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To: "Mariana Potenza" mariana.potenza@ingebi.conicet.gov.ar,marian.potenza@gmail.com
From: "Heinz Mehlhorn" mehlhorn@uni-duesseldorf.de
Subject: Your Submission PARE-D-14-01253R1

Dear Dr Mariana Potenza,

We are pleased to inform you that your manuscript, "Colchicine treatment reversibly blocks cytokinesis but not mitosis in Trypanosoma cruzi epimastigotes", has been accepted for publication in Parasitology Research.

Please remember to quote the manuscript number, PARE-D-14-01253R1, whenever inquiring about your manuscript.

With best regards,

Heinz Mehlhorn
Editor in Chief

The manuscript can be accepted now.

ABSTRACT:

This work analyzes the effect of the alkaloid colchicine on the growth of *Trypanosoma cruzi* epimastigotes, using immunofluorescence microscopy and flow cytometry techniques. We found that colchicine reversibly inhibited cytokinesis but not synthesis or segregation of nuclear and kinetoplastid DNA, in a concentration-dependent manner. We showed that, once colchicine was removed from the growth medium, cytokinesis was restored but abnormal segregation of kinetoplasts and nuclei generated zoids and parasites with two nuclei and one kinetoplast, among other aberrant cells. After drug removal, we also observed a few anucleated cells carrying two kinetoplasts in a stage compatible with the end of cytokinesis. The anomalous sub-cellular localization of the kinetoplast and flagellum observed in treated parasites suggests that the effect of colchicine and its interaction with *T. cruzi* microtubules is cell cycle-dependent. The cross-talk between nuclear and kinetoplastid mitosis, and its incidence on flagellum growth and parasite cell division regulation are discussed.

KEYWORDS:
Colchicine; cytokinesis inhibition; kinetoplast; anucleated parasites

INTRODUCTION:

The flagellated protozoan *Trypanosoma cruzi* is the etiologic agent of the American trypanosomiasis or Chagas' disease. This endemic disease affects approximately 7 to 8 million people and is characterized by acute and chronic phases (World Health Organization, 2014; Rassi et al., 2012). Clinical symptoms occur in 30-40% of human cases and compromise the cardiovascular, gastrointestinal, and/or nervous systems, depending on host and parasite genetic factors (Teixeira et al., 2006; Zingales et al., 2012). Although a vaccine has not been developed yet, current treatments are generally able to cure acute infection and prevent chronic complications but also cause toxic side effects (Urbina 2010).

T. cruzi is transmitted to humans mainly through hematophagous vectors of the family Triatominae. Trypomastigote forms, which circulate in human hosts, are ingested by triatomine insects during a blood meal. This infective, non-proliferative, parasitic stage differentiates into the dividing epimastigote forms in the midgut of the insect. Then, in the posterior end of the intestine tract, parasites transform again into metacyclic trypomastigotes, which are eliminated with insect feces and are able to infect a new host when deposited near the bite site or on mucous membranes. Once in the bloodstream, parasites penetrate mammalian cells and differentiate intracellularly into dividing amastigote forms. After duplication and significant increase in number, parasites transform back into highly motile trypomastigotes, which break the cell by rupture of the plasma membrane. Parasites return to the bloodstream and are free to infect new cells or be ingested by uninfected insects, completing their life cycle (De Souza, 2002). This adaptation to different hosts, alternating between replicative and non-replicative stages, means that the parasite cell cycle must be precisely controlled and closely connected to the metabolic signals present in each environment (Goldenberg and Ávila 2011; Bayer-Santos et al., 2013).

Although several researchers have identified some of the regulators of *T. cruzi* cell cycle (Santori et al., 2002; da Cunha et al., 2005; Muñoz et al., 2006), further studies are necessary to fully understand the control network of this cellular process. The main morphological characteristics have been established for *in vitro* cultured epimastigote cells (Ramos et al., 2011). This differentiation stage is characterized by an elongated shape, with a flagellum emerging from a flagellar pocket, a depression present in the anterior side of the cell body (De Souza, 2002). The flagellum is attached along the cell body by a special connector called the flagellum attachment zone, which is composed of specialized membranes, microtubules and filamentous proteins (Rocha et al., 2006). The synthesis and appearance of a new, short flagellum emerging from the same

