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<https://www.youtube.com/watch?v=vPcSyYwFCcQ>

Evaluation of antiparasitic effect of silver nanoparticles against *Trypanosoma cruzi* in vitro

Evaluación del efecto antiparasitario de las nanopartículas de plata
contra *Trypanosoma cruzi* in vitro



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Abstract

American trypanosomiasis is one the globally neglected tropical disease, and is cause by the protozoan haemoflagellate *Trypanosoma cruzi* (Tc). Currently, the anti-Tc drugs are limited and no vaccine is available. The objective of the study was to determine the effects of silver nanoparticles (AgNPs) against trypomastigotes of the Sylvio X10/4 (Tcl) and Esmeraldo (TcII) strains. The AgNPs were obtained by the reduction method and a structural characterization was carried out. The cytotoxic activity of the AgNPs (anti-Tc activity) was determined by calculating the values of half the maximum inhibitory concentration (IC₅₀) in HaCaT cells and parasites. AgNPs showed no-cytotoxicity against HaCaT cells at the IC₅₀ values assayed in parasites, and the calculated IC₅₀ trypomastigotes was 4.05 µg/ml and 6.16 µg/ml for Tcl and TcII strains, respectively. AgNPs were more active against both *Trypanosoma cruzi* strains than nifurtimox (400 µg/ml) in vitro assays. These results lay the groundwork for further evaluation of AgNPs, to investigate its chemotherapeutic value to fight *T. cruzi* infection in animal models.

Keywords: *Trypanosoma cruzi*, Chagas, AgNP, in vitro, silver.

Resumen

La tripanosomiasis Americana es una de las enfermedades tropicales desatendidas a nivel mundial, y es causada por el protozoario hemoflagelado *Trypanosoma cruzi* (Tc). Actualmente, los fármacos anti-Tc son limitados y no hay vacuna disponible. El objetivo de esta investigación fue evaluar el efecto de las nanopartículas de plata (AgNPs) contra los tripomastigotes de las cepas Sylvio X10/4 (Tcl) y Esmeraldo (TcII). Las AgNPs se obtuvieron por el método de reducción y se les realizó una caracterización estructural. La actividad citotóxica de las AgNPs (actividad anti-Tc) se determinó mediante el cálculo de los valores de la mitad de la concentración inhibitoria máxima (IC₅₀) en las células HaCaT y en los parásitos. Las AgNPs no mostraron citotoxicidad contra las células HaCaT en los valores de IC₅₀ probadas en los parásitos, y la



IC₅₀ calculada en los tripomastigotes fue de 4.05 µg/ml y de 6.16 µg/ml en las cepas TcI y TcII respectivamente. Las AgNPs fueron más activas contra las dos cepas de *Trypanosoma cruzi*, que el nifurtimox (400 µg/ml) in vitro. Estos resultados sientan las bases para la evaluación mayor de las AgNPs, por investigar su potencial quimioterapéutico para combatir la infección por *T. cruzi* en modelos animales.
Palabras claves: *Trypanosoma cruzi*, Chagas, AgNP, in vitro, plata.

INTRODUCTION

Trypanosoma cruzi (*T. cruzi*) is a flagellated protozoan from the Kinetoplastida order (El-Sayed *et al.*, 2005). It is the causative agent of American trypanosomiasis, a vector-borne parasitic disease endemic to South America, Central America and Mexico. *T. cruzi* represents a major public health problem, with approximately 8 million people infected and 25 million at risk of infection (Ibáñez-Cervantes *et al.*, 2018). In 3-5% of acutely affected patients, infection may result in several conditions: 1) myocarditis, which may progress to heart failure and death; 2) destructive lesions of cardiomyocytes and cardiac nerves, which may also progress to heart failure; or 3) meningoencephalitis (James *et al.*, 2005). In chronically infected patients, the disease may go unnoticed for several years, until 20-35% of these patients develop irreversible lesions of the autonomic nervous system. Chagas disease represents the leading cause of cardiac lesions in young, economically productive adults in endemic countries (El-Sayed *et al.*, 2005; James *et al.*, 2005).

