Extracellular vesicles isolated from *Trypanosoma cruzi* affect early parasite migration in the gut of *Rhodnius prolixus* but not in *Triatoma infestans*

Larissa F Paranaiba1,2, Alessandra A Guarneri2, Ana C Torrecilhas3, Maria N Melo1, Rodrigo P Soares2,4

1Universidade Federal de Minas Gerais, Departamento de Parasitologia, Belo Horizonte, MG, Brasil
2Fundação Oswaldo Cruz-Fiocruz, Instituto René Rachou, Belo Horizonte, MG, Brasil
3Universidade Federal de São Paulo, Departamento de Ciências Farmacêuticas, Diadema, SP, Brasil

The protozoan *Trypanosoma cruzi* has the ability to spontaneously secrete extracellular vesicles (EVs). In this paper, *T. cruzi* EVs derived from epimastigote forms were evaluated during interaction with triatomine bugs *Rhodnius prolixus* and *Triatoma infestans*. *T. cruzi* EVs were purified and artificially offered to the insects prior to infection with epimastigote forms. No effect of EVs was detected in the parasite counts in the guts of both vectors after 49-50 days. On the other hand, pre-feeding with EVs delayed parasite migration to rectum only in the gut in *R. prolixus* after 21-22 days. Those data suggest a possible role of *T. cruzi* EVs during the earlier events of infection in the invertebrate host.

Key words: *Trypanosoma cruzi* - triatomines - extracellular vesicles - interaction

Chagas disease is caused by the protozoan *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae). It is estimated that more than eight million people are infected and 25 million are at risk of acquiring the disease. More than 10 thousand people die per year due to complications of clinical manifestations of the disease. Chagas disease was originally found in the Americas, but recently, due to human migration, has expanded to non-endemic countries in North America, Europe and Asia. *T. cruzi* is transmitted by triatomines (Reduviidae: Triatominae) including *Triatoma infestans* and *Rhodnius prolixus*, the most important vectors in Latin America.(1,2,3)

To develop and establish infection within the host environments in the digestive tract of the vector and vertebrate hosts, *T. cruzi* developed a variety of strategies implicating a wide number of molecules. (4) Those are important for attachment and internalisation including Tc-85,(5) glycoinositolphospholipids (6) and glycosylphosphatidylinositol (GPI)-mucins. (7) Some of those molecules can be either shed or expressed in the surface of extracellular vesicles (EVs).

EVs are spontaneously released by any cells including prokaryotic and eukaryotic. (8) Depending on the origin, size and function they can be classified as microvesicles, nanoparticles, apoptotic bodies and exosomes. In general, EVs are composed of a phospholipid bilayer containing lipids, proteins, glycoconjugates and nucleic acids. (9) The role of EVs during interaction with vectors is poorly understood. However, it is already known that in another Trypanosomatid, *Leishmania major*, EVs are released by promastigotes in the midgut of the sand fly vector and further inoculated together with the parasite in the vertebrate host. (10) Altogether, this inoculum will be important for cell attraction and parasite establishment in the vertebrate host. In this context, *T. cruzi* EVs have already demonstrated their pro-inflammatory activity during host innate and chronic immune responses. (11,12) However, no study on the role during the interaction with triatomin vector was performed.

Here, we provide evidence that in the digestive tract of triatomin vectors, *T. cruzi* EVs were able to functionally affect early parasite migration in *R. prolixus* and had no effect on the number of metacyclics in both vector species.

Bug2149 cl10 *T. cruzi* strain (Bug), originally isolated from naturally infected *T. infestans* (Rio Grande do Sul, Brazil) was used. (13) Epimastigote forms were cultured in liver-infusion tryptose (LIT) supplemented with 15% foetal bovine serum (FBS), 100 µg/mL streptomycin, 100 units/mL penicillin (27°C and pH 7.2). *T. infestans* and *R. prolixus* used in this study were obtained from a laboratory colony derived from insects collected in Brazil and Honduras, respectively. Triatomines were reared at 25 ± 1°C, 60 ± 10% relative humidity and natural illumination as previously reported. (13) Fourth instar nymphs, starved for 30 days after ecdysis, were used in the assays.