flagellar pocket as the old one is the first sign for a parasite that has abandoned the G1 phase and started to synthesize its genetic material. At the same time, mitochondrial DNA, namely the kinetoplast DNA, segregates into two new daughter organelles, the flagellar pockets divide, and nuclear mitosis occurs. The new flagellum continues to elongate and grow until reaching its final length during cytokinesis (Elias et al., 2007). In proliferative forms of *T. cruzi*, as well as in related trypanosomatid species, cell segregate by binary fission (Farr and Gull, 2012). This aspect of the cell cycle has been extensively studied in *Trypanosoma brucei*, where cell division takes place longitudinally, starting from the anterior tip of the new flagellum attachment zone toward the posterior end of the cell between the two flagella (Vaughan and Gull, 2008). In trypanosomatid parasites, there is no evidence that the actin-myosin contractile ring formation constricts a cleavage furrow to separate two daughter cells, as typically found in mammalian and fungal cells (García-Salcedo et al., 2004). *T. cruzi* also divides by its longitudinal axis, and the subset of interconnected subpellicular microtubules underlying beneath the plasma membrane have to disassemble, synthesize and rearrange for cell division to proceed (De Souza, 2002).

The microtubule inhibitor colchicine, a natural compound derived from flowering plants of the genus *Colchicum* and *Gloriosa superba* (Roubille et al., 2013), arrests the cell cycle in eukaryotic cells at mitosis. The effect of colchicine is the result of its binding to tubulin subunits and the subsequent disruption of the microtubule dynamics (Chakraborty et al., 2004). Besides its remarkably toxic and adverse effects, colchicine has been used widely for the treatment of Gout, Familial Mediterranean Fever and other inflammatory diseases (Cocco et al., 2010). Colchicine is also used as a second-line treatment for cardiovascular diseases (Norrid and Oliphant, 2014), and research is currently focused on colchicine analogs for potential use as antitumor therapy (Larocque et al., 2014; Satpati et al., 2014). The effect of colchicine has been studied on myocardial injury induced by *T. cruzi* in experimental Chagas' disease in hamsters (Fernandes et al., 2012), in *in vitro* cultured cells (Rosestolato et al., 2002; Tyler et al., 2005), and in *in vitro* cultured parasites (Filho et al., 1978). To better understand the action of colchicine over *T. cruzi* microtubules, here we performed experiments incubating epimastigote cultures in the presence of the drug, with the subsequent analysis of parasite morphology and DNA content using flow cytometry and immunofluorescence microscopy.

MATERIAL AND METHODS

Parasite culture and drug incubations. Epimastigotes from the *T. cruzi* CL Brener strain were grown at 28 °C in liver infusion tryptose medium (LIT) supplemented with 10% Fetal Bovine Serum (FBS, Natocor, Argentina) plus 10 U/mL penicillin and 10 mg/L streptomycin. Different concentrations of colchicine (Sigma-Aldrich, USA), ranging from 2.5 x 10^{-3} to 2.5 mM, were added diluted in distilled, deionized water. Drug removal was achieved centrifuging the parasites at 1000 xg and washing the pellet three times with phosphate buffered saline (PBS), pH 7.4, and then resuspended in pre-warmed LIT medium.

Flow cytometry analysis. To analyze parasites by flow cytometry, samples were taken at different times and processed as follows: approximately 6 x 10^6 cells (drug treated or not) were washed in PBS and fixed with 500 µL of 70% ethanol in PBS at 4 °C overnight. Then, fixed parasites were washed again with PBS and incubated for 30 min at 37 °C with 500 µL of PI staining solution (2 mM EDTA, 10 mg/mL DNAse-free RNaseA and 20 mg/mL propidium iodide in PBS). Samples were analyzed in a flow cytometer (FACSAria, BD Biosciences) using the FACSDiva software. Fluorescence intensity, which is proportional to the DNA content present in parasite populations, was plotted against cell count for each point to monitor the progression of cell cycle. Experiments were carried out in triplicate.

