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FURTHER STUDIES OF DEATH AND PROGRESSIVE ELIMINATION OF INTRACELLULAR TOXOPLASMA GONDII IN PRESENCE OF HYDROXYUREA

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ABSTRACT

Toxoplasma gondii tachyzoite infects and resides within a special parasitophorous vacuole in eukaryotic cells. Viable parasites manipulate the vacuole allowing the uptake of nutrients from the host cell while avoid parasitophorous vacuole fusion with the host cell endocytic pathway. We have shown that hydroxyurea arrests parasite multiplication, leading to parasitophorous vacuole acidification and elimination of the parasite. However, cellular and kinetic events associated with this were not described. In the present study we investigate the successive steps of *Toxoplasma* destruction in presence of hydroxyurea. For these purposes, Vero cells were infected with tachyzoite of *Toxoplasma gondii* and treated with increasing amounts of hydroxyurea for several times. We showed that the arrest of tachyzoite multiplication occurred after 5 hours of treatment, coinciding with the time required for parasite division cycle. After 8 hours of incubation, treated cultures had amorphous parasites-containing parasitophorous vacuole, which fused with host cell lysosome. After 12 h, disrupted parasites-containing autophagic vacuoles were also visible. After 24 h, parasite DNA fragmentation also occurred. Once the tachyzoites had been eliminated, treated cells recovered mitochondrial functionality and distribution on the host cell cytoplasm. These assays suggested that the main target of hydroxyurea is the intravacuolar proliferative tachyzoites and host cells recovered their defense against intracellular *T. gondii* during hydroxyurea treatment. The observations reported in this paper also imply that hydroxyurea would be a useful tool in the study of host–pathogen

interactions, mainly in the study of Apicomplexa cell biology and in the development of more specific therapeutic agents.

KEYWORDS: apoptosis, cell cycle, hydroxyurea, parasitophorous vacuole-lysosome fusion, *Toxoplasma gondii.*

INTRODUCTION

Tachyzoites – the infective and proliferative form of *Toxoplasma gondii* – actively invade all nucleated cells. Parasite invasion is closely associated with the sequential discharge of products from secretory organelles (micronemes, roptries and dense granules), leading to the formation of a permissive parasitophorous vacuole – PV – which shelters this obligate intracellular parasite.[1-3]

The hybrid feature of the parasitophorous vacuole membrane (PVM) underlies the inability of the PV to fuse with the host endolysosomal machinery and also avoiding other microbicide systems.[4-5] However, the PVM imports small molecules < 1.3 kDa[6] and lipids[7-8] from the host cell cytoplasm and associates with the host cell organelles such as mitochondrial membrane, endoplasmic reticulum element[9-12] and microtubules.[13]

During intracellular *T. gondii* development, several studies have provided evidences that infection occurs preferentially during the S-phase of host cell cycle[14-16], interrupting the cell cycle at the G2/M phase.[17-19] Once settled in the PV, haploid intravacuolar tachyzoite undergoes a new cycle of division after 5 to 8 hours (for the RH strain), a process known as endodiogeny.[20-21] Replication proceeds inside the PV until the host cell lyses, approximately 48 hours post-infection[22], resulting in tissue rupture - the effect associated with toxoplasmosis in vertebrate organisms. Traditional anti-toxoplasmosis chemotherapeutics are not efficient to eliminate intracellular *Toxoplasma gondii* and have drastic toxic effects to host organisms. Therefore studies about *Toxoplasma gondii* – host cell interactions may improve the development of efficient compounds against others intravacuolar Apicomplexa parasites as *Theileria sp., Plasmodium sp*., and *Eimeria sp.*

Hydroxyurea (HU) is an antimitotic and antitumoral drug; it is also used to study DNA damage-independent replication fork arrest in prokaryotes and eukaryotes.[23-24] HU inhibits class I ribonucleotide reductase (RNR), an enzyme that regulates the nucleotide balance in cells. Several studies have indicated that RNR is the main target of HU.[25-27] Mammalian

cells in the S-phase of the cycle are more sensitive to HU action due to the high enzyme activity in this phase.[28-29]

The effect of HU on *T. gondii* was first demonstrated by the studies involving the isolation of a HU-resistant mutant strain of the parasite[30] and later by our group.[31] We also have shown the reduction on *T. gondii* infection during treatment with HU and also with the antimitotic compounds of the thiosemicarbazone classe[31-34] and that tachyzoites-containing PV fuses with lysosomes and the parasites are eliminated during the treatments.[35] However, little is known about the progressive events associated with the action of HU on intravacuolar parasites. We propose here to investigate the progressive steps involved in *T. gondii* death and elimination during HU treatment.

METHODS

Host cells

Vero cells (African green monkey kidney fibroblasts) obtained from Adolfo Lutz Institute, São Paulo-Brazil, were grown in plastic Falcon flasks (25 cm^2) in complete Dulbecco´s modified Eagle´s medium (DMEM 1152, SIGMA ALDRICH – St. Louis, Missouri, USA), supplemented with 5% fetal bovine serum (FBS, GIBCO – WALTHAM, MA, USA), 2 mM L-glutamine, 50 U of penicillin/mL, and 50 μg of streptomycin/mL. Cultures were trypsinized when the cell density approached to the semi-confluent monolayer. One day before use in the experiments, approximately $4x10^4$ cells were placed on Linbro tissue plates (24 wells) that contained a round sterile coverslip, or were inoculated into 75 cm^2 flasks (3–$5x10^5$ cells/flask), and maintained at 37 °C overnight, under a 5% CO_2, 95% air atmosphere.

