REVISTA CONTRIBUCIONES A LAS CIENCIAS SOCIALES

A atovaquona afeta o ciclo do ácido tricarboxílico, a oxidação de ácidos graxos e o catabolismo de proteínas de *Toxoplasma gondii*

Atovaquone affects the tricarboxylic acid cycle, fatty acid oxidation, and protein catabolism of *Toxoplasma gondii*

La atovacuona afecta el ciclo del ácido tricarboxílico, la oxidación de ácidos grasos y el catabolismo de proteínas de *Toxoplasma gondii*

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RESUMO
A toxoplasmose é uma infecção altamente prevalente e um problema de saúde pública. A atovaquona é uma droga potencial para o tratamento, mas ainda não faz parte dos protocolos terapêuticos atuais. Este estudo teve como objetivo avaliar, *in vitro*, as concentrações de metabólitos orgânicos do ciclo do ácido tricarboxílico (TCA), do catabolismo de ácidos graxos e do catabolismo de proteínas para analisar o metabolismo dos taquizoítos das cepas RH e ME49 de *Toxoplasma gondii* e sua resposta após a exposição à atovaquona. O parasita foi cultivado em cultura de macrófagos e exposto à atovaquona (10 nM, 50 nM, 100 nM), sendo avaliado nos seguintes períodos: 24, 48, 72 horas e 7 dias. Após os intervalos de tempo, os parasitas foram alicotados, os ácidos orgânicos extraídos e avaliados por cromatografia líquida de alta eficiência e espectrofotometria. Além da ação já descrita no nível da cadeia respiratória, observou-se que a atovaquona atuou nas vias analisadas, intensificando o TCA e, consequentemente, aumentando o fornecimento de acetil-CoA para a via de oxidação de ácidos graxos. Isso pode indicar que *T. gondii* abriga um TCA altamente dinâmico e essencialmente oxidativo que sustenta o crescimento e a diferenciação do parasita. Na cepa RH, também foi observado um aumento na atividade do TCA com um aumento nas concentrações de citrato, uma fonte importante de acetil-CoA para a elongação de ácidos graxos; isso não foi observado na cepa ME49. A atovaquona induziu o parasita a utilizar vias alternativas para a produção de energia.

Palavras-chave: toxoplasmose, metabolismo energético, ciclo do ácido tricarboxílico, oxidação de ácidos graxos, catabolismo de proteínas.

ABSTRACT
Toxoplasmosis is a highly prevalent infection and a public health problem. Atovaquone is a potential drug for treatment, but it is not yet part of the current treatment protocols. This study aimed to evaluate, *in vitro*, the concentrations of organic metabolites from the tricarboxylic acid cycle (TCA), fatty acid catabolism, and protein catabolism to assess the metabolism of tachyzoites of the RH and ME49 strains of *Toxoplasma gondii* and their response after exposure to atovaquone. The parasite was grown in macrophage culture and exposed to atovaquone (10 nM, 50 nM, 100 nM), and evaluated over the following periods: 24, 48, 72 hours, and 7 days. After the time intervals, parasites were aliquoted, organic acids extracted, and evaluated by high-performance liquid chromatography and spectrophotometry. In addition to the already described action at the respiratory chain level, it was observed that atovaquone acted on the pathways analyzed, intensifying the TCA and consequently increasing the supply of acetyl-CoA to the fatty acid oxidation pathway. This may indicate that *T. gondii* harbors a metabolically oxidative highly dynamic and essential TCA that supports the growth and differentiation of the parasite. In the RH strain, an increase in TCA activity was also observed with an increase in citrate concentrations, an important source of acetyl-CoA for fatty acid elongation; this was not observed for the ME49 strain. Atovaquone induced the parasite to use alternative pathways for energy production.

Keywords: toxoplasmosis, energy metabolism, tricarboxylic acid cycle; fatty acid oxidation, protein catabolism.

RESUMEN
La toxoplasmosis es una infección altamente prevalente y un problema de salud pública. La atovacuona es un fármaco potencial para el tratamiento, pero aún no forma parte de los protocolos de tratamiento actuales. Este estudio tuvo como objetivo evaluar, *in vitro*, las concentraciones de metabolitos orgánicos del ciclo del ácido tricarboxílico (TCA), el catabolismo de ácidos grasos y el catabolismo de proteínas para analizar el metabolismo de los taquizoítos de las cepas RH y ME49 de Toxoplasma gondii y su respuesta tras la exposición a la atovacuona. El parásito se cultivó en cultivo de macrófagos y se expuso a atovacuona (10 nM, 50 nM, 100 nM), siendo evaluado en los siguientes períodos: 24, 48, 72 horas y 7 días. Después de los intervalos de tiempo, los parásitos fueron alicuotados, se extrajeron los ácidos orgánicos y se evaluaron mediante cromatografía líquida de alta eficiencia y espectrofotometría. Además de la acción ya descrita a nivel de la cadena respiratoria, se observó que la atovacuona actuó sobre las vías analizadas, intensificando el TCA y, en consecuencia, aumentando el suministro de acetil-CoA a la vía de oxidación de ácidos grasos. Esto puede indicar que *T. gondii* alberga un TCA altamente dinámico y esencialmente oxidativo que respalda el crecimiento y la diferenciación del parásito. En la cepa RH, también se observó un aumento en la actividad del TCA con un incremento en las concentraciones de citrato, una fuente importante de acetil-CoA para la elongación de ácidos grasos; esto no se observó en la cepa ME49. La atovacuona indujo al parásito a utilizar vías alternativas para la producción de energía.

Palabras clave: toxoplasmosis, metabolismo energético, ciclo del ácido tricarboxílico, oxidación de ácidos grasos, catabolismo de proteínas.

1 INTRODUÇÃO

Toxoplasmosis is a highly prevalent infection worldwide and a serious public health problem caused by *Toxoplasma gondii*. The seroprevalence of the infection varies according to region and is directly related to socioeconomic factors. The severity of the manifestations is related to the condition of each individual's immune system, but they are usually more severe in immunocompromised individuals and pregnant women. During pregnancy, the parasite can cross the placental barrier, reach the fetus, and cause multiple complications (Woldegerima *et al.* 2024).