Immunofluorescence microscopy. Cultures of *T. cruzi* epimastigotes were placed on poly-lysine microscope slides, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 and washed twice with PBS. After blocking samples in 2% bovine serum albumin (BSA-PBS), slides were incubated with an anti-paraflagellar rod mouse monoclonal antibody (anti-PFR, kindly provided by Dr. S Schenkman) to reveal the flagellum and with an anti-YL1/2 rat antibody (anti-TUB) to detect the parasite body by its affinity to tyrosinated tubulin (diluted 1:2 and 1:500, respectively, in 1% BSA–PBS). The secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 546-conjugated goat anti-rat were used (both at 1:500). All incubations were made for 1 h each and at room temperature. Slides were mounted in ProLong Gold antifade reagent (Molecular Probes), containing 10 mg/mL of 40,60-diamino-2-phenylindole (DAPI). Cells were observed in an Olympus BX-61 fluorescence microscope.

RESULTS

The effect of colchicine was studied incubating parasites for 24 h with different concentrations (2.5 x 10^{-3} to 2.5 mM) of the drug and then performing flow cytometry to measure the DNA content of each cell population

by PI staining (Fig. 1a). Untreated control cultures exhibited the typical histogram for an asynchronous cell population, with a major peak corresponding to parasites in G0/G1 phases (2C: 1 nucleus, 1 kinetoplast), and a smaller one representing cells at G2-mitosis boundary (4C: 2 nuclei, 2 kinetoplasts). In the presence of increasing concentrations of colchicine, the proportion of cells with double content of DNA increased in comparison to the proportion of cells with single content of DNA (4C and 2C peaks in Fig. 1a, respectively). This suggests that the response of *T. cruzi* epimastigotes to the drug is dose-dependent.

Epimastigote cultures were then incubated with colchicine at the highest concentration previously tested (2.5 mM) and samples taken at different times up to 96 h. The number of parasites did not vary along time, suggesting complete growth arrest (data not shown). Analysis of fixed samples by flow cytometry revealed the emergence of a new population of cells with DNA content of 8C, evident from 72 h of treatment (Fig. 1b). Histograms also showed the appearance of a population with >1C, possibly corresponding to apoptotic cells. To study the morphology and the genetic characteristics of colchicine-treated parasites, samples were taken at different times and subjected to immunofluorescence microscopy (Fig. 1c). Using DAPI staining, anti-tubulin and anti-paraflagellar rod antibodies, it was possible to determine that colchicine inhibited cytokinesis, but not nuclear or kinetoplast DNA synthesis or segregation. After 24 h of drug treatment, almost every epimastigote cell contained two segregated nuclei and two kinetoplasts (2N2K). In function of time, parasites continued to grow and segregate their genetic material without cell division, generating multinucleated cells. Gradually, the elongated shape was completely lost, and after 72 to 96 h of colchicine treatment, cells with multiple nuclei, kinetoplasts and flagella were observed (Fig. 1c, inlets corresponding to 48 to 96 h). In some cells, the kinetoplast, together with the origin of the flagellum, appeared positioned towards the posterior end to the parasite body (Fig. 1c, 12 h). This resulted in a nucleus located at an anterior position in relation to the kinetoplast. Epimastigotes with the DNA-containing organelles duplicated and segregated at this abnormal subcellular disposition were also observed (Fig. 1c, inlets marked with asterisks).

Next, *T. cruzi* epimastigotes were treated with colchicine only for 24 h. After that, drug was removed from the culture, and parasites were washed with PBS and then grown in fresh LIT medium. Flow cytometry histograms revealed that after colchicine removal, the proportion of cells with 4C DNA content decreased, whereas a population of 2C content arose in the first 12 h (Fig. 2a). However, intermediate populations containing genetic material among 2C and 4C appeared after that time. In addition, 24 h after drug removal,

another peak, corresponding to a cell population with 1C DNA content, became evident and increased through time. To further study the morphological and genetic characteristics of the parasites representing the 1C, 2C and 4C populations, samples at 72 h post-drug removal were analyzed by immunofluorescence microscopy (Fig. 2b). At such time, cells exhibiting a plethora of different DNA contents were observed. In concordance with that observed in the flow cytometry histograms, parasites containing two nuclei and two kinetoplasts (2N2K) appeared at low proportion, whereas specimens carrying 2N1K or 1N2K were also present. In addition, parasites also tended to recover the elongated shape, whereas the location of some kinetoplasts appeared in a trypomastigote-like position. Interestingly, anucleated parasites containing two large flagella and two kinetoplasts (0N2K) were observed. In some 0N2K cells, the two kinetoplasts were positioned parallel and facing away from each other, in a disposition similar to that observed in epimastigotes undergoing cytokinesis (Fig. 2b, inlet with asterisk). Anucleated parasites carrying one flagellum and one kinetoplast exhibited normal morphology (0N1K). Cells with only one nucleus were also observed with the two kinetoplasts in the cytokinesis position (1N2K).

