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RESEARCH ARTICLE **OPEN ACCESS**

MODULATION OF GAP JUNCTIONS IN THE HUMAN FIBROBLASTS CELL LINES INFECTED WITH *Toxoplasma gondii*

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ABSTRACT

Toxoplasmosis is a zoonosis of wide worldwide prevalence, caused by the obligate intracellular protozoa *Toxoplasma gondii*. Once infected, the host may present several changes in the organism. Some of these changes are associated with intercellular communication, mediated by gap junctions. The gap junctions are responsible for the exchange of ions and small messengers that assist in the maintenance of tissue homeostasis. Thus, morphological and microenvironmental changes were observed in vitro of human foreskin fibroblast cells infected with *Toxoplasma gondii* over 72 hours through (1) phase contrast micrographs of the culture of cells of the HFF control strain and infected with *Toxoplasma gondii* in the period of 24, 48 and 72h; (2) evaluation of the cell mortality rate and (3) immunofluorescence assays of the Cx43 protein by confocal microscopy and Apotome epifluorescence. HFF cells infected with the parasite *Toxoplasma gondii* showed morphological changes and a significant increase in cell death. It was also observed the alteration of the positioning of Cx43 in infected cells, allowing the debate on a possible positive regulation of Cx43 in the presence of *Toxoplasma gondii* in this cell lineage.

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan that causes toxoplasmosis, a worldwide prevalent zoonosis. This protozoan has felines as its definitive host, however it has the ability to infect and replicate within any eukaryotic cell. (LÜDERA *et al*., 2001; JONES & DUBEY, 2012; BLUME & SEEBER, 2018). In Brazil, the serum prevalence of toxoplasmosis is about 56%, being four times higher than the exposure in the United States, 13% (DUBEY *et al*., 2012). Because it is a pathogen with highly successful infection, it is believed to have infected about 30% of the world's population (HIDE, 2016). Most human *T. gondii* infections are usually mild or asymptomatic, however the infection can result in life-threatening disease in immunocompromised individuals and congenital infection (LIMA & LODOEN, 2019). Faced with parasitic infections, such as toxoplasmosis, a structure that deserves to be highlighted are the cell junctions (CAMPOS DE CARVALHO *et al*., 1998).

With several evasion mechanisms, *Toxoplasma gondii* when entering the cell causes alterations to facilitate its survival (SEABRA *et al*., 2002). Campos de Carvalho *et al*. (1998) showed that the reduction in gap junction could be related to secondary alterations in the cell's metabolism, provided by infection with *Toxoplasma gondii*, therefore the parasite would have the capacity to exert influence on the gap junctions. In papers previously published by our research group, it was seen that *T. gondii* alters the expression and positioning of gap junctions in macrophage cell lineage, J774-G8 (DE CARVALHO *et al*., 2021) and intestinal epithelium lineage, IEC-6 (SOUZA *et al*., 2022) resulting in changes in cell behavior that in the future may be linked to comorbidities of this parasitemia. The gap junctions allow direct communication between tissues being characterized by transmembrane channels (DHEIN, 1998). The gap junction, composed of connexins, is formed by a multigenic family of transmembrane proteins (HERVÉ *et al*., 2004; WONG *et al*., 2017). Each conexon is a hexamer-shaped junctional hemichannel formed by

six connexins (MAKOVSKI *et al*., 1977; DERMIETZEL & SPRAY, 1993; KUMAR & GILULA, 1996; WHITE *et al*., 1999; UNGER *et al*., 1999; SEGRETAIN & FALK, 2004; KIM *et al*., 2016; LILY *et al*., 2016; POGADA *et al*., 2018). The gap junctions allow bidirectional communication, direct from the cytoplasm of two neighboring cells, being a low-resistance pathway for propagation of electrical impulses in mammals and allowing the traffic of molecules up to 1 kDa (FLAGG-NEWTON *et al*., 1979; Hervé *et al*., 2004; BERMUDEZ-FAJARDO *et al*., 2007; KOVAL *et al*., 2014). It is known that, the fibroblasts play an important role in tissue morphogenesis and behavior, exhibiting topographic diversity, tissue-specific functions (CHANG *et al*., 2002). The behavior of fibroblasts in the face of the inflammatory process and lesions includes the interaction with the immunocompetent system and cells and the alteration of gap junctions (Cx43) in the healing process (GABBIANI, CHAPONNIER & HÜTTNER, 1978; SORRELL & CAPLAN, 2009; PISTORIO & EHRLICH, 2011; BUECHLER & TURLEY, 2018). Highlighting the importance of understanding the behavior of the cell line in the process of infection by *T. gondii* and how parasitemia alters the behavior of gap junctions mediated by Cx43.

