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Chapter

Cell Culture and Maintenance of the Evolutionary Forms of *Trypanosoma cruzi* for Studies of Parasitic Biology

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Abstract

This chapter aims to present and discuss the main cell culture techniques used for the growth and maintenance of the different evolutionary forms of the protozoan *Trypanosoma cruzi*, the etiologic agent of the Chagas disease. Chagas disease is a neglected tropical disease endemic in several Latin American countries. Here, we intend to present the main difficulties, advantages, and disadvantages of using *Trypanosoma cruzi* cell culture in parasitic biology. Finally, we present the main research opportunities in the context of *Trypanosoma cruzi* parasitic biology using cell culture techniques, such as drug development, characterization of pharmacological targets, molecular markers for diagnosis, structural biology, and many other biomedical applications.

Keywords: *Trypanosoma cruzi*, Chagas disease, cell culture, in vitro assay, metacyclogenesis

1. Introduction

Carlos Chagas described the American trypanosomiasis through microscope observation of hemoflagellate protozoan in the sample blood and named *Trypanosoma cruzi* [1]. The parasite belongs to the Kinetoplastida order and *Trypanosomatidae* family. This family is characterized by the presence of flagellum and only one mitochondrion, which extends throughout the cell body. The mitochondrion matrix contains a complex array of DNA fibrils structure called kinetoplast (kDNA).

Trypanosoma cruzi (*T. cruzi*) is a digenetic parasite that develops through a complex life cycle involving an insect vector and a mammalian host. Trypanosomatidae family shows, during development stages, several forms, which can be easily identified by light microscopy in Giemsa-stained preparations. In the case of *T. cruzi*, three main morphogenetic forms are recognized, and the transition between each form

	Evolutionary forms			
	Epimastigote	**Trypomastigote metacyclic**	**Trypomastigote**	**Amastigote**
Localization	Intestine of the vector and axenic culture	Rectal ampoule of vector axenic culture	Blood and intracellular spaces of vertebrate-host and cells culture	Inside the cells of vertebrate-host and cells culture
Cell morphology	Elongate	Elongate	Elongate	Spherical
Position of the kinetoplast	Near the nucleus	After the nucleus	After the nucleus	Near the nucleus

These evolutionary forms are identified by the relative position of the kinetoplast in relation to the nucleus.

Table 1.
View in optical microscope of the various developmental stages of Trypanosoma cruzi: epimastigote, intracellular amastigote, trypomastigote metacyclic, and bloodstream forms.

involves morphologic modifications in gene expression and cellular cycle (**Table 1**). Therefore, the evolutionary forms, trypomastigote and amastigote, are found in the vertebrate host, and epimastigote and metacyclic trypomastigotes are found in the invertebrate host [2].

Due to its genetic diversity, the parasite *T. cruzi* represents one of the best model organisms for studies of clonal evolution, with rare events of recombination and/or genetic hybridization. Molecular markers, like the mini-exon genes and the ribosomal DNA 24S, allow the characterization of *T. cruzi* lineages and the subdivision in seven discrete typing units (DTUs, from TcI to TcVI) [3, 4]. Parasite of *T. cruzi* diversity could be associated with adaptation and survives inside different hosts. Therefore, this genotypic variation among the different strains of *T. cruzi* from different geographical regions may explain the frequency of variability in different characteristics of parasitism, such as virulence, subclinical infection rate, clinical symptoms of infection, and susceptibility to antiparasitic drugs [5].

A century of its discovery, Chagas disease remains a cause of early mortality and morbidity in individuals at productive age in endemic areas, generating significant socioeconomic impact. The importance of parasite biology studies is undeniable. In this context, in vitro studies of *T. cruzi* offers the opportunity to understand the coordination of metabolic and cellular change as a response to environmental stimuli in a simple and well-characterized system.

2. How to maintain and cultivate *Trypanosoma cruzi*?

During life cycle, *T. cruzi* alternates among different morphological and functional types and different hosts (mammals and triatomine bugs). Epimastigote replicative form can result in non-replicative and infective trypomastigote forms. Amastigote forms could be achieved by infected mammal cells. The adaptation mechanism of *T. cruzi* to environmental changes and diverse physicochemical conditions represents an important means of survival.

Substantial interest in understanding the cell biology of *T. cruzi* made possible its maintenance and growth in in vitro systems. Several methods have already been described for obtaining the different stages of the parasite, and these can be cultured in non-defined axenic media, defined axenic media, and in various cell types.

The axenic cultures of *T. cruzi* were primarily established in different cell types and tissues with appropriate conditions that allowed the infection process and

its survival [6]. During this period, metacyclogenesis process was observed and characterized through detailing all the cellular and/or behavioral transformations undergone by the parasite. By observing the biological changes undergone, key points during the process became possible to be determined that were later mimicked to facilitate the differentiation process [7].