Parasites

Tachyzoites of *Toxoplasma gondii* (WT) from the virulent RH strain were maintained by intraperitoneal passage in Swiss mice or in cell culture (red fluorescent protein - tagged tachyzoites[37], as describe in.[35] The pellet obtained was washed with PBS, pH 7.2 and suspended to a density of 10^7 parasites/ml in DMEM medium without fetal calf serum. The parasites were used about 30 min after removal from infected animals, and the viability was evaluated using a dye-exclusion test with trypan blue 0.2% (w/v).

Host cell–parasite interaction

Vero cells were infected with tachyzoites of *T. gondii* for 2 h, at 37ºC, using a 5:1 parasite–host cell ratio. After incubation, the cells were washed twice with DMEM medium to remove

extracellular parasites, and incubated for 2, 8, 12 or 24 h at 37 °C, 5% CO_2 – 95% air atmosphere. After that HU was added to the cultures, as described below.

Hydroxyurea treatments

HU was dissolved in DMSO (Dimethylsulphoxide – SIGMA ALDRICH) and added to DMEM; DMSO was used at a final concentration of 1% (V/V), which was not toxic neither for Vero cells nor intracellular *T. gondii* (data not shown). A screen of HU concentrations (0.1, 2, 4, 8 or 20 mM) was performed with infected cultures for 24h. To observe the successive stages of destruction we used the standard concentration of HU – 4 mM[31] for 1-48h of incubation times.

In order to determine the time span required to observe the anti-proliferative effect of HU, a reversibility assay was performed. The medium was removed and infected Vero cultures were treated with 0.1 mM or 4 mM of HU for 5, 12 or 24 h, at 37ºC, 5% CO_2. After that, the medium containing the drug was replaced by fresh culture medium without HU to monitor subsequent tachyzoite proliferation. The samples were fixed at 12, 24 and 48 h, at 37 ºC, 5% CO_2.

Viability assay

To assay the viability of treated and untreated host cells, trypan blue (0.2% W/V) or 6-carboxyfluorescein diacetate (cFDA-SE - SIGMA), a cell-permeant, amine-reactive probe, were used.[38] The viable cells excluded trypan blue, but were positive for cFDA-SE (data not shown). In addition, viability evaluation was also performed by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT - SIGMA) assay. Briefly, $1x10^5$ Vero cells were cultivated overnight in 96-well plates. After that cells were infected at MOI 5:1 with *T. gondii* and incubated with HU (4mM) for 2, 12, 24 and 48h, 37ºC, 5% CO_2. After each treatment point, MTT solution (6mg/mL) was added to each well at final concentration 1mg/mL. The plates were incubated for more 4 hours at 37ºC. Then the medium was taken out and 200 µl of DMSO was added to each well and homogenized. Next, the absorbance of each time point of treatment was obtained at 540 nm. Each experiment was performed in triplicate and the percentage of viable cells was calculated in relation to untreated cultures.

Morphological analyses and quantification

Uninfected and infected cultures treated or not with HU were rinsed with PBS, pH 7.2, at 37ºC. After this, the cells were fixed with Bouin´s solution for 15 min and stained with Giemsa (10% V/V) (SIGMA) for 2 h at room temperature. All preparations were examined using a Zeiss AXIOPLAN photomicroscope equipped with a 40X objective. The images were obtained with AnaliSIS software or Cell*F Image System software with a DP72 Olympus camera.

In the same monolayer, the following parameters were analysed in infected treated or untreated cultures: a) mean number of normal (crescent shaped) intracellular parasites; b) percentage of infected cells containing parasites with normal morphology; c) percentage of infected cells containing parasites with altered morphology; d) mean number of parasitophorous vacuoles containing altered parasites. At least 300 cells in the same sample were scored by counting 10 randomly chosen fields at a magnification of X400.

Ultrastructural analyses

Vero cells were cultivated in culture flasks (75 cm^2) and infected with parasites as described above. After incubation, the cultures were washed with PBS at 37ºC and fixed, at room temperature, in a solution containing 1% glutaraldehyde, 4% formaldehyde, 5 mM $CaCl_2$, and 5% sucrose in 0.1 M cacodylate buffer, pH 7.2. Then, cells were post-fixed for 1 h in a solution containing 2% OsO_4, 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. Samples were rinsed with 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in PolyBed (Polyscience Inc., - WARRINGTON, PA, USA). Thin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss 900 Transmission Electron Microscope, at 80 kV acceleration.

Parasite death

To check parasite death following HU treatment, the impermeant DNA intercalating YOPRO-1 (YP) (Molecular Probes, Eugene, OR, USA) was used as previously described.[39] Briefly, infected Vero cells were treated for 24 h with 4 mM HU. After that, the medium was removed and cultures were incubated with 10 µM YP for 10 min, at 37ºC. Live cells were immediately analysed using a Zeiss Confocal Laser Scan Microscope (CLSM) with a 488 nm argon laser.

