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Morphometric analysis of tachyzoites of the RH strain of *Toxoplasma gondii* after in vitro exposure to atovaquone

Análise morfométrica de taquizoítos da cepa RH de Toxoplasma gondii após exposição in vitro à atovaquona

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ABSTRACT

Toxoplasmosis is a zoonosis with a major impact on public health, and despite this, there is still no effective treatment for all morphological forms of the parasite and stages of the disease. The current treatment protocol uses sulfadiazine and pyrimethamine, which can trigger serious adverse reactions in patients, as well as not being effective in tissue cysts. Atovaquone is an antimalarial drug that has demonstrated anti-*Toxoplasma* activity. This study aimed to evaluate *in vitro* the morphometric changes in tachyzoites of the RH strain of *Toxoplasma gondii* after exposure to atovaquone. The *in vitro* experiment was carried out using RAW 264.7 cells, seeded on plates with supplemented RPMI medium. Simultaneously with the infection of the cells, the tachyzoites were exposed to atovaquone. Exposure was assessed at 2 hours, 48 hours, and 7 days. The experiment was carried out in quintuplicate. The morphometric evaluation was carried out using ImageJ® software, and the statistical analysis was done using GraphPad Prism® 8.2.1. The morphometric changes observed showed that from the first hours of exposure, atovaquone affects the morphology of the tachyzoites.

Keywords: *Toxoplasma gondii*, atovaquone, morphometric analysis.

RESUMO

A toxoplasmose é uma zoonose de grande impacto em saúde pública e, apesar disso, ainda não existe um tratamento eficaz contra todas as formas morfológicas do parasito e fases da doença. O protocolo de tratamento vigente utiliza sulfadiazina e pirimetamina que pode desencadear reações adversas graves aos pacientes, além de não ser eficaz nos cistos teciduais. A atovaquona é um fármaco antimalárico que tem demonstrado atividade anti-*Toxoplasma.* O objetivo do trabalho foi avaliar *in vitro* as alterações morfométricas de taquizoítos da cepa RH de *Toxoplasma gondii* após exposição à atovaquona. O experimento *in vitro* foi feito utilizando células RAW 264.7, semeadas em placas com meio RPMI suplementado. Simultâneo à infecção das células, os taquizoítos foram expostos à atovaquona. A exposição foi avaliada nos períodos de 2h, 48h e 7 dias. O experimento foi realizado em quintuplicata. A avaliação morfométrica foi realizada no software ImageJ® e as análises estatísticas no programa GraphPad Prism® 8.2.1. As alterações morfométricas observadas demonstraram que desde as primeiras horas de exposição, a atovaquona age na morfologia dos taquizoítos.

Palavras-chave: *Toxoplasma gondii*, atovaquona, análise morfométrica.

1. INTRODUCTION

The discovery of *T. gondii* is a milestone in human and veterinary parasitology. In 1908, researchers Charles Nicolle and Louis Manceaux isolated the parasite from the tissues of a North African desert rodent, *Ctenodactylus gundi*, in Tunisia. Initially, they had thought they had found a new species of protozoa from the Leishmania genus. At the same time, Alfonso Splendore isolated the same protozoan in rabbit tissues in Brazil[1,2,3]. *T. gondii* infection, known as toxoplasmosis, has one of the highest prevalence rates in the world and is a serious public health problem. Toxoplasmosis is frequent in developing countries and in regions where hygiene conditions are inadequate, as well as in populations with higher consumption of raw or undercooked meat[4,5]. The infection is usually benign in immunocompetent patients. In humans, it can be symptomatic or asymptomatic, depending on factors inherent to the parasite, such as the parasite load and the strain involved, and also factors involving the host itself, such as the individual's immune response[6].

Despite having been described in 1908, it wasn't until the 1960s that the parasite's life cycle was understood, with felids as definitive hosts and other animals as possible intermediate hosts. Its two main transmission routes were then established: congenital and through the accidental ingestion of different infective stages[3,7]. All forms of the parasite are infectious: sporozoites, tachyzoites, and bradyzoites[8]. Tachyzoite is the fast-multiplying form of the parasite and was given its name in 1973 by Frenkel precisely because of this replication characteristic: in Greek, “tachos” means fast. It is a form found in various cells of intermediate hosts and non-intestinal epithelial cells of felids[9]. It is the evolutionary form that is related to the clinical symptoms presented in the acute phase of *T. gondii* infection[8].