Epimastigote forms are maintained in axenic conditions at 28°C, in the nutritive medium liver infusion tryptose (LIT), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) as described by Camargo [8]. Under chemically defined conditions and suppression of nutrients, the transformation of epimastigotes into metacyclic trypomastigotes is possible in vitro using a process named metacyclogenesis [9].

To obtain intracellular amastigote forms, usually mammal cells, Vero lineage, murine peritoneal macrophages, fibroblast (mouse L929), and primary heart or skeletal muscles cells are commonly incubated with specific medium supplemented with 10% inactivated FBS (v/v) and allowed to adhere usually for 24 h at 37 ± 2°C in 5% CO_2. After this period, adherent cells can infect with trypomastigote metacyclic using a 10:1 ratio of parasites per cell and incubated at 37°C in 5% CO_2, without FBS. Amastigote can be observed in microscope at 4–24 h after infection. Intracellular amastigote can release within 72 h trypomastigote metacyclic from culture derived. Infected Vero cells can be in continuous cultivation during 6–8 weeks [10].

Infected cells with *T. cruzi* can spontaneously release amastigote forms into the supernatant medium. The viable amastigote forms can be collected and produce the axenic amastigote. Firstly, wash cells with phosphate buffer saline (PBS, pH 7.2) through centrifugation, collect, and then resuspend in appropriate medium, usually Grace medium supplemented with nicotinamide and ATP. This addition can neutralize differentiation of amastigote forms, and the culture can be maintained at 37°C in a humid atmosphere containing 5% CO_2. Every 4 days, the media have to be partially removed by centrifugation to remove the supernatant medium and add the fresh medium to maintain the amastigote forms [11].

3. In vitro differentiation of *Trypanosoma cruzi* epimastigote to trypomastigote forms

Metacyclic trypomastigotes (non-replicative) originating from the insect vector or trypomastigotes from infected cells, such as culture derived and bloodstream/tissue, are infective forms of the parasite. Metacyclogenesis is observed in the stationary phase of culture, and metacyclic trypomastigotes are largely absent during logarithmic growth. However, in vitro differentiation of *T. cruzi* is possible according to biologically active factors and essential nutrient depletion or accumulation of certain metabolites [12]. Generally, metacyclogenesis process can be mimicked using chemical differentiation medium or axenic-defined medium and nutrient suppression for the differentiation of the epimastigote forms to occur in trypomastigotes. The omission of medium supplements, such as glucose, was able to lead to flagellar elongation, as observe by Tyler and Engman [13]. The epimastigote-extending flagellum occurs in order to undergo metacyclogenesis.

By contrast, chemical modification is commonly used for differentiation of epimastigote to trypomastigote forms. Epimastigote forms can be collected from the LIT culture, during the stationary phase of the growth curve, and then resuspended in artificial triatomine urine (TAU) supplemented with proline (TAUP medium) or TAU supplemented with L-proline, L-sodium glutamate, L-sodium aspartate, and D-glucose (TAU3AAG medium) [14]. In the first medium, parasite culture can differentiate after 10 days and 72–96 h using TAU3AAG medium. Recent study

demonstrated that reductive environment using urate, a salt or ester of uric acid, could promote epimastigote differentiation with significant increment of trypomastigotes [15]. In addition, Grace medium supplemented with *Triatoma infestans* intestinal homogenate was used for in vitro metacyclogenesis of *T. cruzi* [16].

Metacyclic trypomastigotes are essential for the understanding of host-pathogen interaction and it is remarkable genetic variability between strains. Although, these biological stages in axenic medium are difficult to purify, culture remains with epimastigote stages without showing a high efficiency in the purification of metacyclic trypomastigotes [8].

Complement-mediated lysis of epimastigotes followed by separation of the trypomastigotes by gradient centrifugation through a dense albumin column represents a selective method that exploited to purify viable trypomastigotes from cells culture medium. Alternate pathway activation seems capable by itself generating epimastigote lysis and consequent activation of the classical pathway just in the presence of serum [17].

Other several techniques such as density separation with Percoll [18, 19] and extensive use of chromatography have permitted purifying metacyclic trypomastigotes for the association of specific molecules expressed on this parasite stage membrane separation. Chromatography based on the differential plasma membrane charge between epimastigotes and trypomastigotes using ion exchange chromatography with resins, such as cellulose-DEAE and sephadex, have been developed for the purification of metacyclic trypomastigotes [20, 21]. These previous chromatography techniques presented a major disadvantage such as the decrease in the infective capacity of the obtained trypomastigote [22].