Parasites were grown in LIT medium, washed in hanks’ balanced salt solution (HBSS), centrifuged (1000g/10min, 10°C) and counted. For EVs release, *T. cruzi* in early log phase (1 x 10⁶ parasites/mL) were resuspended in LIT medium without FBS and incubated at 28°C for 2 h. Parasites were fixed and cover slips were prepared for scanning electron microscopy (SEM)
and transmission electron microscopy (TEM) as previously reported.\(^{(1,12)}\) After vesiculation, supernatants were collected, filtered (0.22 μm) and ultra-centrifuged (100,000g/2h, 4°C). Nano-particle tracking analysis (NTA) was performed to determine size, distribution and concentration of EVs as reported elsewhere.\(^{(13)}\) Acquisitions were measured in a Nanosight NS300 instrument (Malvern Instruments Ltd, Malvern, UK) equipped with a 405-nm laser and coupled to a charge-coupled device (CCD) camera (the laser emitting a 60-mW beam at 405-nm wavelength). Data were analysed using NTA software (version 2.3 build 0017). The detection threshold was set to 10. Blur, Min track Length and Min Expected Particle Size were set to auto. To perform the measurements, samples were diluted 1:100 in phosphate-buffered saline (PBS). Readings were taken in triplicates during 30 s at 20 frames per second (three times for each sample), at camera level set to 14 and manual monitoring of temperature (19°C).

Citrate rabbit blood was obtained from Centro de Criação de Animais de Laboratório (CECAL), Fiocruz, RJ. Insects were artificially exposed to EVs and parasites in two consecutive moments: (i) first day, nymphs were artificially fed on citrated heat-inactivated (56°C, 30 min) rabbit blood containing EVs. Each insect was allowed to ingest 20-30 μL of blood (approximately 6.4-9.6 x 10⁴ particles/μL); (ii) second day, the same nymphs were artificially fed to repletion on citrated heat-inactivated rabbit blood containing epimastigotes. Those parasites were obtained from LIT cultures, washed in PBS and resuspended in the rabbit blood at a final concentration of 1 x 10⁷ parasites/mL. Since each insect could ingest 20-30 μL of blood, the number of epimastigotes would range from 2-3 x 10⁷. Control group was fed on blood + parasites with similar amounts of the treated group in the respective days. The midgut and rectum of nymphs and controls were observed (Mann Whitney, p > 0.05; Fig. 4A). After 49-50 days p.i. the number of parasites in the midgut was reduced in both treatments, with no differences between the groups (Mann Whitney, n.s.; Fig. 4B). For T. infestans, no differences in the number of parasites between EVs-exposed nymphs and controls were observed (Mann Whitney, n.s.; Fig. 5A-B). At 28 days p.i. the number of metacyclics in the urine did not vary among controls and EV exposed insects for both triatomine species (t test, p > 0.05, Fig. 6A-B). All T. infestans nymphs released parasites in urine, but in numbers ~10 fold smaller than those found in R. prolixus ones.

Parasites are known release exosome-like EVs that function as cell-to-cell effectors during the host-parasite interaction.\(^{(11,12,15,16,17)}\) One of the initial studies showed that challenge of BALB/c mice with T. cruzi EVs exacerbated parasite load, heart inflammation and mortality.\(^{(14)}\) Later, it was demonstrated that T. cruzi could modulated not only the innate but also the acquired immune events by activating TLR2, triggering cytokine production, MAPKs activation and invasion.\(^{(12,18)}\) It is important to mention that the concentration of vesicles used in those studies ranged from 1-10 μg/mL. Here, pre-feeding with EVs was approximately 5 μg/mL. Although it may be not physiological, this concentration is within a range known to functionally activate vertebrate cells. However, reports regarding the role of EVs with respect to their invertebrate hosts are unknown.