Tachyzoites are disseminated through the host's blood and lymphatic vessels and can reach various regions of the body, as well as being able to cross important biological barriers such as the blood-brain barrier and the transplacental barrier[9]. They have a characteristic elliptical shape, similar to a "half moon" and measure approximately 2 µm x 6 µm. They are obligatorily intracellular evolutionary forms that infect nucleated cells[8]. Three predominant genetic strains of *T. gondii* are currently known, capable of infecting numerous species, including humans[10]. The protozoan strains are classified into: type I, type II and type III[11]. Type I strains are often associated with congenital toxoplasmosis in humans and are related to acute virulence in mice. Type II and III strains generally cause chronic

infections in humans and cyst formation in mice[12]. For experimental studies, the RH strain is the main representative of type I.

Atovaquone has been gaining ground in research into new therapies for the treatment of *T. gondii* infection for the last 20 years[13]. The studies involve new formulations and purposes for their application[13,14]. The studies on *T. gondii* evaluating the use of atovaquone justify its interest, mainly due to its better tolerance and fewer side effects when compared to the use of sulfadiazine and pyrimethamine[13,14,15].Drug repositioning is a strategy in the search for new therapeutic alternatives since it proposes the use of drugs that have already been approved for use in other diseases. The advantages of repositioning include a reduction in the costs and time invested in developing new drugs[16]. In the search for new treatments for toxoplasmosis, much research has been done on drugs with antimalarial activity, since the protozoa, both belonging to the apicomplexa phylum, have biochemical and structural similarities[17].

One of the structures that *T. gondii* and *Plasmodium* spp. What we have in common is the apical complex. This organelle, as well as being essential for the invasion mechanisms of the host cell, is also indispensable for the biochemical and metabolic activities of the parasite, acting in the synthesis of important biomolecules, such as fatty acids. The similarities, mainly biochemical, between *T. gondii* and Plasmodium spp. Arouse interest, from a therapeutic point of view, in using similar drugs to treat both infections[17]. Among the drugs being researched, atovaquone is a promising target, since its action occurs at the mitochondrial level. Atovaquone also stands out for not interfering with folic acid metabolism and can be used by pregnant women[18].

Morphometric studies on *T. gondii* help to understand the structural characteristics of the protozoan and can thus contribute to the development of new treatment strategies against toxoplasmosis. Through these studies, it is possible to determine size and shape in response to the action of drugs. Therefore, morphometric studies are a tool to advance understanding of the development of new treatment strategies. This study aimed to evaluate the morphometric changes triggered by atovaquone in *T. gondii* tachyzoites *in vitro*.

2. METHODS

2.1 Ethical considerations

The project complies with the ethical principles in animal experimentation established by the Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL), approved under protocol no. 063/21 by the Ethics Committee for the Use of Animals at the Federal University of Goiás (CEUA/UFG).

2.2 Thawing and maintenance of RAW 264.7 cells

RAW 264.7 cells kept cryopreserved in liquid nitrogen were used. To carry out the thawing process, the cells were placed in a bain marie, under continuous and slow agitation, at 37ºC. They were then transferred to the laminar flow, previously decontaminated with alcohol, exposed to ultraviolet (UV) light for 15 minutes, and placed in a sterile Falcon tube. Approximately 10mL of RPMI medium was added to the tube and centrifuged at 1500rpm for 10 minutes at 10ºC. The supernatant obtained was discarded and the sediment was resuspended in 1mL of RPMI medium. A culture medium was added to the culture container (small bottles) and the cell suspension was seeded and kept in the CO2 incubator (5%) at 37ºC in the Cell Culture Laboratory of the Multiuser Center for Bioinput and Health Technology Research (CMBiotecs), located at IPTSP/UFG. Each bottle was filled with around 5mL of supplemented RPMI medium (10% SFB, penicillin/streptomycin, 2-mercaptoethanol, HEPES and L-glutamine). The cells were viewed under an inverted microscope.

2.3 Obtaining the RH strain: *in vivo* and *in vitro* maintenance

In vivo maintenance of the RH strain of *T. gondii* took place in BALB/c mice at the Multiuser Center for Animal Production and Experimentation (CMPEA) at IPTSP/UFG. To maintain the strain, the infected animal was euthanized and then washed intraperitoneally with saline solution (0.9%). After inoculation with saline solution and peritoneal massage, the lavage containing tachyzoites was aspirated and quantified so that a new inoculum could be made in new mice. To ensure contaminant-free *in vitro* cultivation, the procedure for obtaining the tachyzoites to be used in the culture was carried out in a laminar flow, with the mice previously sanitized with 70% alcohol. Once they had been taken for *in vitro* cultivation,

maintenance was carried out in the same way as for the cultivation of RAW 264.7 cells, changing the culture medium every two days and adding cells at each maintenance.

