The Importance of Checking *Leishmania* Promastigotes Viability in the Proteomics Analysis of Secretions

Sekresyonların Proteomiks Analizinde *Leishmania* Promastigotların Canlılıklarının Kontrol Edilmesinin Önemi

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ABSTRACT

Objective: The aim of the present study was to compare the efficacy of checking the viability of *Leishmania* promastigote by flow cytometry using propidium iodide (PI) and microscopic method using trypan blue (TB) before proteomics analysis of the secretions.
Methods: The promastigotes (6×109) of *Leishmania infantum* in the exponential growth phase were transferred to serum-free media. Then, the viability of promastigotes was checked and compared with flow cytometry and microscopic method at 0, 2, 3, 4, 5, and 72 h.
Results: Flow cytometry did not show many dead cells at 0 to 4 h, and the viability was approximately 98%. The percentage of the dead promastigotes increased to 8% at 5 h and 17% at 72 h. Meanwhile, the microscopic method using TB did not show any dead cell after 4 and 72 h, and the viability was 100%.
Conclusion: The present study confirms the importance of flow cytometry using PI in checking the viability of *Leishmania* promastigotes, especially before the proteomics analysis of the secretions. It also shows that flow cytometry using PI is more sensitive than microscopic method using TB.
Keywords: Flow cytometry, trypan blue, viability, *Leishmania*, proteomics
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ÖZ

Amaç: Bu çalışmanın amacı, sekresyonların proteomik analizinden önce, Leishmania promastigotunun canlılığının tripan mavisi (TB) kullanılan mikroskopik yöntem ve propidum iyodid (PI) kullanılan akış sitometrisi ile kontrol edilmesinin etkinliğini karşılaştırmaktır.
Yöntemler: Üstel büyüme fazında *Leishmania infantum* promastigotları (6×109) serumsuz ortama aktarıldı. Daha sonra promastigotların canlılığı 0, 2, 3, 4, 5 ve 72nci saatlerde akış sitometrisi ve mikroskopik yöntemle kontrol edildi ve karşılaştırıldı.
Bulgular: Akış sitometrisi 0 ila 4 saat arasında çok ölü hücre göstermedi ve canlılık yaklaşık % 98 idi. Ölü promastigotların yüzdesi 5 saatte %8'e ve 72 saatte %17'ye yükseldi. Diğer yandan, TB kullanan mikroskobik yöntemde 4 ve 72 saat sonra herhangi bir ölü hücre gözlenmedi ve canlılık % 100 idi.
Sonuç: Bu çalışma, özellikle sekresyonların proteomik analizinden önce, *Leishmania* promastigotlarının canlılığının kontrol edilmesinde PI kullanılarak uygulanan akış sitometrisinin önemini doğrulamaktadır. Ayrıca PI kullanılan akış sitometrisinin, TB kullanılan mikroskopik yöntemden daha duyarlı olduğunu da göstermektedir.
Anahtar kelimeler: Akış sitometrisi, tripan mavisi, canlılık, *leishmania*, proteomik
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INTRODUCTION

Leishmaniasis is a major parasitic vector-borne disease caused by a family of obligate intracellular dimorphic protozoa of the genus *Leishmania*. The life cycle of *Leishmania* includes extracellular promastigote in sandflies and amastigote stage that presents in mammals within mononuclear phagocyte cells (1, 2). To our knowledge, there is no effective treatment with reduced side effect for leishmaniasis in the literature. Therefore, improving the diagnosis, treatment, and vaccination of leishmaniasis is urgent for the control and prevention of this disease (3, 4).

Nowadays, most studies on *Leishmania* are performed using promastigote as this form is more adaptable in vitro, and the required technology for its cultivation is not tedious and laborious (5). The antigenic targets in secretions of *Leishmania* promastigotes are suitable sources for designing vaccines and diagnosing leishmaniasis since these antigens are considered as stimulants of the immune system (6, 7). Therefore, the preparation of excretory–secretory antigens from live promastigotes (viable cells ≥98%) is a considerable advantage, especially in the proteomics research on secretions of *Leishmania* parasites.

Proteome refers to a set of proteins that have been encoded by the genome (8) and defined as the analysis of proteins to determine their unique identity, quantity, function, and interaction (9). Evidence also shows that proteomics is practically suitable for the analysis of the proteome of the *Leishmania* genus (10). Recently, researchers have attempted to use this technique to identify immunodominant antigens in *Leishmania* parasites and to introduce new targets for vaccines (11).

