EVIDENCE OF AUTOPHAGY IN TRYPANOSOMA CRUZI CELLS BY QUANTUM DOTS

W. A. Marques[1]; S. A. O. Gomes[2]; D. B. Almeida[3]; J. P. F. Pacheco[1], R. F.S. Menna-Barreto[4]; J. R.Santos-Mallet[5]; C. L. Cesar[2] and D. Feder[1]*

[1]Laboratório de Biologia de Insetos, GBG, Universidade Federal Fluminense-UFF, Rio de Janeiro, RJ, Brazil;
[2] Laboratório de Biodiversidade de Parasitas e Vetores, GBG, Universidade Federal Fluminense-UFF, Rio de Janeiro, RJ, Brazil - CEP:24210-130;
[3]Laboratório de Aplicações Biomédicas de Lasers, Departamento de Eletrônica Quântica, Instituto de Física Gleb Wataghin, Universidade Estadual de Campinas, Campinas, SP, Brazil, CEP: 13083-970;
[4]Laboratório de Biologia Celular, IOC-FIOCRUZ - Rio de Janeiro, RJ, Brazil;
[5]Laboratório de Transmissores de Leishmanioses, Setor de Entomologia Médica e Forense, IOC-FIOCRUZ - Rio de Janeiro, RJ, Brazil, CEP: 21040-360.

*Corresponding author, E-mail: mdfeder@id.uff.br

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ABSTRACT
Nanotoxicity by quantum dots (QDs) has been extensively studied in prokarionte and eukaryonte cells. In this context, we are using QDs capped with mercaptoacetic acid synthetized in an aqueous medium in order to evaluate the nanocytotoxicity effects as cell death by autophagy in *Trypanosoma cruzi*. To check this fact, we used two kinds of autophagy markers: 3 methyladenine autophagy (3-MA) a phosphatidylinositol 3- kinase (PI3k) inhibitor and monodansylcadaverine (MDC) a lisossomotropic fluorescent compound useful for identifying cadaverine protein present in the autophagic vacuoles. The quantification of control parasites, 3-MA treated parasites and/or incubated with two QDs concentrations (2 and 200 μM) was made from the growth curves. Then, the same samples were processed for transmission electron microscopy (TEM) and labeling of autophagic vacuoles by MDC was qualitatively analyzed. The results from the growth curve indicated a decrease in parasite numbers incubated with 200μM QDs when compared to parasites incubated with 2μM QDs and the control group. In contrast, parasites incubated with 200μM QDs and treated with 10 and 15mM of 3-MA showed a dose-dependent reversion of cell death by 10% and 15% respectively. The parasite treated by MDC demonstrated many autophagic vacuoles. Also, we found ultrastructural morphological changes in parasites incubated with 2μM and 200μM QDs when compared to control group. Our results show that high QDs concentrations are toxic to *T. cruzi*, inducing cell death by autophagy.

Keywords: Quantum dots, *Trypanosoma cruzi,* nanocitotoxicity, autophagy.

EVIDENCIA DE AUTOFAGIA EM *TRYPANOSOMA CRUZI* MARCADOS COM QUANTUM DOTS

RESUMO
A toxicidade de nanopartículas como quantum dots (QDs) tem sido extensivamente estudada em células procariontes e eucariontes. Neste contexto, utilizamos QDs cobertos com ácido mercaptoacético, sintetizado em um meio aquoso, a fim de avaliar efeitos nanocitotóxicos como morte celular por autofagia, em *Trypanosoma cruzi*. Foram utilizados dois tipos de marcadores de autofagia: 3-metiladenina (3-MA) um inibidor de fosfatidilinositol 3-kinase (PI3-K) e monodansilcadaverina (MDC) um composto fluorescente lisossomotrópico usado na identificação de cadaverina, proteína presente em vacúolos autofágicos. A quantificação de parasitas tratados com 3-MA e / ou incubados com 2 e 200 μM de QDs foi feita a partir da curva de crescimento. Em seguida, os parasitas foram processados para micorscopia eletronica de transmissão (MET) e marcados com MDC, sendo as vacúolos autofágicos MDC positivas qualitativamente analisadas. Os resultados obtidos da curva de crescimento indicaram uma diminuição do número de parasitas incubados com 200 μM de QDs quando comparados com parasitas incubados com 2 μM de QDs e o grupo controle. Em contrapartida, parasitas incubados com 200 μM de QDs e tratados com 10 e 15 mM de 3-MA, mostraram uma reversão dose-dependente (10% e 15%) da morte celular por autofagia. Os parasitas marcados com MDC demonstraram a presença de muitos vacúolos autofágicos. Além disso, encontramos alterações morfologicas ultraestruturais nos parasitas incubados com 2 μM e 200 μM QDs quando comparado com o grupo controle. Nossos resultados demonstram que altas concentrações de QDs são tóxicos para *T. cruzi*, induzindo morte celular por autofagia.