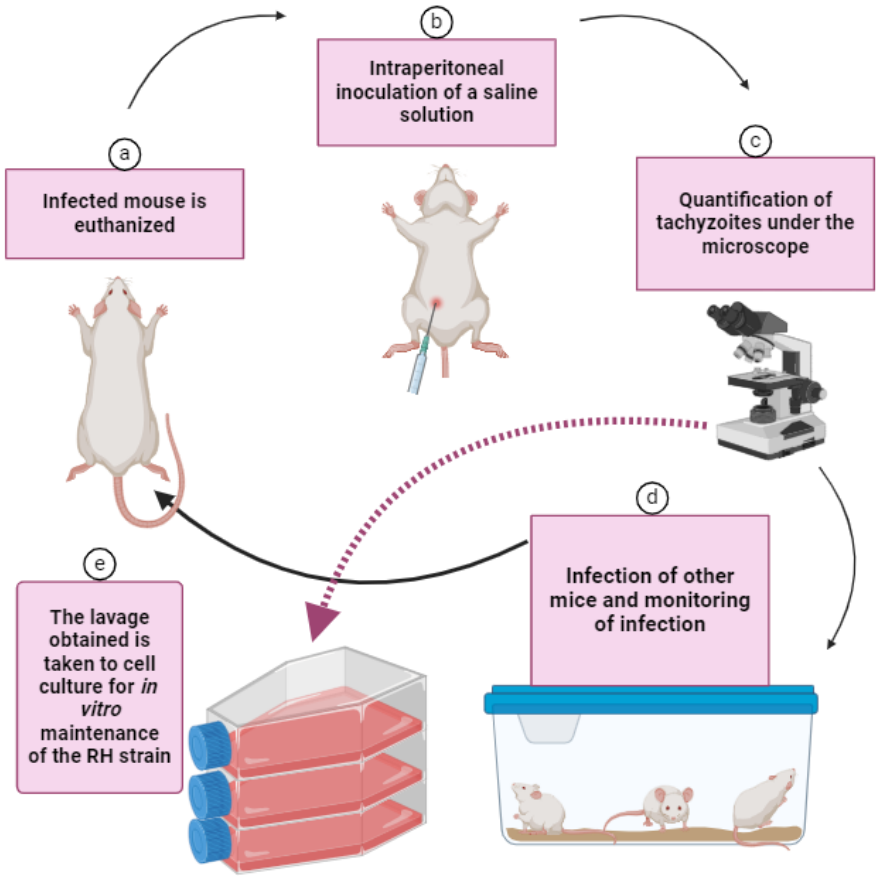

Figure 1. Schematic representation of the in vivo maintenance of the RH strain of *Toxoplasma gondii*. Prepared by the author.

2.4 Placement of cells and exposure of tachyzoites to atovaquone

The experiment was carried out in quintuplicate on plates simultaneously for the two concentrations of the proposed treatment (50nM and 100nM) with atovaquone. Atovaquone was solubilized with Milli-Q water (type I) and the proposed concentrations were determined by the study carried out by Souza[19].

Initially, 200,000 cells (RAW 264.7) were seeded in five wells of the plate containing 3mL of supplemented RPMI medium. They were kept in a CO2 oven at 37ºC for 24 hours. After the time needed for the cells to adhere to the plate, each well that had been seeded with cells was infected with 1x 106 tachyzoites. Simultaneously with infection with the tachyzoites, treatment with atovaquone was also carried out on the 50nM and 100nM atovaquone test plates.

The period of exposure between the parasite and atovaquone was evaluated at 2h, 48h, and 7 days, without replacement of the drug or RPMI medium. At the end of each period, a sample was taken from each well to make slides. In short, the groups referred to as control were made up of cells and tachyzoites and the treated groups were made up of the same content, but with the addition of the respective concentrations of atovaquone (50nM and 100nM).

2.4 Morphometric analysis of tachyzoites after exposure with atovaquone

Slides were made with the samples obtained from the *in vitro* culture at all the proposed times (2h, 48h and 7 days) of treatment with atovaquone (Figure 2). After drying at room temperature, the slides were fixed with absolute methanol and stained with Giemsa. A photomicroscope (Leica® DM750) was used in the Microscopy and Bioprocesses Laboratory at CMBiotecs/IPTSP/UFG and images of the slides were obtained using Leica® image acquisition software.