Currently, treatment options are limited and no vaccine is available to prevent or control *T. cruzi* infection. The drugs approved by WHO for the treatment of Chagas disease, such as nifurtimox (NFMX) and benznidazole (BNZ), have at least four disadvantages (Colantonio *et al.*, 2016). First, due to the harsh side effects of these drugs, about 40% of patients drop out of treatment. Secondly, these drugs work most effectively during the acute phase of the infection; however, as it is difficult to detect, most people start drug treatment until the chronic phase of the disease has been established, with a consequent reduction in the antiparasitic efficacy of the drugs (Colantonio *et al.*, 2016). Third, public access to therapeutic drugs is limited (Chaves *et al.*, 2017). Fourth, some *T. cruzi* strains are resistant to NFMX and BZN, which makes their efficacy suboptimal (Toledo *et al.*, 2004). These factors emphasize the need to develop better anti-*T. cruzi* drugs and/or new treatment strategies for American trypanosomiasis.

Current medical trends include nanomaterials, especially noble metals such as gold and silver. These materials have had biomedical applications since ancient times (Ge *et al.*, 2014). Recent research regarding gold nanoparticles has been oriented to the treatment of cancer, while silver nanoparticles (AgNPs) have been used to fight pathogens (bacteria, fungi, viruses and protozoa) (Ge *et al.*, 2014).



Crystalline silver nanostructures are available in many shapes and sizes (Sun *et al.*, 2002) that can be used as medical treatments (Dankovich *et al.*, 2011; Dubas *et al.*, 2006; Kokura *et al.*, 2010; Lee *et al.*, 2006), but the effect of silver nanoparticles on parasitic protozoa is still incompletely understood (Mao *et al.*, 2016). In this sense, two studies have reported that traditional method to synthesize the AgNPs may have cytotoxic properties against the protozoa pathogens *Leishmania tropica* and *Toxoplasma gondii* (Allahverdiyev *et al.*, 2011; Adeyemi *et al.*, 2017) and although recently Brito *et al.*, 2020 biosynthesized AgNPs from corn cobs containing xylans were able of causing death of the Y strain of the *T. cruzi*.

The aim of the present study was to evaluate the cytotoxic potential of AgNPs on trypomastigotes of two *T. cruzi* biotypes in vitro. To our knowledge, this is the first attempt to define how the dose and structural characteristics of AgNPs affect *T. cruzi* cultures in vitro, in an effort to find a safe alternative for the treatment of Chagas disease.

MATERIALS AND METHODS

Synthesis of silver nanoparticles

Inorganic silver (AgNO_3 , $\geq 99.0\%$), and sodium borohydride (NaBH_4 , $\geq 99.99\%$) were purchased from Sigma-Aldrich, USA. All reactions were carried out using deionized water ($18\text{M}\Omega$; Millipore). The chosen method induces the dispersion of nanoparticles through a stable colloidal solution, generating a concentration gradient. Sodium borohydride was used as a reducing agent under a cold bath with magnetic stirring (Mulfinger *et al.*, 2007). The AgNP solution (Figure 1) was produced using $0.1\text{ mM AgNO}_3/50\text{ mL (H}_2\text{O)}$.

AgNPs prepared in aqueous solution facilitated the study of the interaction between the nanoparticles and the parasite, without the intervention of any surfactant or dispersion medium other than deionized water. This was achieved after several pilot syntheses with two reducing agents (citrate and borohydride). The borohydride produced a more stable and concentrated synthesis (0.1 mM AgNPs) that served to achieve the doses of $1\text{-}12\text{ }\mu\text{g}$ used by Allahverdiyev *et al.*, 2011 against *Leishmania tropica*. Sterility of AgNPs was tested by incubating the nanoparticles in sterile Luria Bertani broth ($200\text{ }\mu\text{L/L}$) at 37°C for two weeks.