The strains of *T. gondii* are classified based on the genotypic, biochemical, and origin criteria of the isolates from animals and humans. They have a wide variety of hosts and, as a result, different types of host cells. Even so, they have low genetic variability and are grouped into three types: type I (such as the RH strain), type II (e.g., Prugniaud and ME-49), and type III

(such as C56 and 76K) (Dubey, 1988). Type I parasites are highly virulent and are generally associated with congenital toxoplasmosis (25%) (Fuentes *et al*. 2001). Type II strains account for more than 70% of human isolates. *In vitro* systems, these strains differentiate from the tachyzoite to the bradyzoite form and are associated with a high cystic load and the chronic phase of infection. Type III strains account for around 45% of parasites isolated from animals and are infrequent in isolates from humans who show symptoms (Weiss, 2000).

Although the cited strains are genetically very similar, they have 1–2% differences in their nucleotides, as well as phenotypic differences, which influence their growth, virulence, and ability to form cysts. Polymorphism analyses indicate that the different strains arose after a genetic crossover, which caused their expansion (Howe; Sibley, 1995; Dubey *et al.*, 2020).

The combined therapy of sulfadiazine and pyrimethamine has been used to treat toxoplasmosis for over half a century. In pregnant women, in addition to these drugs, the administration of spiramycin is recommended, which can prevent congenital transmission. It is an effective therapy during the acute phase of the disease, as it acts against tachyzoites. However, it is ineffective in the chronic phase as it is unable to combat bradyzoites (Eyles; Coleman, 1952; Konstantinovic *et al*. 2019). For this reason, there is still a need to jointly search for new treatments for toxoplasmosis.

Sulfadiazine and pyrimethamine are drugs that act on folate metabolism. Another drug that can be used with the duo is clindamycin, especially in cases of patients with AIDS and cerebral toxoplasmosis. To optimize the treatment of toxoplasmosis, much research has been done into the use of new drugs, and *in vitro* tests are a key step in identifying these compounds (Konstantinovic *et al*. 2019).

The plastid genome of *T. gondii* is similar in both organization and gene content to that found in *Plasmodium falciparum* and other apicomplexa species (Konstantinovic *et al.* 2019). Atovaquone is a naphthoquinone with broad-spectrum antiprotozoal and antimicrobial activity, with effective action on parasites with phylogenetic proximity to *T. gondii*, such as for the treatment and prevention of malaria. Atovaquone is a considerably lipophilic drug with low water solubility. It began to be marketed in 1992, and its trade name is Mepron®. Used in combination with other drugs, it gives better results. Atovaquone acts on complex III of the electron transport chain, as an analog of ubiquinone (Baggish, 2002).

The present study evaluated concentrations of organic metabolites from the tricarboxylic acid cycle, fatty acid catabolism, and protein catabolism to assess the parasite's basal metabolism and its response to challenge with atovaquone. In this way, this study can contribute to understanding the parasite's metabolic pathways and how they are affected by atovaquone under the conditions evaluated.

2 METHODS

The work complied with the ethical principles in animal experimentation recommended by the Brazilian Society of Science in Laboratory Animals. It was approved by the Ethics Committee in Animal Use of the Federal University of Goiás, protocol number 086/17.

2.1 OBTAINING TACHYZOITES OF THE RH STRAIN

To obtain tachyzoites of the RH strain, 1 mL of BALB/c mouse peritoneal lavage was used 48 hours after prime infection. The tachyzoites were collected by intraperitoneal washed in saline solution. A concentration of 4.8×10^6 tachyzoites per mL was used to infect RAW 264.7 murine macrophages. The culture was kept in a CO_2 incubator at 37 ºC in 25 cm^2 flasks (Corning®) containing 3 mL of supplemented RPMI medium (Gibco®). For every 87 mL of RPMI medium, 1 mL of 200 mM L-glutamine, 1 mL of 1M Hepes, 1 mL of streptomycin/penicillin (10,000U/mL strep and 10,000 µg/mL), 100µl of 2-Mercaptoethanol (50mM), 10mL of inactivated fetal bovine serum (SFB) (Cultilab®) were added.

Every 72 hours, the flasks were washed with medium to eliminate dead cells. For maintenance, 2 mL of the supernatant was discarded, and 2 mL of supplemented RPMI medium was added every three days. Tachyzoites of the RH strain was obtained from the supernatant of the cell culture flasks.

2.2 OBTAINING TACHYZOITES OF THE ME49 STRAIN

BALB/c mice were infected with the ME49 strain, and, after 7 days of infection, blood was collected by cardiac puncture. This blood was inoculated into a culture of RAW 264.7

macrophages and kept in a CO_2 incubator under the same conditions as for the RH strain. For 30 days, the cells were washed with medium every 72 hours, 1 mL of supplemented RPMI was mixed with 1 mL of the used medium and added to the cultures. At this time, extracellular tachyzoites were firstly observed. After this time point, tachyzoites of the ME49 strain were obtained from the culture supernatant. For both strains, these cultures were expanded into new culture flasks.

2.3 TACHYZOITES EXPOSURE TO ATOVAQUONE

For this study, the atovaquone's concentrations followed a previous study by Souza *et al.*[10]. The experiment was carried out in quintuplicate. Using culture plates of six wells, 2 x 10^5 RAW 264.7 macrophages were seeded with 3 mL of supplemented RPMI medium, maintained at 37 ºC and 5% CO_2, for 24 hours, to promote cell adhesion to the plate. The culture of macrophages was then infected with 1x10^6 parasites per well and exposed to 10 nM, 50 nM, and 100 nM of atovaquone. Incubation took place for 24, 48, 72 hours, and 7 days, without drug replacement. Thus, the treatment groups were defined by infected macrophages treated with atovaquone and the control was defined by infected macrophages without atovaquone.

2.4 PREPARATION OF SAMPLES

At the end of the incubation periods, each well of the culture plate was washed to remove cells and parasites. Using a Pasteur pipette, the adhered macrophages and tachyzoites were removed from the plates and aliquoted. The content of the each well of the plate were stored in a tube with phenylmethylsulfonyl fluoride. The aliquots were lysed by freezing and thaws five times in liquid nitrogen and a water bath at 90°C. The sample obtained was frozen to extract the organic metabolites.

