célula hospedeira pelo tripomastigota inicia-se com o reconhecimento de várias moléculas de superfície, encontradas tanto no parasito quanto na sua célula hospedeira, e o desencadeamento de vias de sinalizações dependentes de cálcio (SOUZA; CARVALHO; BARRIAS, 2010). Como mostrado no trabalho intitulado *Life and death of Trypanosoma cruzi in the presence of metals*, a incubação dos tripomastigotas com o cádmio, especificamente, inviabilizou a infecção

celular, evento que não aconteceu quando somente as células hospedeiras foram tratadas com o mesmo metal (Tabela 5, Figura 13). O cádmio é reconhecidamente um metal que substitui cálcio e zinco devido às semelhanças físico-químicas (MOULIS, 2010), o que indica que o cádmio pode estar interferindo no desencadeamento das vias de sinalizações dependentes de cálcio no *T. cruzi* ou bloqueando as moléculas de superfície do parasito, impedindo a adesão celular e evitando, assim, a invasão.

Os efeitos dos metais no estabelecimento intracelular da infecção também foram investigados. Normalmente, após a invasão celular, os parasitos permanecem por algumas horas (8-16 h) dentro de um vacúolo com características endocíticas ou fagocíticas onde o parasito inicia seu ciclo intracelular. O escape para o citoplasma e a multiplicação parasitária só é possível após a fusão do vacúolo com lisossomos e o desencadeamento de vários fatores que formam poros causando a ruptura da membrana deste vacúolo, liberando os parasitos no citoplasma do hospedeiro (CARVALHO; SOUZA, 1989). No presente estudo, investigamos a influência dos metais em cada etapa do ciclo de vida intracelular detalha mais frase com geral da tese.

No trabalho *Intracellular development of Trypanosoma cruzi in the presence of metals* foi mostrado que as incubações da cultura infectada com tripomastigotas com os metais cádmio e mercúrio a 1 μM e zinco a 20 μM por 24 h não foram suficientes para impedir o escape para o citoplasma (Figuras 3 e 4). Entretanto, quando estas mesmas condições foram testadas na etapa posterior quando os amastigotas estavam em multiplicação, ocorreu uma redução no número de parasitos em relação ao controle (*Life and death of Trypanosoma cruzi in the presence of metals*, Tabela 2, Figuras 5, 6 e 7). Estes resultados sugerem que os estágios morfológicos do *T. cruzi* possuem diferentes níveis de tolerabilidade a presença dos metais. Este fato está de acordo com o descrito em outros trabalhos onde mostram que as necessidades de mudanças bioquímicas e celulares que os parasitos sofrem para se adaptarem a diferentes microambientes em seus hospedeiros (OSÓRIO *et al.*, 2012). Os tripomastigotas, também presentes na porção final do intestino do vetor e na corrente sanguínea dos mamíferos, precisam se adaptar rapidamente as diferentes condições e a lidar com os mecanismos microbicidas

moleculares do seu hospedeiro (KOLLIEN; SCHAUB, 2000). Possivelmente, esta plasticidade o tornou mais resistente aos metais.

O fim do processo de transformação do tripomastigota em amastigota no ambiente citoplasmático ocorre, aproximadamente, 18 horas após a infecção. Desta forma, os amastigotas entram no ciclo de divisão celular estabelecendo a infecção. Nesta fase, os parasitos foram novamente incubados com os metais a fim de avaliar suas influências no ciclo de multiplicação do parasito. Como mostrado em *Life and death of Trypanosoma cruzi in the presence of metals*, os metais não essenciais foram capazes de reduzir significativamente o número de amastigotas tratados em relação aos controles com 2 horas de incubação (Figuras 5 e 6, Tabela 2), enquanto o zinco precisou de 24 h para poder reduzir o número de parasitos significativamente. Para que ocorra a redução do número de parasitos, os metais precisam estar controlando o ciclo de divisão celular ou levando a eliminação destes parasitos de alguma maneira, ou ainda, induzirem estes dois eventos ao mesmo tempo. Neste contexto, possíveis alvos celulares e eventos relacionados com a eliminação dos amastigotas foram investigados através da incubação dos metais nas infecções estabelecidas e o uso de marcadores fluorescentes específicos para diferentes eventos celulares.

