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ORIGINAL PAPER

Human urine stimulates in vitro growth of *Trypanosoma cruzi* and *Trypanosoma rangeli*

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Abstract Previous studies conducted in *Leishmania* led us to test the hypothesis that addition of human urine (HU) to the Liver Infusion Tryptose (LIT) medium would stimulate the in vitro growth of *Trypanosoma cruzi* and *Trypanosoma rangeli* strains. Herein, we show that the addition of 3% HU to LIT medium (LIT-HU3) significantly stimulated the growth of all the *T. rangeli* strains studied when compared with the parasite growth in conventional LIT medium ($p<0.05$), and it was equivalent to the growth observed in LIT supplemented with fetal calf serum (FCS) in two parasite strains. Four out of the six *T. cruzi* strains analyzed showed a significant increase in parasite multiplication in LIT-HU3 ($p<0.05$). However, two parasite strains presented good growth in both LIT and LIT-HU, suggesting differences in the parasite's ability to grow in vitro. Furthermore, we have not observed differences in *T. cruzi* growth in LIT-HU3 and LIT supplemented with heat-denatured HU and in the metacyclogenesis of parasite strains cultured in LIT-HU3. These results allow concluding that the addition of HU to LIT medium stimulates the in vitro growth of *T. rangeli* and *T. cruzi* and can replace FCS as a supplement in culture medium.

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Introduction

Trypanosoma cruzi is the etiological agent of Chagas disease or American trypanosomiasis, a debilitating disease that affects approximately 16 million people, mainly in Latin America (WHO 2002). Human infections caused by *Trypanosoma rangeli* have already been described in Central America and in Northwest South America, where this species is sympatric with *T. cruzi* (D'Alessandro and Saravia 1992; Coura et al. 1996; Cuba Cuba 1998; Ramirez et al. 2002). Distinctly from *T. cruzi*, *T. rangeli* is nonpathogenic to humans and other domestic and sylvatic mammals; however, it is pathogenic to the triatomine vector (Guhl and Vallejo 2003).

Parasitological tests, such as blood culture and xenodiagnosis, are highly specific for Chagas disease diagnosis; however, they lack sensitivity because of the low parasitemia observed in chronic Chagas' disease (Luz et al. 1994). In the case of *T. rangeli*, reports on the direct demonstration of the parasite in the peripheral blood are scarce (D'Alessandro and Saravia 1992), and there is no data in the literature about the diagnostic sensitivity of these methods. Serological tests possess high sensitivity, but they lack specificity, as *T. cruzi* and *T. rangeli* share approximately 60% of their soluble antigens, leading to cross-reactivity in serological tests and making difficult the specific diagnosis of Chagas disease in some endemic areas (Saldaña and Sousa 1996), where a nonestimated number of individuals are infected with *T. rangeli* (D'Alessandro and Saravia 1992; Vasquez et al. 1997).

In vitro cultivation of some trypanosomatids are hampered by the difficulty in culture media standardization and by the lack of detailed information about the nutritional requirements of these protozoa. Some media used for the cultivation of trypanosomatid species (Hendricks et al.

1978; Childs et al. 1979) require the addition of high concentrations (10–30%) of fetal calf serum (FCS), an expensive supplement with an undefined and varying composition. In the attempt to partially or totally replace the FCS by other components in culture media, several researches have added nutrients to the culture media, such as amino acids, vitamins, hormones, and bovine serum albumin (Chaudhuri et al. 1986; O'Daly and Rodriguez 1988; Kar et al. 1990; Ali et al. 1998; Merlen et al. 1999). The utilization of sodium urate, uric acid, and cysteic acid, known components of the urine of the insect vector, for in vitro growth and differentiation of *Leishmania donovani* (Howard et al. 1987), has motivated the use of human urine (HU) as a constituent of *Leishmania* spp. culture media. Despite the fact that HU is also a complex and variable supplement, it is free, easily available anywhere, and has been successfully used for cultivation of the several *Leishmania* spp. studied (Howard et al. 1991; Armstrong and Patterson 1994; Shamsuzzaman et al. 1999).

