

## Growth of *Leishmania tropica* Promastigotes in Urine Samples of Different Genders and the Role of Citrate in Culture

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### Abstract

*Leishmaniasis is a zoonotic disease caused by an obligatory intracellular protozoon, which threatens 350 million people in 98 countries. Cultivation of Leishmania parasites plays an important role in developing vaccines and drugs against leishmaniasis, in diagnosis and treatment of disease. Even though some studies showed stimulatory effect of human urine in culture, gender differences, standard living and nutrition conditions haven't been considered. There is also insufficient information about which urine components are responsible for stimulating the growth of parasites. Citrate is one of the most important urine components but the effect of citrate on development of parasites hasn't been investigated. In this study, it was determined that urine taken from male and female donors with identical nutritional backgrounds stimulated the proliferation of Leishmania tropica promastigotes and despite the fact that female urine was more effective; urine of some male donors increased the proliferations of parasites too much. In addition we showed that citrate may be one of the urine components which are responsible for parasites development.*

**Keywords.** Human urine, *L. tropica*, citrate, cultivation

### Introduction

Leishmaniasis, which is one of the biggest public health problems of 98 countries or territories around the world including Turkey, is caused by *Leishmania* species, obligatory intracellular parasites of mammals. It is known that nearly 350 millions are under risk of infection. Every year approximately 1-1.5 million cases of cutaneous leishmaniasis and 500.000 cases of visceral leishmaniasis occur worldwide [1, 2].

*In vitro* cultivation of *Leishmania* parasites plays an important role in vaccine and drug development studies and also in diagnosis and treatment of leishmaniasis [3] *Leishmania* parasites can be cultured in different media, such as Novy, Nicolle and MacNeal's (NNN) medium, Schneider's insect medium or RPMI-1640 medium [4]. These culture media require some growth factor, such as fetal calf serum (FCS), to provide for the parasites' development. However FCS is expensive and not readily available in endemic areas [3]. Therefore, other growth factors have been trialed as substitutes for FCS. Some studies showed the stimulatory effect of human urine as an alternative to FCS [3, 5-7]. Components of urine are not standard and related to nutrition and other factors. However in these studies, gender differences in urine, standard living and nutrition conditions have not been considered. Also there is not enough study about which component of urine stimulates development of *Leishmania* parasites *in vitro*. Only in a study it was shown that xanthine as a factor in urine stimulates

parasites development *in vitro* [8]. Sodium citrate is also one of the most important urine components and it is shown that citrate is effective in stimulation of the differentiation process for trypanosome parasites [9]. But the effect of sodium citrate on development of *Leishmania* parasites was not investigated.

Therefore the aim of this study was the investigation of growth of *L. tropica* promastigotes in culture medium supplemented with sodium citrate and urine samples taken from different genders under identical nutritional conditions.

## Materials and Methods

**Parasite culture.** *L. tropica* promastigotes (MHOM/TR/99/EP39) were cultured as previously described [10]. Cultures were passaged after 4 days of incubation. The growth of promastigotes was monitored every day using an inverted microscope (Olympus CK 40). Subpassaging of cultures was done once a week. Samples of promastigote culture ( $2 \times 10^6$  promastigotes/mL of medium) were transferred to culture flask with 7 mL of RPMI 1640 + 10% FCS.

**Parasite counting.** The parasites were counted using a hemocytometer with a 20x objective under standard light microscopy.

**Urine sample collection.** Urine samples were collected from the students of Private Darussafaka High School. The parents of all children were informed of the project and written consent was obtained prior to participation. Samples were obtained from 46 adolescent volunteers (28 females, 18 males, age group 15-17) under identical nutritional conditions. The school is a boarding school; therefore the living conditions of children (nutrition, sleeping, sporting activities) were comparable. All participants underwent a health-check prior to collection of urine samples. Urine samples were taken in the morning, before any food or drink was consumed. All the urine samples were sterilized by filtration (disposable sterile filters 0.22  $\mu\text{m}$ , Millipore Corporation) and stored in sterile falcon tubes at 4°C. Cultivation with urine samples of different genders. Each urine samples divided into 3 tubes in which 2 mL medium ( $3 \times 10^5$  parasites/mL) was found. Experiments were repeated 3 times. After 24 hours incubation, mediums of experiment groups were supplemented with 2%, 5%, 10%, 15%, 20%, 25% urine and phosphate buffered saline (PBS) was used as control. Parasites were counted at 24<sup>th</sup>, 48<sup>th</sup>, 72nd hours of the experiment. The results of female and male urine were compared with medium supplemented with same concentration of PBS solution.