Palavras chaves: Quantum dots, *Trypanosoma cruzi,* nanocitotoxicidade, autofagia.

INTRODUCTION

The most commonly used method for the detection and quantification of biomolecules are still the fluorescent markers [1].

Luminescent semiconductor nanocrystals or quantum dots (QDs) have received great attention in the research and biomedical applications for the last decade. QDs are nanoparticles whose size can range 2-10 nm in diameters. These kinds of nanoparticles have optical and electronic properties controlled by their size, morphology and interfaces, with exceptional resistance to both photobleaching and chemical degradation. Such nanoparticles have the ability to be bioconjugate with various biomolecules (proteins, antibodies, peptides, DNA, etc.) and therefore, consistently used in biological research and in biomedical applications. This type of fluorescent probes are used widely in biosensing applications including immunoassays, nucleic acid detection, resonance energy transfer, clinical / diagnostic assays, cell labeling and many others. However, the possibility of becoming cytotoxic nanoparticle precludes their widespread use. The toxicity of QDs is currently an area of extensive study in view of the biological and medical application of this marker [2]. Some studies have assessed this issue in prokaryotic and eukaryotic models [3]. Vieira et al. [4] tested the toxicity of nanoparticles in CdTe in parasitic protozoa, such as *Trypanosoma cruzi*, etiologic agent of Chagas disease. These authors showed that the cell damage occurred only with the use of higher concentrations of quantum dots. Several limiting factors are Associated to QDs toxicity in cellular organisms. The cytotoxicity of QDs can be linked to the photochemical process resulting from its irradiation under aerobic conditions *in vivo* [5]. This process seems to involve a transfer of an electron to an excited QD O_2 molecule that produces a superoxide anion. Thus, there may oxidation and corrosion of the surface of the nanoparticles [6]. Excited QDs can also transfer energy to neighboring molecules by a process called Energy Transfer leading to formation of reactive singlet oxygen species (ROS) may cause changes in cell signaling pathway [7]. Concomitantly, in the case of QDs made with cadmium cytotoxicity is a consequence of the release of free cadmium ions (Cd2 +) that are highly toxic [8]. Both cadmium (Cd) and selenium (Se) and tellurium (Te) (CdSe and CdTe), elements are toxic to humans, causing toxicity in the kidneys, lungs, nervous system, in addition to DNA damage [9]. Some toxic changes in the cells have been observed, necrosis and apoptosis characterized by rupture of the membrane, chromatin condensation and extrusion membrane. Most of these changes were observed in *T. cruzi* marked with high concentrations of CdTe QDs [4]. Furthermore, from ultrastructural analysis was found a process of cell death suggesting autophagy in *T. cruzi* labeled CdTe [4]. Autophagy comprises a plurality of processes involved in catabolism and cell biogenesis.

Literature data indicate that, in eukaryotic microorganisms, which undergo differentiation during its life cycle (eg, amoeba, and parasites kinetoplastid) autophagy is essential for the development of the cell which allows changes to adapt to a new host. [10]. In this study, we investigated the toxicity of CdTe quantum dots in epimastigotes of *T. cruzi* in order to show the induction of death by autophagy.

MATERIALS AND METHODS

2.1 Maintenance of *T. cruzi*:

Trypanosoma cruzi parasites, strain Dm28c were kept at 28 ° C in LIT medium (liver infusion tryptose) + NNN [11, 12, 13, 14] supplemented with 10% fetal serum inactivated bovine.