2.5 ORGANIC METABOLITES EXTRACTION

The organic metabolites were extracted using an ion exchange column (Bond Elut® Agilent®) with a Manifold box coupled to a vacuum pump. The column was activated with 1 mL

of 0.5M HCl, 1 mL of methanol, and 2 mL of type I water. Then, 250 µL of the previously frozen sample was added, plus 2mL of Type I water. The box column was removed, 250µL of 0.5 M H2SO4 was added, and the column was centrifuged at 626 g for 5 minutes at 4°C (Souza *et al*. 2024; Vinaud *et al*. 2007; Vinaud *et al*. 2009). After extracting the organic metabolites, the samples were analyzed using high-performance liquid chromatography (HPLC).

2.6 STATISTICAL ANALYSIS

Statistical analysis was carried out using Graph Pad Prism 8.2.1. Descriptive statistics were used to determine the mean and standard deviation, and to assess the differences between the groups analyzed, the variables were tested for normal distribution and homogeneous variance. Since they had a normal distribution, analysis of variance (ANOVA) was used, followed by Dunn's post-test. The differences observed were considered significant at $P < 0.05$.

3 RESULTS

The first results presented in each topic is about the basal metabolism of *T. gondii* observed in these experiments (control group, without treatment with atovaquone). The results for the treated groups are shown after these.

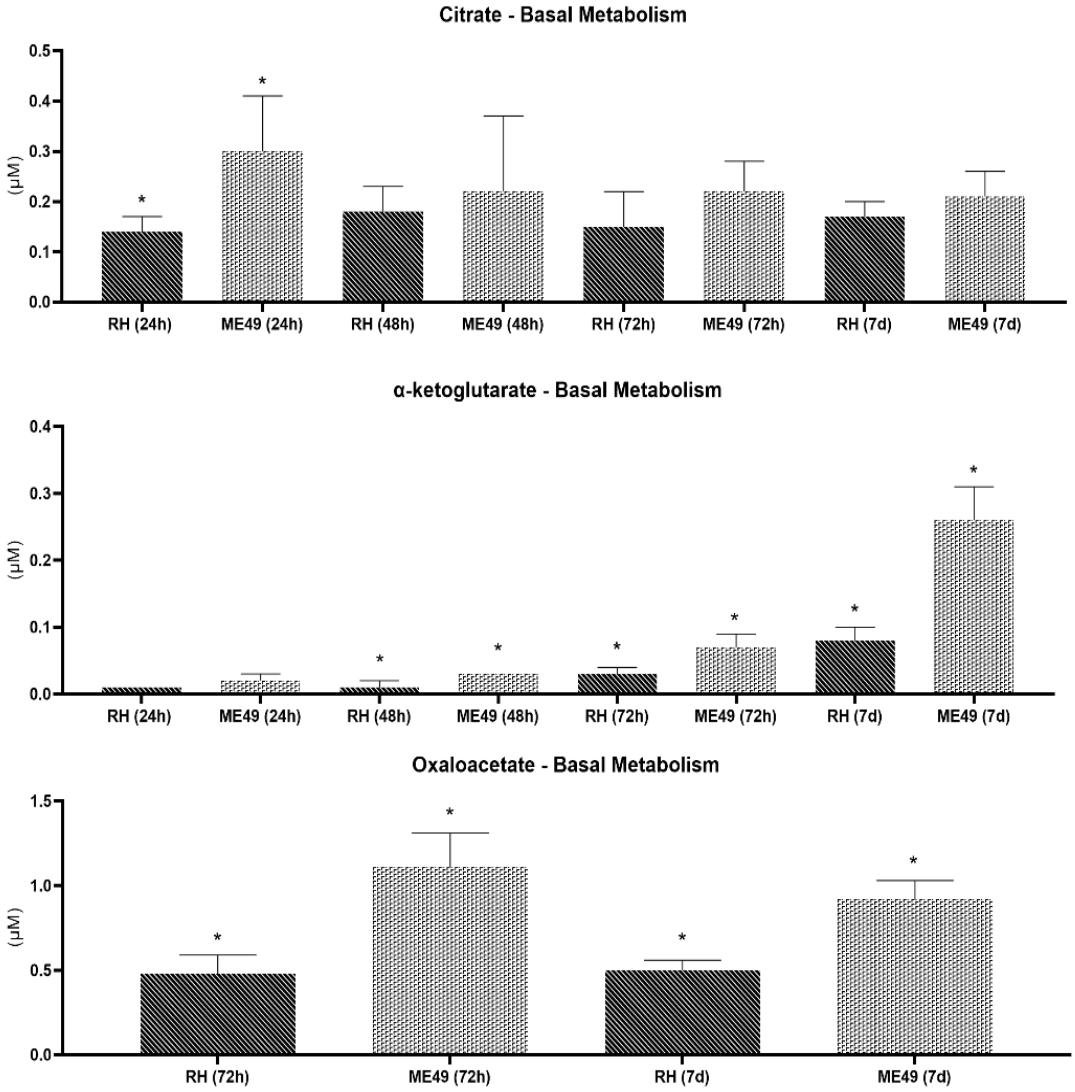
3.1 THE TRICARBOXYLIC ACID CYCLE (TCA)

3.1.1 Basal metabolism: both strains

The basal metabolism of both strains of *T. gondii* were analyzed in the different periods of this study. In ME49 strain, a gradual increase in citrate and α-ketoglutarate was observed over the days. This indicates an active TCA throughout the experiment, which may have been due to the replacement of precursors for this cycle through the glycolytic pathway or other energy pathways carried out by the parasite. When comparing RH with ME49 at 7 days, ME49 showed a higher concentration of α-ketoglutarate ($P = 0.015$) and oxaloacetate ($P = 0.027$) than RH

(Figure 1). This indicates an active TCA and a probable replenishment of the precursors and intermediates of this cycle (Figure 1).

Figure 1. Organic acids of the tricarboxylic acid cycle (TCA) from the basal metabolism of the RH and ME49 strains of *Toxoplasma gondii* at 24 hours, 48 hours, 72 hours, and 7 days of experiment. No oxaloacetate was detected in any strain at 24 hours and 48 hours. *: *P* <0.05 when comparing the basal metabolism of the RH group with the ME49 group.