R. prolixus and T. infestans are the most used triatomine models due to due to existence of laboratory colonies. Both models were successfully used in our procedures and produced the expected infection pattern as previously reported.\(^{(14,15,16,17,18,19)}\) However, the abil-

---

Fig. 1: basic diagrammatic representation of a digestive tract from a triatomine bug.
ity of *T. infestans* to take a blood meal in the artificial system was much lower than that of *R. prolixus*. Pre-feeding with EVs delayed the early migration of *T. cruzi* parasites to the rectum (21-22 days p.i.) in *R. prolixus*. This effect was not observed after 49-50 days p.i., suggesting a transient effect of the EVs during the initial days of infection. However, this effect was not detected in *T. infestans* in both periods. The number of metacyclics in EV exposed and controls did not vary for both vectors reinforcing their role only in the initial events of infection. Interestingly, the number of metacyclic try- pompastigotes recovered in the urine of *R. prolixus* was 10-fold higher than in *T. infestans*. This result was very surprising since Bug strain was originally isolated from *T. infestans* in Brazil, whereas the population of *R. prolixus* used in this study was from Honduras. *R. prolixus* belongs to the tribe Rhodniini, whereas *T. infestans* is from the Triatomini tribe. We thus believe that such differences may be attributed to the species rather than the amount of ingested blood. Although in our model, pre-feeding with EVs affected early parasite migration only in *R. prolixus*, their role in transmission was not as-

---

**Fig. 2:** extracellular vesicles (EVs) of *Trypanosoma cruzi*. (A) Scanning electron microscopy (SEM) of *T. cruzi* membrane shedding (bars: 1-5 µm), Magnification 32,657 x and (B) Scanning electron microscopy (SEM) of *T. cruzi* membrane shedding (bars: 1-5 µm) Magnification 80,000 x. Nanoparticle tracking analysis (NTA) (C) and (D) of *T. cruzi* EVs.

**Fig. 3:** extracellular vesicles (EVs) of *Trypanosoma cruzi*. Transmission electron microscopy (TEM) of *T. cruzi* membrane shedding (A) (lower magnification, bar: 200 nm) and (B) (higher magnification, bar: 100 nm).

**Fig. 4:** parasites found throughout the digestive tract of *Rhodnius prolixus*. Dissections at 21-22 (A) and 49-50 (B) days after the infective feeding. Each dot represents the quantification of parasites from one individual nymph and each horizontal bar corresponds to the median of the group evaluated.
sessed. Since the number of total parasites and metacyclics in later days of infection did not vary, it is not likely that EVs will affect transmission in triatomines. This is different from *L. major*, where EVs were important for parasite transmission during the sand fly bite.\(^\text{(10)}\) Despite the presence of EVs, we showed that both vectors were able to develop metacyclics. However, in nature those parasites are released in the feces and urine and are not inoculated as in *Leishmania*.

In vertebrate cells, it is already known that fusion of EVs is an important mechanism that promotes parasite internalisation.\(^\text{(11,18)}\) Our results suggest that *T. cruzi* EVs could be fusing to the epithelium of the midgut and somehow promoting early parasite retention in *R. prolixus*. This effect was transient and did not affect the number of metacyclics in both vectors.

**AUTHORS’ CONTRIBUTION**

RPS, AAG, ACT and MNM conceived and planned the experiments; LFP, AAG and ACT performed experiments and analysed data. All authors wrote and corrected the manuscript.

**REFERENCES**

1. Pérez-Molina JA, Molina I. Chagas disease. Lancet. 2018; 391(10115): 82-94.
2. Antinori S, Galimberti L, Bianco R, Grande R, Galli M, Corbellino M. Chagas disease in Europe: a review for the internist in the globalized world. Eur J Intern Med. 2017; 43: 6-15.
3. Schofield CJ, Dias JC. The Southern Cone initiative against Chagas disease. Adv Parasitol. 1999; 42: 1-27.
4. Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J Cell Sci. 1999; 112(Pt 17): 2799-2809.

Fig. 5: parasites found throughout the digestive tract of *Triatoma infestans*. Dissections at 21-22 (A) e 49-50 (B) days after the infective feeding. Each dot represents the quantification of parasites from one individual nymph and each horizontal bar corresponds to the median of the group evaluated.

Fig. 6: metacyclic trypomastigotes found in the urine of *Rhodnius prolixus* (A) and *Triatoma infestans* (B) at 28 days p.i. Each dot represents the quantification of parasites in the urine sample of one nymph and each horizontal bar corresponds to the mean of the group evaluated.