PV–lysosome fusion

Infected treated or untreated cultures were incubated with a solution of the dye acridine orange (AO) (SIGMA), *Lysotracker red* (LTr) (MOLECULAR PROBES – EUGENE, OREGON, USA); anti-LAMP-2 antibody of hybridoma supernatants (Development Studies Hybridoma Bank – IA) was gently provided by Dr. Renato Mortara from UNIFESP-Brazil. Acridine orange and *Lysotracker red* were incubated in live cells, respectively, at a final concentration of 5 µg/mL and 50 nM, for 30 min, at 37ºC, 5% CO_2. Both dyes were diluted in DMEM without FBS and incubated in the cultures in the absence of light. Subsequently, the samples were washed with culture medium and examined in a Zeiss Confocal Laser Scan Microscope (CLSM), using a 488 nm or 543 nm argon laser. The presence of the LAMP-2 antigen associated with PV was evaluated by immunofluorescence in cultures fixed at different times after infection and treatment. Infected treated or untreated cells were washed with PBS and fixed with 4% formaldehyde for 15 min; cultures were then washed three times with calcium- and magnesium-free PBS and permeabilized with 0.1% saponin (BDH, Amersham, United Kingdom) in PGN (PBS containing 0.2% gelatin and 0.1% NaN_3). Cells were then incubated with anti-LAMP-2 (ascitic fluid diluted 1:50 in PGN) for 1 h at room temperature, washed three times with PBS and developed with fluorescein-labeled goat anti-mouse IgG (SIGMA), diluted 1:100 in PGN for 1 h. After three washes with PBS, coverslips were mounted in Dako fluorescent mounting medium (DAKO NORTH AMERICA, VILA REAL CARPINTERIA, CALIFORNIA, USA).

Mitochondrial functionality

To ensure the viability and functionality of cells during treatment with HU, the mitochondrial potential was analysed by incubating cells with Rhodamine 123 (SIGMA), a dye which accumulates in functional mitochondria, at 5 µg/mL for 30 min, at 37ºC, 5% CO_2. Live cells were analysed in a Zeiss Confocal Laser Scan Microscope (CLSM).

Detection of autophagic vacuoles

Infected treated (12 or 24 h of HU incubation at 4 mM) or untreated cultures were incubated with a solution of the dye Monodansylcadaverine (SIGMA), an *in vivo* marker for autophagic vacuoles, at 50 µg/mL for 10 min, at 37ºC.[40] The cells were visualized at Zeiss Axioplan microscope with an 335 nm excitation filter.

Ethics statement

Animal experimentation was conducted according to SBCAL/COBEA ethic guidelines (Sociedade Brasileira de Ciências de Animais de Laboratório/Colégio Brasileiro de Experimentação Animal). The present study was approved by CEUA-UENF (Comissão de ética de uso de animais - Universidade Estadual do Norte Fluminense) under the protocol number 104.

Statistical analyses

The results were analysed with the software Prism 4.0 package, using one- or two-way analysis of variance (ANOVA) and the Bonferroni post-test. Graphs display means and standard errors. The data shown are representative of three experiments in triplicate or quadruplicate. Results were considered significant at $p<0.05$.

RESULTS

Progressive toxic effect of HU on uninfected and infected host cells

In previous reports we had shown that after Hydroxyurea incubation at 4mM concentration intravacuolar tachyzoites were completely disrupted, resulting in decreasing of infection *in vitro*.[31,35] However the events that contribute to the progressive tachyzoite destruction were not described. Thus, we are proposing here to analyze the relationship between parasite proliferation and HU action aiming to understand the biology of the host cell-parasite interaction.

Infected Vero cell cultures were incubated with crescent doses of HU (0.1–20 mM) for 24 h. Initially, parasite proliferation was arrested at 0.1 mM and after that, the infections progressively decreased (Fig. 1A, B) with increasing the dose (2–20 mM). The viability of infected and uninfected cultures by MTT assay using HU at 4mM was performed (Fig 1 C, D). The treatments did not reduced significantly the cell number after a long of the time of incubation (2, 12, 24, 48h) in both uninfected (Fig C) or infected Vero culture (Fig D). Nonetheless, untreated cultures showed a significant increase in the absorbance measure after 48h (Fig C, D) while treated host cell did not. This result suggest that the proliferation of treated cells did not occur in presence of HU. We also confirmed the Vero cells viability by assays with trypan blue and cFDA-SE staining, which treated cells were negative for trypan blue and positive for cFDA-SE (data not shown). Together, these results suggest that HU was efficient in arresting *Toxoplasma* multiplication from 0.1 mM concentration with

intravacuolar parasite disruption; the relationship between the duration of drug incubation and the particular progressive disorganization of the parasite is described below.