Structural characterization of AgNPs

The nanoparticles were characterized by UV-Vis spectroscopy, transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HRTEM) using a 1% dilution of AgNPs suspended in deionized water (v/v). A Genesys 10S VIS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to perform UV-Vis spectroscopy and verify the characteristic surface plasmon resonance wavelength (400-



450 nm) of AgNPs (Mulfinger *et al.*, 2007). Aliquots (40 μ L) of AgNPs dispersed in methanol (1:5) were placed on 400 carbon-coated copper grids, dried in a desiccator at 25 °C for 24 h, and then observed in a JEM-ARM200F-TEM at 120 kV. To determine the crystallographic direction of AgNPs facets by fast Fourier transform (FFT), interatomic distances were observed in a JEM-ARM200F-TEM at 200 kV (HRTEM) and analyzed with Garantia Microscopy Suite Digital Micrograph Software.

Parasite culture

Trypomastigotes from the *T. cruzi* strains Sylvio X10/4 (Tcl) and Esmeraldo (TclII) were cultured in monolayers of Vero cells, which were kept in DMEM medium supplemented with 2% fetal bovine serum [FBS] and 1% penicillin–streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) under controlled conditions (37°C, 5% CO₂, and saturated humidity) (Aparicio-Burgos *et al.*, 2015). As trypomastigotes started bursting out of cells, free parasites were collected and prepared for the AgNP toxicity assay.

Trypomastigotes were placed in 15 mL conical tubes and centrifuged at 704 \times g for 7 min. Supernatants were discarded and pellets were re-suspended in 1 mL of fresh DMEM medium. The number of parasites was estimated with a hemocytometer and adjusted to 1×10^6 per well in a 96-well tissue culture dish for the toxicity assay.

AgNPs toxicity assay for *T. cruzi* *in vitro*

Trypomastigotes (1×10^6 per well) were placed in 96 well plates (Sarstedt, USA) in DMEM supplemented medium and cultured at 37°C, 5% CO₂, and saturated humidity, with different AgNP doses (1, 5, 10 or 12 μ g/100 μ L). The assay was performed by triplicate with untreated (- Ctrl) or NFMX (400 μ g/100 μ L, Lampit®, Bayer) treated trypomastigotes as negative and positive controls. NFMX was prepared as described by Rolón *et al.*, 2006. Briefly, one tablet of the commercial presentation of NFMX (120 mg) was ground in a sterile mortar and pestle and resuspended in 1 mL of dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture media never exceeded 0.3% (v/v).

Plates were incubated for 24 hours under the same controlled conditions. After drug treatment, parasite and cell viability were estimated by MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, internal salt) of CellTiter 96 kit® Aqueous One Solution (Promega, USA), following the manufacturer's instructions, by colorimetry at 490 nm wavelength. For this assay, optical density (OD) values correlated positively with cell viability. All experiments were performed in triplicate. The half-maximal inhibitory concentration (IC₅₀) for each *T. cruzi* biotype was calculated from the dose-response curve (Attarde *et al.*, 2020; Barile & Hopkinson, 1993).



***In vitro* cytotoxic assays in a human keratinocyte cell line (HaCaT)**

HaCaT cells (an immortal cell line of non-cancerous human keratinocytes) (2×10^4 /well) were cultured in a 96-well plate (Sarstedt, USA) in supplemented DMEM (2% FBS, penicillin 10,000 units/mL and streptomycin 10,000 $\mu\text{g}/\text{mL}$) and AgNPs at 1, 3, 5, 10 or 12 $\mu\text{g}/\mu\text{L}$. For each experiment, cells from at least three wells were left untreated (control). The plate was incubated for 48 h under controlled conditions (37 °C, 5 % CO₂ and saturated humidity). After treatment, cell viability was estimated by MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethylthioxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium, internal salt) of CellTiter 96 kit® Aqueous One Solution (Promega, USA), following the manufacturer's instructions. The metabolic activity of MTS-treated cells was estimated by colorimetry at a wavelength of 490 nm.

Statistical analyses

ANOVA was used to analyze results from the *in vitro* viability assay. Mean differences for all assays were assessed using a Tukey test. Differences were considered significant at $P < 0.05$. Statistical analyses were conducted with the GraphPad Prism 5.0 software package (GraphPad Software Inc., USA).