Imagej® software was used to carry out the morphometric analysis of the tachyzoites[20]. For each evaluation period, 4 plates were used (2 for the controls and 2 for the treated groups). Two slides were made for each of the five wells of the plates, and 10 tachyzoites were evaluated on each slide, totaling 1,200 tachyzoites at the end of the analysis. The characteristics evaluated among the tachyzoites were: longitudinal length (distance from one end to the other); distance between the nucleus and the anterior end of the parasite (region that houses the apical complex); and area (delimited around the tachyzoite). The Imagej® software was calibrated before the analyses were carried out.

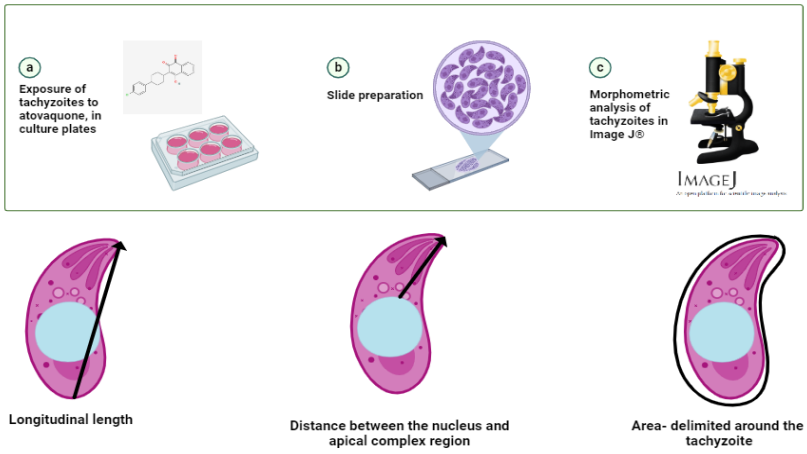

Figure 2. Schematic representation of the experiments and measurements. Prepared by the author.

2.6 Statistical analysis

The tachyzoite morphometry data was subjected to statistical analysis using GraphPad Prism® version 8.2.1. The software determined the mean and standard deviation. Analysis of variance (ANOVA) and the T-test were also carried out. Differences were considered significant when $p<0.05$.

3. RESULTS

3.1 *In vitro* evaluation of morphometric changes in tachyzoites

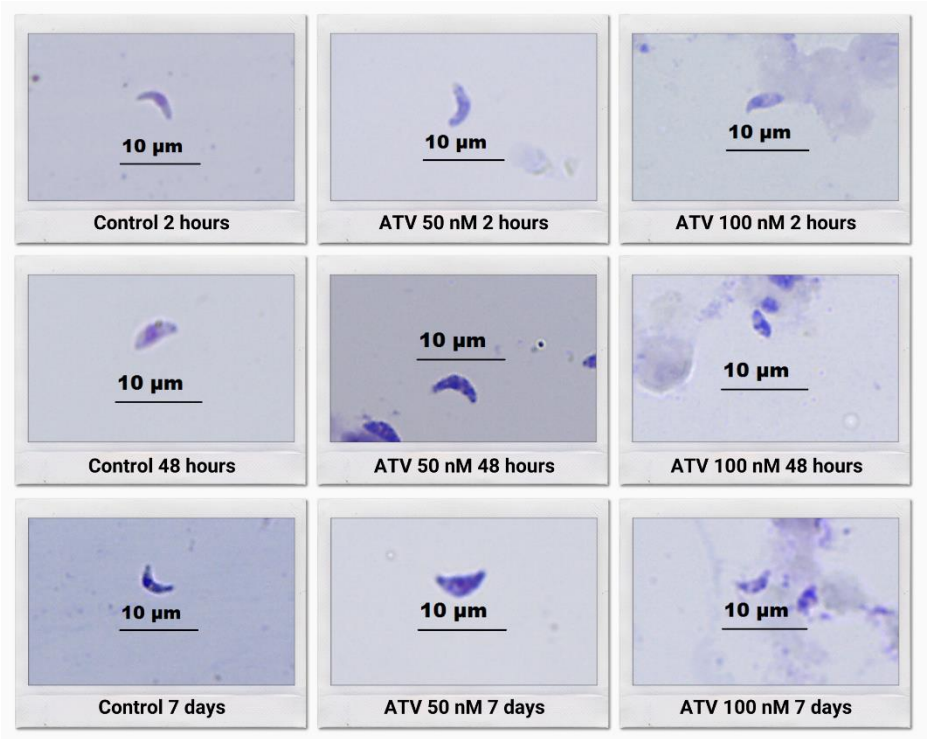

Figure 3. Photomicrographs showing the kinetics of morphology observed at three time points (2 hours, 48 hours and 7 days) of *Toxoplasma gondii* tachyzoites treated with 50 nM and 100 nM atovaquone, on Giemsa-stained slides, observed at 1000x magnification.

