Tunnelling Nanotube Projections May Interfere with *Toxoplasma gondii* Adhesion to Host Cells

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Article

Tunnelling Nanotube Projections May Interfere with *Toxoplasma gondii* Adhesion to Host Cells

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Abstract

Toxoplasma gondii, the causative agent of toxoplamosis, a disease widely distributed, is an intracellular parasite that invades host cells of different tissues using specialized endocytic activity. Recent studies suggest that tunneling nanotubes (TNTs), thin cell surface projections, may participate in the parasite-host cell interaction process. We report results on the involvement of host cells TNTs in the adhesion and internalization of *T. gondii* tachyzoites to epithelial LLC-MK^2 cells. Microscopy analysis showed that incubating cells in 0.45 M sucrose induces reversible assembly of TNTs without affecting cell viability. The presence of extended TNTs correlated with increase on parasite adhesion and reduction of parasite entry, suggesting a structural or signaling role in mediating adhesion. TNTs assembled following sucrose incubation contain both actin and tubulin components. These results highlight the functional relevance of TNTs in *T. gondii* host cell interaction, especially in parasite adhesion, opening new perspectives for understanding *T. gondii*-host cell interaction.

Keywords: *Toxoplasma gondii*; parasite-host cell adhesion and interaction; fluorescence microscopy; electron microscopy; tunneling nanotubes

1. Introduction

Toxoplasma gondii is an intracellular parasitic protist belonging to the Apicomplexa phylum and is the etiological agent of human and animal toxoplasmosis, widely distributed worldwide that is capable of infecting nearly all warm-blooded animals, including humans. It is estimated that approximately 30% of the global human population is infected with this parasite. It actively invades host cells, using highly specialized mechanisms known as induced endocytosis, involving parasite motility, adhesion to the surface of the host cell, extrusion of the conoid and release of proteins existing in the micronemes and rhoptries of the parasite, culminating in the formation of a parasitophorous vacuole located within the host cell [1–4]. Using high-resolution scanning electron microscopy we showed previously that occasionally we observed the presence of structures known as Tunneling Nanotubes (TNTs), emerging from the surface of the host cell and establishing contact with the parasite itself, even at long distances from the host cell surface [5], suggesting a possible participation of TNTs in the *T. gondii*-host cell interaction process. TNTs are plasma membrane protrusions containg actin filaments and microtubules, displaying variable diameters and reaching distances as long as 400 micrometers, establishing contact with another cell forming a kind of nanotube that allow cell-to-cell communication and transference of macromolecular complexes, and even organelles, between them. TNTs were initially described by Kornberg (1999) in Drosophila [6], and later, they were found in many cell types [7], including immune cells [8]. The thin and elongated extensions of the plasma membrane are based on actin, and depending on the cell type, tubulin. TNTs differ from other cellular structures by the absence of contact with the substrate and by the direct

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connection of the cell cytoplasm [9]. It is an additional way to connect cells, being extremely relevant in intercellular signaling and allowing the direct cell-t-cell transfer of biological materials. In this manuscript, we further analyze the interaction of *T. gondii* tachyzoites with cells that have been previously stimulated to assemble many TNTs. Our initial morphological information show a close association of *T. gondii* tachyzoites with TNTs, with their presence increasing parasite adhesion but decreasing parasite invasion of host cells.

2. Materials and Methods

2.1. Host Cells

The host cell lineage used in the experiments was the epithelial cells LLC-MK² (ATCC - CCL7, Rockville, MD/USA) from the monkey kidney Rhesus (*Macaca mullata*), grown in RPMI (Roswell Park Memorial Institute) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated bovine fetal serum (FBS; GIBCO, Thermo Fisher Scientific, Waltham, MA, USA). The cell lineage was grown on glass coverslips or in culture flasks and maintained at 37 °C in a 5% CO^2 atmosphere.

2.2. Parasite Maintenance

T. gondii tachyzoites (RH strain) were maintained by passages in LLC-MK² cells at confluence. After 1 day of infection, parasites were obtained from the supernatant, centrifuged at 1,000 x g for 10 minutes, and resuspended in 10 mL of RPMI medium. The number of parasites was quantified using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany).