DISCUSSION

In higher eukaryotic cells, colchicine interacts with αβ-tubulin heterodimers, inhibiting microtubule polymerization by impairing the assembly of new tubulin dimers to the microtubule ends (Terkeltaub, 2009). At high concentrations, colchicine causes microtubule depolymerization by prevention of lateral contacts between protofilaments (Bhattacharyya et al., 2008). Disruption of the dynamics of the microtubules that compose the mitotic spindle results in the cell cycle arrest at mitosis in mammalian cells, by impairment of chromosome segregation during cell division (Bhattacharyya et al., 2008). Processes that require cytoskeleton changes, including intracellular transport of molecules and vesicles, cell motility, etc, are also affected.

Trypanosomatids do not exhibit the typical colchicine-induced mitotic arrest frequently observed in other eukaryotes, differing in their response to the drug (Dostál and Libusová, 2014). By *in silico* analysis of β-tubulin sequences from *Trypanosoma brucei* and *Leishmania guyanensis*, some researchers have hypothesized that differences in amino acid composition between trypanosomes and bovine tubulin proteins at key positions are responsible for conformational changes that prevent colchicine interaction with its putative binding pocket (Lama et al., 2012; Luis et al., 2013). However, some reports with experiments incubating

trypanosomatid parasite cultures in the presence of colchicine have shown an inhibitory growth effect (Ochola et al., 2002). In *Trypanosoma brucei*, colchicine treatment at 0.1 mM is cytotoxic and causes programmed cell death in bloodstream forms (Rosenkranz and Wink, 2008), also inhibiting parasite growth in a dose-dependent manner, with an IC50 of 3.5 µM (Ochola et al., 2002). Colchicine has also been used in combination with the alkaloid vinblastine and the lectin concanavalin A on *Trypanosoma brucei gambiense* (Ono and Nakabayashi 1979; 1987), where fusion of the extracellular flagellum with the plasma membrane and induction of anucleated forms of the parasite were observed. Treatment of *T. cruzi* epimastigotes with colchicine for 60 min does not affect parasite morphology (Souto-Padron et al., 1993), whereas incubation for seven days with 0.5 mM of the drug causes partial reduction (30%) of parasite growth (Filho et al., 1978). Here, we used higher concentrations of colchicine to treat *T. cruzi* epimastigotes with the aim to better understand the effect of colchicine over this parasite. We found that 2.5 mM of colchicine inhibited parasite multiplication completely by blockage of cytokinesis. Daily parasite counting revealed that the cell number remained constant, at least until 96 h after drug addition. This observation, together with histograms and immunofluorescence microscopy showing that parasites are duplicating the DNA content, suggests that although synthesis of genetic material is not impaired, segregation into daughter cells cannot be achieved. We consider that the differences between the phenotypic response of epimastigotes to colchicine treatment found in this work and that observed in other studies (Filho et al., 1978; Souto-Padron et al., 1993) are due to the different concentrations and incubation times used. This was demonstrated here with the progression of the phenotypic response to drug concentration and time shown in the histograms of Fig. 1a. *T. cruzi* giant cells (possessing many nuclei, kinetoplasts, and flagella), naturally occur in the intestinal tract of infected triatomines (Kollien and Schaub, 1998), due to nutritional changes on the insect diet. Interestingly, generation of multinucleated *T. cruzi* parasites, as a result of cytokinesis inhibition without mitotic arrest, is a phenotype that has also been found using the alkaloids vincristine or vinblastine (Grellier et al., 1999). Similarly, epimastigotes incubated with sub-lethal concentrations of Piperine (a compound derived from plant alkaloids) presented a round shape, multiple flagella, kinetoplasts and nuclei, but impaired division into daughter cells (Freire-de-Lima et al., 2008). Rounded cell shape with blocked cytokinesis has also been found in *T. brucei* when tubulin or flagellum adhesion glycoprotein 1 expressions are deleted (Ngô et al., 1998; LaCount et al., 2002). As shown in Fig. 1c, the appearance of rounded forms, together with multiple flagella and

