Study of *Trypanosoma cruzi* epimastigote cell death by NMR-visible mobile lipid analysis

DIEGO BENITEZ[1], HORACIO PEZAROGLO[2], VERÓNICA MARTÍNEZ[2], GABRIELA CASANOVA[3], GONZALO CABRERA[4], NORBEL GALANTI[4], MERCEDES GONZÁLEZ[1] *and* HUGO CERECETTO[1]*

[1] *Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Montevideo, Uruguay*
[2] *Laboratorio de Resonancia Magnética Nuclear, Facultad de Ciencias-Facultad de Química, Universidad de la República, Montevideo, Uruguay*
[3] *Unidad de Microscopía Electrónica de Transmisión, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay*
[4] *Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*

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SUMMARY

Cell death mechanisms in *Trypanosoma cruzi* have not been disclosed in detail though different conventional techniques have been used in the classification of parasite-cell death type. Nuclear magnetic resonance (NMR) has successfully been used as a tool to evaluate the onset of apoptosis in a number of higher eukaryote-cell models analysing the ratio of CH_2/CH_3 integration from the visible mobile lipids (VML). Surprisingly, this versatile non-invasive spectroscopy technique has never been employed with this purpose in *T. cruzi*. In the present study it is shown that under different parasite death-conditions the ratio CH_2/CH_3 varied drastically. Thus, *T. cruzi* epimastigotes in apoptotic conditions increase significantly this ratio while in necrotic as well as in autophagic situations the parasites maintain the VML, CH_2/CH_3 ratio, in normal values. Additionally, other VML markers commonly used in these studies, such as the change in the region of methyl-choline moiety, $-N^+(CH_3)_3$, exhibited different particular patterns according to the type of cell death. Our results suggest that the ^{1}H NMR-VML technique is an adequate tool to discriminate different *T. cruzi* death pathways.

Key words: *Trypanosoma cruzi*, apoptosis, necrosis, autophagy, NMR, visible mobile lipids, choline.

INTRODUCTION

Cellular death types and involved pathways in unicellular parasites have great relevance to the understanding of the development and evolution of infections (Besteiro *et al*. 2006). Programmed cell death (PCD) is a regulated process allowing organisms to eliminate cells, avoiding an inflammatory response (Hengartner, 2000). PCD, including apoptosis and autophagy, is well characterized in higher eukaryotes. Apoptosis (type I PCD) is a regulated mechanism of self-killing, involved in many biological processes, that is characterized in mammalian cells by cell volume reduction, extracellular phosphatidylserine exposure, caspase activation, internucleosomal DNA fragmentation, blebbing of the plasma membrane, formation of apoptotic bodies and loss of mitochondrial membrane potential with cytochrome *c* release into the cytoplasm (Menna-Barreto *et al*. 2009*a*). *Trypanosoma cruzi* (*T. cruzi*) was the first unicellular organism in which apoptosis was described (Ameisen *et al*. 1995). Autophagy (type II PCD) is the major mechanism used by eukaryotic cells for bulk degradation of proteins and organelles and is essential for maintaining cellular homeostasis and for cellular development during differentiation, metamorphosis, and aging (Alvarez *et al*. 2008*a*). Type II PCD is characterized by the formation of autophagosomes, portions of cytoplasm engulfed in double-membrane vesicles, cytoskeletal preservation and absence of inflammatory response (Levine and Yuan, 2005). Considering that *T. cruzi* differentiation inside the human host is mediated by autophagy it has been proposed that the inhibition of this process could be an alternative to control the infection (Alvarez *et al*. 2008*b*). In protozoa, PCD may play a role in the regulation of parasite number in biological vectors and in definitive host tissues, in the maintenance of clonality, as a mechanism of immunomodulation and of parasite differentiation (Welburn *et al*. 1997; Freire-de-Lima *et al*. 2000; Alvarez *et al*. 2008*a*). On the other hand, necrosis is defined as a non-apoptotic cell death process, displaying some aspects of programmed cellular suicide. It is characterized by dramatic alterations in mitochondria, ATP depletion, generation of reactive

* Corresponding author: Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay. Tel: +598 2 525 86 18 (7-216). Fax: +598 2 525 0749. E-mail: hcerecet@fq.edu.uy

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oxygen species, loss of calcium homeostasis, cytoplasmic vacuolization and, finally, breakdown of the plasma membrane with an inflammatory response (Zong and Thompson, 2006). Different techniques have been employed in the study of *T. cruzi* cell death, i.e. destructive techniques such as flow cytometry, fluorescence microscopy, Western blot, agarose-gel electrophoresis, and ultrastructural analysis or less destructive ones like protease inhibition analysis (Alvarez *et al*. 2008*a*, *b*; Jiménez *et al*. 2008; Menna-Barreto *et al*. 2009*b*; Irigoín *et al*. 2009).

Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive method that can be used to study the metabolism of cells and tissues as well as to monitor the effects of drugs both *in vitro* and *in vivo* providing dynamic information for the study of biological systems (Griffiths *et al*. 2000; Boiani *et al*. 2008; Caterina *et al*. 2008). The phenomenon of mobile lipid accumulation following the induction of either apoptosis or cytostasis could be studied by NMR because of its capability to visualize these lipids (visible mobile lipids, VMLs) as a more appropriate tool to denote some types of cellular death. Several lipid-associated signals are visible in ^{1}H NMR spectroscopy, the most important ones being the methylene ($-CH_2-$) signal near to 1·3 ppm, and the methyl ($-CH_3$) signal at 0·9 ppm. Increased methylene resonances in ^{1}H NMR spectra resulting from the accumulation of VMLs correlate with the onset of apoptosis in a number of drug-treated cell models (Blankenberg *et al*. 1996) reflected in an increase in the CH_2/CH_3-ratio (Mikhailenko *et al*. 2005), and in some cases increment in the signals from polyunsaturated fatty acids (PUFA) at 2·8 and 5·4 ppm (Hakumäki *et al*. 1999). Other modified signals are those from the choline-containing compounds (choline –Cho-, phosphatidylcholine –PTC-, phosphocholine –PC-, as well as taurine and myo-inositol) near to 3·2 ppm which have been associated with apoptosis and cell loss (Milkevitch *et al*. 2005). However, changes in these signals appear not to be universal, at least in the early stages of apoptosis (Lehtimäki *et al*. 2003). The biogenesis, biochemical nature and subcellular localization of the lipids that originate these VML in NMR are still under debate; however, the ^{1}H NMR spectroscopy has been accepted as a reliable tool to elucidate lipid biochemistry mechanisms involved in eukaryotic cells and tissues (Hakumäki and Kauppinen, 2000; Quintero *et al*. 2007).

The aim of this study was to determine whether the ^{1}H NMR-VML technique is able to distinguish different epimastigote-*T. cruzi* death processes. Our results show that the CH_2/CH_3 ratio and changes in the region of the methyl-choline moiety, $-N^+(CH_3)_3$, but not modifications in the PUFA signals, could be used to distinguish between the types of *T. cruzi* death pathways applying this non-invasive technique.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma-Aldrich unless otherwise indicated. Nifurtimox was purchased from Bayer.

Cell cultures

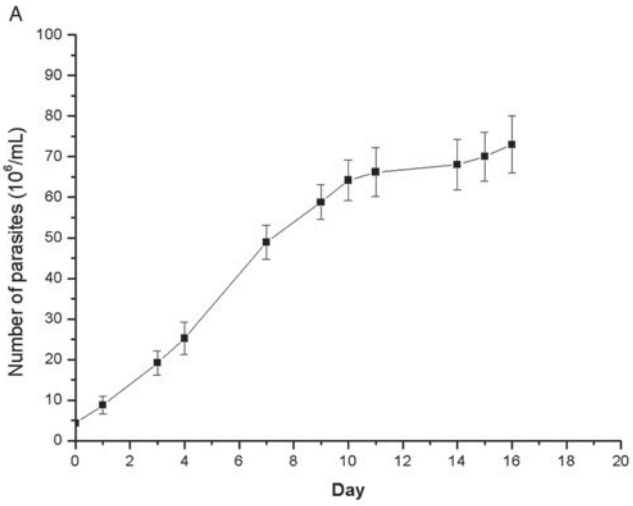
For ^{1}H NMR studies, *T. cruzi* epimastigote forms (Y strain) were cultured at 28 °C for 5–7 days (exponential phase of growth) under aerobiosis in axenic BHI-tryptose milieu (33 g/l brain-heart infusion, 3 g/l tryptose, 0·02 g/l hemin, 0·3 g/L D-(+)-glucose, supplemented with 10% (v/v) calf serum, 200 000 units/l penicillin and 0·2 g/l streptomycin). For TUNEL and propidium iodide- staining studies, *T. cruzi* epimastigote forms (Y strain) were cultured at 28 °C for 5–7 days (exponential phase of growth) under aerobiosis in axenic Diamond milieu (12·5 g/l yeast extract, 12·5 g/l tryptose, 12·5 g/l tryptone, 106 mM NaCl, 29 mM $H_2K_2PO_4$, 23 mM HK_2PO_4, pH 7·2, 7·5 mM hemin supplemented with 10% (v/v) calf serum, 75 units/ml penicillin and 75 mg/l streptomycin).