METHODOLOGY

Human Foreskin Fibroblast Cell Culture: The HFF cell line is a foreskin fibroblast cell and has characteristics similar to the tissue of origin is also provided by the Cell Bank of Rio de Janeiro. The cultures were maintained in DMEM medium (Dulbecco´s modified Eagle´s medium), supplemented with 10% Bovine Fetal Serum (GIBCO-Life Technologies, Rockville, MD), penicillin 1000IU/mL and streptomycin 100IU/mL (Sigma Chemical Company, St Louis, MO). The cells were kept at 37°C in a humid atmosphere at 5% CO_2 (Culture CO_2 Incubator, model CCL-170B-8, Sigapore).

Parasit-Cell Interaction: The cells were grown in cover slips and organized in the plate of 24 wells plates. *T. gondii* taquizoite was resuspended in DMEM medium and placed in contact with the cell in the proportion of ten parasites for each cell (10:1). After 1 hour of interaction in the greenhouse at 37°C, in humid atmosphere and 5% CO_2 (Culture CO_2 Incubator, model CCL-170B-8, Sigapore), the medium was discarded and replaced by supplemented DMEM and returned to the greenhouse within 24, 48 and 72 hours. After that, the cells were submitted to an evaluation of cell growth and its morphology by means of images obtained under inverted microscope (Axiovert 40 CFL, light field, phase contrast, ZEISS, Germany) at increases of 100x, 200x and 400x with the AxioVision program.

Cell Death Index: HFF cells control and infected with *Toxoplasma gondii* at the rate of 10 parasites per cell at 24, 48 and 72 hours and stained with trypan blue solution (50% trypan blue and 50% PBS) for 10 minutes. Then, the reagent excess was removed and the number of live and dead cells was counted. Then, in 100 total cells, the number of stained and unstained cells was calculated. Where the cell death index is represented by the value resulting from the number of dead cells divided by the total number of cells. Counting was performed using an inverted microscope (Axiovert 40 CFL, light field, phase contrast, ZEISS, Germany) and data were analyzed using the GraphPad Prism program for graph acquisition and statistical analysis.

Immunofluorescence: The cells were fixed with 4% formaldehyde for 30 min at room temperature. After this stage, the cells were washed with PBS-TRITON X-100R at a concentration of 1% for 30 minutes and then immediately incubated with bovine serum albumin (BSA-Bovine Serum Albumin, SIGMA), diluted in PBS at 2% for 30 minutes. After blockade, were incubated with anti-Cx43 monoclonal antibody (AB 11369 - abcam), in a 1:500 dilution over night the cells were washed with PBS three times for 10 min, then incubated with