3.1.1 Longitudinal length

The two hours it took for the tachyzoites to adapt to the plate showed that a short period of exposure to atovaquone was enough for the tachyzoites to start undergoing morphometric changes (Figure 4). In the evaluation carried out 2 hours after infection, the size of the tachyzoites treated with 100nM of atovaquone stood out significantly ($p< 0.02$)

compared to the control. At the end of 7 days of treatment, all the groups analyzed showed a significant reduction in their longitudinal length ($p<0.01$).

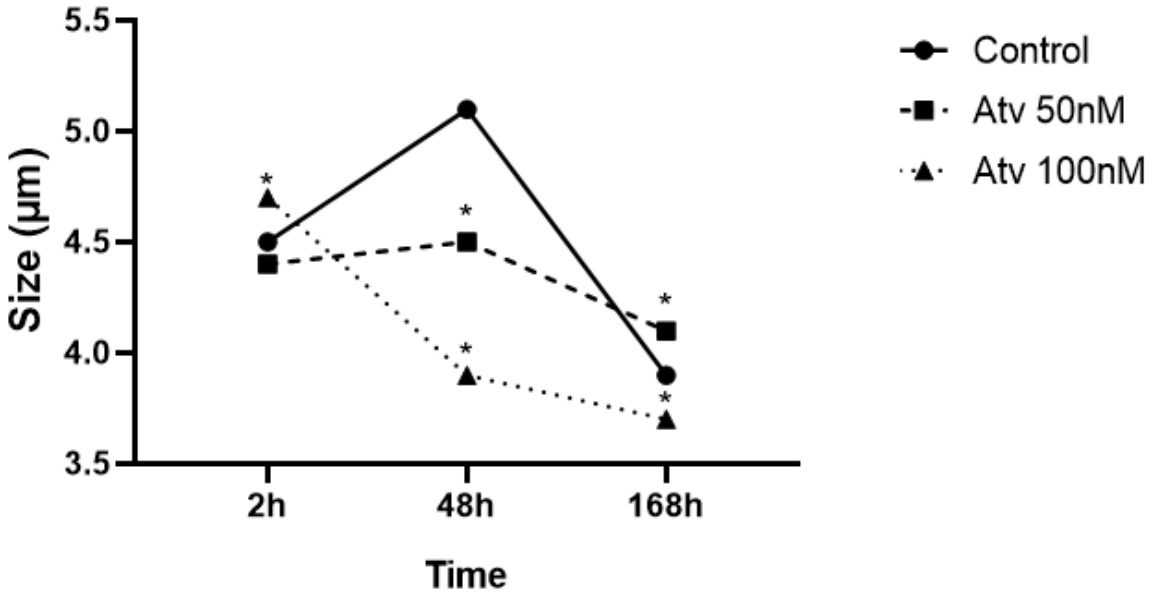

Figure 4. Longitudinal length of tachyzoites of the RH strain of *Toxoplasma gondii* exposed to atovaquone.

3.1.2 Area

In this assessment parameter, the control itself also underwent significant changes ($p<0.01$) over the days, showing significant growth at 48 hours compared to the assessment made at 2 hours of infection. There was a considerable reduction at the end of the 7 days compared to the 48 hours (Figure 5). At 48 hours of infection, the groups treated with the two concentrations of the drug, 50nM, and 100nM, showed a significant reduction compared to the control ($p<0.01$). On the seventh day, the group treated with 100nM of atovaquone showed a significant reduction when compared to the control ($p=0.0003$), which was not detected in the group treated with 50nM. When evaluating all the periods, what happened with the control group throughout the experiment was: a significant increase at 48h compared to the 2h evaluation and a significant reduction on the seventh day ($p<0.01$). On the seventh day of evaluation, all groups showed a lower value compared to the 2h control, but not significantly with their control.