RESULTS

Characterization of AgNPs

The UV-Vis spectrum of the aqueous silver colloid (Figure 1) showed a strong peak at 396 nm, a typical surface plasmon resonance (SPR) band of AgNPs. The narrow and symmetric absorption peak indicates a homogeneous size distribution of AgNPs. Micrographs of AgNP samples show hemispherical nanoparticles accompanied by some elongated structures of size 52 nm (Figure 2a and 2b). High-resolution transmission electron microscopy (HRTEM) showed that ~80% of the particles were decahedrons (Figure 2c) with {111} facets (Figure 2d).

Cell viability for the MTS assay

The trypomastigote viability percentages for both *T. cruzi* strains showed a dose-dependent response; this response was most evident for the Sylvio X10/4 strain, which showed a 50% reduction in parasite viability at the 1 μg dose of AgNPs and a 95% reduction in viability at the highest experimental dose of 12 μg after 24 hours of treatment. However, the Esmeraldo strain showed an 83% reduction in viability at the highest dose

(12 μg), showing statistical differences between the Esmeraldo strain and the Sylvio X10/4 strain. Statistical analysis of the results showed differences between the negative control and all other treatments, including the different doses of AgNPs and the positive control (NFMX) (Figure 3).

IC₅₀ estimation

AgNPs showed no different toxicity between IC₅₀ from the studied strains of *T. cruzi*. The IC₅₀ for trypomastigotes for Sylvio X10/4 and Esmeraldo strains were 4.05 and 6.16 $\mu\text{g}/\text{mL}$, respectively (Figure 4).

HaCaT cells treated with AgNPs

The viability of HaCaT cells was assessed 48 h after treatment (Figure 5). The toxicity of AgNPs on HaCaT cells was assessed by MTS at different concentrations: 1, 3, 5, 10 or 12 $\mu\text{g}/100 \mu\text{L}$, which resulted in an IC₅₀ of 10 $\mu\text{g}/\text{mL}$ ($P < 0.05$), indicating that AgNPs were approximately 2-fold less toxic to HaCaT cells than trypomastigotes. The results showed that AgNPs were not cytotoxic at the effective dose (IC₅₀ values = 4.05 and 6.16 $\mu\text{g}/\text{mL}$) against TcI and TcII strains of *T. cruzi*.

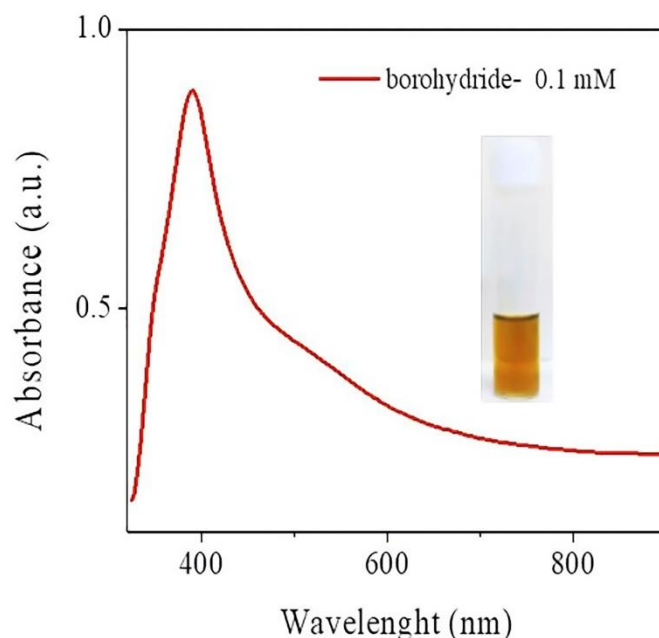


Figure 1. Characterization of AgNPs by UV-Vis Spectroscopy. Absorbance spectra of AgNPs ($\lambda_{\text{max}} = 396 \text{ nm}$). The inserted figure displays the photograph of the silver nanoparticles colloid.



DISCUSSION

Chagas disease is a vector-borne disease caused by *T. cruzi*, a hemoflagellate parasite that often causes debilitating and fatal heart disease. BNZ and NFMX are the only available drugs approved for medical treatment (Chaves *et al.*, 2017; Toledo *et al.*, 2004). However, these drugs induce harsh side effects that prevent patients from concluding treatment. In many cases, *T. cruzi* is resistant to these drugs, making them unable to eliminate the parasite from infected tissue (Colantino *et al.*, 2016; Toledo *et al.*, 2004). Therefore, public health programs require the development of more effective and less toxic therapeutic drugs for the treatment of Chagas disease.