In this article, we describe the effect of Liver Infusion Tryptose medium (LIT) supplementation with HU in the in vitro growth of *T. cruzi* and *T. rangeli*. Results presented here show great potential to improve the sensitivity of blood culture as a method for parasite isolation in American trypanosomiasis.

Materials and methods

Trypanosome strains

We have used the P02, P07, P18, P19, and P21 strains of *T. rangeli* (Ramirez et al. 2002) and the Alv, AQ2, Mut, PV, RNL1, and RNL2 strains of *T. cruzi*, previously isolated and characterized in the Parasitology Laboratory of the Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brazil. Their genotypes, geographical origins, and hosts are presented in Table 1.

Culture conditions and growth curves of *T. cruzi* and *T. rangeli*

Parasite strains were maintained in LIT medium, prepared as previously described (Camargo 1964). The LIT medium was used for posterior supplementation with 10% (v/v) inactivated FCS (LIT-FCS) and distinct concentrations of HU (LIT-HU) obtained from a male donor and sterilized by filtration in a 0.22-μm membrane. Laboratorial analysis of the urine samples showed pH 6.0, density of 1.029, negativity for nitrite, ketonic bodies, and hemoglobin, and normal values for glucose, urobilinogen, and leukocytes, using the reactive stripes (Roche Diagnostics, Mannheim, Germany).

Growth curves of the different parasite strains were conducted in triplicate, using conic disposable polypropylene tubes of 50 ml (CRAL, Cotia, Brazil) containing 10 ml of the culture medium (LIT, LIT-HU, and LIT-FCS). Epimastigotes (1.0×10^6) from the exponential phase of growth were inoculated and kept at 28°C for 20 days. Parasite number was estimated daily in a hemocytometer. Supplements added to the LIT medium were: HU in concentrations of 1, 2, 3, and 5% (v/v), 3% (v/v) HU denatured by heating at 80°C for 15 min (LIT-dHU3), and inactivated 10% FCS (v/v). Despite not observing significant differences in parasite densities reached in LIT supplemented with the different concentrations of HU, we adopted the concentration 3% (LIT-HU3) to determine the growth curves for all the other *T. rangeli* and *T. cruzi* strains selected, as high concentrations of HU inhibit *Leishmania* growth (Shamsuzzaman et al. 1999).

Table 1 Characteristics of *T. cruzi* and *T. rangeli* strains studied

Species	Strain	Genotype	Host	Origin (state of Brazil)
T. rangeli	P02	KP1+	*Didelphis albiventris*	Minas Gerais
T. rangeli	P07	KP1+	*Didelphis albiventris*	Minas Gerais
T. rangeli	P18	KP1+	*Didelphis albiventris*	Minas Gerais
T. rangeli	P19	KP1+	*Didelphis albiventris*	Minas Gerais
T. rangeli	P21	KP1+	*Didelphis albiventris*	Minas Gerais
T. cruzi	AQ2	I	*Triatoma sordida*	Bahia
T. cruzi	Alv	I	*Panstrongylus megistus*	Minas Gerais
T. cruzi	RNL1	II	*Homo sapiens*	Goiás
T. cruzi	RNL2	II	*Homo sapiens*	Goiás
T. cruzi	PV	NC[a]	*Homo sapiens*	Rondônia
T. cruzi	Mut	NC[a]	*Panstrongylus megistus*	Minas Gerais