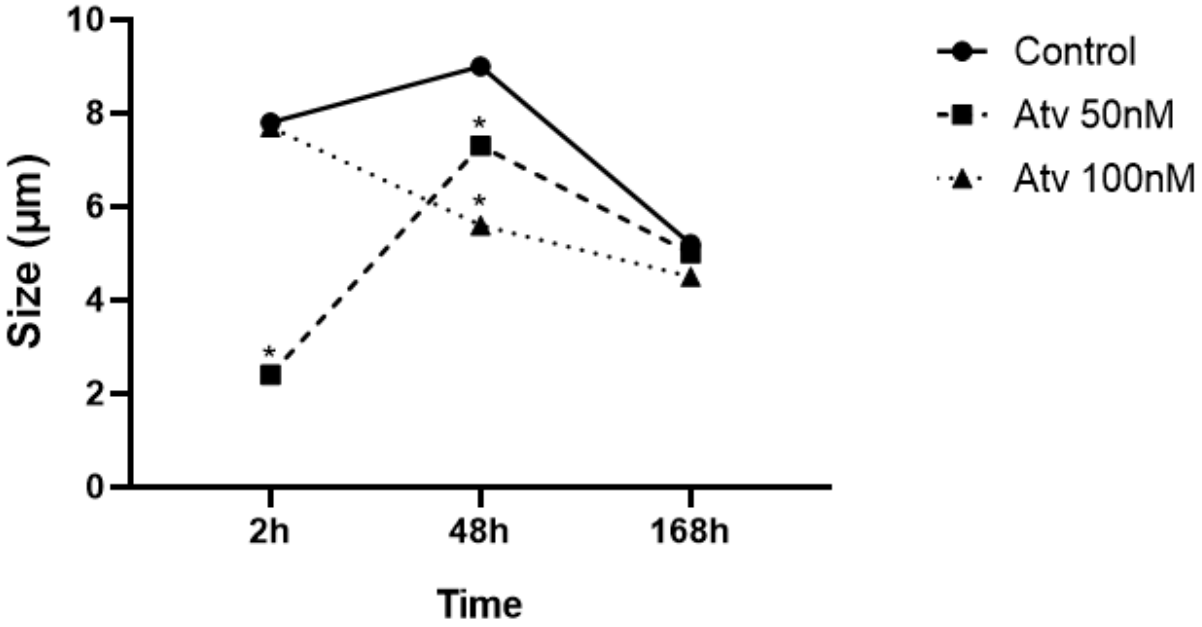

Figure 5. Measured area of tachyzoites of the RH strain of *Toxoplasma gondii* strain exposed to atovaquone.

3.1.3 Distance between the nucleus and apical complex region

Regarding the changes observed in the distance between the nucleus and the anterior, tapered end of the tachyzoites, where the apical complex is located, significant results were found ($p<0.01$) when comparing all the control groups. The distance between the nucleus and the anterior region of the parasite increased at 48 hours compared to 2 hours and decreased on the seventh day. In the group treated with 100nM, the distance decreased significantly ($p<0.01$) at 48 hours and remained so until the seventh day (Figure 6). At the end of the 7 days, the value found for the distance was the same in all groups, control, and test, showing no statistical result when comparing the treated groups with the control.

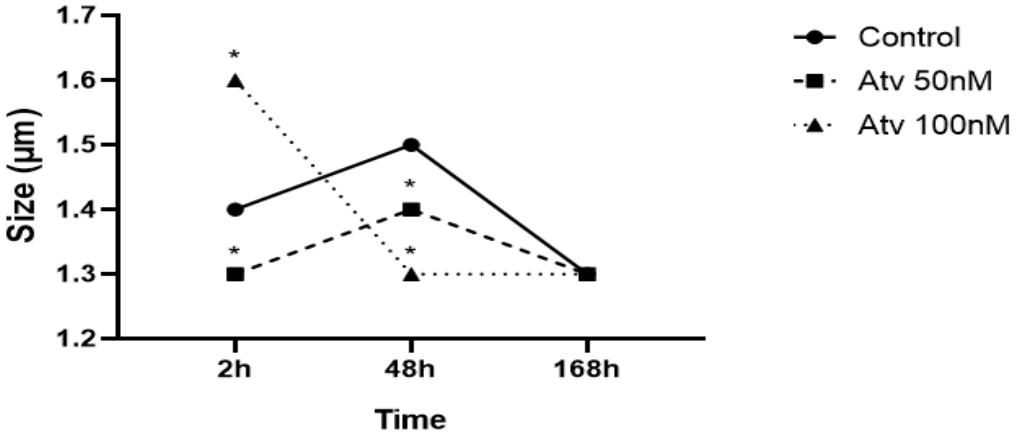

Figure 6. Distance between the nucleus and region of the apical complex of tachyzoites of the RH strain of *Toxoplasma gondii* exposed to atovaquone

4. DISCUSSION

A study published in 2018 by Silva[22] demonstrated the relationship between morphological changes and the behavior of different isolates of *T. gondii,* showing the importance of this correlation for a better understanding of the parasite's biology. A few studies in the literature describe morphometric changes in parasites after exposure to drug treatment, and the data is even scarcer when it comes to *T. gondii*. In this study, morphometric changes were observed in the tachyzoites within the established evaluation parameters.