Trypanosoma cruzi *treatments*

Growth in axenic culture. Cultures of Y strain epimastigotes in the exponential growth phase (5×10^6 parasites/ml) were washed with PBS, re-suspended in BHI-tryptose milieu and then subjected to culture for 14 days. Samples (10 μl) were fixed in 90 μl of formalin (3·8%, w/v) and the number of epimastigotes was determined by counting in a Neubauer chamber. For ^{1}H NMR studies-processes see section 2.6.

H_2O_2 or nifurtimox. Cultures of Y strain epimastigotes in the exponential growth phase (150×10^6 parasites/ml) were incubated with 2·6–530 μM (per 10^6 parasites/ml) freshly prepared H_2O_2 (for 30 min at 28 °C) or with 2 μM nifurtimox (per 10^6 parasites/ml, for 48 h at 28 °C). Treatment was stopped by centrifugation at 3000 ***g*** and the sediment was re-suspended in PBS. *T. cruzi* epimastigotes under the same conditions but without treatment were included as controls. For ^{1}H NMR studies-processes see below.

Starving of cells. Cultures of Y strain epimastigotes in the exponential growth phase (150×10^6 parasites/ml) were washed twice with PBS, re-suspended in PBS at 50×10^6 cells/ml, and incubated for 24 h at 28 °C. Treatment was stopped by centrifugation at 3000 ***g*** and the sediment re-suspended in PBS. *T. cruzi* epimastigotes at the same conditions but



B

Day of culture	CH_2/CH_3 ratio[a]
4	1.00 ± 0.03
7	1.29 ± 0.04*
14	1.47 ± 0.01*

[a] Expressed respect to the ratio of 4th day. * $p < 0.05$ (Student's t-tests).

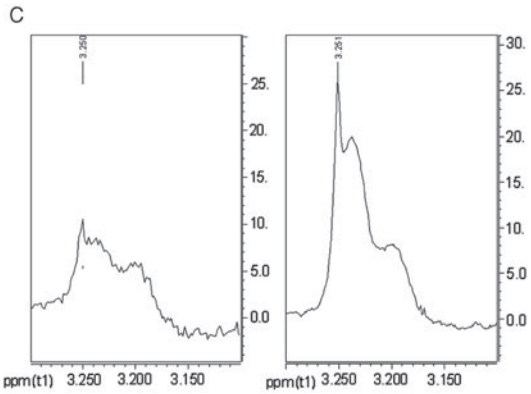


Fig. 1. Changes in the VML evaluated by ^{1}H NMR spectroscopy for *Trypanosoma cruzi* epimastigotes (Y strain) in axenic milieu as a function of time. (A) Growth curve. (B) CH_2/CH_3 ratios for VMLs. (C) Choline region: parasites on the 4th (left) and 7th (right) days of incubation.

without treatment were included as controls. For ^{1}H NMR studies-processes see section below.

TUNEL assays

Parasites after H_2O_2 or nifurtimox treatments were collected by centrifugation at 3000 ***g***, washed twice in PBS and re-suspended in the same buffer. Once placed on a slide and dried at room temperature, the cells were fixed with methanol (70%) and washed in PBS. After permeabilization with 0·2% Triton X-100, the cells were incubated with a reaction mix containing dUTP-FITC (fluorescein isothiocyanate). Nuclei were counterstained with DAPI (4′,6-diamidine-2-phenylindol) (1 mg/ml). Fluorescence was observed using Nikon Eclipse E400 microscopy and pictures were captured with a Nikon Coolpix 4500 digital camera. Results were quantified counting 200 cells in duplicate from 3 independent experiments. Apoptotic index was obtained from the ratio between TUNEL positive cells and total cells.