disorganization of organelles containing genetic material, suggests that the microtubule dynamics is compromised by the addition of colchicine into the growth media, similar to the effect caused when tubulin stability is disrupted, either by use of alkaloids or by key structural protein deletions. In our experiments, neither nuclear mitosis nor flagellum growth were compromised. The fact that mitosis in trypanosomatids is closed (without disruption of the nuclear membrane) and that axonemal microtubules are surrounded by membranes could act as a mechanical barrier protecting or delaying the anti-tubulin polymerization action of colchicine molecules. It is also interesting to point out that, at least with the incubation times performed in our work, several rounds of mitosis can occur in the absence of cytokinesis. However, we also found that cytokinesis blockage by incubating the parasites for 24 h in the presence of colchicine is reversible. After drug removal and addition of fresh media, epimastigotes recovered their cell division machinery, although this has probably resulted in non viable or apoptotic parasites, as can be observed from the FACS profile in Figure 2a. This reversible effect of colchicine on the cytokinesis of eukaryotic cells has been previously observed using low drug concentrations. Treatment of canine epidermal fibroblasts with 2.5×10^{-4} mM of colchicine blocked the cell cycle, although removal of the drug and replacement with fresh medium completely restored cell division (Khammanit et al., 2008). This reversibility in the effect observed with colchicine treatment on different cell types may be the direct result of the intrinsic dynamic nature of microtubule assembly-disassembly (Desai and Mitchison, 1997). However, the different colchicine concentrations needed to maintain this reversibility could be in relation to the level of drug affinity for tubulin isotypes from different organisms (Luis et al., 2013). In addition, it is important to note that the piperine and vinka alkaloid treatments referred above also exhibited a reversible effect (Freire-de-Lima et al., 2008; Grellier et al., 1999), in which blockage of cytokinesis was reversed upon drug removal. These similar phenotypic effects found when growing *T. cruzi* epimastigotes in the presence of drugs that address different targets could be the consequence of the morphological remodeling capacity of an organism which must respond to external stimuli to survive, as it occurs in the trypanosomatid differentiation process (Jimenez, 2014).

In addition to cytokinesis blockage, samples of epimastigotes treated with colchicine revealed the presence of several cells containing the kinetoplast positioned toward the posterior end of the parasite body. This unusual localization was better evidenced by immunofluorescence microscopy at 12 h post-treatment, before

duplication of the genetic material (Fig. 1c, inlet 12 h). Here, the position of the kinetoplast and the flagellum, related to the nucleus localization, resembles the distribution these organelles in trypomastigote forms, rather than in epimastigote forms. It is noteworthy that, although this atypical organelle disposition is similar to the sub-cellular localization of those found in the infective, non-replicating forms, the morphology of the kinetoplast is elongated and the nucleus is rounded, as seen in non-treated epimastigote forms (Elias et al., 2007). In contrast, in the infective trypomastigote stage, the kinetoplast exhibits a rounded shape, while the nucleus appears elongated (Ferreira et al., 2008). In this regard, we suggest that the kinetoplast movement is due to an alteration of the microtubules present in the structures connected directly or indirectly to it, like the flagellar attachment zone or basal body, disrupting the organization or the strength of these components, rather than promoting the differentiation, as postulated by Filho et al. (1978). These authors observed that the presence of trypomastigote forms in an epimastigote culture increased up to 20 % when parasites were grown in the presence of colchicine at 0.5 mM. Taking into account that parasites exhibiting a kinetoplast located posteriorly to the nucleus were considered as being at the trypomastigote stage, we believe that this could be an erroneous conclusion of the authors due to technical limitations regarding microscopy at that time. In contrast, we found no differentiation facilitated in our work, with poor to null presence of trypomastigote stages. In addition, we consider that the different observations between our work and that performed by Filho et al. may be due to the different drug concentrations used, being the former work an "incomplete" effect of colchicine. This is evidenced by the histograms in Fig. 1a, which show how the population compatible with 2N2K (4C DNA content) increases according to the amount of colchicine added. Nevertheless, the reason why the kinetoplast and flagellum (and associated structures) migrate is the most interesting concern and remains to be elucidated. In *T. cruzi*, organelle remodeling has been demonstrated in several cellular processes. Morphological and biochemical changes take place during differentiation from the insect non-infective epimastigote to the mammalian infective trypomastigote stages (Goldenberg and Avila, 2011). Microscopy allow observing that the kinetoplast and flagellum are repositioned during *in vitro* metacyclogenesis (Ferreira et al., 2008), demonstrating that there is an active movement of these organelles. In other trypanosomatids such as *T. brucei*, basal body repositioning is necessary to drive cytokinesis during the cell cycle (Lacomble et al., 2010). Trypanosomes build a new flagellum whose distal tip is connected to the side of the old flagellum by a discrete structure, the flagella connector. During this process, the basal body