Silver nanoparticles (AgNPs) have antimicrobial effects not only against fungi (Soliman *et al.*, 2018; Mousavi *et al.*, 2015), Gram-positive (Adibhesami *et al.*, 2017) and Gram-negative bacteria (Pal *et al.*, 2007), but also against protozoa such as Apicomplexa (*Toxoplasma gondii*) (Adeyemi *et al.*, 2017) and Trypanosomatida (*Leishmania tropica* and *Leishmania infantum*) (Baiocco *et al.*, 2011). Here we report the *in vitro* trypanolytic effects of AgNPs against two different strains of *T. cruzi*.

For this study, we produced hemispherical, decahedral AgNPs with a mean diameter of 52 nm (Figures 1 and 2), and used them at 5 different doses (1, 3, 5, 10 or 12 $\mu\text{g}/\text{mL}$) to test toxicity in HaCaT cells and trypanolytic activity in trypomastigotes produced in co-culture with Vero cell monolayers. We found that silver nanoparticles significantly reduce cell viability in a dose-dependent manner in HaCaT cells. This observation is in agreement with other studies (Chen *et al.*, 2019; Perde-Schrepler *et al.*, 2019). Importantly, AgNPs are not cytotoxic at doses effective against *T. cruzi* strains, and the toxicity of AgNPs on trypomastigotes was indirectly assessed by an MTS proliferation assay, which showed a negative effect of AgNPs on trypomastigote viability. Figure 3 shows that nanoparticles significantly reduced cell viability compared to the negative control, and that AgNPs had similar trypanolytic power to NFMX at different doses. Although no significant differences in *T. cruzi* viability were observed with different doses of AgNPs, a dose-dependent trend in viability was observed. These data suggest that higher doses of AgNPs may have additional effects on the viability of *T. cruzi* trypomastigotes. Interestingly, a relatively low dose (12 $\mu\text{g}/100 \mu\text{L}$) of AgNPs killed up to 96.3% (with an $\text{IC}_{50} = 4.05 - 6.16 \mu\text{g}/100 \mu\text{L}$) of trypomastigotes *in vitro*, whereas NFMX (400 $\mu\text{g}/100 \mu\text{L}$) killed only 70% (Figures 3 and 4). This also suggests that AgNPs could be a more efficient trypanolytic agent *in vitro* than NFMX, as observed in the present study, but also in comparison to BNZ nanoparticles (with an $\text{IC}_{50} = 36 \mu\text{g}/\text{mL}$) (Scalise *et al.*, 2016).