Como observado no trabalho *Cellular events related to Trypanosoma cruzi elimination in the presence of $ZnCl_2$, $CdCl_2$ and $HgCl_2$*, os tratamentos com os metais levaram a uma diminuição da função mitocondrial (Figura 2) e condensação nuclear (Figura 3), eventos capazes de induzirem a morte parasitária por apoptose. No entanto, foi observada uma diminuição do número de amastigotas, indicando que o parasito morto foi eliminado por mecanismos já existentes nas células hospedeiras. Neste contexto, algumas estruturas e eventos celulares do hospedeiro também foram investigados. As células eucarióticas possuem duas principais vias de degradação que incluem os proteossomos e os lisossomos (MIZUSHIMA; KOMATSU, 2011). A degradação por proteossomos é altamente seletiva e ocorre apenas com substratos ubiquitinados como proteínas de vida curta. Por outro lado, materiais extracelulares e proteínas de membrana quando internalizados, componentes citoplasmáticos e organelas são direcionadas aos lisossomos para a degradação através da autofagia (MIZUSHIMA;

KOMATSU, 2011). A formação dos vacúolos autofágicos é um processo de múltiplas etapas incluindo a utilização de membranas do retículo endoplasmático e mitocôndrias e a fusão com endossomos primários e secundários assim como lisossomos, o que diminui o pH intravacuolar, como revisado em ESKELINEN, 2005; TOOZE; YOSHIMORI 2010. De acordo com estas etapas, foi observado neste mesmo trabalho a acidificação tanto no citoplasma da célula hospedeira quanto da estrutura que circundava parasitos estruturalmente alterados (Figura 4), assim como uma proliferação citoplasmática e a presença dos lisossomos no interior desta estrutura (Figura 5). Testes usando marcadores fluorescentes específicos como a monodansilcadaverina que se acumula em vacúolos autofágicos (Figura 6) e citoquímica para a enzima glicose-6-fosfatase, presente exclusivamente no lúmen do retículo endoplasmático (BURCHELL, ALLAN, HUME, 1994) confirmaram a via autofágica como meio de eliminação do parasito (Figura 9). Estes eventos mostram que mesmo o *T. cruzi* em fase de replicação no citoplasma não está livre dos mecanismos microbicidas inatos do hospedeiro e que, para estabelecer uma infecção, o parasito precisa, continuamente, evadir destes mecanismos. Ao mesmo tempo, estes eventos mostraram que mesmo na presença de metais não essenciais como cádmio e mercúrio, as células conseguiram manter suas funções básicas e reagir a uma infecção no seu citoplasma, mostrando que permaneceram viáveis e que foram capazes de eliminar os patógenos a partir do momento que a multiplicação destes foi controlada. A grande importância da autofagia no processo de eliminação de um parasito intracelular é que esta possibilita a apresentação de antígenos na superfície celular, o que também auxilia no controle da infecção (GLICK; BARTH; MACLEOD, 2010). Estes mesmos eventos também foram observados quando estudados em outro modelo de infecção celular como no caso do *Toxoplasma gondii* (CARVALHO; MELO, 2018). O desenvolvimento intracelular do *T. gondii* acontece completamente no interior do vacúolo parasitóforo, estrutura que também evita o reconhecimento dos sistemas microbicidas das células hospedeiras (BLADER; SAAEIJ, 2014). No entanto, sobre as mesmas condições, os mesmos eventos observados na eliminação do *T. cruzi* também foram vistos na eliminação do *T. gondii*, sem efeitos nas células hospedeiras.

Os vários mecanismos de defesa celulares contra os efeitos dos metais nas células de mamíferos podem ter sido essenciais para a manutenção da viabilidade celular, o que possibilitou a recuperação do controle da infecção. Estes mecanismos também podem ter sido responsáveis pela capacidade da célula reverter os efeitos tóxicos causados por baixa concentração de metais. No entanto, o *T. cruzi* possui mecanismos bem menos desenvolvidos, o que os tornou tão susceptíveis a ação tanto do metal essencial zinco quanto aos metais não essenciais cádmio e mercúrio.

5. CONCLUSÃO

- ✓ Os três metais foram capazes de eliminar as principais formas do *T. cruzi* em concentrações não tóxicas as células hospedeiras;
- ✓ As células hospedeiras reverteram os efeitos tóxicos dos metais quando incubados até 5 μM por 24 h, mas os parasitos não;
- ✓ A incubação dos tripomastigotas com $CdCl_2$ inibiu a infecção celular;
- ✓ O escape do parasito do vacúolo endocítico não foi prejudicado pelos metais, mas houve um atraso no desenvolvimento citoplasmático;
- ✓ A pré-incubação com $ZnCl_2$ diminuiu significativamente os efeitos tóxicos do $CdCl_2$, mas não do $HgCl_2$ nos parasitos;
- ✓ Os amastigotas perderam a funcionalidade mitocondrial e apresentaram DNA bastante condensado; eventos que podem causar a morte do parasito;
- ✓ Eventos ocorridos na célula hospedeira como acidificação citoplasmática, aumento de lisossomos e autofagia estão envolvidos na eliminação do parasito;
- ✓ A ocorrência destes eventos mostrou que as células estavam viáveis após os tratamentos com os metais e que seus mecanismos microbicidas naturais foram capazes de eliminar os parasitos;