Ultrastructural analysis

Starved parasites were processed for transmission electron microscopy. After washing 3 times in PBS, the parasites were fixed in 2·5% glutaraldehyde (40 min/room temperature) and post-fixed in a solution containing 1% OsO_4, 0·8% potassium ferricyanide and 2·5 mM $CaCl_2$ (30 min/room temperature). Afterwards the cells were dehydrated in an ascending alcohol series following by acetone and embedded in Epoxy-resin (Araldita Durcupan, FLUKA). Thin sections, 0·5 μm, were stained with methyleneboraxic blue (1%) and examined in a Nikon Eclipse E200 microscope. Ultrathin sections were stained with uranyl acetate and lead citrate during 10 min and examined in a Jeol JEM 1010 transmission microscope operated at 80 kV.

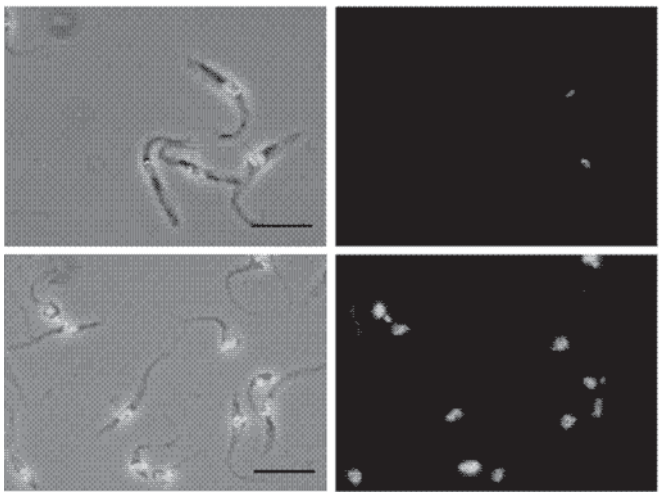
NMR experiments

Cell sample preparation. Treated or un-treated (control) cells were harvested and centrifuged for 10 min at 3000 ***g***. The pellet was washed 3 times in PBS, re-suspended in PBS (500 μL), transferred to a 5 mm NMR tube (ALDRICH, USA) and D_2O (90 μL) was added. The mixture was homogenized prior to acquire the spectrum.

NMR spectra acquisition

^{1}H NMR experiments were recorded at 20 °C in a Bruker Avance DPX-400 spectrometer, operating at 400·132 MHz, with a 5 mm broadband inverse geometry probe. The acquisition parameters included: 90° pulse (zgpr, avance-version v 1.7.10.2,

A



B

$[H_2O_2]$ (μM)[a]	CH_2/CH_3 ratio[b]
2.6	0.99 ± 0.04
5.3	1.01 ± 0.05
53	1.50 ± 0.30*
530	1.00 ± 0.30

[a] Per 10^6 parasites/mL, treated during 30 min. [b] Expressed respect to untreated parasites. * $p < 0.05$ (Student's t-tests).

Fig. 2. Effect of H_2O_2 on *Trypanosoma cruzi* epimastigotes. (A) *T. cruzi* epimastigotes (12×10^6/ml) were incubated with PBS (upper panels) or with 500 μM H_2O_2 (42 μM H_2O_2 per 10^6 parasites/ml) (lower panels) for 30 min at 28 °C. Bars: 10 μm. (B) CH_2/CH_3 ratios for VMLs.

1D sequence with f1 presaturation), 128 scans, and spectral width of 14·983 ppm. The acquisition time was 1·3664 s. Signal intensities were calculated by performing appropriate baseline corrections and then integrating the area under each of the resonances using MestRe-C NMR software (http://mestrelab.com/). Spectra were analysed using the Topspin 1.3 software package.

The integrated regions were: 1. for CH_2: 1·20–1·35 ppm; 2. for CH_3: 0·80–0·90 ppm. The visualized regions were: 3. for Cho: 3·10–3·30 ppm; 4. for PUFA: 2·80 and 5·40 ppm.