2.2 Quantum dots Preparation:

The nanoparticles were synthesized in collaboration with Diogo Almeida Burigo Laboratory of Biomedical Application of Lasers Institute of Physics Gleb Wataghin UNICAMP. The CdTe nanocrystals were passivated and functionalized with mercaptoacetic acid (AMA) for application in living cells. The pH was adjusted to 7.2 with addition of more AMA. The final solution and the diameter of CdTe nanoparticles utilized were 1.3 mM and 2 nm, respectively.

2.3 Parasites labelling:

For the in vitro assay, 5 µL of *T. cruzi* suspension ($3,7X10^6$ parasites/mL) was incubated with 50 µL of diluted yellow-emitting CdSe QDs (10 µL CdSe to 90 µL of a 0.14 M PBS/0.01 M NaCl phosphate buffer, pH 7.2) and stabilized with MAA, for 60 min at 28°C [15]. The parasites were then observed by confocal microscopy.

2.4 Cell proliferation:

To determine the effect of QDs on live parasite cells, we constructed a dose-response lethality curve using several concentrations. On the fifth day of culture, parasites were harvested in sterile tubes, centrifuged at 1,500 gat 4ºC for 10 min and washed twice with the cold buffer solution [phosphate buffered saline (PBS)] described above. The cell density was estimated using a haemocytometric chamber and the growth curve was initiated with 3.7×10^6 cells followed by seven days of incubation with different CdTe concentrations: 2 and 200 µM. Cell proliferation was verified at 0, 24, 48, 72, 96 and 120h after incubation with the nanoparticles.

2.5 Autophagy assay:

For this assay, was used monodansylcadaverine (MDC) (Sigma- Aldrich) [16] as autophagic vacuoles marker. *T. cruzi* parasites from 5 days of culture were divided into three groups: control and incubated with 2 and 200 mM CdTe QDs concentrations. After treatment the tests were made in the third and fifth days after incubation with different QDs concentrations. Parasites were washed 3X in PBS and incubated with MDC, 100 µM for 1 hour at 28°C. Then, the parasites were fixed in 2% PFA for 40 minutes at room temperature. The analyses were made by Zeiss Axioplan Microscope (fluorescence) in order to quantify the percentage of MCD +parasites.

2.6 Phosphatidylinositol 3-kinase (PI3-k) inhibition assay by 3-methyladenine autophagy (3-MA):

For the PI3-k inhibition, the groups tested with were the following: control groups (*T. cruzi*); 3-MA (Sigma-Aldrich) (10mM and 15 mM) + *T. cruzi*; 3-MA (10mM and 15 mM) + QD 2µM + *T. cruzi*; 3-MA (10mM and 15 mm) + QD 200 µM + *T. cruzi*; QD 2µM + *T. cruzi*; QD 200 µM + *T. cruzi*. All of them were quantified to make a growth curve as described above and the result was statistically plotted on the graph.

2.7 Fluorescence microscopy analysis:

After treatment, the parasites were washed and incubated with 100 µM monodansylcadaverine (MDC) (Sigma-Aldrich) for 1 h at 28 °C. After fixation in 4% paraformaldehyde (40 min/room temperature), the analysis was performed by Zeiss Axioplan Microscope (fluorescence) to qualify parasites that were MDC+.

2.8 Transmission Electron Microscopy (TEM):

Aliquots treated with 3-MA were collected for TEM (72h and 144h). Parasites were incubated with 2µM and 200 mM of QDs and centrifuged at 2000 rpm for 10 minutes. Then, all of the aliquots were washed twice in PBS and then fixed for 24 hours at 4°C with Karnovsky (2.5% glutaraldehyde in cacodylate buffer purified 0.1 M pH 7.2, 4% PFA 0.08M and 0.5 mM $CaCl_2$, 4% Formaldehyde in 0.08M buffer.