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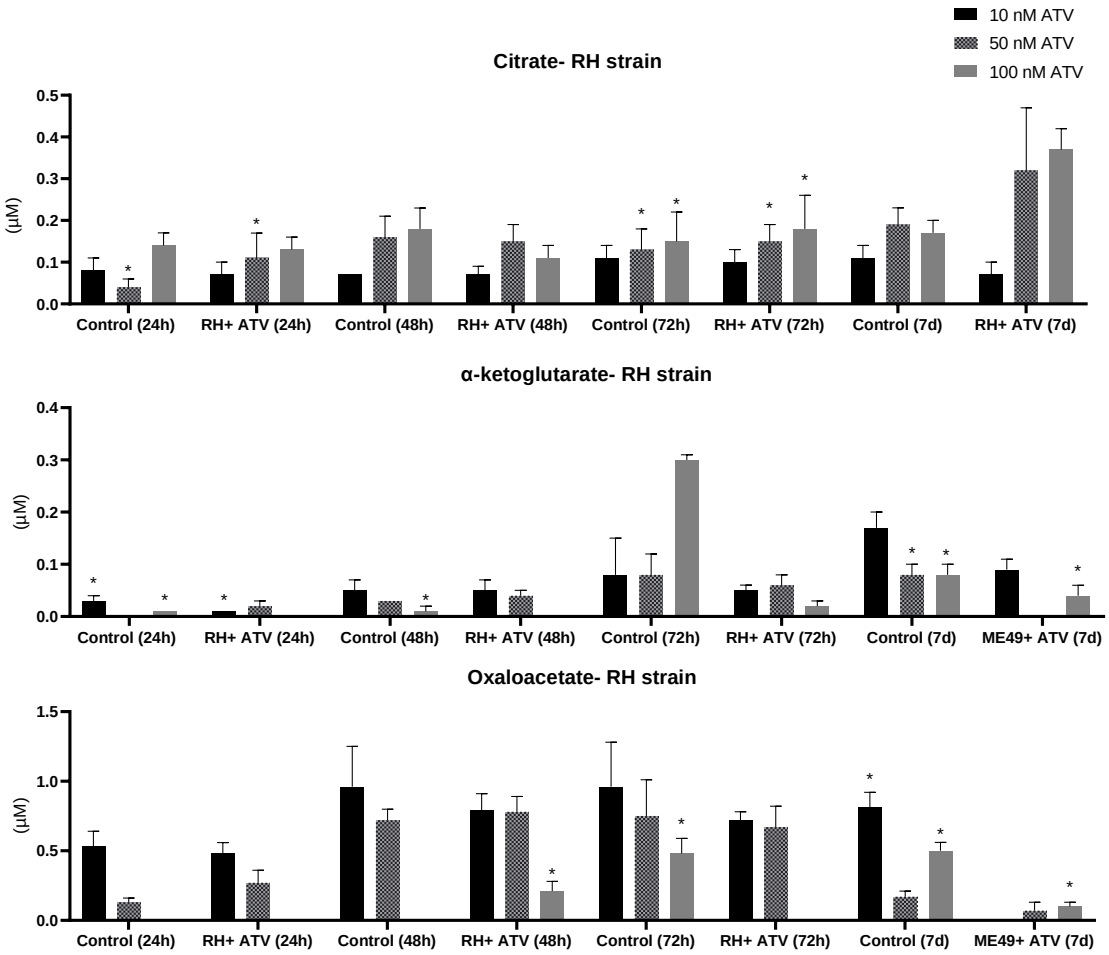
3.1.2 RH Strain: treated groups

In the analysis of the organic acids of the TCA cycle, it was possible to detect significant changes in the concentrations of citrate, α-ketoglutarate, fumarate, and oxaloacetate. Succinate

and malate were not detected in the groups analyzed. The detection of propionate in some groups may indicate the consumption of succinate for its formation.

When comparing the RH treated with atovaquone with the control, at 7 days, there was a decrease in the concentrations of α-ketoglutarate and oxaloacetate in all groups. The RH group treated with 100 nM atovaquone showed a higher concentration of citrate at 7 days compared to the control group. These results suggest that the accumulation of citrate is related to the blocking of the metabolization of this organic acid, which is reinforced by the simultaneous decrease in the concentrations of α-ketoglutarate and oxaloacetate (Figure 2).

Figure 2. Organic acids of the tricarboxylic acid cycle in the RH strain of *Toxoplasma gondii*, treated or not with atovaquone. ATV: atovaquone, *: *P* <0.05 when comparing the RH test group to its respective control.



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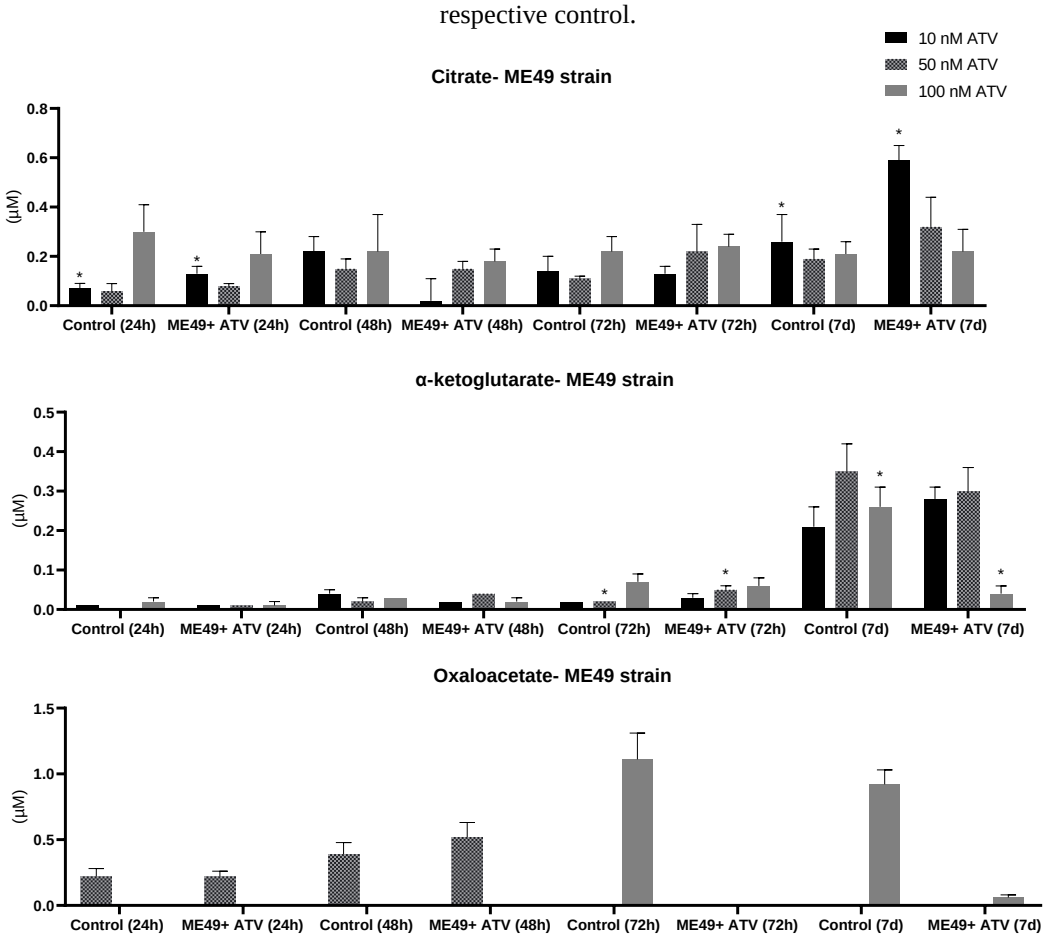
3.1.3 ME49 Strain: treated groups