In another group experiments, growth of *L. tropica* promastigotes were studied in medium (RPMI 1640 + 10% FCS) supplemented with urine taken from menstruating females and females in secretory phase of the endometrial cycle and compared with the medium (RPMI 1640 + 10% FCS) that did not contain urine.

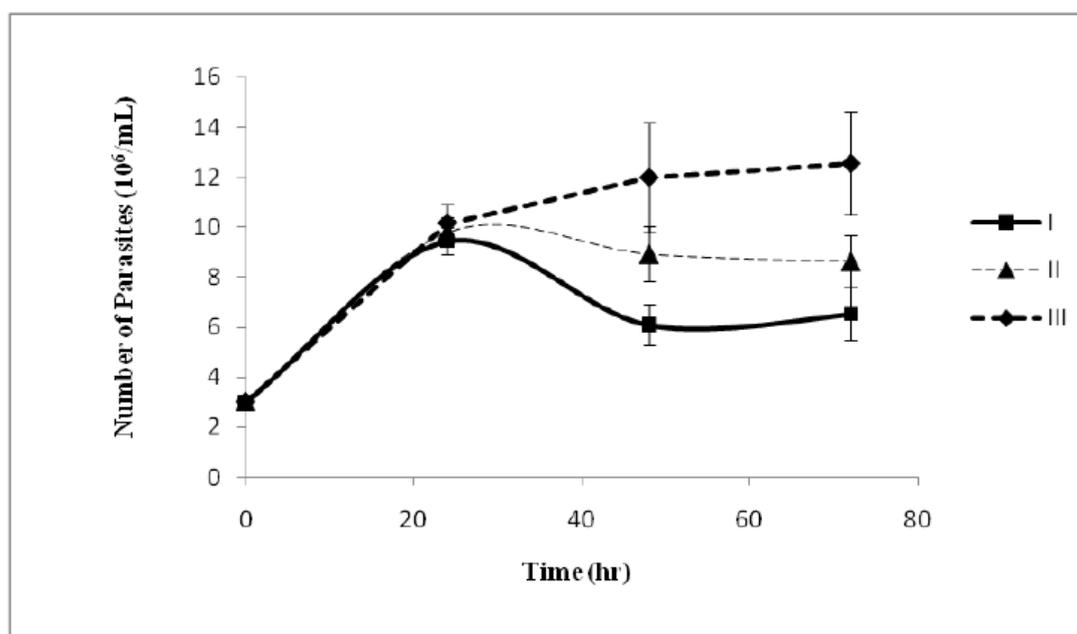
**Cultivation with sodium citrate.** Sodium citrate solutions (Sigma, St. Louis, MO) were prepared by dissolving sodium citrate in distilled water. The solution was stirred for a few minutes and sterilized with filtration (0.22  $\mu\text{m}$ , Millipore filter). *Leishmania* parasites ( $1.5 \times 10^5$  parasites/mL) were incubated in RPMI 1640 medium (10% FCS) supplemented with citrate solutions. Parasites were counted at 48<sup>th</sup> and 96<sup>th</sup> hours of incubation. The final concentrations of sodium citrate in the culture were 10  $\mu\text{g/mL}$ , 20  $\mu\text{g/mL}$ , 40  $\mu\text{g/mL}$ , 60  $\mu\text{g/mL}$  and 80  $\mu\text{g/mL}$ .

**Data processing and statistics.** All experiments were repeated 3 times or more. The results were expressed as mean  $\pm$  SD. Levene's test was used to determine the homogeneity of the variances. A parametric test (t student, analysis of variance, F, and Tukey's post-hoc test) was used to evaluate the significance of the results. All data were analyzed using the Statistical

Packages of Social Sciences (SPSS, version 16.0 for Windows), and values of  $p < 0.05$  were considered statistically significant.

## Results and Discussion

In our studies at first, effects of different concentrations of urine (2%, 5%, 10%, 15, 20%, 25%) on the growth of *Leishmania* promastigotes were investigated. It was observed that development of parasites was better in 25% urine concentration. Therefore this concentration was used for other group experiments. The average number of parasites in medium supplemented with male and female urine on the growth of *L. tropica* promastigotes was shown in figure 1. The number of parasites in the cultures supplemented with male and female urine was significantly higher than the control group ( $p < 0, 05$ ). The group with female urine consistently contained more parasites than the male urine groups for all incubation times ( $p < 0.05$ ). In culture groups both with male (II) and female (III) urine, there were higher numbers of parasites in contrast to control groups (I).