After fixation time the parasites were centrifuged at 800 g for 12 minutes and washed three times for ten minutes in 0.1 M cacodylate buffer pH 7.2. The parasites are then

post-fixed in osmium tetroxide 1% in acetone dehydrated gradually. Subsequently, the material was infiltrated in Epon following inclusion in final plastic forms, to polymerization in an oven at 60 °C for 48 hours. Of the blocks obtained by this process were made ultrathin (50-80 nm thick) sections, collected in copper grids and contrasted with uranyl acetate at 5% in water for 15 minutes and then lead citrate 2% in water 2 minutes for further observation on Transmission Electron Microscope (Jeol JEM 1011) operated at 80 kV.

2.9 Statistical analysis:

The comparison between control and treated groups was done using the ANOVA and Student test. The values that represent $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The semiconductor, Quantum Dots (QDs) are nanoparticles of various compositions (CdTe / CdS, CdSe / ZnSe and ZnSe / ZnS), with typical dimensions ranging from 2 to 10 nm. In general, QDs have been successfully used as fluorescent labels to cells and can be used to biological applications (Weng *et. al.* 2006). The properties of nanomarkers to monitor ultrastructural interactions have been demonstrated by several authors [17, 18] observed that the markers colloidal semiconductors have high efficiency for marking cytoskeleton, nucleus and other cytoplasmic structures, when combined with specific molecules [18]. They also demonstrated that colloidal QDs bioconjugates are valuable for monitoring and imaging of DNA in vivo [18]. Despite the success of QDs as fluorescent probes, some drawbacks still exist (especially when the application is focused on living cells and small animals), little is known about the mechanisms of *in vivo* interaction of QDs with biological systems and how these mechanisms can affect cellular functions.

In this work were used the fluorescent nanoparticles of cadmium and tellurium (CdTe), synthesized in water, that were effective in marking the parasitic cell for a long time and did not interfere until a certain concentration in the normal physiology of *T. cruzi*. [19,15,4].

For this study, we evaluated the cytotoxicity of CdTe QDs in *T. cruzi* epimastigotes, using two concentrations, 2μM and 200μM in order to verify the induction of parasite death by autophagy using as markers 3- methyladenine autophagy (3-MA) acting as an inhibitor of a protein involved in the initial processes of autophagy, the phosphatidylinositol 3-kinase (PI3-k) and monodansylcadaverine (MDC) that shows the cadaverine protein present in the autophagic vacuoles. Many studies have shown that the QDs uptake by cells occurs by endocytosis [20, 21, 22]. The QDs can bind nonspecifically to the cell surface via electrostatic interaction or receptor [18]. Generally, nanoparticles are not degraded, but rather stored in compartments surrounded by membranes. Chaves *et. al.* [22] showed that QDs enter into *T. cruzi* by endocytosis through the cytostome and are delivered to reservosomes. In addition, Feder *et. al.* [15] showed that the uptake of QDs-SNA (*Sambucus nigra agglutinin*)- conjugated in *T. cruzi* occurs by endocytosis, since this process was blocked at 4 °C as reported in many eukaryotic cells [23,24,25,26]. Still based on the work of Feder *et. al.* [15], we found that the fluorescent labeling in *T. cruzi* was more intense in the vesicles present in the posterior region of the parasite.

Vieira *et. al.* [4], showed the presence of QDs dispersed in the cytoplasm and in cytoplasmic vesicles, sometimes closer to reservosomes. This result is an indicative that the nanoparticle would be endocytosed by a mechanism not yet fully understood.

Despite this fact, Vieira *et. al.* [4] did not observe the presence of QDs within reservosomes, suggesting that probably it is not stored in this organelle, unlike the results obtained by Chaves *et. al.* [22]. In our study, we observed the presence of QDs across the parasite within the cell as well as around and within vacuoles. In this work, CdTe QDs nanoparticles were analyzed kinetically

and its location in the parasites cells were identified by transmission electron microscopy.