2.3. Interaction Assay

1 day before the interaction, 5 × 10 ^5 Cells were seeded on coverslips placed in a 24-well plate or directly on a 96-well plate. Then, the cells were washed 3 times with phosphate-buffered saline (PBS), pH 7.2. Subsequently, the samples were incubated at 37 °C in a 5% CO2 atmosphere. The parasites were added at a 50:1 ratio (parasites per host cell) for 30 minutes. After interaction, with the parasites, the cells were again washed three times with PBS to remove non-adherent parasites, in order to confirm persistent adhesion. The samples were fixed in a solution containing 3% freshly prepared formaldehyde and 0.1% glutaraldehyde in PBS for 15 minutes, or in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for light and electron microscopy, respectively. To determine adhesion and internalization indexes after the interaction, the cultures were fixed in a 4% formaldehyde solution in PBS, pH 7.2, and stained with 10% Giemsa. The samples were dehydrated in different concentrations of acetone-xylol, and the coverslips were mounted on Entellan ® (Merck Millipore, Darmstadt, Germany) drops and analyzed under a light microscope using an Axion Zeiss microscope (Axio Imager, Zeiss, Oberkochen, Germany) and a 100x objective with oil immersion. To distinguish adhered from internalized parasites, we carefully examined the samples and used the presence of a parasitophorous vacuole as a criterion for identifying internalized parasites. The adhered parasites were easily identified in the samples since they are located on the host cell surface. The adhesion index was obtained by multiplying the mean number of parasites adhered to the host cell and the percentage of cells with adhered parasites. The internalization index was calculated by multiplying the mean number of internalized parasites per infected cell and the percentage of infected cells [10]. The data were obtained after analysis of three different coverslips per experiment. The data were plotted using GraphPad Prism 9.0. The presented results represent the means ± standard deviations from at least three independent experiments, and differences were considered statistically significant at $P < 0.05$.

2.4. Induction of Tunneling Nanotube Assembly

Nanotubes were induced by incubating the cells with 0,45 M sucrose for 30 minutes at 37 °C, washing with PBS (pH 7.4), and then supplementing with RPMI medium [11]. They were allowed to

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interact with the parasites, as described above. In order to distinguish the effects of sucrose treatment from TNT formation, all assays included appropriate controls without sucrose exposure.

2.5. Cell Viability

To assess the effect of sucrose treatment and the reversibility of the TNT induction process, LLC-MK^2 cells were seeded at 2.5 × 10^^{5} cells/well in a 96-well plate. They were incubated in RPMI supplemented with 10% FCS. After 24 h of growth, they were incubated for 1 hour in 0.45 M of sucrose in RPMI. To assess cell viability after incubation, they were washed with PBS (pH 7.2), incubated in RPMI supplemented with 10% FCS for 20 minutes, and cell viability was then assessed using the MTS/PMS assay. Quantification was made by measuring optical density at 490 nm using a BioTek Synergy H1 spectrofluorometer (BioTek Instruments, Winooski, VT, USA). Three independent experiments were performed in triplicate. The values were calculated by fitting them to a percentage viability analysis after reversal of the sucrose treatment. Regression analyses were performed using SigmaPlot 10.

2.6. Fluorescence Microscopy

For fluorescence microscopy analysis, the interaction was performed as described above. First, the cells were fixed in a solution containing 4% freshly prepared formaldehyde in PBS (pH 7.2) for 20 min, washed with PBS (pH 7.2), and permeabilized in a solution containing 0.05% saponin and 3% bovine serum albumin (BSA) in PBS for 10 min. Subsequently, the samples were washed three times with PBS (pH 7.2) and sequentially incubated in a solution containing 50 mM ammonium chloride (NH_4Cl) in PBS for 30 min, followed by a blocking solution (0.05% saponin and 3% BSA in PBS) for 30 min at room temperature. The cells were then incubated with the mouse anti-tubulin polyclonal antibody (1:2000 dilution) (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Subsequently, the cells were washed with blocking solution and incubated with Alexa Fluor 488 anti-rabbit secondary antibody (1:800 dilution) (Molecular Probes, Thermo Fisher Scientific, Eugene, OR, USA) for 1 h, protected from light. After incubation, the cells were washed with a blocking solution. Samples were incubated for one h with fluorescent phalloidin 546 (1:40 dilution) (Molecular Probes, Thermo Fisher Scientific, Eugene, OR, USA), a fungal peptide that binds to the actin filaments, washed, and mounted with ProLong® Gold Antifade Reagent with DAPI (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with 4',6-diamidino-2-phenyl-indole (DAPI). The observations were made using the confocal mode of a Zeiss Elyra PS.1 Light Scanning Microscopy 710 light microscope (Germany).

2.7. Scanning Electron Microscopy

For morphological analysis of the initial events of the interaction by scanning electron microscopy, the interaction assay was performed, as described above. After 15 minutes of interaction, the samples were washed once with PBS (pH 7.5) and fixed with a solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Then, the cells were washed in PBS, postfixed in 1% OsO4 (Osmium Tetroxide), and dehydrated in ethanol (30%-100%) before being critically-point dried with liquid CO2. The samples were coated with a 5 nm thick platinum layer and observed in a Zeiss Auriga 40 scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany) operating at 4kV. For Ion microscopy observation, the samples were not coated and observed in an ORION model Helium Ion Scanning microscope (HIM) (Zeiss Nanofab, Peabody, MA, USA) with tungsten filament and operated at 35 kV, with a working distance of 8.7 mm, pressure of 5.5 x 10-6 Torr, and beam current of 0.8 pA.