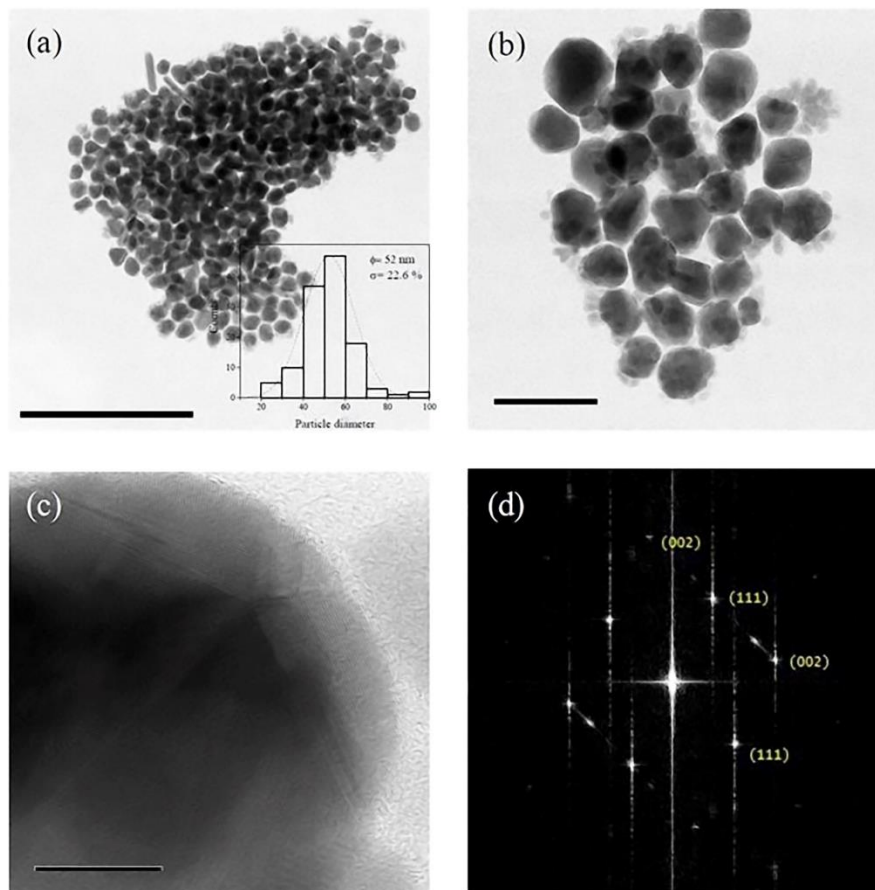


Figure 2. Micrographs show silver nanoparticles produced when borohydride was used as a reducing agent at a concentration of 0.1 mM. (a) Transmission Electron Microscopy (TEM) image of silver nanoparticles with an average diameter of 52 nm; (b) Particles are mostly semi-spherical, and some elongated structures are also observed; (c) High Resolution Transmission Electron Microscopy (HRTEM) showed decahedral particles and (d) Fast Fourier Transform (FFT) displayed {111} and {002} facets.

T. cruzi has already developed some resistance to BNZ and NFMX, the only clinically approved drugs against Chagas disease (Mejia *et al.*, 2012; Molina *et al.*, 2000). Different *T. cruzi* biotypes have differential resistance to BNZ. TcI strains of *T. cruzi* are more resistant to BNZ, whereas TcII strains are more sensitive to this drug (Toledo *et al.*, 2004; Mejia *et al.*, 2012). Here, we found no differences in sensitivity to AgNPs between the two *T. cruzi* strains tested (Sylvio X10/4 and Esmeraldo) belonging to different biotypes (TcI and TcII, respectively). Although further studies are needed, our data regarding the broad spectrum of antiparasitic activity of AgNPs suggest that nanoparticles could be an interesting option to solve the problem of parasite resistance to other drugs, either used as an alternative drug or in combination with those currently in use.