Recently, a new protocol in LIT medium cultures was implemented using sepharose-DEAE as a resin during purification process, which could recover metacyclic trypomastigotes for two different DTUs (I and II). DTU TcI was the one recovered a greater amount of these forms, and parasite infectivity in vitro and in vivo was maintained [20]. Metacyclogenesis with different DTUs exhibited significantly different morphologies and metacyclogenesis pattern [23].

Still it remains crucial to have easy, fast, and reliable tools to obtain purified metacyclic trypomastigote forms of *T. cruzi*. Thus, results acquired with these purified parasites should be interpreted with caution in order to validated biological, chemical, or immunological studies.

4. Advantages and limitation of in vitro *Trypanosoma cruzi* culture

The entire *T. cruzi* cycle can be studied in vitro, and it is one of the easiest to keep in the laboratory. A crucial point, which is considered to be a basic requirement for all the biological assays in vitro system using *T. cruzi* is the selection of methods and adoption of biosafety procedures, including good laboratory facilities and laboratory practices and the use of collective and individual protection tools. Therefore, the training of personnel involved in the manipulation and cultivation of *T. cruzi* strains is required, especially on the handling of drug-resistant strains.

Probably, the main advantages of the use of in vitro systems for maintenance and cultivation of *T. cruzi* are related to costs and biosafety. The in vitro system of *T. cruzi* cultivation presents some characteristics that deserve to be highlighted: (i) reduction of the number of animals used in experimental models of infection, (ii) ethical aspects in animal replacement, (iii) less need for laboratory infrastructure, (iv) reduced time of experiments compared to in vivo methods, and (iv) lower risk of human infection during culture procedures [15].

As mentioned above, the use of *T. cruzi* in in vitro assays allows the researcher to access a variety of strains with different biological and genetic profiles, in addition to the different stages of parasite differentiation. Furthermore, *T. cruzi* clones exhibit a greater rate of multiplication in culture medium.

One of the possibilities of in vitro system is to obtain large amounts of metacyclic trypomastigotes (derived culture or chemical modification medium) with biological properties similar to those of the insect-derived forms, which facilitates the study of the biology of *T. cruzi*. On the other hand, there is a controversy that the strains are kept in artificial culture media, transformed into true "laboratory strains," with total or partial loss of their original characteristics, despite their morphological similarities. Contreras and colleagues demonstrated that culture-derived metacyclic forms of *T. cruzi* can decrease their capacity to invade cells [16].

The great potential of the in vitro models is the study of the interaction *T. cruzi*-host cell throughout amastigote culture. It has been demonstrated the in vitro capacity of invasion in all cell types was tested [10, 24]. As yet, in vivo models, infected animals, as well as in human, show evidence of a parasite tropism by cells of the mononuclear phagocytic system and by muscle and nerve cells, contrary to what is observed in vitro as mentioned before. On the other hand, maintained trypomastigote metacyclic in vivo (mice) condition generally increased virulence [14].

However, the in vitro assay does not provide full information on the behavior and characteristics about *T. cruzi*. The complexity of in vivo system is bigger than in parasite cultivation system. The mechanisms of parasite-host interaction are controlled by cells and innumerable proteins that trigger numerous genetic and physiological signals in the parasite. These interactions cannot be accurately evaluated in in vitro systems.

5. Applications in the cell culture of *Trypanosoma cruzi*

Despite decades of research in *T. cruzi* biology, many questions remain to be solved. The cultivation of *T. cruzi* has been explored in several biomedical researches, mainly as a strategy for the understanding of parasite biology and parasite-host interaction. Thus, these in vitro methodologies have been used to understand mechanisms of parasite invasion, mechanisms of resistance and susceptibility to drugs, identification of virulence factors, signaling, cellular metabolism, etc. Our research group has been using the *T. cruzi* cultures to evaluate the potential of new compounds with antiparasitic activity for the treatment of Chagas disease [25]. The cultivation methods also allow to isolate and characterize the circulating *T. cruzi* genotypes, present in triatomines captured in the natural environment of transmission of Chagas disease [26]. In addition, other robust molecular and structural biology tools can be applied to the study of the biology of *T. cruzi* using cell culture, namely, functional genomic and proteomic studies [27, 28] and structural cryo-microscopy [29].

6. Conclusion

In this chapter, we have shown the importance of cell culture techniques as a complementary tool for studies of parasitic biology. Through the cultivation of different evolutionary forms of *T. cruzi*, we can answer important scientific questions in the context of parasitic diseases, more specifically in Chagas disease. In conclusion, this work shows a reflection about the advantages and limitations of *T. cruzi* cultivation techniques in studies of parasitic biology.

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Conflict of interest

The authors declare no conflict of interest.

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