The control and treated ME49 groups showed a decrease in α-ketoglutarate (P =0.002) and oxaloacetate (P = 0.0005) at a concentration of 100nM. The ME49 group treated with 10 nM atovaquone showed a higher concentration of citrate compared to the control group (P < 0.001). As for the concentrations of oxaloacetate and α-ketoglutarate in the ME49 groups treated with 100 nM compared to the control groups, there was a decrease in their concentrations (Figure 3). These data show TCA activity over the days, indicating that there may have been a replacement of precursors for this cycle via the glycolytic pathway, resources taken from the culture medium, or other energy pathways carried out by the parasite.

Figure 3. Organic acids of the tricarboxylic acid cycle were detected in the ME49 strain of *Toxoplasma gondii*, treated or not with atovaquone. ATV: atovaquone, *: P <0.05 when comparing the ME49 test group to its respective control.



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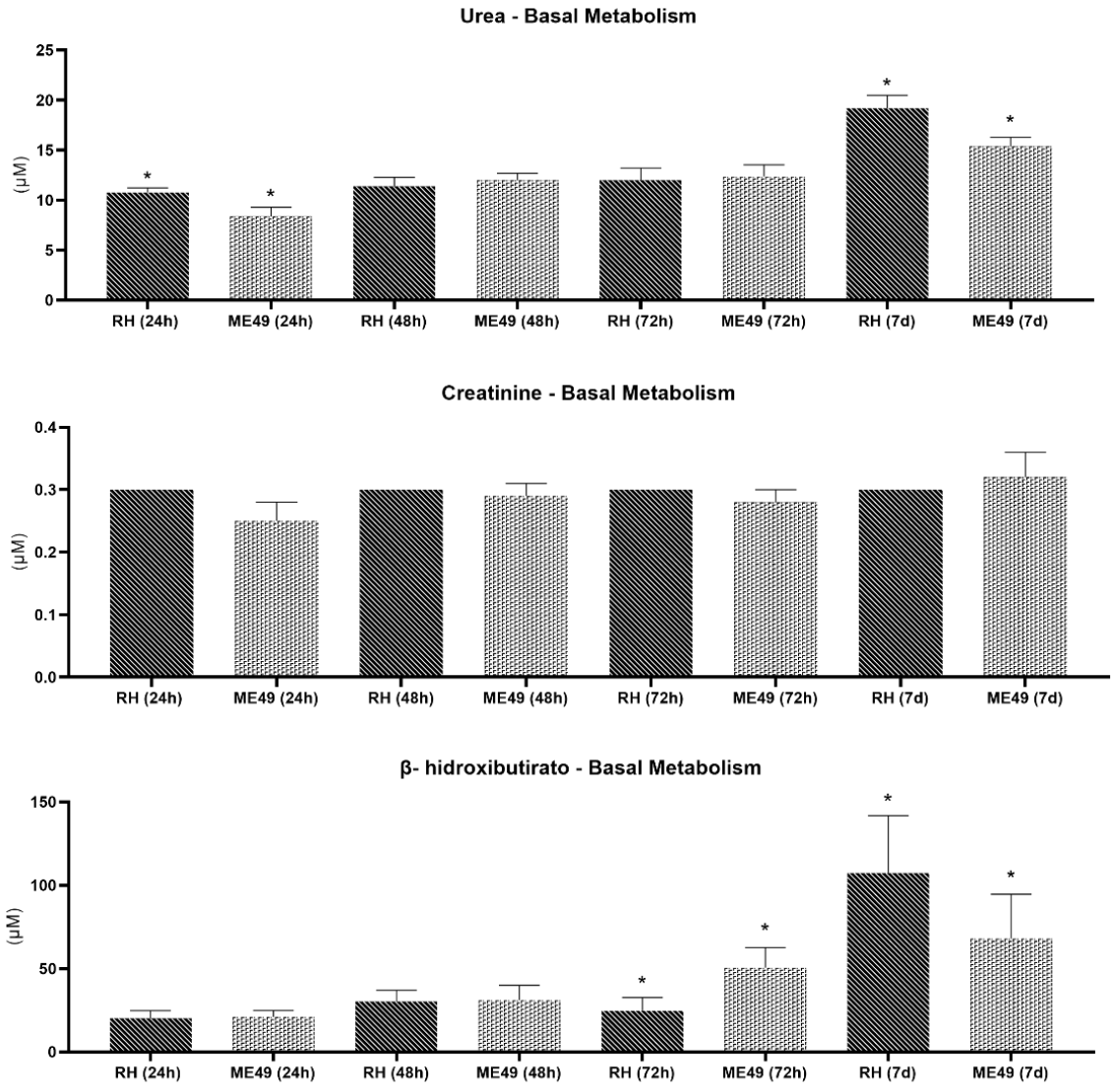
3.2 PROTEIN CATABOLISM AND FATTY ACID OXIDATION