2.8. Transmission Electron Microscopy

Parasite-host cell interaction was carried out as described above. The cells were fixed in a solution containing 2.5% glutaraldehyede in 0.1 M cacodylated buffer, pH 7.2, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedde in Epoxi Resin. Thin sections were stained

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with uranyl acetate and lead citrate and observed in a transmission electron microscope (Hitachi or FEI) operating at 100 kV. 3. Results

We decided to use the method of incubating cells in the presence of 0.45 M sucrose as an inducer of tunneling nanotubes [12] since we previously obtained similar results with those using other treatments [11,13]. After pretreatment of LLC-MK² cells with 0.45 M sucrose for 1 hour, followed by washing and incubation in medium supplemented with 10% fetal bovine serum, the cytotoxic effect of sucrose on the cells was evaluated. Under these conditions and at the concentration described, the cells showed 94% viability compared to untreated cells. After the removal of sucrose from the medium, the TNTs decreased in both number and length. Data is the average of three independent experiments in triplicate (***$p < 0.001$).

We also chose to use the expansion microscopy process aiming at a better visualization of the two main components of TNTs: actin filaments and microtubules. Labeling for actin and tubulin was clearly seen throughout the cell and within the TNTs (Figure 1).

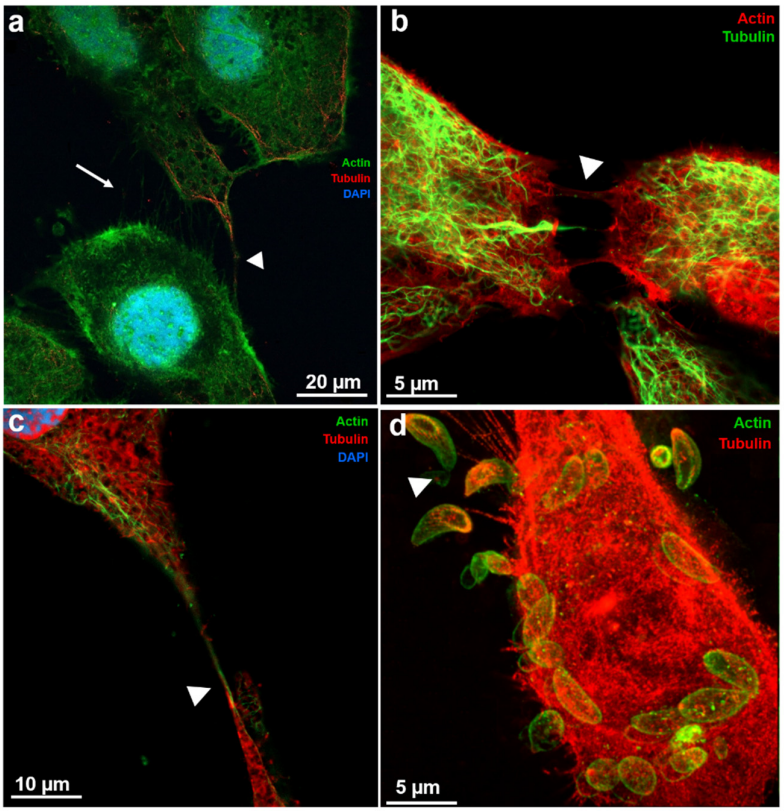

Figure 1. Morphology of the LLC MK² cell line, with and without sucrose treatment. a. Untreated control cell showing uniform actin distribution. b. Untreated cells exhibiting short TNTs between adjacent cells. c. Cells treated with sucrose for 30 minutes, showing TNT (arrow) labeled for actin and tubulin. d. Interaction between the host cell and tachyzoites, showing TNTs and direct contact between parasites and host cells.

Using scanning electron microscopy, we observed that the LLC MK² cells presented a well-preserved morphology, with a slight rounding shape, compared to the untreated cells (Figure 2). It is important to point out that cell viability assay indicated that that the sucrose treatment did not affect the cell and that the induction of TNTs assemble was reversed after transference of the cells to the culture medium.

The results presented in Figure 2 reveal significant morphological changes on the surface of LLC-MK² cells induced by sucrose treatment, as observed by scanning electron microscopy. In Figure 2a, a classical epithelial organization is evident, with elongated or polygonal cells and well-defined membrane projections, representing the basic morphology of untreated cells. Figure 2b highlights the

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presence of short TNTs, suggesting that these structures are present under physiological conditions, albeit in limited number and length. However, sucrose treatment leads to notable morphological alterations: in Figure 2c, a clear increase in TNT length is observed, with structures exceeding 5µm. This effect is further intensified as shown in Figure 2d, where longer TNTs are observed