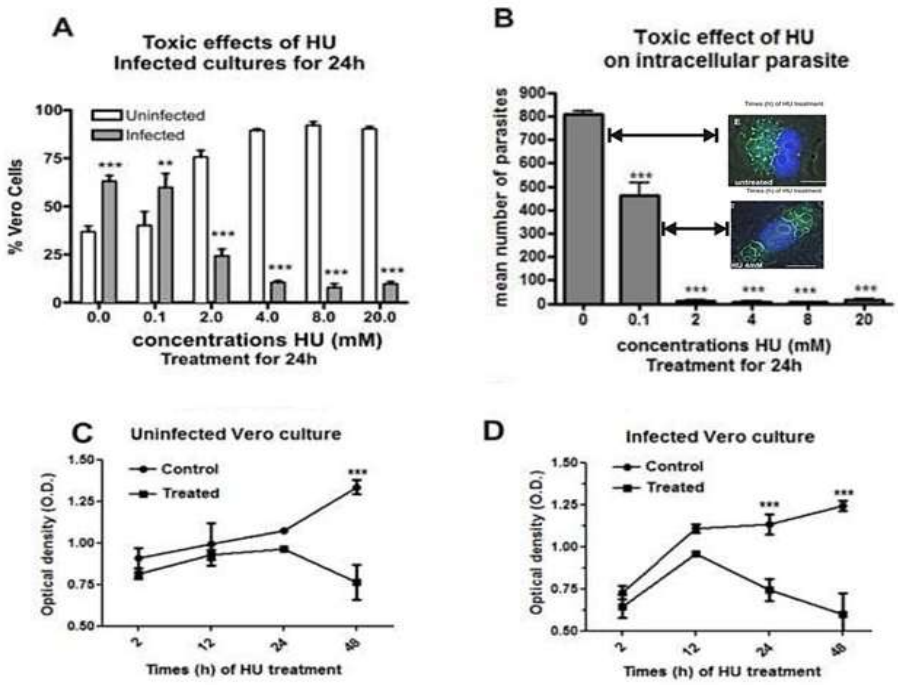

Figure 1. Effect of HU concentrations on infected culture. (A) Percentage of infected (gray bars) and uninfected (white bars) cells in cultures infected with *T. gondii* tachyzoites for 24 h, and incubated with HU at 0, 0.1, 2, 4, 8, and 20 mM for 24 h. (B) Mean number of intracellular tachyzoites after incubation with HU for 24 h. (C) and (D) absorbance of MTT assay in uninfected and infected culture, respectively. DAPI: nucleus. A, B: **P<0.01;***P<0.001. C, D: ***P<0.001 of control related to the point of treatment. Bar: 20 µM.

Progressive stages of structural disorganization of intravacuolar tachyzoite

We analyzed intracellular parasite disorganization and elimination in progressive times (1-36 h) of HU incubation (4mM) (Fig. 2). Tachyzoites of the RH strain multiply at 5 h 20 min post infection.[21] Then, we analyzed the effect of HU on different stages of parasite division, recording three parameters: 1) the percentage of uninfected cells; 2) the percentage of infected cells containing morphologically normal parasites; and 3) the percentage of infected cells containing altered parasites.

Until the first 5 h of infection, the incubation with HU did not reduce the mean number of intracellular parasites (Fig 2 A). Nonetheless, after the first replication cycle (8 h) the cells had non-proliferative and morphologically disorganized intravacuolar tachyzoites and a

significant elimination of parasites (Fig. 2A). The parasite were almost totally eliminated with 12 h of HU incubation (the equivalent of two replication cycles), while the control increased continually. The morphological features of these effects were observed using light microscopy. Following endodiogeny interruption, Altered tachyzoites-containing PVs (Fig. 2B) were observed, while a decrease in the number of PV containing 2 parasites also occurred (Fig. 2B, inset).

To observe whether the parasite viability were compromised after 5 h of HU incubation, the drug-containing medium was replenished by fresh medium (without HU) for 24 and 48 h (Fig. 2C, D). After 5 h, the percentage of infected cells (Fig. 2C) and the mean number of intracellular parasites were similar to those observed in untreated cultures (Fig 2 A). Even in the absence of HU, the majority of parasites underwent alterations and did not recover their ability to multiply being eliminated from the cell culture (Fig. 2D), while some unaltered tachyzoites returned to cell cycle division in the PV (Fig. 2C, D). These results can be associated to the assynchronic infection in the culture, resulted from tachyzoite host cell invasion at different times.

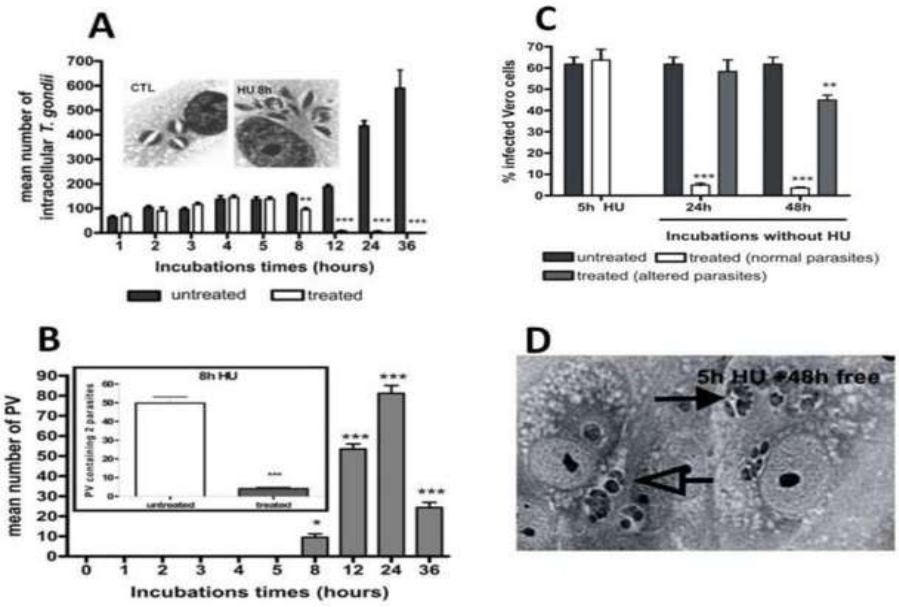

Figure 2. Quantification of successive stages of tachyzoite destruction in infected Vero cell cultures. (A) Mean number of normal parasites in untreated (black bars) and treated Vero cells (white bars) with HU. Light microscope images in the same figure show the morphology of the intravacuolar parasite in untreated cultures (left side control-CTL) and those treated with HU for 8 h (right side). (B) The mean number of altered parasites-containing parasitophorous vacuole in treated cultures determined from the same experiment

shown in A. Inset B: number of vacuoles containing two parasites in the untreated (white column) or treated cultures (gray column) after 8 h of treatment. (C) Untreated Vero cells infected for 2 h (black bar), treated culture containing normal parasites (white bar) and altered parasites (gray bar). The 5 h-incubated cultures (left side) were reincubated with fresh medium for 24 and 48 h (right side). (D) Morphological appearance of intravacuolar parasites treated with HU for 5 h and incubated for more 48 h in fresh medium without drug. All treatments with HU were at 4 mM. Open arrow: disrupted intravacuolar parasite; Black arrow: normal intravacuolar parasites. *P<0.05; **P<0.01; ***P<0.001.