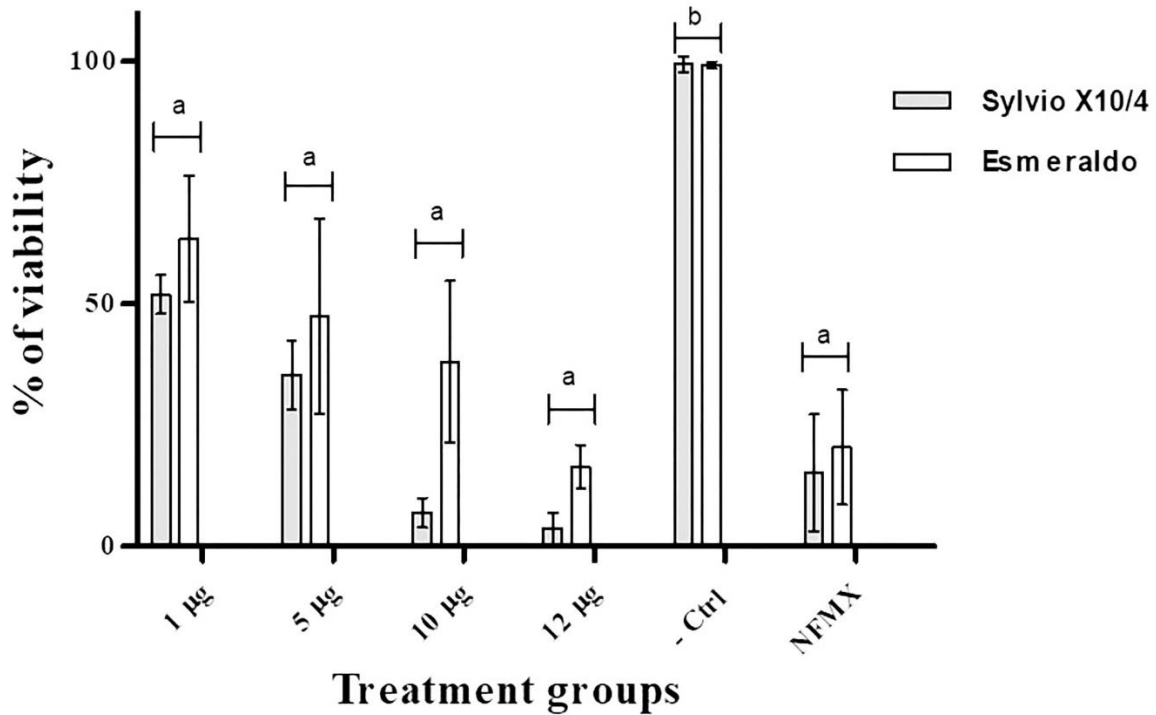


Figure 3. *T. cruzi* survival after treatment with any of three different doses of AgNPs (1, 5, 10, 12 µg/100 µl), Nifurtimox (NFMX, 400 µg/100 µl) or without treatment (-Ctrl, used as a negative control for the MTS viability assay). Samples were evaluated after 24 hours of treatment. Each bar represents the absorbance mean value ± SD. Differences ($P < 0.05$) among groups, according to Tukey's test, are indicated by characters on top of treatment bars.

Further studies should address the effects of AgNPs as alternative drugs against *T. cruzi*, especially to understand whether these nanoparticles can exert a detrimental effect on amastigotes, the intracellular developmental stage of the parasite in mammals. *Leishmania tropica* amastigotes have been reported to be more sensitive to AgNPs than promastigotes at a dose of 10 µg/0.1 mL (Allahverdiyev *et al.*, 2011). If that hypothesis holds for *T. cruzi*, the use of AgNPs *in vivo* for therapeutic use against Chagas disease would become an interesting topic of study.