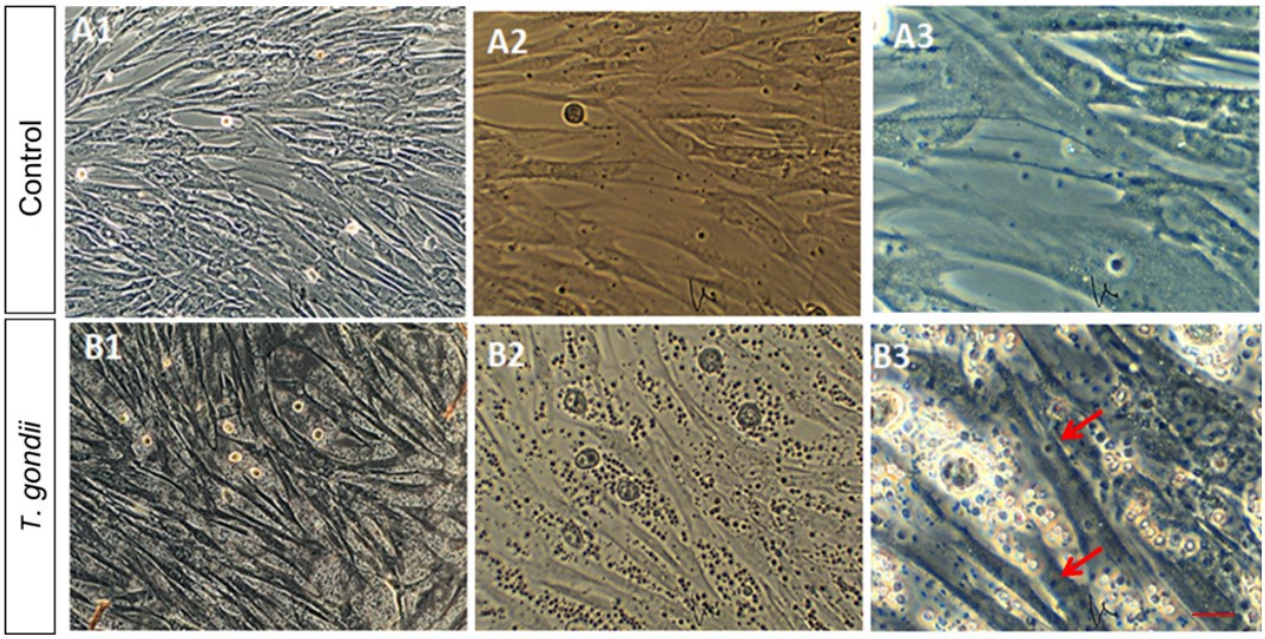


Figure 1. Micrographs in phase contrast microscopy of the HFF cell line in the period of 24 hours. In group A the uninfected control is observed and in B the cell line infected with *Toxoplasma gondii,* groups 1, 2 and 3 demonstrate the field in the increase of 100x, 200x and 400x. In image B1 the microenvironment of the culture is observed in a broad way, in B3 we see in more detail the cell structure and its morphology, as well as the presence of the intracellular parasite indicated by the arrows. Calibration bar: 50µm

Obtaining Taquizoite from Toxoplasma gondii: Tachyzoite form of *T. gondii* (RH Strain) were provided by the Technology Laboratory in Cell Culture (LTCC) by UERJ -ZO, Brazil-RJ. These parasites were obtained through the number of the Ethics Committee of the State University of Northern Fluminense Darcy Ribeiro (ID 124396). Tachyzoites of *T. gondii* (RH strain) were inoculated intraperitoneally into BALB/c mice. After 2-3 days, tachyzoites were harvested using intraperitoneal wash with phosphate buffered saline (PBS; pH 7.3) and then were counted.

dilution of 1:400 (A-11008, invitrogen). After incubation, wash three times with PBS for 10 min and then incubated with the cytoskeleton Phalloidin (F-actin) (Alexa Fluor® 546 phalloidin (A22283), and in then labeled with DAPI (4',6-diamidino-2-phenylindole) to mark the nucleus cells after washing and mounted on slides with Prolong Gold^R. The markings on the slides were observed uses the Optical Fluorescence Microscope Inverted Zeiss Axio Observer with Apotome system (Axio Vision Software) and theEpifluorine Microscope AXIOVERT 40 Plus Carl ZEISS (Photo microscope),

excited with illumination by high pressure mercury lamp, HBO 50W, (ʎ = 490 nm) and the emission monitored using a set of emission filters for fluorescence fluoresceína (ʎ = 525nm). The experiments were submitted to observation and simple analysis or sections in plane Z (three-dimensional reconstruction) using the confocal microscope LSM 710 QUASAR (Carl ZEISS, Oberkochen, Germany).