Statistical analysis

Values are expressed as means ± S.E.M. of at least 3 independent experiments. Statistical comparisons were performed with unpaired Student's *t*-tests by using OriginPro 8 software. $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Detection of apoptosis in the stationary phase of Trypanosoma cruzi *epimastigote growth*

In a previous study, it was shown that natural death of *T. cruzi* epimastigotes occurs by PCD at the beginning of the stationary phase of growth in axenic cultures or under nutrient deprivation (Jimenez *et al.* 2008). Consequently, we studied the modifications in the VMLs profiles, using ^{1}H NMR spectroscopy, in cultures of *T. cruzi* epimastigotes maintained in axenic milieu for 16 days (Fig. 1A). The best indicator of apoptosis evolution was the increment in the CH_2/CH_3-ratio as a function of the time of incubation. Thus, this ratio increased 29% from day 4 to day 7 of culture (Fig. 1B) reaching 47% at the stationary phase (14 days of culture). These results are in agreement with the apoptotic index previously obtained at the same times of incubation using the TUNEL technique (Jimenez *et al.* 2008). In the PUFA-signal regions, 2·8 and 5·4 ppm, we did not detect any relevant modifications; however, the 'choline region' clearly changed as a function of time (Fig. 1C). Though the 'choline region' changes were not as notorious as after treatment with H_2O_2 (see next section), the appearance of new peaks in this region could be indicative of the transformation of PTC into PC, Cho, or lysophosphatidylcholine. Those are well-known phenomena that play an important role in the membrane perturbations related to the apoptotic cascade (Hakumäki and Kauppinen, 2000; Lutz, 2006).

Analysis of the effects of H_2O_2 on Trypanosoma cruzi *epimastigotes by ^{1}H NMR*

As previously described (Piacenza *et al.* 2001; Deolindo *et al.* 2010; Vilchez Larrea *et al.* 2011) hydrogen peroxide (H_2O_2) was able to induce different types of *T. cruzi* epimastigote cell death in a dose-dependent manner. Indeed, incubation of 150×10^6 parasites/ml for 30 min with H_2O_2 (30–85 μM per 10^6 parasites/ml) clearly provoked PCD as shown by the TUNEL assay (Fig. 2A). However, when the H_2O_2 concentration was increased up to near to 530 μM, a necrotic process took place as evidenced by light microscopy, i.e. trypan blue staining, mobility, and morphology (data not shown). These *T. cruzi* treatments encouraged us to investigate the VMLs modification by ^{1}H NMR spectroscopy. Consequently, we studied the VMLs