As reviewed by Pelley *et. al.* [17], the toxicity of QDs is associated with their physicochemical properties and the studied model. Once the diversity of nanoparticles (CdTe, CdSe and others) and the use of different forms of capping (MAA, ZnS, MSA) do not contribute to standardization of results. Therefore it is not possible to interpret and extrapolate the toxicity mechanisms uniformly because the concentrations used in each models is very variable. Hardman [27] in his previous work already suggested ways to reduce the toxicity of QDs by selecting capping of nanoparticles [28], using lower doses [29] and modulating the size of the nanoparticles [30]. Mainly, the size of the nanoparticle is also an essential factor in the toxicity analysis, since smaller QDs appear to be more toxic to the cells [31], which was verified by Lovric *et. al.* [32] studying CdTe with emission in the red (6nm) and green (2nm).

In our previous work [4], were observed the nanotoxics effects of CdTe nanoparticles on *T. cruzi* epimastigotes, showing changes in the morphology of these forms followed by death with high concentrations of QDs treatment. In our work, we take as a parameter the possible induction of *T. cruzi* death by autophagy treated with high QDs concentrations. Thus, we investigated the *T. cruzi* cell death process by autophagy incubated with 200µM QDs like seems by Vieira *et. al.* [4].

In this study, in order to evaluate the possible toxic effects on *T. cruzi* were performed growth curves in a time scale of *T. cruzi* control and incubated with different concentrations of CdTe QDs (2 and 200 µM) to determine the occurrence of a change in the development of *T cruzi in vitro*. The quantification of 3-MA-treated parasites and/or incubated with two concentrations of QDs (2 and 200µM) was made from the growth curves (Figure 1). The results of the growth curve indicated a decrease in the number of parasites incubated with 200 µM QDs when compared to parasites incubated with 2 µM QDs and the control group. In contrast, parasites incubated with 200 µM QDs and treated with 3-MA showed a reversion of cell death by 20% (Figure 2). We emphasize that 3MA-treated groups (10 and 15mM) showed a pattern of autophagic inhibition of approximately 10 and 15% respectively (Figure 3), thus reversing the process of autophagy caused by high concentration of nanoparticles (200 mM). These results indicate that the toxic effects of CdTe but were not dose dependent and time dependent.

Statistical analyzes shows significant difference between *T. cruzi* + 200 µM QDs and *T. cruzi* + 200 µM QDs + 3-MA compared with 3-MA control ($P < 0.0001$) however the group *T. cruzi* + 3-MA + 2 uM QDs showed no significant difference when compared to the control group ($P < 0.1$).

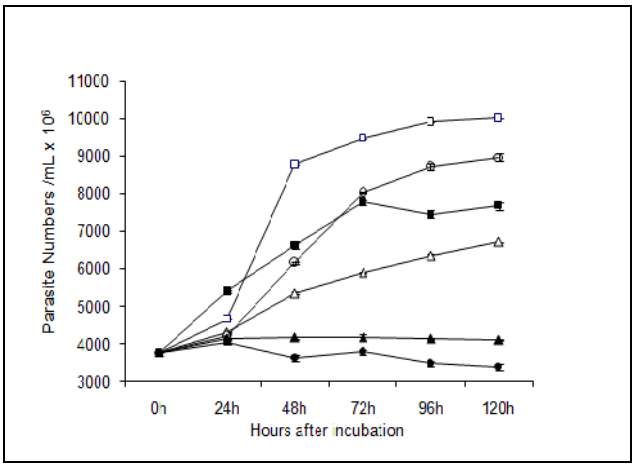

Fig. 1. Growth Curve Assay of Autophagy Inhibition with 3-metiladenine (3-MA) (10mM) of the control group *T. cruzi* (open square); *T.cruzi*+3-MA (closed square); 2 µM QDs + 3-MA (open triangle); and 200 µM QDs + 3-MA (closed triangle); 2µMQDs (open circle); 200µM QDs (closed circle).

To confirm the hypothesis by autophagy death of parasites cells that were incubated with 200 µM and marked with MDC, the flagellates were taken to fluorescence microscopy and the images revealed the presence of many autophagic vacuoles within the parasites cells (Figure 4), showing the process of autophagy developing.