RESULTS

HFF cell line infected with *T. gondii:* We analyzed the gap junction profile and the alteration of the acute infection microenvironment generated by the *T. gondii* parasiteduring 24, 48 and 72 hours (respectively figures 1, 2 and 3).

decrease in the number of cells in culture and an increase in the amount of parasites free by cell lysis. Highlighting in the period of 72 hours the greatest damage when compared to the other interaction times (24 and 48 hours) and to the control, because the initially infected cells were lysed by the parasite.

Cell Death Index: To evaluate the cell death index, the dead cell count of the control culture and infected with *T. gondii* at the rate of 10 parasites per cell at was performed at 24h, 48h, 45h and 72h, obtaining the data for Graph 1.

Location of Connexin 43 and interaction with the cytoskeleton: Figure 5 shows the labeling of the cell nucleus in blue, the marking of the f-actin filaments by the phalloidin in red and the marking for connexin 43 in green in the cellular cytoplasm of the control HFF

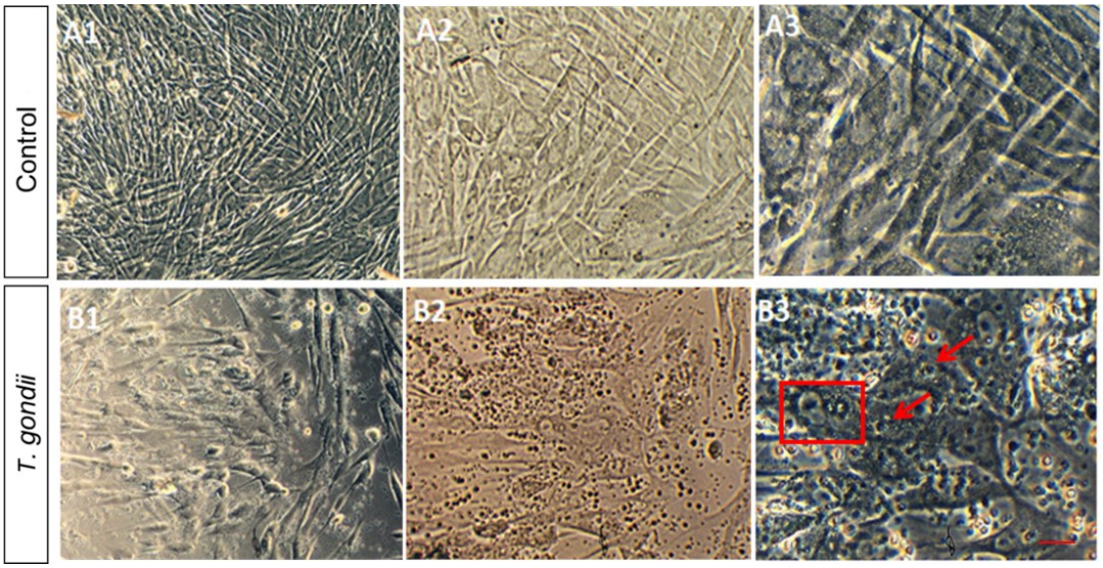


Figure 2. Images of the HFF cell line in the period of 48 hours of interaction. Group A is the uninfected control. Group B, we see the cells infected with *Toxoplasma gondii* in the period of 48 hours. In B1 the infected culture is observed in the increase of 100x, in B3 it is noted the cells infected with *T. gondii* in the increase of 400x highlighted by the arrow the protozoan and the rosacea inside the cell is highlighted by the square. Calibration bar: 50µm