of the new flagellum migrates towards the posterior end of the cell (Absalon et al., 2007). These observations clearly demonstrate that the flagellum attachment zone and its components are a motile structure. However, which is the motor triggering such repositioning in physiological conditions is still unknown and thus deserves further research. Nevertheless, it is well established that the microtubule dynamics severely influences organelle positioning. In the protozoan *Giardia lamblia*, colchicine treatment at 200 µM also affects cytokinesis but not karyokinesis, whereas flagella appear irregular in location, number and shape (Mariante et al., 2005). Taken together, results from our work and others suggest that flagellum movement with cytokinesis blockage could be assumed as a typical effect of colchicine over flagellated protozoans. We found kinetoplast migration in around 10 % of parasites. Why this movement takes place is out of the scope of the present work, although it is conceivable that this could depend on the cell cycle stage of each single cell. The cytopharynx, a component connected to the cytostome that goes inside the epimastigote cell, is surrounded by specialized microtubules and modifies its length and orientation through the passage from G1 to G2 phases of the cycle (Ramos et al., 2011; Alcantara et al., 2014). Whether the uptake of colchicine molecules, and hence its effect on kinetoplast localization, is related to the morphology of the main site of endocytosis in *T. cruzi* epimastigotes in different cell cycle phases is a hypothesis that has to be proved.

On the other hand, upon colchicine removal, an increasing proportion of anucleated cells containing one kinetoplast were observed by microscopy, in agreement with the increasing population exhibiting 1C DNA content found in PI histograms (Fig. 2a). The presence of anucleated parasites was also observed by Filho et al. (1978), but more strikingly is the fact that we observed the presence of anucleated epimastigotes carrying two kinetoplasts and two flagella in a disposition seeming to be in process of cell division. Although we do not know whether those species are directed to cell death, this finding highlights and reinforces the main dependence on kinetoplastid and flagellum synthesis and segregation as the forces driving the cytokinesis process. In addition, this also suggests that nuclear and kinetoplast duplication and segregation are governed by two different (and maybe also disconnected) regulatory pathways.

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CONFLICT OF INTEREST

Authors declare that there are no conflicts of interest.

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FIGURE LEGENDS:

Figure 1: Dose-dependent effect of colchicine on DNA content in *T. cruzi* cells and phenotypic analysis of drug-treated parasites. **a**: The figure shows the flow cytometry analysis of epimastigote cultures grown in the presence of different drug concentrations. CONTROL: untreated culture. mM: millimolar. **b**: Flow cytometry analysis of *T. cruzi* epimastigotes in the presence of colchicine over time. The figure shows the histograms from a representative experiment with samples taken 24, 48, 72 and 96 h after drug addition. **c:** Immunofluorescence microscopy of epimastigotes incubated with colchicine over time. Parasite cells were fixed and flagella detected with an anti-PFR antibody and cell body detected with an anti-TUB antibody, followed by an Alexa Fluor 488-conjugated goat anti-mouse and an Alexa Fluor 546-conjugated goat anti-rat antibodies. Nuclei and kinetoplasts were detected using DAPI staining. Asterisks (*) indicate parasites exhibiting DNA organelles improperly positioned.

Figure 2: Phenotypic analysis of *T. cruzi* epimastigotes after colchicine removal. **a**: Flow cytometry analysis of parasites grown for 24 h in the presence of colchicine. The figure shows the histograms from a representative experiment with samples taken 12, 24, 48 and 72 h after drug removal. **b:** Immunofluorescence microscopy of epimastigotes 72 h after drug removal. Parasite cells were fixed and flagella and cell body detected with the anti-PFR and anti-TUB antibodies, respectively, followed by Alexa Fluor conjugates. Nuclei (N) and kinetoplast (K) were detected using DAPI staining. Asterisk (*) indicates 0N2K zoids with parallel kinetoplasts.