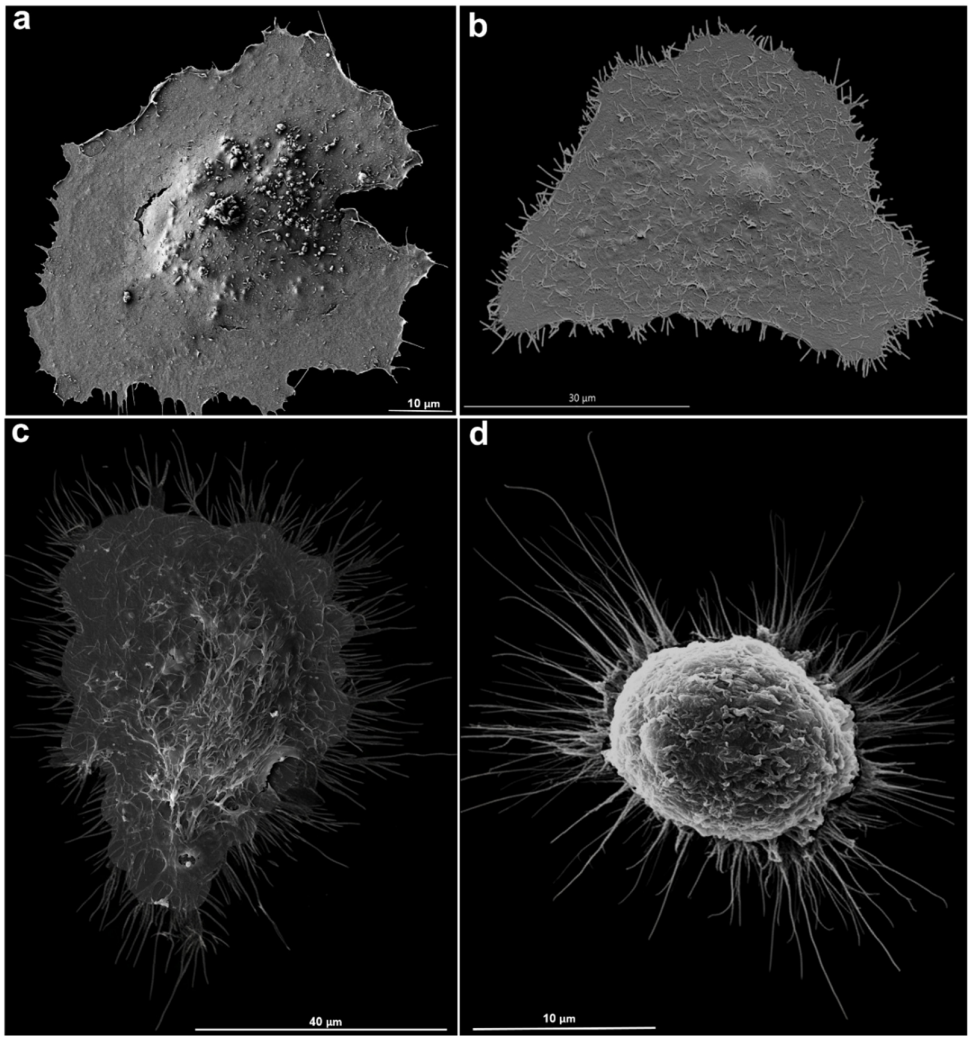

Figure 2. Morphological aspects of the surface of the LLC MK² cell line observed by scanning electron microscopy. a. Classical epithelial organization, with elongated/polygonal cells and well-defined membrane projections. b. Short TNTs, with lengths between 900 nm and 5 µm. c. Cell treated with sucrose: TNTs longer than 5 µm. d. rounding of the de cellular body, and the presence of TNTs up to 20 µm long.

We used hundreds of SEM images to measure the length of the TNTs to determine their frequency distribution as shown in Figure 3. We observed that in the control cells, most of the TNTs presented lengths ranging from 0.9 to 5µm, while in the cells previously treated with sucrose, TNTs reached a length of 20µm

Similar experiments were conducted using control cells and cells treated with sucrose, incubated with *T. gondii* tachyzoites. We observed that parasite adhesion was significantly higher in cells treated with sucrose compared to control cells. On the other hand, the internalization index in control cells was approximately twice as high as in treated cells, reaching values similar to their respective adhesion indices. Adhesion and internalization indices were calculated based on the absolute number of events per 100 host cells; these values were subsequently normalized and expressed as a

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percentage for comparative purposes. The data underlying these absolute counts are available in Figure 4.