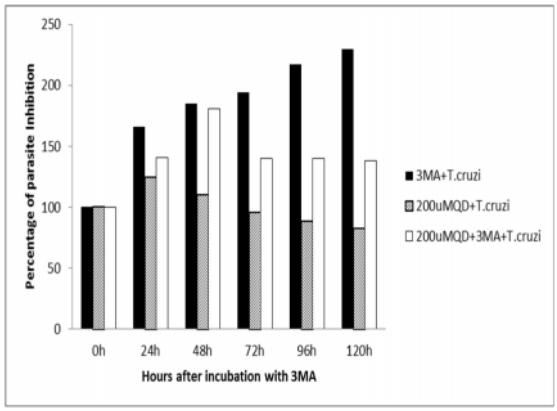

Fig. 2. Percentage of *T. cruzi* Autophagy 3-metiladenine (3-MA) (10mM) Inhibition after 120h post-incubation. The bars represents: black bar = *T. cruzi* with 3-MA (10mM); hachure bar = *T. cruzi* + 200µM QDs and white bar = *T. cruzi* + 200µM QDs + 3-MA.

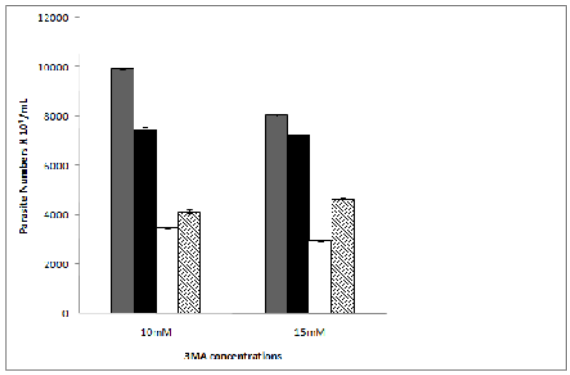

Fig. 3. *T. cruzi* Autophagy dose-dependent 3-metiladenine (3-MA) (10mm and 15mM) Inhibition after 120h post-incubation. The bars represents: grey bar = *T. cruzi*; black bar = *T. cruzi* with 3 MA; white bar = *T. cruzi* + 200µM QDs and hachure bar = *T. cruzi* + 200µM QDs + 3 MA.

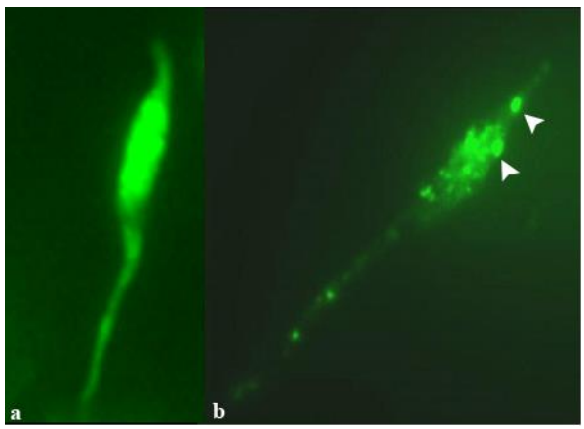

Fig. 4. a- *T. cruzi* control; b- *T. cruzi* treated with 200µM of QDs + 100 µM of monodansylcadaverine. These head arrows represent vacuoles MDC (+) expressing the cadaverine protein labeled, visualized by Zeiss Axioplan Microscope (fluorescence).

In trypanosomatids, the autophagy process is a major mechanism for degradation of large quantities of proteins and organelles and is essential for maintaining homeostasis for cell growth and cell differentiation [33]. Thus, we highlight the *T. cruzi* epimastigotes autophagy marked with 200µM QDs and treated with 3-MA (early autophagic inhibitor) comparing with untreated, however, incubated with the same QDs concentrations. This fact led us to believe that the parasites incubated in 200 µM QDs were dying by autophagy. This process can be induced by pro-autophagic signals as toxins, nanoparticles or nutrient deprivation. In this regard, the beginning of the process, there is the development of a double liprotein membrane structure called phagocytic vacuoles from pro-phagocytic vacuoles, which initiates the engagement of cytoplasmic cellular components such as proteins and organelles. This is the stage of nucleation /initiation of autophagy and some of the proteins involved at this early stage are phosphatidylinositol 3 (PI 3)-kinases [34, 35].