[a] Not conclusive

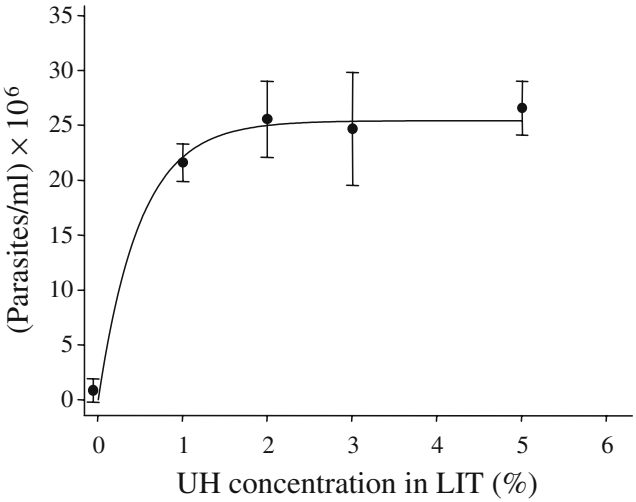

Fig. 1 Maximum parasite density of *T. rangeli* strain P07 in LIT medium without HU and LIT supplement with different concentrations of HU (1, 2, 3, and 5% v/v)

Statistical analyses

For the application of statistic tests, we determined the day in which each strain individually reached the highest parasite density. Data were submitted to the normality tests of Komolgorov–Smirnov, Lillefors, and Shapiro–Wilks. The Levene test was used to determine the homogeneity of the variances. Then, we have used parametric (*t* student, analysis of variance, *F*, and the Tukey's post-hoc test) and nonparametric tests (Mann–Whitney, Kruskal–Wallis, and the Dunn's post-hoc test) to evaluate the results significance.

In vitro differentiation of *T. rangeli*

Parasite samples from P07 and P18 strains of *T. rangeli* cultured in LIT and LIT-HU3 media were collected each 10 days until 60 days of culture for the elaboration of glass slides. Giemsa-stained samples were analyzed by light microscopy (1,000×), and the percentage rate of differentiation was determined by the morphological classification of 100 parasites as epimatigotes or tripomastigotes forms.

Results

Determination of the effect HU in the growth of *T. cruzi* and *T. rangeli*

The addition of HU to LIT increased, approximately, tenfold the maximum parasite density of the P07 strain (2.4×10^6 parasites/ml in LIT and 2.6×10^7 parasites/ml in LIT-HU; Fig. 1). Parasite multiplication was significantly higher in LIT-HU when compared to the growth in LIT, irrespective of the concentration of HU employed ($p<0.001$).

Strains PV and Mut of *T. cruzi* showed similar maximum parasite densities when cultivated in LIT supplemented with different concentrations of HU. Maximum parasite densities were higher in LIT-HU than in LIT. However, these differences were not significant for both strains (data not shown).

In vitro growth of *T. cruzi* strains in LIT-HU3

Peaks of maximum parasite density in LIT-HU3 were reached at the tenth day of culture for *T. cruzi* Alv, AQ2, and RNL1 strains, at the 12th day for Mut, and PV strains, and at the 14th day for RNL2 strain (Fig. 2b). The maximum growth of AQ2 and Alv strains was 7.8×10^6 and 9.3×10^6 parasites/ml in LIT and 3.6×10^7 and 5.3×10^7 parasites/ml in LIT-HU3, respectively. RNL1 and RNL2 strains (*T. cruzi* II) reached a low parasite density in LIT, 1.6×10^6 and 1.2×10^6 parasites/ml, whereas they reached 5.7×10^7 and 1.3×10^7 parasites/ml in LIT-HU3, respectively ($p<0.05$). Mut and PV strains did not show differences in parasite growth when cultivated in LIT (2.0×10^7 and