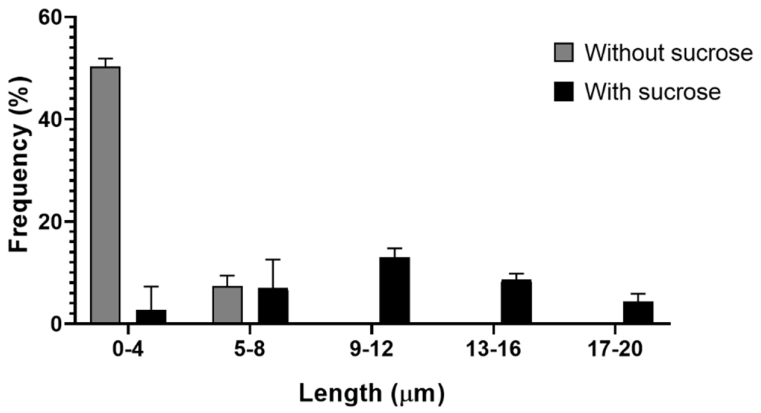

Figure 3. Frequency of length in micrometers of LLC MK² cells. In gray, cells without sucrose treatment, and in black, cells treated for 30 minutes with sucrose.

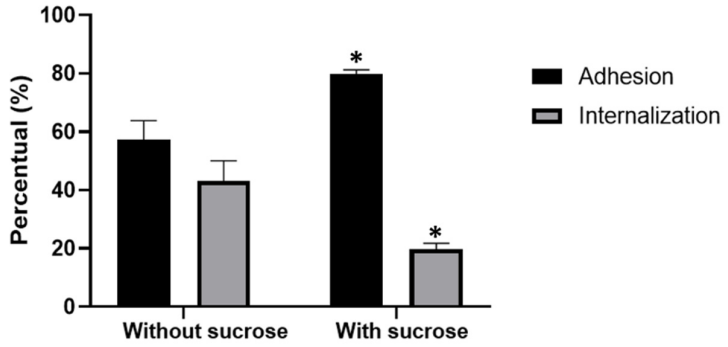

Figure 4. Quantification of *T. gondii* interaction with LLC MK² cells. The interaction was performed under two conditions: i) initially at 4 °C for 30 minutes and then maintained at 37 °C for an additional 20 minutes before adhesion (synchronized condition); or ii) parasites interacted with host cells that had been pretreated with sucrose for 30 minutes prior to interaction. Adhesion and internalization indices were obtained as described in Materials and Methods. Data was obtained from three independent experiments. Student's t-test was used to compare adhesion and internalization at the entry site of *T. gondii* into host cells. Values were considered statistically significant when *$p \leq 0.01$(*).

Scanning electron microscopy images of tachyzoites allowed to interact for 15 min with control cells showed images that confirm previous studies on the parasite-host cell interaction. Figure 5 shows images where parasites attached to the host cell surface are evident, with the presence of surface projections in contact with the tachyzoites. Short TNTs were seen in association with the tachyzoites. Several TNTs were observed throughout the interaction, possibly initiating the sequential process of recognition, adhesion and subsequent internalization, with the participation of the host cell.

In the overview figures 2, multiple TNTs can be observed projecting from the host cell, with tachyzoites positioned in close proximity. This spatial organization suggests a potential role for these structures in attracting or guiding parasites. The higher magnification figures 2 reinforces this hypothesis by showing a tachyzoite in close contact with the projections, appearing to engage with them in a directed manner. This pattern may indicate that TNTs function not only as structural elements of the host cell but also potentially as mediators of intercellular communication or as guiding structures that facilitate parasite approach and cellular recognition. In comparison of both

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assays previously treated with sucrose, without the presence of the tachyzoite and in the presence of the parasite, there were TNTs that reached a size of up to 24 μm (Figure 6), our findings, under the conditions previously reported.