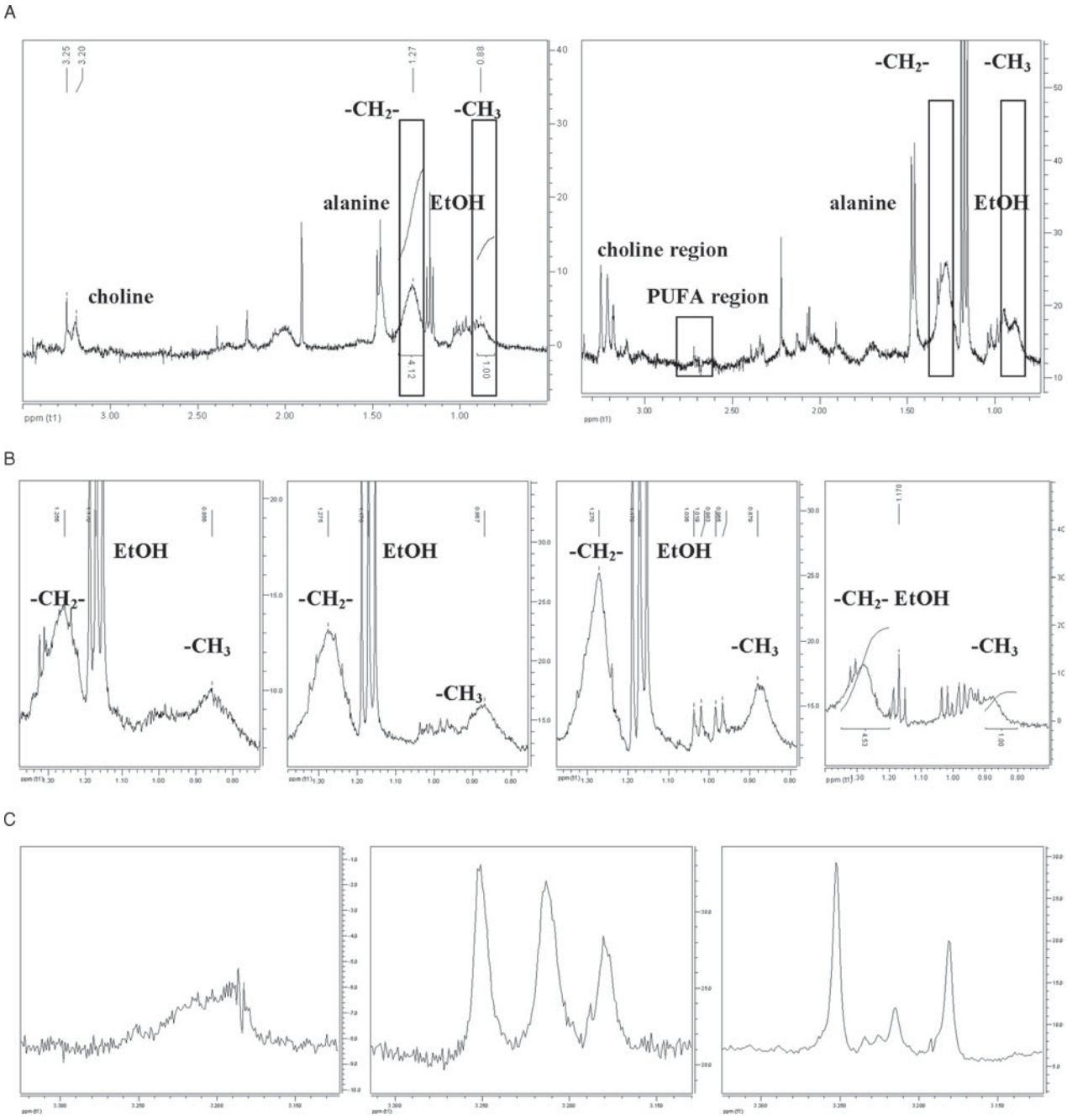


Fig. 3. *Trypanosoma cruzi* epimastigotes were treated with different H_2O_2 concentrations and examined by ^{1}H NMR. (A) Control (PBS, left) and H_2O_2 (right) 53 μM/10^6 parasites/ml (30 min). (B) CH_2/CH_3 regions for VMLs, left to right: untreated parasites (control, PBS), H_2O_2–parasites incubated during 30 min with 2·6, 53 and 530 μM/10^6 parasites/ml, respectively. (C) Choline region, left to right: untreated parasites (control, PBS, left), parasites exposed 30 min to 53 (centre), and 530 H_2O_2 μM/10^6 parasites/ml (right).

changes at different H_2O_2 concentrations. Applying H_2O_2 concentrations lower than those required to induce apoptosis, i.e. 2·6 or 5·3 μM, the CH_2/CH_3 ratios were similar to the values for untreated-parasites (Fig. 2B, Fig. 3B). Similarly, when the conditions were necrotic, i.e. 530 μM H_2O_2, the ratio was 1·0; however, when the conditions where apoptotic the CH_2/CH_3 ratio increased to 50%.

In addition to the relevant changes on CH_2/CH_3 ratios, from VMLs, other significant modifications were observed after the different H_2O_2-treatments (Fig. 3A). Interestingly, in these ^{1}H NMR experiments the whole parasites were associated with free alanine, ethanol, and glycine (for glycine signal see Fig. 6B–D) metabolites, confirmed by standards additions, which were not eliminated after exhaustive washing. The most important change was in the 'choline region'. Under apoptotic the conditions of 53 μM of H_2O_2/10^6 parasites/ml for 30 min, new peaks were evident in this region (Fig. 3C) probably