3.2.1 Basal metabolism: both strains

Regarding the urea concentrations in the basal metabolism of the two strains, we observed higher dosages for the RH strain when compared to the ME49 strain at practically all times of the experiment (Figure 4). Of the metabolites related to fatty acid oxidation analyzed, only β-hydroxybutyrate was detected. In the control groups of both strains, it was observed that the 7-day group had a higher concentration of β-hydroxybutyrate when compared to the other days (Figure 4), indicating intense use of this metabolic pathway. When comparing the strains, it was observed that at the end of 7 days, RH had a higher concentration of β-hydroxybutyrate than ME49 ($P < 0.001$). This may be related to the more intense activity of this pathway by the RH strain, which is related to the extraordinary multiplication capacity of this strain (Fuentes *et al.* 2001). As for creatinine, there was no significant difference in any group. Acetate and acetoacetate could not be detected in any of the groups analyzed.

Figure 4. Organic acids from β-oxidation and protein catabolism were detected in the basal metabolism of *Toxoplasma gondii* strains RH and ME49 at 24 hours, 48 hours, 72 hours, and 7 days of the experiment. *: *P* <0.05 when comparing the basal metabolism of the RH group with the ME49 group.



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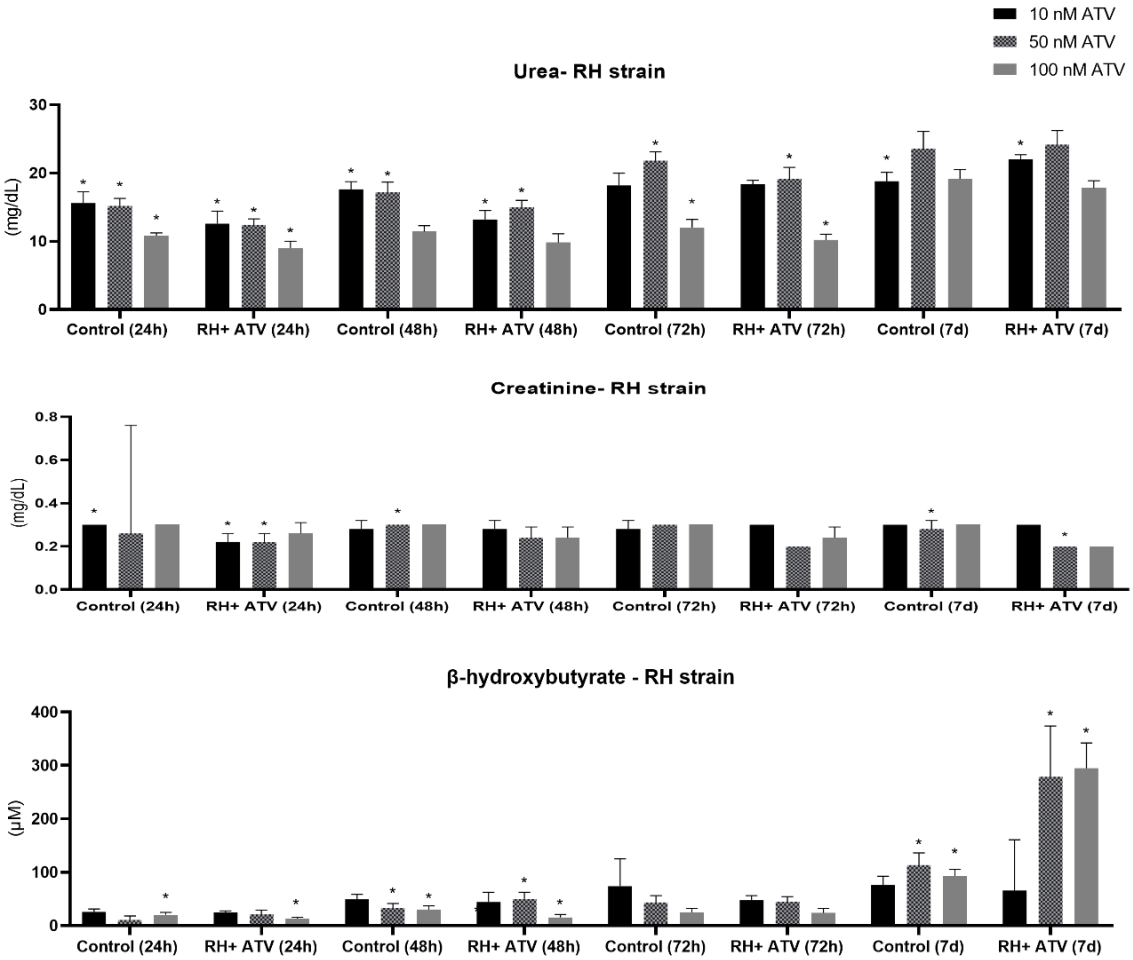
3.2.2 RH strain: treated groups

When comparing the basal metabolism of RH with the metabolism of the treated groups, it was observed that the concentration of β-hydroxybutyrate at the end of 7 days was higher in the treated groups, 50 nM (*P* = 0.009) and 100 nM (*P* = 0.001) (Figure 5), doubling its concentration. This may indicate a greater activation by this pathway for energy, highlighting the importance of fatty acid catabolism in *T. gondii*.

Figure 5. Organic acids from β-oxidation and protein catabolism were detected in the RH strain of *Toxoplasma gondii* treated or not with atovaquone. ATV: atovaquone, *: *P* <0.05 when comparing the RH test group to its respective control.