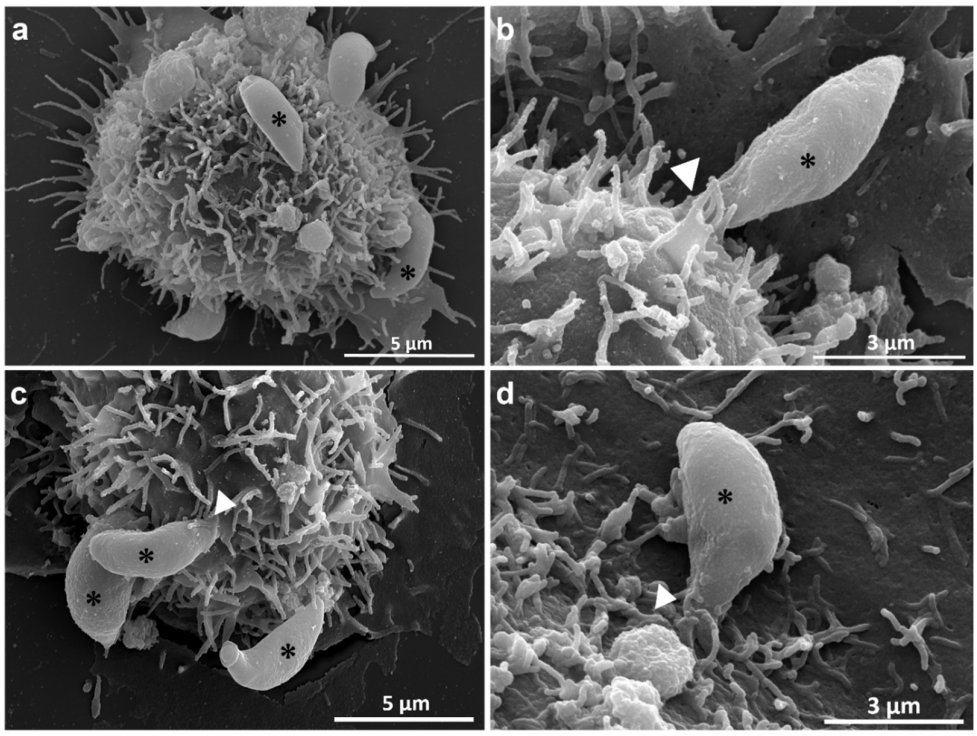

Figure 5. Surface of the untreated host cell in contact with the tachyzoites. a. Several tachyzoites (*) are associated with the cell surface. b. Cytoplasmic projections (arrowhead) of the host cell can be seen surrounding the posterior part of the parasite (*). c. Host cell membrane (arrowhead) surrounding the conoid region of the tachyzoite (*) during the interaction. d. 80 nm thick projections (arrowhead) surrounding the apical pole of the tachyzoite (*).

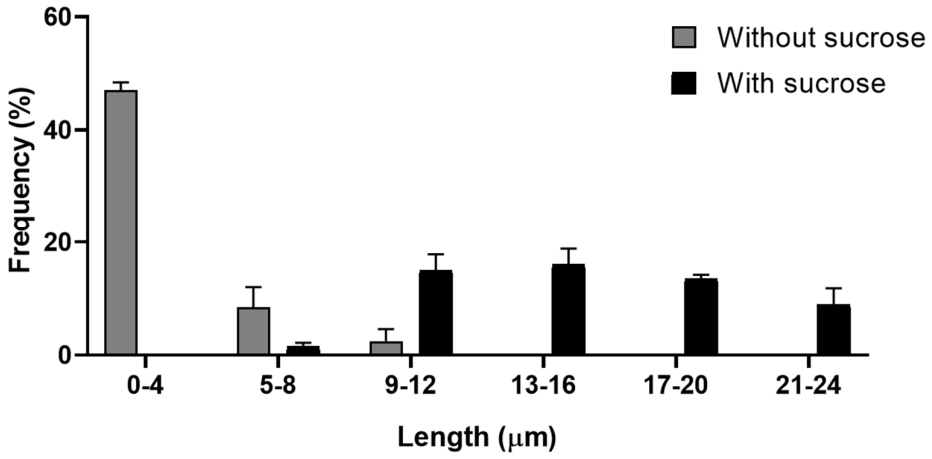

Figure 6. Frequency of the length in micrometers of the TNTs of the LLC MK2 cell line. In blue, cells without sucrose treatment and with interaction with the *T. gondii* tachyzoite; in black, TNTs of the cells treated for 30 minutes with sucrose and with interaction with the tachyzoite.

In comparison of both assays previously treated with sucrose, without the presence of the tachyzoite and in the presence of the parasite, there were TNTs that reached a size of up to 24 μm

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(Figure 6), under the conditions previously reported. Scanning microscopy was used to analyze the projections of the TNTs in the various experimental conditions. In the presence of the tachyzoite, the previously treated cells showed a greater diameter in the length of the TNTs, and the parasites remained in contact with these projections in order to reduce the internalization rates (Figure 7).