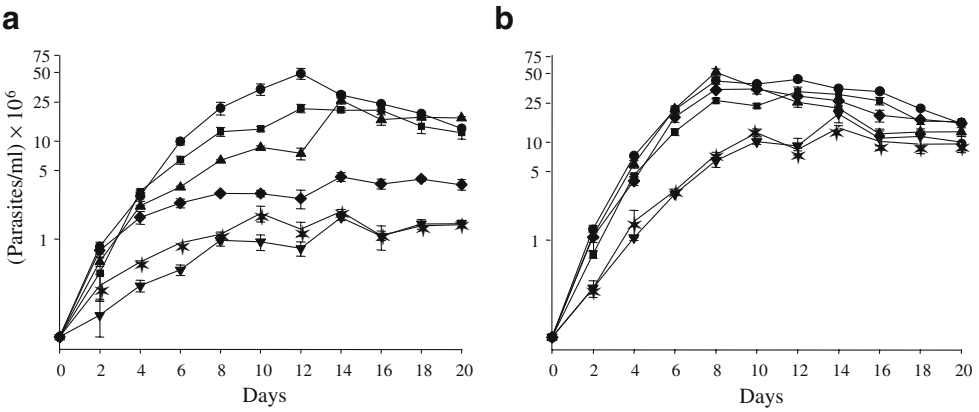

Fig. 2 Growth curves of *T. cruzi* strains Mut, AQ2, PV, Alv, RNL1, and RNL2 in LIT medium (**a**) and LIT supplemented with 3% HU (LIT-HU3) (**b**). *T. cruzi* strains are: Mut (*squares*), AQ2 (*diamonds*), PV (*circles*), Alv (*triangles*), RNL1 (*stars*), and RNL2 (*inverted triangles*)

4.8×10^7 parasites/ml) or LIT-HU3 (2.9×10^7 and 4.4×10^7 parasites/ml), respectively (Fig. 2a,b).

The growth of the *T. cruzi* RNL1 strain in LIT-HU3 and in the medium prepared with a heat-denatured sample of HU (LIT-dHU3) was equivalent in both media and significantly higher ($p<0.05$) than the parasite growth observed in LIT (Fig. 3).

In vitro growth of *T. rangeli* strains in LIT-HU3

In LIT-HU3, P19 strain reached the maximum growth in the tenth day of culture. Maximum parasite density was observed for P07 and P21 strains at the 16th day of culture and for P02 and P18 strains at the 18th day of culture. HU significantly stimulated growth of all *T. rangeli* strains, as compared with parasite growth in LIT ($p<0.05$). *T. rangeli* strains P07, P18, P02, P19, and P21 when cultivated in LIT-HU3 presented an increase of 16.7×, 36.6×, 32.8×, 6.7×, and 92.8×, respectively, in maximum parasite density, as compared with parasite growth in LIT (Fig. 4a,b, $p<0.05$).

The growth of P02 and P21 strains was equivalent in both LIT-HU and LIT-FCS (Fig. 4b,c). Parasite strains P07, P19, and P18 presented a superior growth in LIT-FCS than in LIT-HU3 (Fig. 4a,c, $p<0.05$).

Metacyclogenesis

The kinetic analysis of *T. rangeli* differentiation determined with P07 and P18 strains revealed that the presence of epimastigote forms was predominant in the first 20 days of culture (97 to 100%) both in LIT and LIT-HU. The metacyclogenesis analysis of P07 and P18 strains after 50 days of culture revealed 60 and 53% of metacyclic trypomastigotes, respectively, in LIT and 62 and 56% of metacyclic trypamastigotes in LIT-HU3, respectively. We have not observed viable parasites at the 60th day of culture. Therefore, we have not observed significant differences in parasite differentiation in both media.