The preparation of a high-quality sample is an important issue in the proteomics approaches (12). Therefore, it is very essential to provide a reliable method that can determine the viability of *Leishmania* promastigotes before proteomics analysis of the secretions.

For performing some experimental procedures, checking cell viability is the first step (13). Most of the viability tests are based on the integrity of the cell membranes. Vital dyes, such as trypan blue (TB), can penetrate selectively into the dead cells, but the viable cells do not permit these dyes to penetrate the cells so the live cells remain unstained. Therefore, optical microscopy methods are used to count the number of stained and unstained cells using a Neubauer chamber (14).

One of the major practical assays widely used to check the viability is flow cytometry with propidium iodide (PI) staining (15). The PI function is similar to TB and penetrates the dead cells that produce a complex with DNA (16). The advantages of the PI assay are its high accuracy and the possibility of running many samples in a short time. There are only few studies in the literature that have been conducted to improve the TB exclusion test by making the adaptations with flow cytometry (17).

To the best of our knowledge, few studies have checked the viability of *Leishmania* promastigotes before proteomics analysis of the secretions (18-20). Based on various results in this regard, the aim of the present study was to evaluate and to check the viability of *Leishmania infantum* promastigotes by flow cytometry using PI in comparison to microscopic method using TB.

METHODS

Sample preparation and promastigote culture

L. infantum strain (MCAN/IR/07/Moheb-gh) was provided by the Department of Parasitology and Mycology of Medical Sciences, Shiraz, Iran. *L. infantum* promastigotes were mass cultivated at 25 °C in the Schneider's insect culture media (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 20% (v/v) bovine serum (heat inactivation requires the serum to be at 56 °C for 50 min), 100 U/mL penicillin, and 100 µg/mL streptomycin. Then, 6×10^9 promastigotes were collected in the exponential growth phase (day 3) by centrifugation at 2000×*g* for 10 min at 4 °C and washed three times with RPMI-1640 (Shellmax Co., China) serum-free media (21).

Washed promastigotes were transferred to 10 mL RPMI-1640 serum-free media for secretions assay. The number of promastigotes was determined by a Neubauer hemocytometer (22). Then, the viability of promastigotes was indicated at 0, 2, 3, 4, 5, and 72 h by flow cytometry using PI and microscopic method using TB.

Flow cytometry assay using PI

Ten thousand promastigotes in 500 µL of the RPMI-1640 serum-free media were incubated in the presence of 5 µL PI solution (1 mg/mL; Sigma Chemicals Co.) for 5 min at 23-25 °C (19). Suspended promastigotes were run by flow cytometry (BD FACSCalibur®, USA) set to excitation at 493 nm and emission at 636 nm wavelengths. The results were analyzed by the FlowJo software, version 7 (LLC, USA) at 0, 2, 3, 4, 5, and 72 h.

Microscopic method using TB

In the TB exclusion test, 10 µL of TB was added to 10 µL of RPMI-1640 serum-free media containing 10,000 promastigotes, and the viability was immediately determined at 0, 2, 3, 4, 5, and 72 h (23). In PI and TB experiments, the promastigotes that were exposed to methanol were used as a positive control.

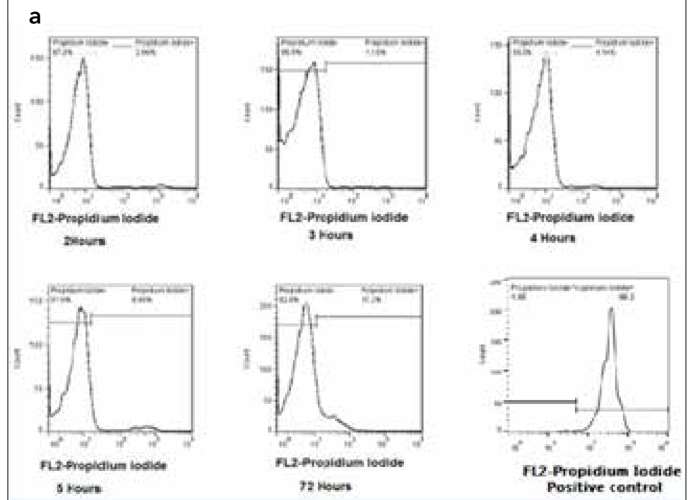
Statistical Analysis

Data were expressed as mean±SD of at least three independent experiments. The statistical significant difference between the groups was evaluated using the GraphPad software (GraphPad San Diego, CA, USA). The statistical test was evaluated using the one-way analysis of variance. A $p<0.05$ was considered statistically significant.