Given that each mitotic cycle of the virulent strain RH (Type I) occur between 6 and 8 h and that the tachyzoite does not have a G2-phase[42-43], the results described here suggest that 5 h is the minimum period of time required for the effective action of HU on parasites. After 5 h, the dead parasites initiated progressive morphological alterations.There is strong evidence that HU acts mainly on the intravacuolar parasite. Indeed, this hypothesis is supported by the identification of a single aquaporin gene in *T. gondii* that codifies the aquoporin protein, a bifunctional membrane pore that is permeable to hydroxyurea.[44] Once *Toxoplasma* is exposed to HU, the effect of the drug is irreversible, suggesting that unlike mammalian cells, tachyzoites have no check-point for such damage.

Ultrastructural aspects associated with progressive *T. gondii* elimination

To observe the destruction associated with *T. gondii* elimination, we used the transmission electron microscopy (TEM). The 2 and 12 h-infected nucleated cultures (the beginning and an established infection, respectively) were treated with HU at 4 mM for 4 and 8 h. After 2 hours of infection the parasites showed usual morphology (Fig. 4 A); however after 8 h of treatment they were highly destroyed (Fig. 4 B). The 12 h-infected cultures were treated for 4 h (Fig. 4 C) and 8 h (Fig. 4 D) with HU at 4 mM. The short time of incubation (4 h) did not change the parasite morphology, contrasting with the results observed after 8 h, when intracellular tachyzoites were completely disorganized (Fig 4 D). The host cell's organelles such as mitochondria remained associated with the membrane of the PV, as observed in control cultures. Taken these results together, we suggest that after 8 h of incubation with HU, intravacuolar parasites underwent a progressive digestion process.

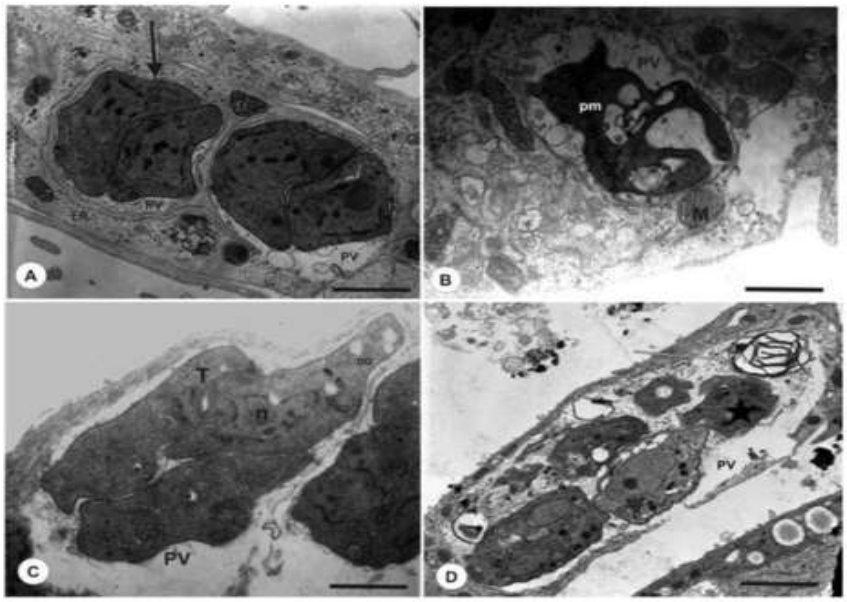

Figure 4. Ultrastructural analyzes of intravacuolar tachyzoites after treatment with HU at 4mM. (A) The eletron microscopy of untreated cells showed normal tachyzoites inside the PV. (B) Vero cells infected for 2 h and treated for 8 h with HU or infected for 12 h (C, D) and treated for 4 (C) and 8 h (D) with HU. PV: parasitophorous vacuole; ER: endoplasmic reticulum; pm: parasite mitochondria; M: host cell mitochondria; T: tachyzoite; DG: dense granules. Bar: 3 µm.

Progressive events involved in parasite elimination

The toxic effects on the parasite began with gradual and progressive changes in structural organization and they drastically increased when the parasite started its mitotic division (Fig. 5 A1-12). The mechanism involved with this structural disorganization is the fusion of acid compartments as lysosomes with the parasite-containing PV, which also began soon after the parasite starts its division cycle (Fig. 5 A6-12).

Initially, we investigated PV acidification and the PVM–LAMP-2 association in infected cultures. After 5 h of treatment, normal parasites-containing vacuoles were not acidic as well as the control cultures (Fig. 5 B, C), as shown by live cell staining with acridine orange (data not shown) and *Lysotracker red* after 5 h of treatment. However, after 8 h of treatment, some altered parasites-containing vacuoles were positive for *Lysotracker red* (Fig. 5 D, E). Contrasting with the acidification results, the measurement of PVM-LAMP-2 showed a contant increase in 5, 8 and 24 h of treatment with HU (Fig. 5 F, G, H). These results suggest that the association of LAMP-2 and PV starts around 5h after HU incubation, before the acidification, just seen at 8 hours. Besides, the parasite disorganizations were not dependent

of the acidification of the PV, as observed in the culture treated for 8 h and stained with acridine orange, suggesting that parasite disorganizations are resulted of the parasite death.