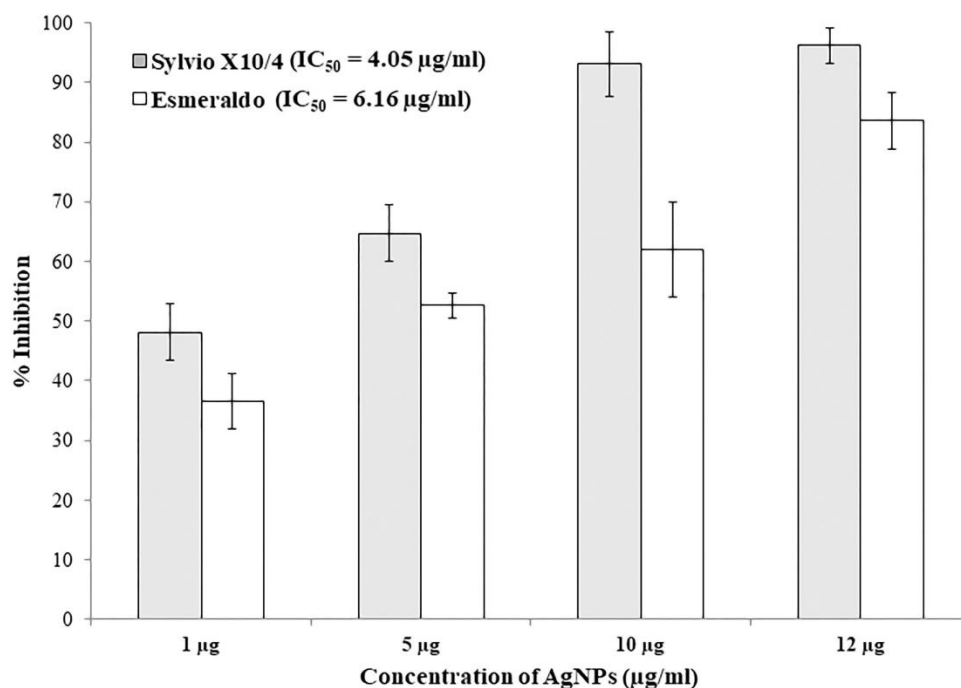


Figure 4. Inhibition of *T. cruzi* after 24 hours of AgNPs treatment (1, 5, 10, 12 µg/100 µl). Each bar represents the absorbance mean value ± standard deviation (n=6). Within the same time point, statistical differences ($P < 0.05$) among groups are shown with different characters above the bars according to Tukey test.

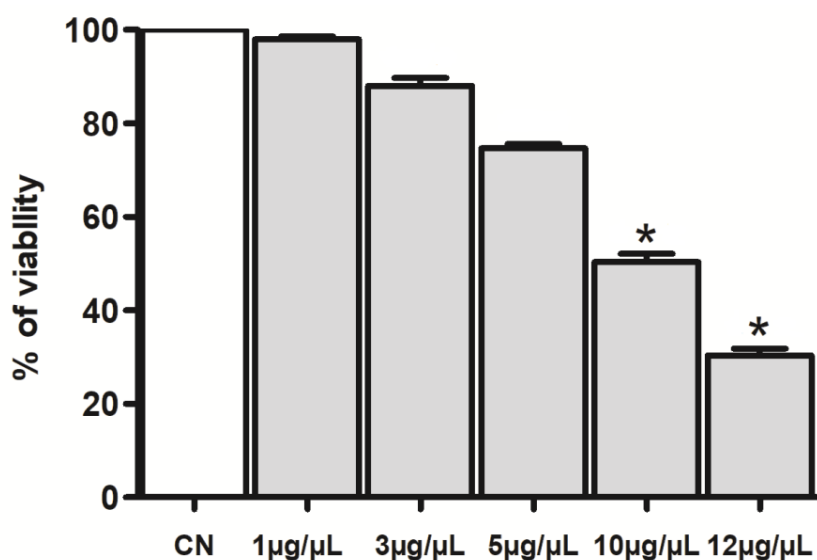


Figure 5. The viability of HaCaT cells was examined by MTS assay. HaCaT cells were stimulated with various doses of AgNPs (1, 3, 5, 10, 12 µg/100 µl) for 24 hours. The asterisks (*) indicate statistical significant differences compared to control (CN) ($P < 0.05$).



AgNPs possess unique physical and electronic properties, which make them excellent candidates for medical applications. The antimicrobial mechanism of AgNPs seems to be related to the formation of free radicals that damage the microorganisms' membrane (Kim *et al.*, 2007). These properties depend on size, shape, and nanoparticle coating (Dubey *et al.*, 2015). In the present study, we synthesized AgNPs with an average diameter of 52 nm; most of them were hemispherical (decahedral), had {111} and {110} facets, and had acceptable trypanolytic activity. However, we believe that the trypanolytic activity of AgNPs could be improved, if the shape and size of the nanoparticles were adjusted to triangles with a size of 1-10 nm. This type of nanoparticles shows higher antibacterial activity than larger AgNPs with hemispherical shapes due to the higher density on the facets {111}. This allows triangular nanoparticles to freely permeate the plasma membrane and thus induce more damage to microorganisms. In addition, truncated triangular AgNPs require lower doses (1-10 µg/0.1 µL) to exert bactericidal properties than larger spherical and nanorodal AgNPs (12.5 and 50-100 µg/0.1 µL, respectively) (Ge *et al.*, 2014; Dubey *et al.*, 2015; Morones *et al.*, 2005).

CONCLUSIONS

A dose of 12 µg/100 µL dose of AgNPs with an average diameter of 52 nm was cytotoxic for trypomastigotes from TcI and TcII *T. cruzi* biotypes *in vitro*. Future studies test triangular shaped AgNPs with {111} facets to improve the antiprotozoal effects of AgNPs on different biotypes of *T. cruzi*.

Conflict of interest statement

We declare that we have no conflict of interest.

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