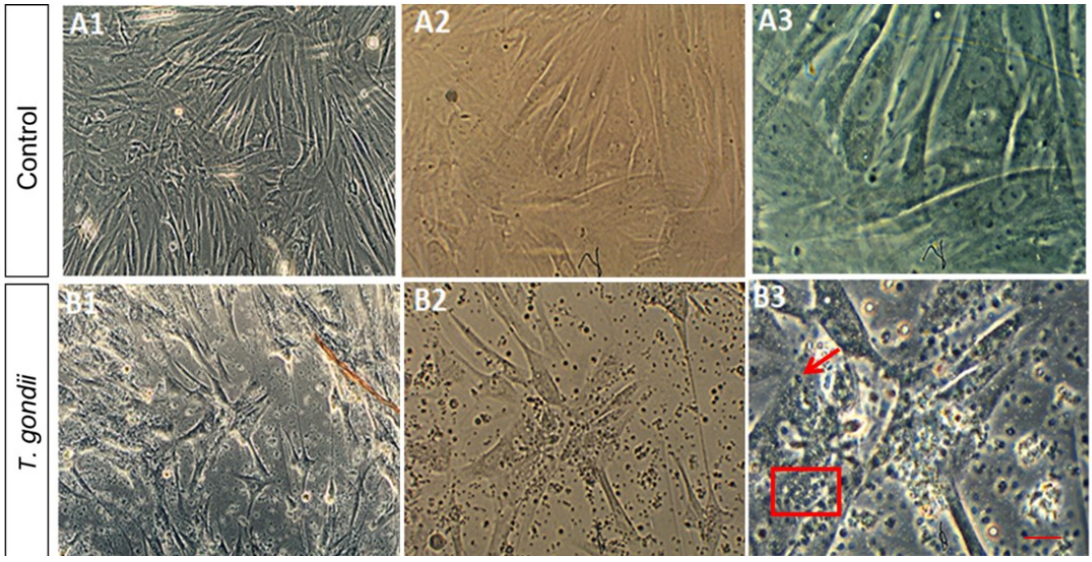
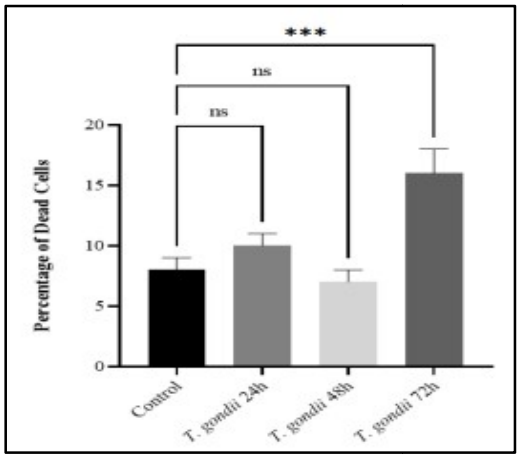


Figure 3. Images of the HFF cell line infected with *T. gondii* within 72 hours. In A, uninfected control is observed. In B, cells infected with *T. gondii* are observed. Highlighting in B1 the reduction in the number of cells in the microenvironment, in B3 we observed parasites inside and outside the cells in culture. Calibration bar: 50µm

It was possible to observe the cellular changes in a phase contrast microscope in the infection process, involving the maintenance of the survival of the parasite in the intracellular environment, compared with the control cells in increments of 100x, 200x and 400x to observe the cell structure and behavior in the microenvironment. It was observed that there were changes in all incubation times, with a

lineage. It is possible to observe in Figure 6 the immunofluorescence staining of the HFF cell line infected with *T. gondii* for 48h, in which it is possible to highlight in A the marking for the cell nucleus in blue and in B the green marking for Cx43 located in the cell membrane. The formation of the junctional plate highlights the location of Cx43

in its morphofunctional site, showing greater communication between cells in the presence of the protozoan.



Graph 1. Data on the percentage of dead cells in control and infected 24h, 48h and 72h. It is possible to observe that the control presented 8% of cell death represented by the first column. In the column on the side, 10% of dead cells can be seen in the interaction of the HFF cell line with *T. gondii* within 24 hours. In the third column, the interaction of 48 hours is observed, indicating 7% of cell death. In 72 hours of infection, represented by the last column, it presents an index of 16%, which is the highest proportion of cell death.. P<0.05 (*); P<0.01(**), P<0.001(***); P<0.0001 (****)

Location of Connexin 43 and interaction with the cytoskeleton: Figure 5 shows the labeling of the cell nucleus in blue, the marking of the f-actin filaments by the phalloidin in red and the marking for connexin 43 in green in the cellular cytoplasm of the control HFF lineage.