Munafó & Colombo [36] and Fernandes et al. [37] used monodansylcadaverine (MDC) as a fluorescent marker of autophagic vacuoles. Based in these studies, we use this marker for *T. cruzi* autophagic vacuoles. Our results have shown qualitative differences between the control parasites and parasites treated with 200µM of QDs and marked with MDC, which showed numerous autophagic vacuoles within the parasites cell and can thus confirm the developing of autophagy process observed in the 3-MA assays. However, Rohloff et al. [38,39], emphasized that young epimastigotes of *T. cruzi* have a lot of organelles called acidocalcisomes and a robust contractile vacuole located near the flagellar pocket region responsible for the mechanism of cell osmoregulation. Therefore, it is not possible to say that all vacuoles that appear in the cell are vacuoles of death, instead of which were marked with the MDC. MDC (+) positive structures containing lysosomal enzymes, but not mark autophagosomes, that containing LC3 protein responsible for late events of autophagy [40].

These results revealed differences between the parasites treated with 200µM QDs compared with the control group without treatment and control labeled with 2µM QDs (data not shown). The parasites incubated with 200µM QDs and treated with 3-MA showed a dose-dependent reversal of cell death by approximately 15% and 10% of 10mM and 15mM (3-MA), respectively (Figure 3). This reversal is confirmed by TEM results. Samples were processed at different times of the growth curve to highlight possible cell damage and the final destination of the QDs in *T. cruzi* cells (Figure 5). The analysis revealed that untreated parasites showed normal ultrastructural morphology of organelles such as the mitochondrion, nucleus, kinetoplasts, and reservosomes (Fig. 5- a, b, c).

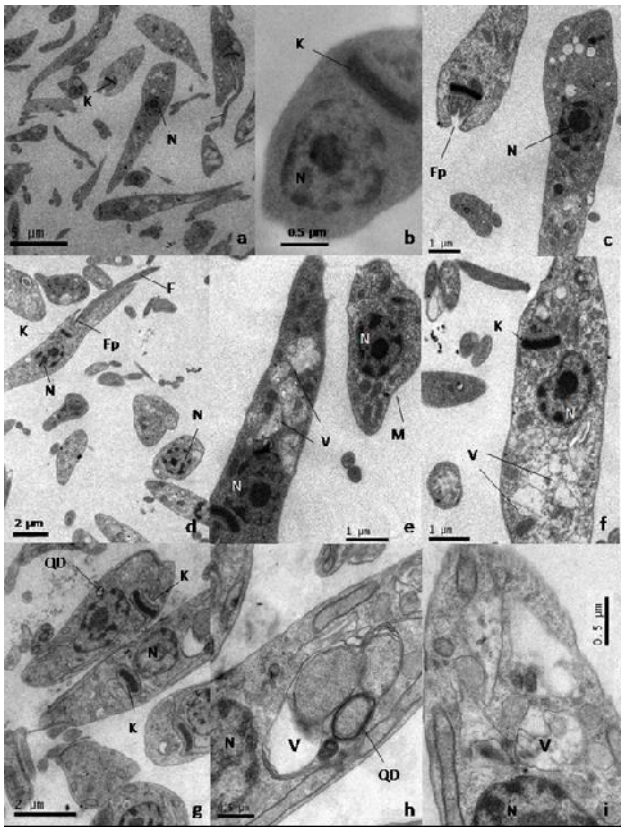

Fig. 5. Transmission Electron Microscopy of inhibition assay of autophagy: *T. cruzi* treated with 3-methyladenine autophagy (3-MA) and 2µM QDs. A, b, c – control; D,e,f – 2µM QDs at 72 hours; G, h ,i - 2µM QDs at 120 hours. The arrows represent QDs evident vacuoles inside and wrapping. QDs- quantum dots; V- vacuoles; M- membrane; N-nucleus; K- kinetoplast; F- flagellum and FP- Flagellar pocket . Bars: 0.5-5.0µm.

The treatment of epimastigotes with 200µM QDs led to the appearance of well-developed endoplasmic reticulum profiles surrounding different subcellular structures; large vacuoles or atypical vacuoles inside the parasite body; blebbing of the flagellar membrane; strong disorganization in the Golgi apparatus (Figure 6).