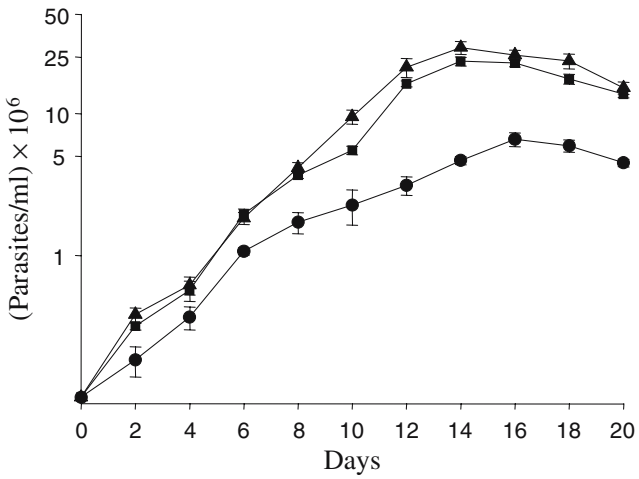

Fig. 3 Growth curves of *T. cruzi* strain RNL1 in LIT medium (*circles*), in LIT supplemented with 3% HU (LIT-HU3) (*squares*), and LIT supplemented with 3% of heat-denatured HU (LIT-dHU3) (*triangles*)

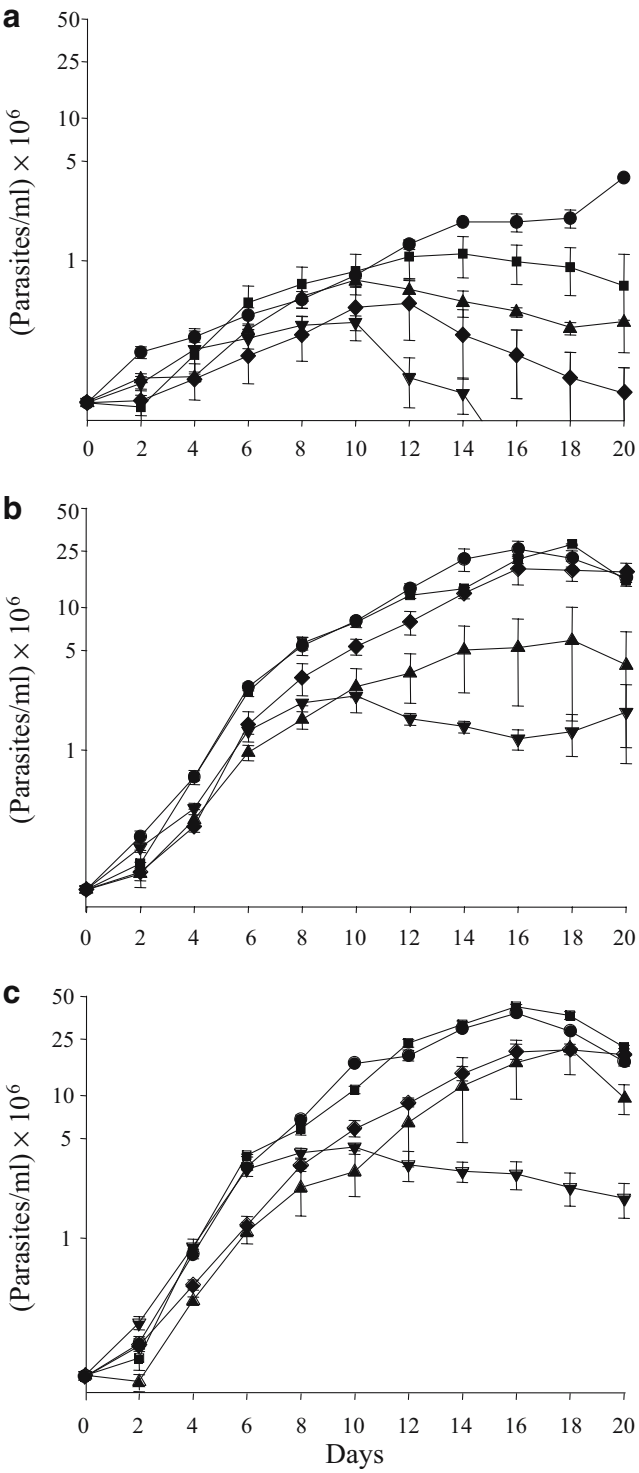

Fig. 4 Growth curves of *T. rangeli* strains P02, P07, P18, P19 and P21 in LIT (**a**), in LIT supplemented with 3% HU (LIT-HU3) (**b**), and LIT supplemented with fetal calf serum 10% (LIT-FCS) (**c**). *T. rangeli* strains are: P02 (*triangles*), P07 (*circles*), P18 (*squares*), P19 (*inverted triangles*), and P21 (*diamonds*)