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7. APÊNDICE

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The toxic effect of *Vu*-Defr, a defensin from *Vigna unguiculata* seeds, on *Leishmania amazonensis* is associated with reactive oxygen species production, mitochondrial dysfunction, and plasma membrane perturbation

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ORIGINAL PAPER

Further aspects of *Toxoplasma gondii* elimination in the presence of metals

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Abstract

Toxoplasma gondii, the etiological agent of toxoplasmosis, infects nucleated cells and then resides and multiplies within a parasitophorous vacuole. For this purpose, the parasite secretes many virulence factors for the purpose of invading and subverting the host microbicidal defenses in order to facilitate its survival in the intracellular milieu. Essential metals are structural components of proteins and enzymes or cofactors of enzymatic reactions responsible for these parasitic survival mechanisms. However, an excess of non-essential or essential metals can lead to parasite death. Thus, infected host cells were incubated with 20 μM $ZnCl_2$ in conjunction with 3 μM $CdCl_2$ or $HgCl_2$ for 12 h in order to investigate cellular events and organelle damage related to intracellular parasite death and elimination. In the presence of these metals, the tachyzoites undergo lipid uptake and transport impairment, functional and structural mitochondrial disorders, DNA condensation, and acidification of the parasitophorous vacuole, thus leading to parasite death. Additional research has suggested that lysosome-vacuole fusion was involved in parasite elimination since acid phosphatases were found inside the parasitophorous vacuole, and vacuoles containing parasites were also positive for autophagy. In conclusion, low concentrations of $CdCl_2$, $HgCl_2$, and $ZnCl_2$ can cause damage to *Toxoplasma gondii* organelles, leading to loss of viability, organelle death, and elimination without causing toxic effects to host cells.

Keywords Cadmium · Essential metal · Mercury · Nonessential metals · *Toxoplasma gondii* · Zinc

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In Vitro Anti-*Toxoplasma gondii* and Antimicrobial Activity of Amides Derived from Cinnamic Acid

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Cell toxicity by ricin and elucidation of mechanism of Ricin inactivation

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ABSTRACT

Castor cake is a by-product of the extraction of oil from from seeds of castor plants (*Ricinus communis*). This by-product contains high levels of proteins, but a toxic protein, ricin, limits its use as an animal feed. Ricin can be efficiently inactivated by treatment with calcium oxide (CaO), which can be evaluated by a cytotoxicity assay using *LLC-MK2* cells. The mechanism by which the CaO treatment inactivates ricin, however, is unclear. We report the structural changes responsible for ricin inactivation. Purified ricin was treated with 0.6% CaO and then analyzed by mass spectrometry. This treatment degraded the ricin at preferential sites. The aqueous CaO solution had a pH >12, which preferentially cleaved asparagine residues, followed by glutamine, serine and glycine residues. The alkaline pH affected the tertiary structure of the ricin, cleaving its polypeptide chains and thereby eliminating its cytotoxic activity.

ORIGINAL ARTICLE

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records of natural products

Chemical Composition and Anti-*Candida* and Anti- *Trypanosoma cruzi* Activities of Essential Oils from the Rhizomes and Leaves of Brazilian Species of *Renealmia* L. fil.

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Abstract: This work aimed to determine the chemical composition of essential oils from rhizomes and leaves of *Renealmia chrysotricha* Petersen, *R. breviscapa* Poepp. & Endl. and *R. nicolaioides* Loes., and to evaluate the biological activities of these oils on three *Candida* species and the parasite *Trypanosoma cruzi*. The rhizomes and leaves were collected in the Atlantic and Amazon rainforests. Essential oils were isolated and characterized by gas chromatography. *Beta*-Caryophyllene was found to be the most predominant compound in the essential oils of rhizomes and leaves of *R. breviscapa*, and the rhizomes of *R. nicolaioides*, whereas (*E*)-nerolidol was the most abundant compound in the oils of leaves of *R. nicolaioides*. In *R. chrysotricha*, α-terpineol, coronarin-E and 1,8-cineole were found to be the most predominant compounds in the essential oils of rhizomes, whereas *cis*-3-hexenol was predominant in the leaves. The tested oils did not inhibit *C. albicans* growth at 1000 µg/mL, whereas leaf oils from *R. chrysotricha* and *R. nicolaioides* inhibited the growth of *C. buinensis* and *C. tropicalis* by about 50%. Essential oils from the rhizomes and leaves of *R. chrysotricha* exhibited efficient antiparasitic activity against *Trypanosoma cruzi*. Damage to *T. cruzi* epimastigotes was confirmed by LM and TEM.

Keywords: Zingiberaceae; rhizomes; leaves; terpenoids; anticandidal activity; antiparasitic activity.