Soon afterward, we incubated a 24 h-infected and 24 h-treated cells with YOPRO-1(YP), a marker for fragmented DNA, to analyse the main features of parasite death. It was verified that intravacuolar parasites were positive stained for YP, an indicative of occurrence of apoptosis (Fig. 5 I). Untreated and uninfected cells were not stained with YOPRO-1 (not shown). Finally, infected untreated (J-L) and treated (N-Q) cultures (12 and 24 h of HU at 4 mM) were incubated with monodansylcadaverine to verify the formation of autophagic vacuoles (Fig. 5 J – Q). Since 12 h of incubation it was possible to observe positive stainings circumventing destroyed parasites (Fig. 5 J-M), which also continued until 24 h of incubation (Fig. 5 N-Q).

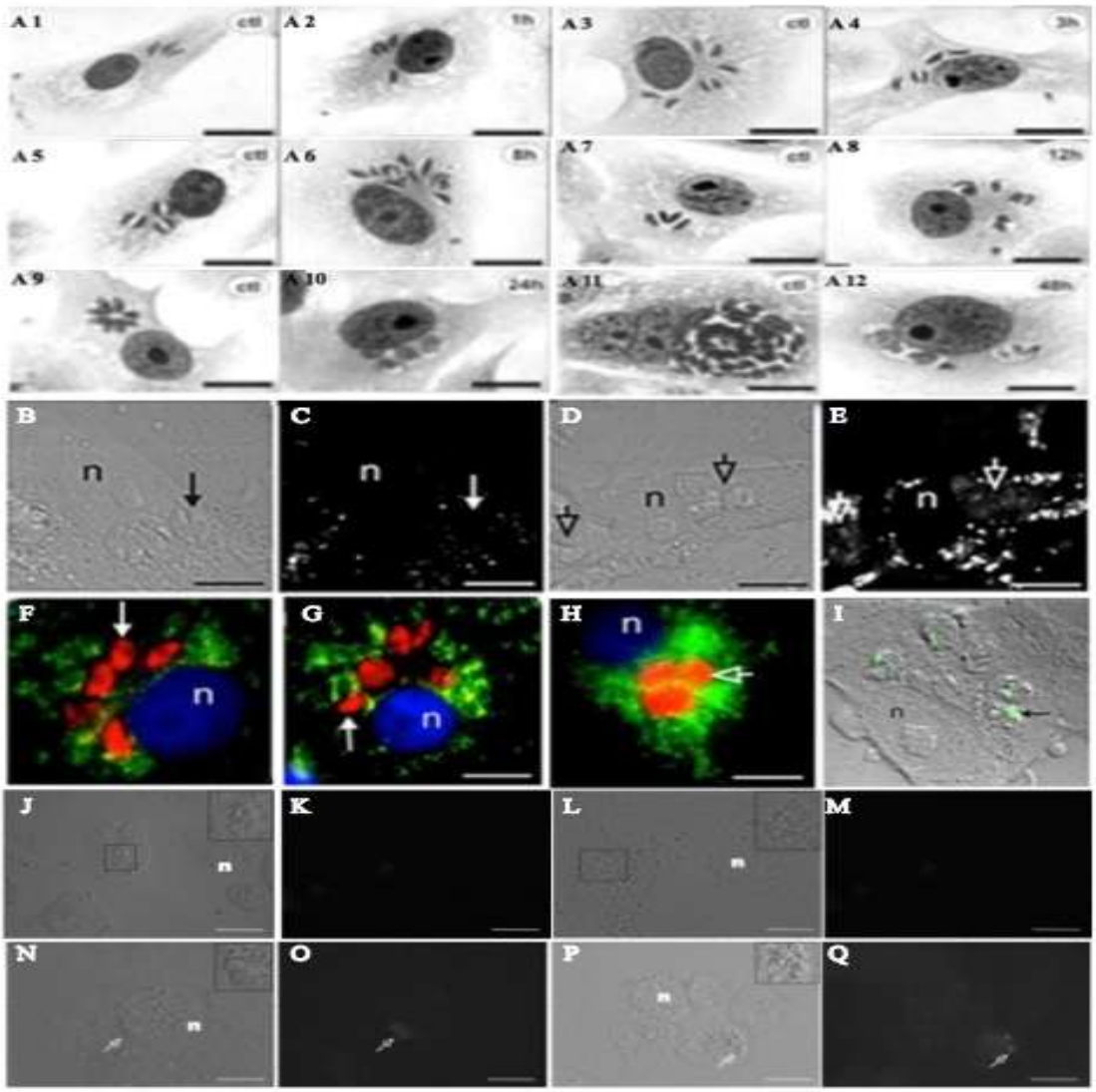

Figure 5. The morphological aspects of progressive destruction of *T. gondii.* (A) Light

microscopy of Vero cells infected for progressive time with *T. gondii*, showing the successive stages of parasite destruction after HU incubation. The untreated (control) cultures are shown in A1, A3, A5, A7, A9 and A11, while cultures treated with 4 mM HU during several time points (1, 3, 8, 12, 24 and 36 h) are shown in A2, A4, A6, A8, A10, A12, respectively. (B and C) The 12 h- infected cells were treated with HU for 5 h or (D and E) 8 h and incubated with LisoTracker Red to visualize lysosomes-PV fusion. PV-integral parasites were not positive for LisoTracker Red (B and C – arrow), while at 8 h (D and E) they were acidified (D – arrowhead). The 12 h- infected cells were treated with HU for 5 h or (G) 8 h or (H) and immunostained with LAMP-2, showing changed parasites-containg PV with LAMP attached to it. (I) YOPRO-1 stain in 24 h-infected cells and treated with HU for 24 h contained fluorescent altered parasites indicating DNA fragmentation. (J and K) The 12 h-infected untreated cells, (L and M) 24 h-infected untreated cells, (N and O) 12 h-infected treated cells and (P and Q) 24 h-infected treated cells, all labelled with monodanasylcadaverine to monitor the autophagic vacuole formation, which was seen since 12 h of HU incubation. All HU treatments were at 4 mM. N: host cell nucleus. Bar A and I: 25 μm. Bar B-H: 10 μm. Bar J-Q: 50 μm.