5. Alves MJ, Colli W. *Trypanosoma cruzi*: adhesion to the host cell and intracellular survival. IUBMB Life. 2007; 59: 274-9.
6. Nogueira NF, González MS, Gomes JE, de Souza W, Garcia ES, Azambuja P, et al. *Trypanosoma cruzi*: involvement of glycoinositolphospholipids in the attachment to the luminal midgut surface of *Rhodnius prolixus*. Exp Parasitol. 2007; 116(2): 120-28.
7. Soares RP, Torrecilhas AC, Assis RR, Rocha MN, Moura e Castro FA, Freitas GF, et al. Intraspecies variation in *Trypanosoma cruzi* GPI-mucins: biological activities and differential expression of α-galactosyl residues. Am J Trop Med Hyg. 2012; 87(1): 87-96.
8. Szempruch AJ, Dennison L, Kieft R, Harrington JM, Hajduk SL. Sending a message: extracellular vesicles of pathogenic protozoan parasites. Nat Rev Microbiol. 2016. 14(11): 669-75.

9. Campos JH, Soares RP, Ribeiro K, Andrade AC, Batista WL, Torrecilhas AC. Extracellular vesicles: role in inflammatory responses and potential uses in vaccination in cancer and infectious diseases. J Immunol Res. 2015; 2015: 832057.

10. Atayde VD, Aslan H, Townsend S, Hassani K, Kamhawi S, Olivier M. Exosome secretion by the parasitic protozoan *Leishmania* within the sand fly midgut. Cell Rep. 2015; 13(5): 957-67.

11. Torrecilhas ACT, Tonelli RR, Pavanelli WR, da Silva JS, Schumacher RI, de Souza W, et al. *Trypanosoma cruzi*: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. Microbes Infect. 2009; 11(1): 29-39.

12. Nogueira PM, Ribeiro K, Silveira ACO, Campos JH, Martins-Filho OA, Bela SR, et al. Vesicles from different *Trypanosoma cruzi* strains trigger differential innate and chronic immune responses. J Extracell Ves. 2015; 4: 28734.

13. Palace-Berl F, Pasqualoto KFM, Jorge SD, Zingales B, Zorzi RR, Silva MN, et al. Designing and exploring active N'-[(5-nitrofuran-2-yI) methylene] substituted hydrazides against three *Trypanosoma cruzi* strains more prevalent in Chagas disease patients. Eur J Med Chem. 2015; 96: 330-9.

14. Ferreira RC, Kessler RL, Lorenzo MG, Paim RMM, Ferreira LDL, Probst CM, et al. Colonization of *Rhodnius prolixus* gut by
Trypanosoma cruzi involves an extensive parasite killing. Parasitology. 2016; 143(4): 434-43.

15. Torrecilhas AC, Schumacher RI, Alves MJM, Colli W. Vesicles as carriers of virulence factors in parasitic protozoan diseases. Microbes Infect. 2012; 14(15): 1465-74.

16. Marcilla A, Martin-Jaular L, Trelis M, de Menezes-Neto A, Osuna A, Bernal D, et al. 2014. Extracellular vesicles in parasitic diseases. J Extracell Ves. 2014; 1: 1-15.

17. Torró LMP, Moreira LR, Osuna A. Extracellular vesicles in Chagas disease: a new passenger for an old disease. Front Microbiol. 2018; 9: 1190.

18. Ribeiro KS, Vasconcellos CI, Soares RP, Mendes MT, Ellis CC, Aguilera-Flores M, et al. Proteomic analysis reveals different composition of extracellular vesicles released by two Trypanosoma cruzi strains associated with their distinct interaction with host cells. J Extracell Ves. 2018; 7(1): 1463779.

19. Castro DP, Moraes CS, González MS, Ratcliffe NA, Azambuja P, García ES. Trypanosoma cruzi immune response modulation decreases microbiota in Rhodnius prolixus gut and is crucial for parasite survival and development. PLoS One. 2012; 7(5): e36591.

20. Lent H, Wygodzinsky PW. Revision of the triatominae (Hemiptera, Reduviidae), and their significance as vectors of Chagas disease. Bull Am Mus Nat Hist. 1979; 163: 125-520.