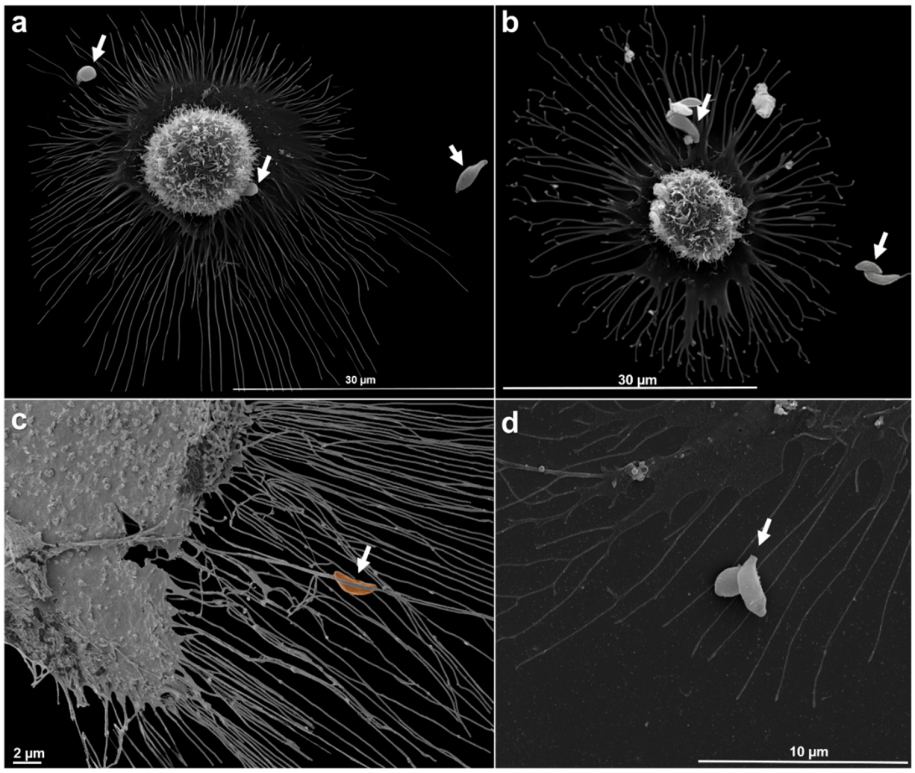

Figure 7. TNTs from the host cell pretreated with sucrose for 30 minutes and the tachyzoite during recognition. a. LLC MK² emitting up to 22µm TNT. b. Tachyzoites dispersed among the TNT projections from the host cell. c. Numerous TNTs projected from the host cell and tachyzoites during recognition. d. Tachyzoite in contact with the apical pole with the TNT.

The results obtained through high resolution scanning ion microscopy demonstrate a remarkable interaction between *T. gondii* tachyzoites and tunneling nanotubes (TNTs) originating from the host cell (Figure 8).

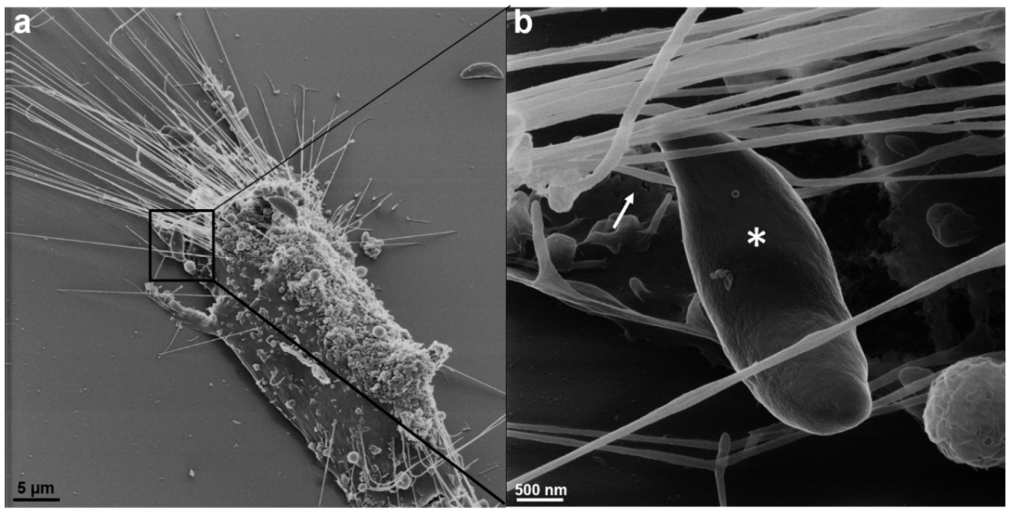

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Figure 8. Interaction between *T. gondii* and host cell observed by ion microscopy. a. Overview of the host cell displaying numerous tunneling nanotubes and surrounding tachyzoites. b. Higher magnification of a selected region from panel (a), showing a tachyzoite (*) closely interacting with host cell projections.

Transmission electron microscopy reveals differences between the TNTs of untreated cells compared to the treated ones, and that *T. gondii* tachyzoites interact closely with these projections, suggesting that TNTs may guide or facilitate the parasite's entry into the host cell, possibly acting as pathways for communication or cellular recognition (Figure 9).

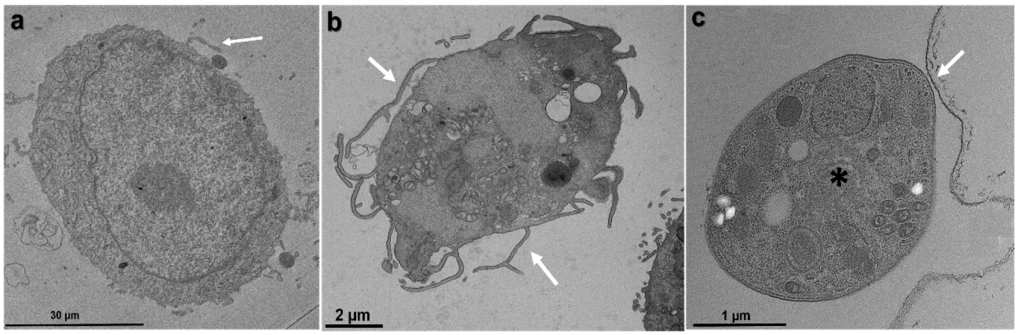

Figure 9. Cell morphology comparing the control cell with the treated one and the interaction of the tachyzoite with TNT. a. Cell of the LLC MK² line without any treatment. The arrow indicates a site of assemble of a TNT. b. Cell of the LLC MK² line treated with sucrose for 30 minutes with several TNTs (arrow). c. Tachyzoite (*) in close contact with a TNT (arrow).