After 7 days of treatment, tachyzoites treated with 100nM atovaquone showed a similar finding to that published by Portes[21] using pterocarptoquinone. Tachyzoites had a rounder shape, losing part of their usual “half-moon” shape. Again, there is a lack of studies of this type in the literature, but the data found in this study can be compared with what Portes detected in 2012 when testing pterocarptoquinone.

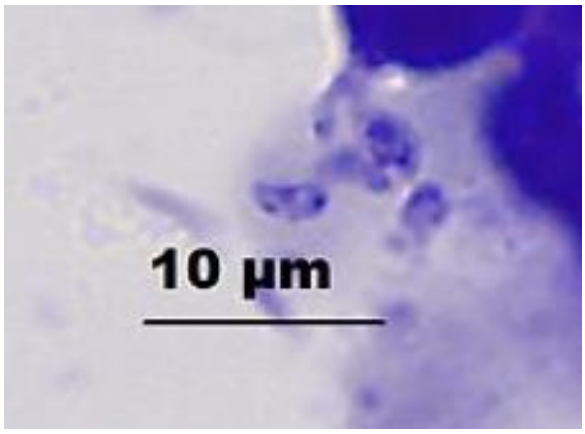

Figure 7: Tachyzoite of *Toxoplasma gondii* from the group treated with 100nM atovaquone on the seventh day of the experiment, on a slide stained with Giemsa, with a rounded shape, observed at 1000x magnification.

The study by Portes described changes similar to those found here, such as a reduction in size and volume, supporting the hypothesis that quinones contribute to these changes. The compound also caused tachyzoites to lose their usual shape. This alteration could be due to the oxidative stress triggered by the drug. Here, the control also changed. It can be inferred that as the days pass in the microenvironment of the plate, without the replacement of the culture medium, the parasites change normally, and the changes would only be accentuated by the presence of the drug.

For the distance between the nucleus and the apical complex, the values initially found were compatible with those detected by Júnior[21] for the RH strain. Throughout the experiment, as with the other parameters, the value was significantly reduced in all the groups evaluated.

5. FINAL CONSIDERATIONS

This study evaluated the morphometric changes that atovaquone caused in *T. gondii* tachyzoites since the first hours. Despite the limited literature on the subject, the morphometric changes found in tachyzoites can be considered suggestive of transformations that later result in the cell death of the protozoan. Although morphometric studies are rare in the literature, they provide a basis for further research. To complement the results obtained in this study, it is important to continue the research, for example, with electron microscopy. It is also important to carry out studies at other concentrations of the

drug and at different evaluation times. Studies are still needed on the potential of atovaquone to be used in the treatment of *T. gondii* infection.

REFERENCES

1 Nicolle C, Manceaux L 1908. Sur une infection a corps de Leishman (ou organismes voisons) du gondi. C R Acad Sci 147: 736.

2 Splendore A 1908. Un nuovo protozoa parassita deconigli incontrato nelle lesioni anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell'uoma. Nota preliminare pel. Rev Soc Sci Sao Paulo 3: 109-112

3 Dubey JP. History of the discovery of the life cycle of *Toxoplasma gondii*. International Journal for Parasitology. 2009 Jul;39(8):877–82.
4 Maldonado YA, Read JS. Diagnosis, Treatment, and Prevention of Congenital Toxoplasmosis in the United States. Pediatrics. 2017 Jan 30;139(2):e20163860.

5 Torgerson PR, Mastroiacovo P. The global burden of congenital toxoplasmosis: a systematic review. Bulletin of the World Health Organization. 2013 May 3;91(7):501–8.

6 Boyer K, Hill D, Mui E, Wroblewski K, Karrison T, Dubey JP, et al. Unrecognized Ingestion of *Toxoplasma gondii* Oocysts Leads to Congenital Toxoplasmosis and Causes Epidemics in North America. Clinical Infectious Diseases. 2011 Oct 21;53(11):1081–9.

7 Ferguson DJP, Hutchison WM, Dunachie JF, Siim JChr. ULTRASTRUCTURAL STUDY OF EARLY STAGES OF ASEXUAL MULTIPLICATION AND MICROGAMETOGONY OF *Toxoplasma gondii* IN THE SMALL INTESTINE OF THE CAT. Acta Pathologica Microbiologica Scandinavica Section B Microbiology and Immunology. 2009 Aug 15;82B(2):167–81.