Discussion

The low densities obtained with *T. rangeli* cultivation in the conventional LIT medium led us to test the effect of HU in parasite growth in vitro, as this supplement had already been successfully used for *Leishmania* spp. in vitro cultivation (Howard et al. 1991; Armstrong and Patterson 1994; Shamsuzzaman et al. 1999). D'Alessandro and Saravia (1992) suggested the LIT medium (Camargo 1964) supplemented with FCS 10% or Eagle's medium supplemented with 20% FCS for in vitro propagation of *T. rangeli*. Our results show that maximum parasite densities obtained with *T. rangeli* strains cultured in LIT-HU3 were approximately tenfold higher than those described for the San Augustin strain, cultured in Eagle's medium supplemented with L-glutamine and FCS 20% (Takle and Young 1988). We also show that parasite multiplication in LIT-HU3 and in LIT-FCS was equivalent in both media only for two parasite strains, and the other strains grew better in LIT-FCS. These results are distinct from that described in *Leishmania*, where parasite growth was equivalent both in media supplemented with HU or FCS (Armstrong and Patterson 1994; Shamsuzzaman et al. 1999).

T. cruzi PV and Mut strains grew well in both LIT and LIT-HU. All the other *T. cruzi* strains studied show significant higher parasite densities in LIT-HU3 ($p<0.05$), pointing to important differences in nutritional requirements and the ability of parasite strains to grow in vitro. Engel et al. (1982) demonstrated parasite heterogeneity by their ability to grow in the LIT medium. Other authors have already reported distinct nutritional requirements for *T. cruzi* and *T. rangeli* strains (Zeledon 1959; Avila et al. 1983).

HU is a complex supplement, making difficult the isolation and characterization of the component, which is capable of promoting the growth of members of the Trypanosomatidae family. Parasites of the genus *Trypanosoma* possess a receptor for the epidermal growth factor (EGF), a protein usually found in HU (Hide et al. 1989) and implicated as the factor that would lead to parasite growth in vitro. In our experiments, however, the ability of HU to promote in vitro growth has not been lost by the heat denaturation of the HU sample, excluding the possibility that EGF would be the responsible to stimulate parasite growth. Another possible candidate is biopterin, an enzymatic cofactor known to be essential for kinetoplastid growth (Trager 1969) and found in approximate concentrations of 6.7 nM in samples of HU from healthy individuals (Slazyk and Spierto 1990). From our results, we cannot exclude the possibility of biopterin as one of the components of HU that promote trypanosome growth.

In previous studies, a solution named "triatomine urine" and its supplementation with proline induced in vitro metacyclogenesis of *T. cruzi* (Contreras et al. 1985). However, such an effect is not observed in *T. rangeli*, and only the amino acid glutamine has shown positive results in the parasite metacyclogenesis (Koerich et al. 2002). Our data show that HU does not interfere in *T. rangeli* differentiation.

Trypanosomatids in vitro cultivation is hampered by the difficulty in standardization of culture media and by the lack of information about nutritional requirements of these protozoa. The risk of parasite subpopulation selection after in vitro culture can be minimized by the correct utilization of nutritional supplements, which would allow the proper isolation of all parasite populations present in a sample. The use of HU in culture media for *T. cruzi* and *T. rangeli* show several advantages, such as the availability and absence of costs in obtaining the urine samples and the lack of inhibitory effects in parasite growth. Furthermore, improvements in parasite culture will have a direct impact on increasing blood culture sensitivity, consequently facilitating parasite maintenance in the laboratory and allowing conducting genetic characterization studies that more closely resemble the parasite populations found in their hosts.

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