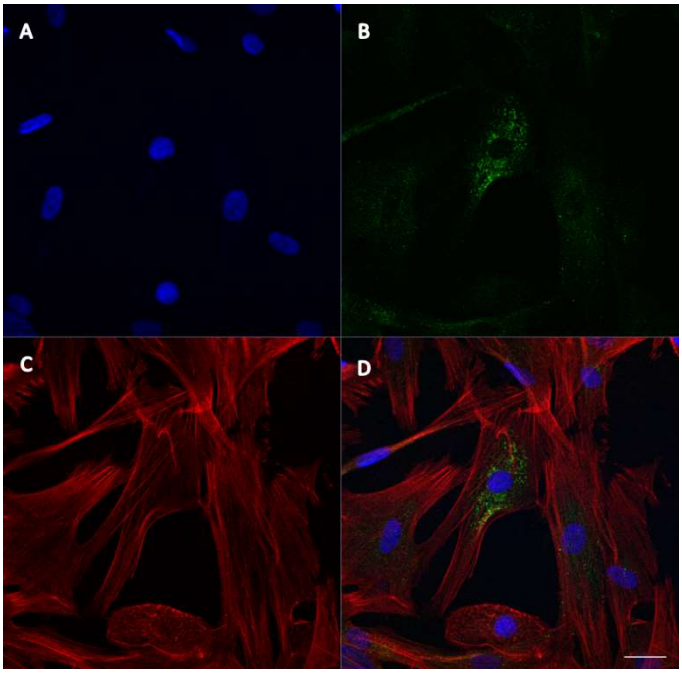


Figure 5: Immunofluorescence demonstrated in confocal microscopy (LSM710, Zeiss, Germany), indicating the tag for protein connexin 43 (Cx43) and Phalloidin (marker actin filaments) in HFF cell line control. In micrograph A is demonstrated nucleus of the cells were labeled with DAPI.The labeled for Phalloidin is observed in the micrograph B and Cx43 can be observed in the cellular cytoplasm cells in the micrograph C. In micrograph D is demonstrated interposing images. Calibration bar: 50µm

It is possible to observe in Figure 6 the immunofluorescence staining of the HFF cell line infected with *T. gondii* for 48h, in which it is possible to highlight in A the marking for the cell nucleus in blue and in B the green marking for Cx43 located in the cell membrane. The formation of the junctional plate highlights the location of Cx43 in its morphofunctional site, showing greater communication between cells in the presence of the protozoan.

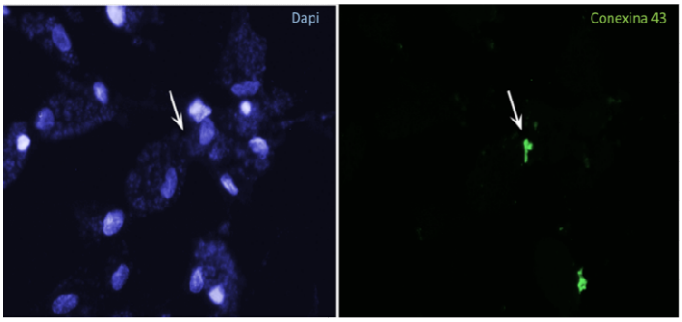


Figure 6. Fluorescence microscopy images of the HFF cell line observed with Apotome. In A is visualized the nucleus of the HFF cell and the nucleus of *Toxoplasma gondii* marked by Dapi, in B we can observe in green the labeling for the protein connexin-43 located in the cell membrane forming the junctional plate. Calibration bar: 50µm