4. Discussion

It has been assumed that the surface of host cells play an important role in the interaction with parasitic protozoa, especially with those that require cell invasion by the parasite in order to divide. In the case of tachyzoites of *T. gondii* it has been shown that tachyzoites penetrate the host cell via endocytic processes induced in part by secretion of parasite molecules that may activate almost all types of endocytosis. In a previous study we showed that during parasite-host cell interaction tachyzoites of *T. gondii* may attach to thin surface tubular structures known as TNTs [5]. Our present morphological observations add new information on this topic using an experimental approach where assemble of TNTs in the host cell surface was induced by incubation in the presence of 0.45M sucrose that promotes hypertonic pressure, interfering with the mechanosensitive ion channel, inducing changes in the cytoskeleton and interfering with clathrin-mediated endocytosis [11,13,14]. We further characterized this experimental system by showing that it can be reversed by washing the cells and incubation in normal culture medium. During the experiments cell viability remained around 94%.

Here, we confirmed that sucrose promoted the induction of TNTs. After removal of this medium, subsequent washing, and incubation with medium supplemented with fetal bovine serum, there was a reduction in TNT elongation, as well as a 94% rate of viability of these cells. These results revealed that sucrose treatment was not detrimental to cell viability, and the cell projections were reversible, with no other ultrastructural changes observed.

There are several terms used to describe membrane protrusions involved in cell-cell contact. Two types of membrane protrusions that may be confused are cytonemes and TNTs. The main difference between them is that cytonemes are extremely thin, and their interaction with another cell ends with the membranes coming into proximity [7]. In contrast, TNTs can exhibit vesicular trafficking and, upon contacting another cell, fuse their membranes to connect the cytoplasm of both cells [15]. However, various organizations of TNT-like structures can be observed, where membrane fusion and cytoplasmic connection may not necessarily occur. Mechanisms of interaction between

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TNT-like structures and cytonemes of parasites with host cells have been reported [15,16]. It has been shown that contact of *Leishmania donovani* with immune cells, such as B cells and macrophages, stimulates the formation of TNT-like structures in these immune cells, facilitating parasite transmission between them [16]. The results of this study show that parasites can slide between the TNT-like structures of a macrophage to B cells and between B cells. This indicates that during in vitro infection with *L. donovani*, such transmission of parasites from infected macrophages to B cells and then dissemination among B cells, leading to activation of polyclonal B cells, may occur [16]. On the other hand, there are several reports dealing with assemble of TNT-like structures in parasitic protozoa such as *Trichomonas vaginalis, Tritrichomonas foetus, Giardia intestinalis,* and *Trypanosoma brucei* [15,17,18]

The biogenesis of TNTs is not yet fully understood and probably depends on the cell type and microenvironment, allowing sharing of intracellular material, up to the capture of microvesicles [7,19]. Previous studies described the emergence of TNTs a few hours after the start of cultivation, remaining stable in semiconfluent cultures with acidic pH and hyperglycemic medium, favoring the formation of TNTs [12]. In the present work we used a medium supplemented with sucrose, which allowed us to morphologically characterize and propose data involving the projections of TNTs interfering in the adhesion and internalization of *T. gondii* tachyzoites in host cells. Our present observations show that while the induction of TNTs significantly increased the adhesion of the parasites to the host cell surface the process of invasion was reduced. Indeed, scanning electron microscopy revealed many parasites associated to the TNTs and previous studies showed that incubation in the presence of sucrose interferes with clathrin-mediated internalization of *Toxoplasma gondii* [13] and *Trypanosoma cruzi* [11] by the host cells. Based on the observed decrease in parasite internalization and consistent association with TNTs, our data suggest that TNTs may act as physical barriers, potentially limiting parasite access to the central domains of the host cell bodywhere the entry process takes place.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Meaning
TNT	Tunneling Nanotube(s)
T. gondii	*Toxoplasma gondii*
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

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Abbreviation	Meaning
LLC-MK²	Rhesus monkey kidney epithelial cell line
RPMI	Roswell Park Memorial Institute medium
PBS	Phosphate-Buffered Saline
pH	Hydrogen Ion Concentration
CO_2	Carbon Dioxide
FCS	Fetal Calf Serum
MTS/PMS	MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium / PMS: Phenazine Methosulfate
BSA	Bovine Serum Albumin
DAPI	4′,6-diamidino-2-phenylindole
OsO_4	Osmium Tetroxide
nm	Nanometer

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