RESULTS

In the present study, the results showed that the PI can precisely discriminate dead promastigotes against viable promastigotes. Interestingly, the percent of dead cells using PI was shown to be time dependent. The cell viability using PI was approximately 98% at 0, 2, 3, and 4 h. However, the viability declined to 92% and 83% after 5 and 72 h, respectively (Figures 1a, b).

Furthermore, the same experiment was performed in regard to TB to evaluate the viability. The percent of the viability of promastigotes in respect to TB was not inconsistent with the PI results. Surprisingly, all promastigotes were viable after 5 and 72 h. In the positive control, the dead cells were measured at approximately 98% by flow cytometry using PI (Figures 1a, b) and 100% by microscopic method using TB.



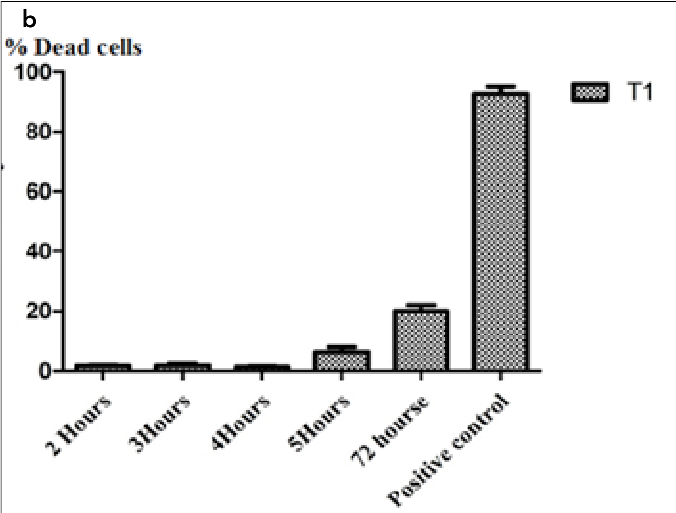


Figure 1. a, b. The results of flow cytometry with PI for checking the viability of L. infantum promastigotes at 2, 3, 4, 5, and 72 h and positive control (a) Values are expressed as mean±SD of three independent experiments (b).

DISCUSSION

Some proteomics studies in *Leishmania* parasites present different antigens that could be effective in the development of vaccines, drugs, and diagnosis of leishmaniasis (7, 24-27). Obtaining secretions from the viable cells is pivotal for having a good interpretation of the results of proteomics experiments (28). Since the contamination of secretions with dead parasites leads to some unreliable data, checking the viability is necessary in this regard.

A proteomics research on the secretions of *Leishmania* promastigotes did not use a precise method to check the viability of promastigotes (29). The findings by Cuervo et al. (18) showed that the best time point for collecting secretions is 3 h based on the TB method. However, our findings indicated that the microscopic method using TB cannot detect dead cells at 3 h, and the accuracy of the results will probably be decreased.

In 2015, Kumar et al. (19) detected the immunostimulant antigens in soluble exogenous antigens in *Leishmania donovani* and used the microscopic method to check the viability of promastigotes. Their findings identified that the appropriate time for the evaluation of viability is approximately 72 h. Although our microscopy results were in accordance with their study, the flow cytometry results showed that 17% of promastigotes are not viable after 72 h. The microscopic method using TB appears to be imprecise and can lead to an overestimation of the live cells, especially in cases in which we intend to check the viability of many cells (30).

Our findings for PI are somehow in line with the study by Braga et al. (20). They used the flow cytometry assay to check the viability of promastigotes within 2–8 h and proposed that the optimum time for collecting the promastigote secretions is 6 h (19).

To our knowledge, few studies have been conducted to improve the TB exclusion test using flow cytometry (17). They suggested that using TB in flow cytometry could also be precise. However, the use of TB in flow cytometry based on the literature is not common. In addition, they did not compare their results with PI.

CONCLUSION

The present study proposes and confirms that flow cytometry using PI staining should be prioritized for checking the viability of promastigotes in the proteomics analysis of *Leishmania* secretions. In addition, our findings proved that the optimum incubation time is 4 h for obtaining secretions without many dead cells.

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