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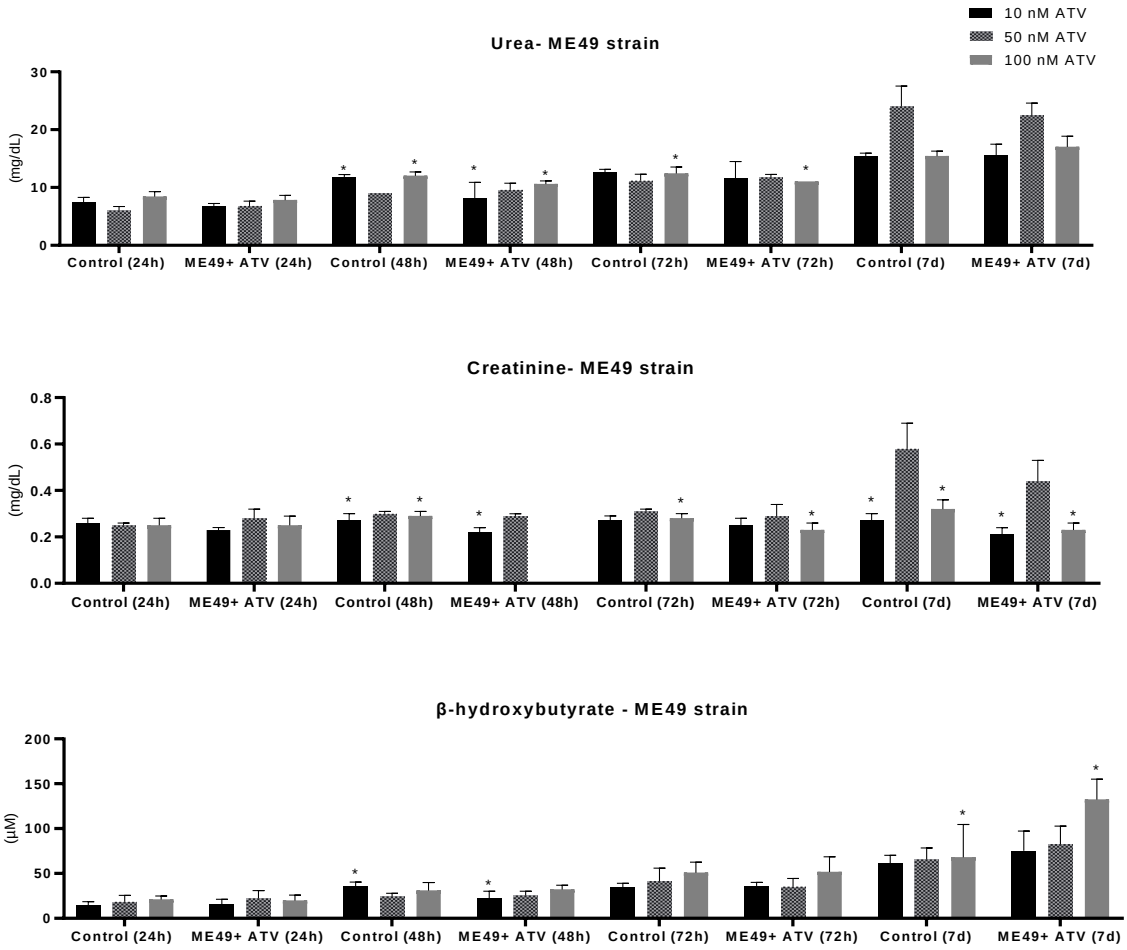
When analyzing the ME49 strain, a significant increase in urea concentration was observed throughout the experiment, which may indicate that there was greater activity in this pathway over the days. As for the fatty acid catabolism pathway, the group treated with 100 nM atovaquone showed a higher concentration of β-hydroxybutyrate than the untreated group (*P* = 0.0035) (Figure 6).

Figure 6. Organic acids from β-oxidation and protein catabolism were detected in the ME49 strain of *Toxoplasma gondii* treated or not with atovaquone. ATV: atovaquone, *: *P* <0.05 when comparing the ME49 test group to its respective control.



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4 DISCUSSION

T. gondii tachyzoites encode all the genes for a complete tricarboxylic acid (TCA) cycle and a mitochondrial respiratory chain. They lack a mitochondrial isoform of pyruvate dehydrogenase that normally links glycolysis to TCA, which is the main pathway for converting pyruvate to acetyl-CoA (Usey; Huet, 2022; Chen *et al.* 2024; Hamid *et al.* 2024; Niu *et al.* 2022)[1]. It has been shown that *T. gondii* can reuse a branched-chain ketoacid dehydrogenase (BCKDH) to connect glycolysis to the TCA cycle. BCKDH converts glucose-derived pyruvate into acetyl-CoA and appears to replace pyruvate dehydrogenase, which is located in the parasite's apicoplast rather than in the mitochondria. So BCKDH would be the enzyme that facilitates acetyl-CoA

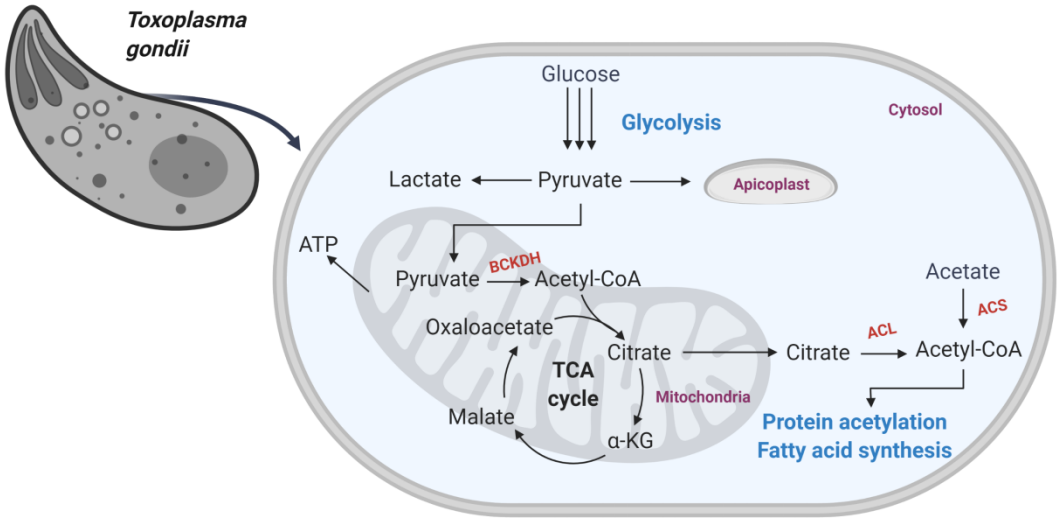
synthesis in the mitochondria of parasites such as *Toxoplasma* and *Plasmodium* (Santos *et al.* 2023; Nair *et al.* 2023; Lyu *et al.* 2024; Walsh *et al.* 2022; Guo *et al.* 2024).