Others important intracellular parasite protozoan such as *L. amazonensis* and *T. cruzi*[45], HU led to the arrest of multiplication, but the cellular mechanism responsible for this was unknown. In the case of the prokaryotic organism *Escherichia coli* exposured to HU produced deleterious hydroxyl radicals, which contributed to the majority of the bacteria death[46]. We have shown that after intracellular *Toxoplasma gondii* treatment with HU, for at least 12 h, the destroyed parasite-containing PV becomes acidified.[35] Here we demonstrated that this event occurs within a short period of time, at 8 h of HU incubation, suggesting that from this time the parasites are no longer able to maintain the PV as a safe environment. *Toxoplasma* death included morphological alteration and DNA fragmentation, as demonstrated using YOPRO-1. This dye is usually used to detect apoptosis events and it is not toxic for viable cells.[39,47] In fact, the plasma membrane becomes slightly permeable during apoptosis and this modification allows the uptake of YOPRO-1 but not propidium iodide (PI).[48-49] In the present study, the majority of infected cells treated for 24 h with HU incorporated YOPRO-1, which stained the intravacuolar parasite, but not the host cell, suggesting that the host cells were viable and did not become apoptotic. Once the parasite was disrupted, PV–lysosome fusion occurred (8 h of HU incubation), suggesting that parasite

death was not caused by lysosome fusion, but resulted from the inability of *T. gondii* to maintain its vacuole as a safe environment.

Kinetic studies using the LysoTracker Red to monitor the PV – lysosome fusion showed that the minimum time required to occur the fusion is 8 h of HU incubation, which combines with the time necessary to drastically reduce the number of intracellular parasites.

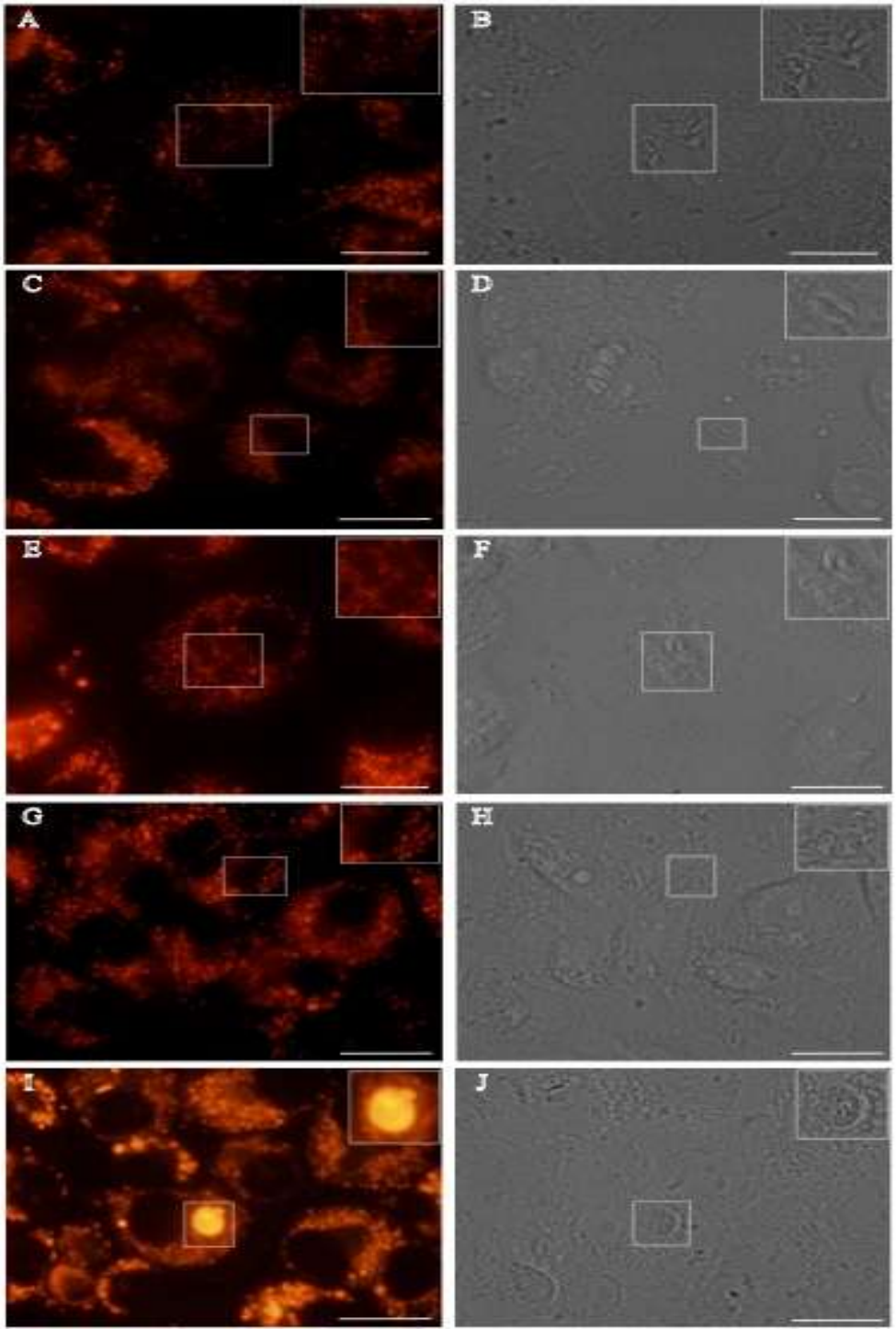

Figure 6. Kinetic study of parasitophorous vacuole-lysosome fusion in presence of 4 mM

of Hydroxyurea. (A, B) Infected untreated cultures. (C and D) Infected treated cultures incubated for 6 h, (E and F) for 7 h, (G and H) for 7,5 h and (I and J) for 8 h. Scale bars: 50 μm.