8 Dubey JP, Lindsay DS, Speer CA. Structures of *Toxoplasma gondii* Tachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts. Clinical Microbiology Reviews [Internet]. 1998 Apr 1 [cited 2019 Apr 14];11(2):267–99. Available from: https://cmr.asm.org/content/11/2/267.

9 Sanchez SG, Besteiro S. The pathogenicity and virulence of *Toxoplasma gondii*. Virulence [Internet]. 2021 Dec 1 [cited 2022 Feb 25];12(1):3095–114. Available from: https://pubmed.ncbi.nlm.nih.gov/34895084

10 Khan A, Su C, German M, Storch GA, Clifford DB, Sibley LD. Genotyping of *Toxoplasma gondii* Strains from Immunocompromised Patients Reveals High Prevalence of Type I Strains. Journal of Clinical Microbiology. 2005 Dec 1;43(12):5881–7.

11 Souza W, Toxoplasmose C, *Toxoplasma*. SciELO Books / SciELO Livros / SciELO Libros. Available from: https://www.arca.fiocruz.br/bitstream/icict/42081/3/souza-9788575415719.pdf

12 Howe DK, Sibley LD. *Toxoplasma gondii* Comprises Three Clonal Lineages: Correlation of Parasite Genotype with Human Disease. Journal of Infectious Diseases. 1995 Dec 1;172(6):1561–6.

13 Sordet F, Aumjaud Y, Fessi H, Derouin F. Assessment of the activity of atovaquone-loaded nanocapsules in the treatment of acute and chronic murine toxoplasmosis. Parasite. 1998 Sep;5(3):223–9.

14 Sanaz Jafarpour Azami, Amani A, Keshavarz H, Roqya Najafi-Taher, Mehdi Mohebali, Mohammad Ali Faramarzi, et al. Nanoemulsion of atovaquone as a promising approach for treatment of acute and chronic toxoplasmosis. European Journal of Pharmaceutical Sciences. 2018 May 1;117:138–46.

15 Haile LA, Flaherty JF. ATOVAQUONE: A Review. 1993 Dec 1;27(12):1488–94.

16 Andrews KT, Fisher G, Skinner-Adams TS. Drug Repurposing and Human Parasitic Protozoan Diseases. International Journal for Parasitology: Drugs and Drug Resistance [Internet]. 2014 Aug;4(2):95–111. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4095053/

17 Secrieru A, Costa ICC, O'Neill PM, Cristiano MLS. Antimalarial Agents as Therapeutic Tools Against Toxoplasmosis—A Short Bridge between Two Distant Illnesses. Molecules [Internet]. 2020 Mar 30 [cited 2021 Nov 30];25(7):1574. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7181032/

18 Hayward JA, Rajendran E, Zwahlen SM, Faou P, van Dooren GG. Divergent features of the coenzyme Q: cytochrome c oxidoreductase complex in *Toxoplasma gondii* parasites. Soldati-Favre D, editor. PLOS Pathogens. 2021 Feb 1;17(2):e1009211.

19 De Y, Batista G, Felipe R, Daher S, Da L, Maria A, et al. Exploring the energetic metabolism of *Toxoplasma gondii*: evaluating the impact of atovaquone. Contribuciones a las ciencias sociales. 2024 Jan 15;17(1):2306–30.

20 Júnior, A. R. G., Análise da virulência e de alterações morfométricas de isolados de *Toxoplasma gondii* obtidos de galinhas caipiras na região metropolitana de Goiânia, Goiás, Brasil. Trabalho de Conclusão de Curso, Universidade Federal de Goiás, Curso de Biomedicina. 2018.

21 Portes JA, Netto CD, Silva AJM, Costa PRR, DaMatta RA, Santos, et al. A new type of pterocarpanquinone that affects *Toxoplasma gondii* tachyzoites in vitro. Veterinary Parasitology. 2012 May 1;186(3-4):261–9.

22 Gontijo M, Fuad Moares Ibrahim, José G, Omar Franklin Molina, João Bartholomeu Neto, Eugênio E, et al. MORPHOMETRIC ANALYSIS AND VIRULENCE OF ISOLATES OF *Toxoplasma gondii* RECOVERED FROM PREGNANT AND NEWBORN MICE IN GOIÂNIA-GO. Amazônia. 2018 Mar 1;6(1):48–53.