Studies using isotope labeling to assess central carbon metabolism in *T. gondii* show that these parasites catabolize glucose and glutamine for basal growth conditions. In addition, they have shown that TCA is essential for cell replication, linking this cycle to the extraordinary replication capacity of these parasites, and impairment of this cycle may interfere with their multiplication. Studies with succinyl-CoA synthetase knock-out parasites and TCA impairment showed a 30% reduction in cell replication of this parasite (MacRae *et al.* 2012).

When analyzing the basal metabolism of the RH and ME49 strains at the different incubation times, there was an increase in citrate and α-ketoglutarate on the seventh day compared to the 24 hours (Figure 2). These data show that TCA is active over time, corroborating data in the literature on the importance of this metabolic pathway for *T. gondii*. Tachyzoites of the RH strain treated with 50nM and 100nM concentrations of the drug showed a significant increase in citrate concentration compared to their respective controls (Figure 2), with a simultaneous increase in β-hydroxybutyrate concentrations (Figure 5). The pathway of this metabolite is directly related to the apicoplast, since the acetyl-CoA generated in this organelle by the degradation of pyruvate is probably destined for the catabolism of fatty acids (MacRae *et al.* 2012). Biochemical evidence of the importance of beta-oxidation of fatty acids by *T. gondii* tachyzoites is still scarce, even though several enzymes of this pathway are present in the genome of this parasite (He *et al.* 2024).

Intracellular tachyzoites use host-derived glucose and glutamine to fuel a canonical TCA cycle. It is also possible that the TCA cycle plays a role external to the mitochondrion, exporting citrate for acetyl-CoA production via ATP-citrate lyase (ACL) activity, thereby fueling fatty acid elongation (Maclean *et al.* 2022). The extent to which this occurs is currently under investigation, but this study reinforces this data by showing the increase in metabolite concentrations as shown in Figure 7.

Figure 7: Schematic representation of the acetyl-CoA supply pathway from TCA to fatty acid oxidation in *Toxoplasma gondii*. Enzymes are in red. ACL: ATP-citrate lyase; ACS: Acetyl-CoA synthetase; BCKDH: branched-chain ketoacid dehydrogenase.



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In addition to the already described action at the respiratory chain level, it was observed that atovaquone acts on the pathways analyzed in the present study, with a significant intensification of TCA and consequent increase in the supply of acetyl-CoA for the oxidation of fatty acids. The literature suggests that *T. gondii* harbors a metabolically oxidative, highly dynamic, and essential ATP that supports the growth and differentiation of the parasite (Maclean *et al*. 2022). TCA impairment in turn alters the intensity of pathways such as fatty acid catabolism and protein oxidation. The results of this study suggests that atovaquone shifts the parasite's metabolism toward a greater reliance on fatty acid catabolism, possibly as a survival mechanism when ATP production via the respiratory chain is disrupted.

Comparing the basal metabolism of ME49 to that of the RH strain (Figure 1), there are higher concentrations of TCA metabolites in ME49. However, the concentrations of β-hydroxybutyrate are lower for this strain when compared to RH, which may indicate that the citrate produced in TCA by ME49 is not used to the same extent as RH as a carbon source for fatty acid catabolism. Another plausible source of acetyl-CoA for TCA in *T. gondii* is via amino acid degradation. Consistent with this assumption, this parasite has mitochondrial pathways for amino acid degradation (Krishnan; Soldati-Favre, 2021).

Research by Olson *et al.*, further elucidates the understanding of the TCA cycle and its role in energy production, such as that the mitochondrial source of acetyl-CoA is from BCKDH

and that a GABA shunt also allows *T. gondii* to use glutamine to fuel its TCA cycle. Furthermore, *T. gondii* citrate dehydrogenase, which catalyzes the rate-limiting step of the TCA cycle, appears not to be NAD+-dependent like the human enzyme, but rather NADP+-dependent. TCA intermediates were more abundant when the number of parasites was greater, and the greater intensity of TCA in the RH strain may be related to this strain's greater ability to replicate.

Glutaminolysis contributes to the flow of carbon to the TCA and helps to replenish glycolysis intermediates via gluconeogenesis. This is proven by the enzyme phosphoenolpyruvate carboxykinase, which catalyzes the first step of gluconeogenesis and is essential for the parasite's survival in the absence of glucose, is still used even under glucose-rich conditions. The fact that the concentrations of TCA intermediate metabolites and protein degradation products increased after the incubation time reinforces data in the literature on the importance of protein catabolism for this parasite (Lin *et al.* 2011).

As a result of the possible influence of atovaquone on TCA, it is believed that, like mammals, the parasites of the RH strain have used alternative routes of energy production, such as fatty acid oxidation. Thus, the detection of β-hydroxybutyrate in increased concentrations in the treated groups of both strains suggests that *T. gondii* uses fatty acid oxidation when exposed to the drug as an alternative source of energy. Protein catabolism pathways are also more active in the treated groups, proposing that they use protein catabolism to obtain partial metabolites from glycolysis and TCA.

5 CONCLUSION

The findings of this study indicate that *Toxoplasma gondii* maintains a metabolically active basal tricarboxylic acid (TCA) cycle throughout the experiment. The use of atovaquone enhanced the parasite's reliance on alternative energy production pathways, particularly the oxidation of fatty acids. In the RH strain, this metabolic shift was accompanied by an increase in TCA cycle activity, which correlated with elevated citrate concentrations. Since citrate serves as a key precursor for acetyl-CoA, this suggests that fatty acid elongation was also intensified under drug treatment. This suggests that *T. gondii* makes greater use of fatty acid oxidation when exposed to the drug. Protein catabolism pathways were also shown to be more active in the treated groups, suggesting that it uses glutaminolysis to obtain partial metabolites from glycolysis

and TCA. This metabolic adaptation likely provides essential intermediates for both glycolysis and the TCA cycle, further supporting the parasite's survival under stress conditions induced by the drug.

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