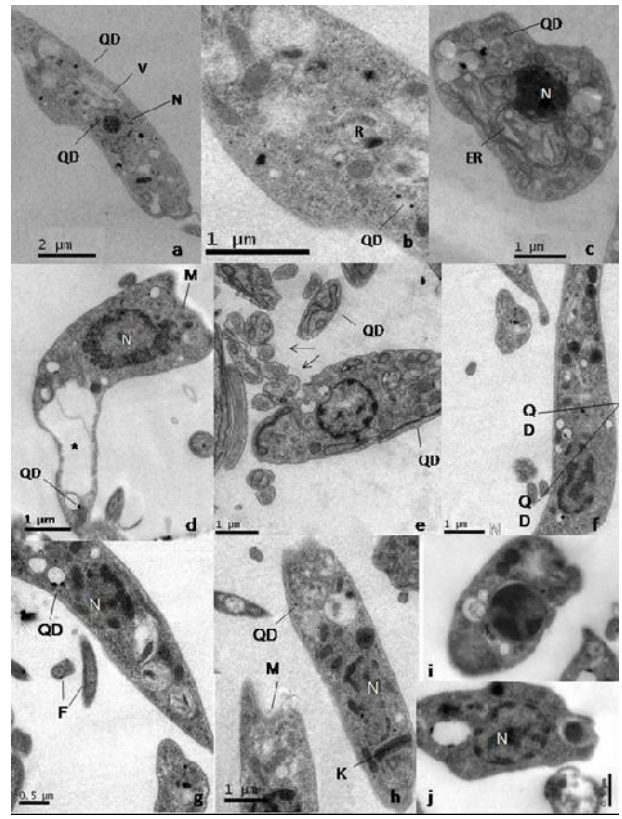

Fig. 6. Transmission Electron Microscopy of the inhibition assay of autophagy: *T. cruzi* treated with 3-methyladenine autophagy (3-MA) and 200µM QDs. A, b - 200µM QDs at 72 hours; C, d, e - 200µM QDs at120 hours; F,g, h - 200µM QDs + 3MA at 72 hours; I, j - 200 µM QDs + 3-MA at 120 h. The arrows represent QDs evident vacuoles inside and wrapping. QDs- quantum dots; V- vacuoles; M- membrane; N-nucleus; K- kinetoplast; F- flagellum; R-reservossomes; ER- Endoplasmatic reticullum. (d) Asterisk inside a large vacuole in parasite cell and head arrows pointed at excluded vesicles from the parasite cell membrane. Bars: 0.5-2.0µm.

Vieira *et. al.* [4] showed that the use of CdTe at a concentration of 2µM did not alter *T. cruzi* morphology. However, several changes were observed in parasites incubated with 200µM of the same nanoparticle. Lovric *et. al.* [41] found that mitochondria are organelles very

sensitive to "stress" induced by QDs. These results support those reported in ultrastructural analyzes by Vieira *et. al.* [4], which was shown to be a mitochondrial swelling induced by incubation with 200μM of CdTe, and the presence of many vacuoles in the parasites incubated with this QDs concentration. Our results confirm the findings of Vieira *et. al.* [4], where we can suggest that, besides the size, high concentrations of QDs can lead to morphological changes in the cell parasite, among them the remarkable presence of double membranes containing concentric structures and intense vacuolization, that also characterize a process of cell death by autophagy. The literature data shows that some of the ultrastructural changes observed in *T. cruzi*, the formation of membrane structures and concentric profiles of endoplasmic reticulum involving structures are suggestive of autophagy [10]. Moreover, the programmed cell death protozoon is still controversial [42]. However, the molecular features of this process have not been characterized yet.

CONCLUSIONS

To understand the interconnections between the cellular and QDs dynamics (including mechanisms for intracellular uptake and distribution) is important to study all of nanotoxic effects for safety biological and biomedical applications. Proteomic studies are required to understand the cell death induced by high QDs concentrations in *T. cruzi*. Thus, we intend to deepen the studies with *T. cruzi* parasites labeled with QDs and evaluate other toxic effects.

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