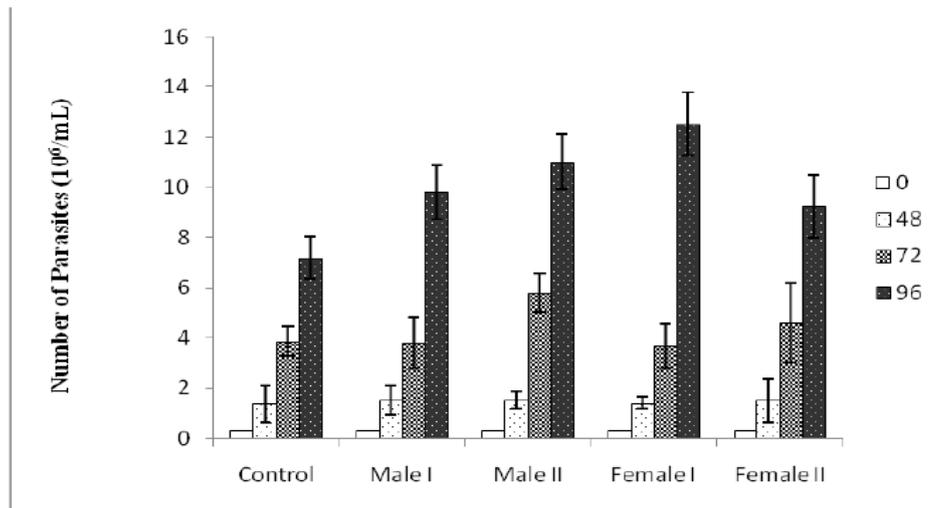


**Figure 1.** Growth of *L. tropica* promastigotes *in vitro* culture medium (RPMI-1640+10% FCS) supplemented with 25% urine concentration of female urine (III), male urine (II) and without urine (I).

Thus urine samples taken from both of male and female stimulated growth of *L. tropica* promastigotes, and generally female urine were more effective than male urine that were taken from healthy individuals with identical nutritional background. The stimulatory effect of human urine on growth of *Leishmania* parasites *in vitro* has been reported by previous researchers, whose findings supported those of the present study [3, 5-7, 11-13].

Although the average number of parasites in medium with female urine is statistically more than the number of parasites in medium with male urine, in our study urine samples taken from 2 of 18 male donors provided parasite development more than some female's.

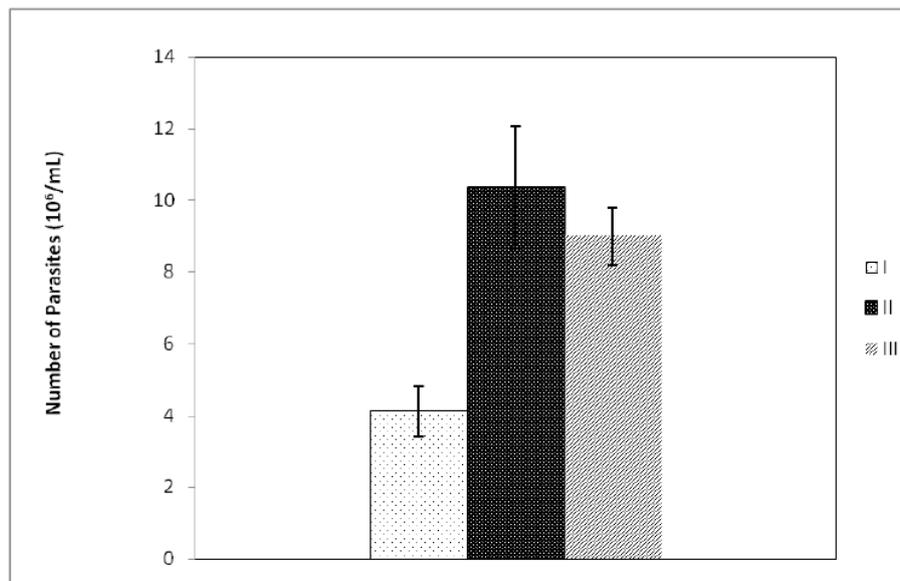
The individual effects of female and male urine on the growth of *L. tropica* promastigotes were shown in Figure 2. The urine of female-I has affected parasite development more than male and other female urine. However male-II stimulated parasite development more than female-II.



**Figure 2.** Growth of *L. tropica* promastigotes *in vitro* culture medium (RPMI-1640+10% FCS) supplemented with 25% concentration of female and male urine to hours.

This results showed that even if the average number of parasites in culture supplemented with female urine are more than male urine, some male urine stimulate growth of parasites more than female's. In this study for the first time we showed that different stimulating effect of urine depends on donors under standard nutritional conditions.