DISCUSSION

Morphology and Microenvironment: Fibroblasts have a wide role in human physiology, being present in different systems, but their main role is to maintain skin functions, being responsible for synthesizing and secreting molecules in the extracellular matrix (FANG *et al*., 2016), also acting in healing, inflammation and fibrosis (HÄKKINEN *et al*., 2012). To evaluate the HFF cell line in homeostasis, morphology analysis, microenvironment and immunostaining of uninfected cells were performed. Among the various interaction studies using the human foreskin fibroblast (HFF) cell line and the protozoan *Toxoplasma gondii*, few have investigated possible changes caused by the parasite in specific proteins of host cells (GAIL & BOHNE, 2004; SCHMIDT *et al*., 2013). Furthermore, in these studies there are no reports of analysis and follow-up of morphological and microenvironment changes caused by parasitemia by microscopy in the description of the infection process by temporality in HFF. Therefore, the present work presents these data for the first time in the literature. It was observed that the morphological and microenvironmental changes during *T. gondii* infection found in this study corroborate with previously published works by our research group (DE CARVALHO *et al*., 2021; SOUZA *et al*., 2022), where infected cells at a ratio of 10 parasites per cell exhibit morphological swelling with the progression of parasitemia. Within 48 hours, a significant increase in parasites can be visualized both in the intracellular environment, as previously shown by Dubey *et al*. (1998). This is attributed to the parasite's replication process through endodyogeny, as well as the increase of Toxoplasma gondii in the extracellular medium due to the rupture of some infected cells, leading to the re-infection of surviving cells. After 72 hours of infection, the microenvironment shows a significant number of dead cells.

Several studies have been carried out in order to unravel the actions of *T. gondii* in the host cell. Among these studies, it is essential to highlight those involving gap junctions, since gap junctions play a crucial role in the homeostasis of several organ systems, mainly in studies associated with parasitic infections (DE CARVALHO *et al*., 1998). Figure 5, shows the organization of the cellular cytoskeleton seen by f-actin labeled with phalloidin, confirming that this cell lineage maintains the general characteristic of fibroblasts, having a well-organized cytoskeleton with intermediate filaments well distributed in an extensive system in the cytoplasm, as reported in the literature by Junqueira & Carneiro (2008). This strain presents a discreet labeling for connexin-43, corroborating the data by Louault*et al*. (2018) who state that fibroblasts express Cx40 and Cx43 with distinct patterns, depending on the conditions and stage of development. To understand the importance of Cx43, some studies stimulate the presence of this protein in the cell membrane, with the aim of improving healing by increasing communication between cells

(WRIGHT *et al*., 2009). Leading to the questioning of the role and behavior of this protein in a parasitemia in this cell lineage. It was reported by De Carvalho *et al*. (2021) that the expression of the Cx43 protein was increased in macrophage cells infected with the parasite *Toxoplasma gondii*. Thus, it is possible to suggest that in response to parasitemia in the HFF cell line it stimulates the positioning of Cx43 to the cell membrane.

Therefore, we propose that the cell can position the connexin to the cell membrane in order to signal the presence of the protozoan to the immune system and try to improve the tissue protection process. As the increase of Cx43 in this cell line improves the direct response between cells in a pathology, as demonstrated in the article by Wright *et al*. (2009). However, it is known that *T. gondii* secretes substances into the intracellular environment that are involved in the interaction with the host. These substances include the release of cysteine protease and cathepsins (exopeptidase), which play an important role in cell invasion and the digestion of host proteins (SIQUEIRA-NETO *et al*., 2018). Additionally, both the tachyzoite in the parasitophorous vacuole and the bradyzoite in the cyst interact strongly with the host cell (BLUME & SEEBER, 2018). With this, we can also suggest that the parasite may be inducing the positioning of Cx43 through these secretions to send information to neighboring cells that increase their susceptibility to infection by the extracellular parasite, improving the infection process, since Cx43 can be modulated. (WRIGHT *et al*., 2009). Although the reports of this parasitemia cause the disarrangement of the cellular cytoskeleton (DE CARVALHO *et al*., 1998) and the proteins that form the gap junctions are directed and positioned by components of the cytoskeleton through microtubules (SEGRETAIN & FALK, 2004), the effects of parasitemia in this lineage can be differentiated. From these findings we can infer that Cx43-mediated communication in fibroblastic cells infected with *T. gondii* plays an important role in this parasitemia. More experiments are needed to clarify the origin of the induction of Cx43 positioning, and whether it also has an increase in protein synthesis.

CONCLUSION

In the HFF cell line, Cx43 is labeled within the cell cytoplasm. However, after in vitro infection with *T. gondii* for 48 hours, there is a promotion of this protein's positioning to the cell membrane, facilitating the formation of junctional plaques. This change is attributed to the repositioning of Cx43 in the host cell's membrane. Nevertheless, further studies are still required to determine the exact role of this alteration.

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