Host cell mitochondrial functionality during *T. gondii* elimination

It is well known that host cell mitochondria is associated with tachyzoites-containing PV during parasite infection.[9,41] So, we investigated whether the host cell can recover mitochondrial functionality and distribution during the parasite destruction using the marker Rhodamine 123. Confirming previously reports, during *T. gondii* infection the mitochondrias were found around the PV (Fig. 7 C and D). After HU incubation during 12 h (Fig. 7 E, F), 24 h (Fig. 7 G, H) and 48 h (Fig. 7 I, J), when the parasites were destroyed, the mitochondrias were gradually distributed into the host cytoplasm. After 48 h of treatment, the distribution of the functional mitochondrias (Fig. 7 I, J) was similar to uninfected cells (Fig. 7 A, B).

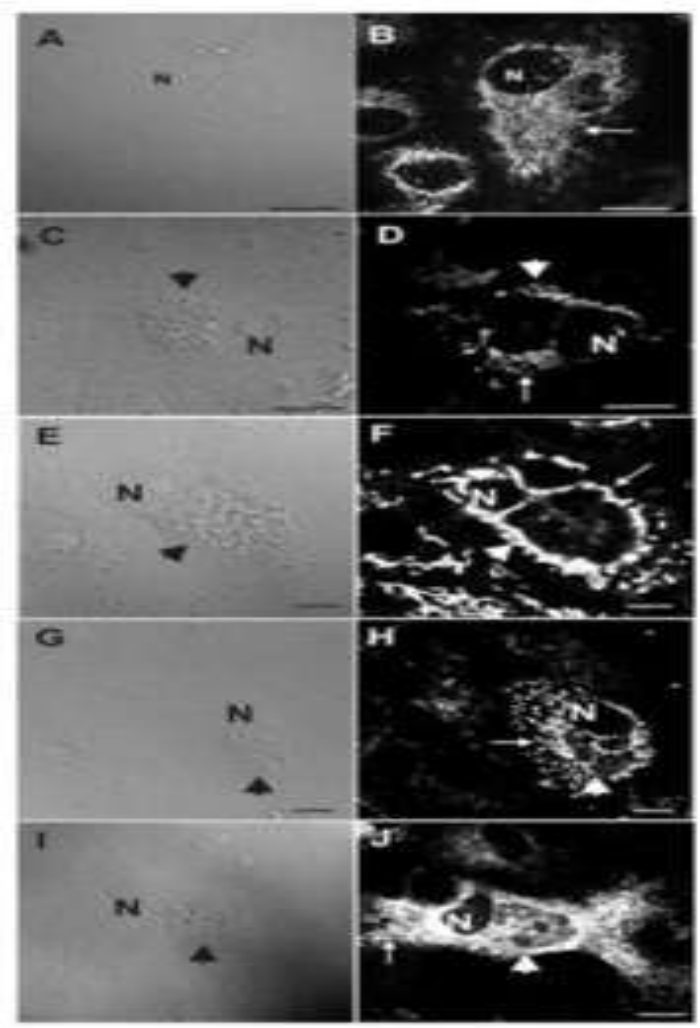

Figure 7. Mitochondrial functionality and distribution during *T. gondii* destruction. Uninfected Vero cells (A, B), or infected cells for 24 h (C, D) were treated with HU at 4mM

for 12 h (E, F), 24 h (G, H) and 48 h (I, J) and incubated with vital fluorescent dye – Rhodamine 123. Arrow: functional mitochondria; arrowhead: PV containing tachyzoites and N: host cell nucleus. Bar: 10 μM.

During *T. gondii* infection the host cell mytochondrias are close related with the Parasites-containing parasitophorous vacuole and their functionality decrease as infection develops.[9] So, finally we demonstrated that host cells resume their cytoplasmic distribution of functional mitochondrias during parasite elimination. In fact, the association of mitochondria with PV was suggested to arise from the stable association between ROP2 and the protein domain in the mitochondria[50]; however, *Toxoplasma* may utilize other as yet unidentified factors to recruit host mitochondria to its PV; in any case, this ability seems to be missing after HU treatment. We also have to consider that mitochondria–PV disassociation could facilitate lysosome–PV fusion.

Overall, the results presented in this paper suggest that *T. gondii* is the main target of HU in infected cells and that the parasite has to be viable to remain in a safe environment. PV manipulation seems to be necessary throughout parasite development and not only at the point of invasion.

CONCLUSION

These results suggest two interesting insights to the use of HU. Firstly, HU may be a useful chemical basis for the development of effective drugs against *Toxoplasma gondii* since HU arrests the parasite replication and its elimination by the host cell. Another point is that by using HU as a modulator of parasite development associated with molecular tools, it could be possible to find key steps in the control of the parasite cell cycle and also in the host cell microbial defense system including others Apicomplexa parasites or even *Leishmania sp*. and *Trypanosoma cruzi*.

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