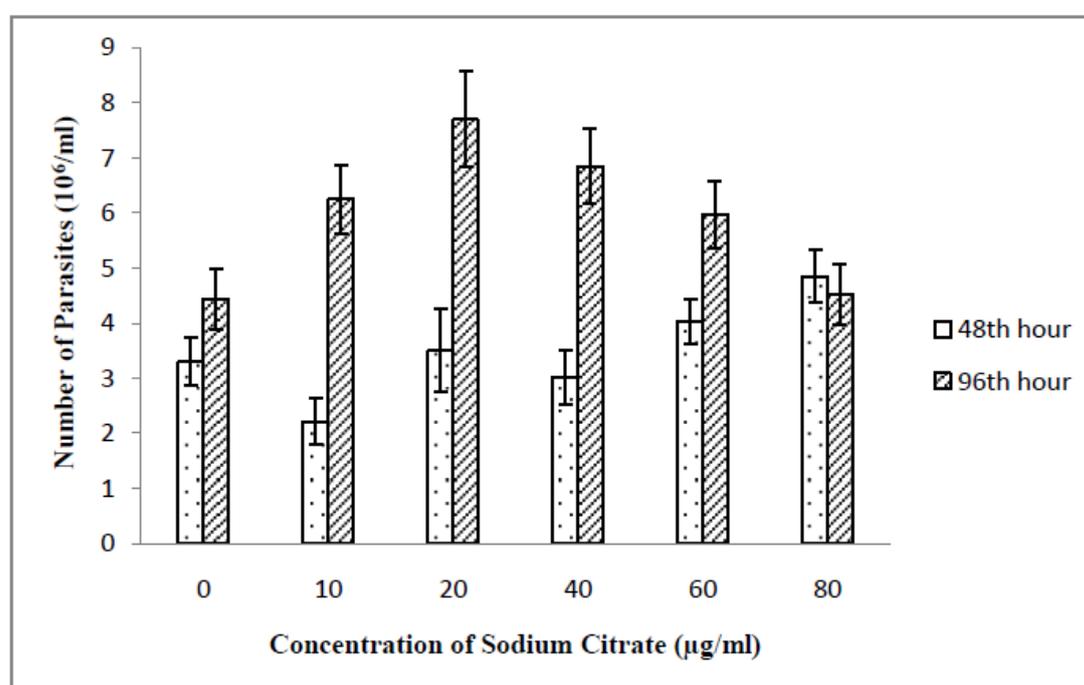
We thought that hormones of menstruating female's urine may affect parasites' development. Thus, in next group experiments we studied the growth of *L. tropica* promastigotes in medium (RPMI 1640+10% FCS) supplemented with urine taken from menstruating females (III) and females in secretory phase of the endometrial cycle (II) and without urine. As it can be seen in Figure 3, there is not any significantly difference in number of parasites between in urine of menstruating females and females in secretory phase of the endometrial cycle. However number of parasites in both groups was significantly higher than the control group ( $p < 0,05$ ). As a result, obtained results from menstruation females don't support the opinion that is about the stimulating effect of hormone.



**Figure 3.** Growth of *L. tropica* promastigotes *in vitro* culture medium (RPMI-1640+10% FCS) supplemented with 25% urine taken from menstruating females (III) and females in secretory phase of the endometrial cycle (II) and without urine (I) (48<sup>th</sup> hour).

As mentioned before, sodium citrate is also one of the most important urine components and the effect of citrate on the conversion from trypomastigote to epimastigote stages for trypanosome parasites [9].

Therefore we studied effect of citrate on growth of *Leishmania* promastigotes *in vitro*. Figure 4 shows the effect of different concentrations (10- 80  $\mu\text{g/mL}$ ) of sodium citrate on parasite growth. The number of parasites within the culture medium supplemented with sodium citrate in the concentrations of 20  $\mu\text{g/mL}$ , 40  $\mu\text{g/mL}$  and 60  $\mu\text{g/mL}$  were significantly higher than the control group at 96<sup>th</sup> hours of incubation ( $p < 0.05$ ). The maximum parasite development occurred within the culture medium supplemented with 20  $\mu\text{g/mL}$  of citrate. In this study we also showed that sodium citrate provides parasite proliferation *in vitro*. Thus citrate may be one of the urine components which are responsible for the development of *Leishmania* promastigotes.



**Figure 4.** The effect of different concentrations of sodium citrate on growth of *L. tropica* promastigotes *in vitro*.

Consequently the results obtained from urine samples of donors that live in same conditions showed that nutrition conditions and genders of donors and also the differences between individuals must be considered in case of using urine in culture of *Leishmania* parasites.

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