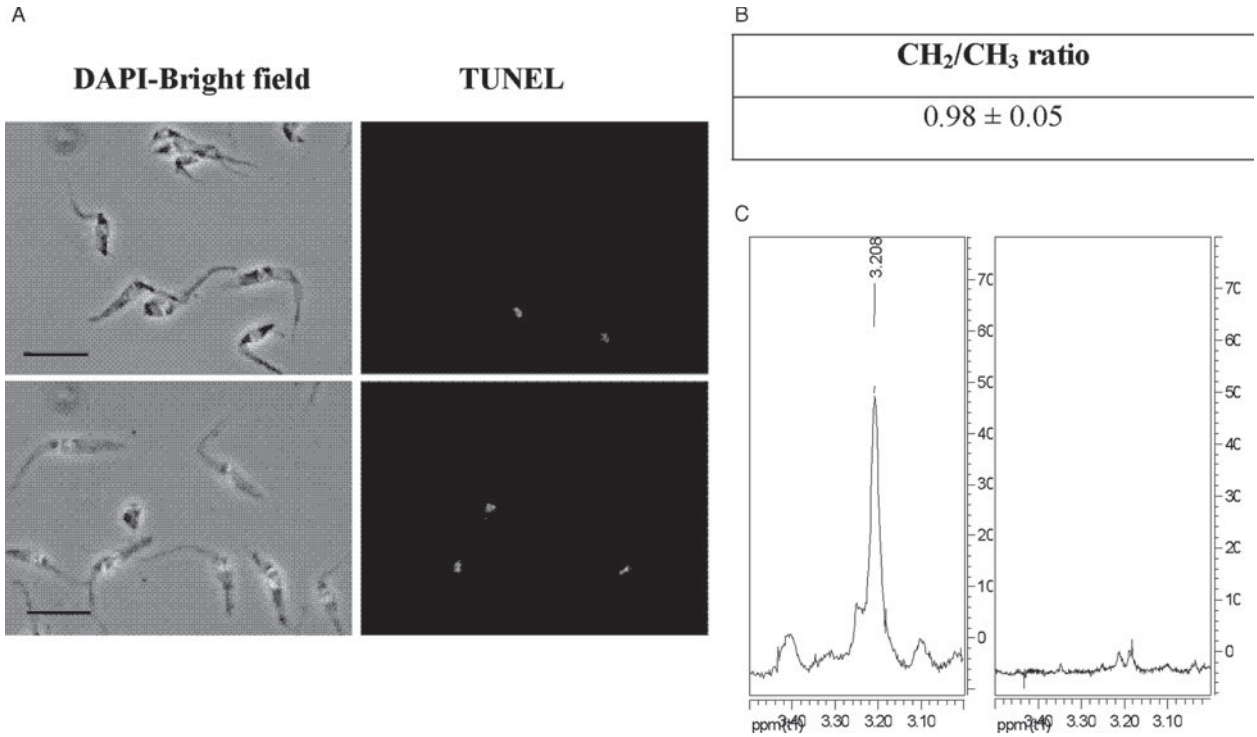


Fig. 4. Effect of nifurtimox on *Trypanosoma cruzi* epimastigotes. (A) *T. cruzi* epimastigotes (12×10^6/ml) were incubated with PBS (upper panels) or with 8·3 μM nifurtimox (per 10^6 parasites/ml) (lower panels) for 24 h at 28 °C. Scale bars: 10 μm. NMR-VML results for *T. cruzi* treatment with nifurtimox at necrotic conditions (2μM/10^6 parasites/ml, during 48 h at 28 °C). (B) CH_2/CH_3 ratio. (C) Choline region: control (left) and nifurtimox-treated (right) parasites.

indicative of the presence of PTC and related compounds, i.e. lysophosphatidylcholine, associated with the phospholipase A2 activity during the apoptotic cascade (Hakumäki and Kauppinen, 2000; Lutz, 2006). In contrast, the PUFA-regions showed a weak signal in the region of 2·8 ppm (Fig. 3A), but not at 5·4 ppm (data not shown), although with lower significance than the other MLVs. Under necrotic conditions, 530 μM of $H_2O_2/10^6$ parasites/ml for 30 min, the profile in the 'choline region' was similar to the profile under apoptotic conditions, with little modifications in the relative signals intensities (Fig. 3C) while the PUFA-peaks were undetectable.

^{1}H NMR-VML spectroscopy analysis of Trypanosoma cruzi *epimastigotes treated with nifurtimox*

First we tested by TUNEL assay (Fig. 4A) and by light microscopy, i.e. trypan blue staining, mobility, and morphology (data not shown), whether nifurtimox, one of the drugs used for Chagas disease treatment (Cerecetto and González, 2010), produced necrotic death on *T. cruzi* epimastigotes. Then, we investigated the changes of VMLs by ^{1}H NMR when *T. cruzi* was treated with nifurtimox under necrotic conditions, i.e. 2 μM (per 10^6 parasites/ml) during 48 h at 28 °C. Results were similar to those observed when epimastigotes were treated with H_2O_2 at 530 μM (necrotic conditions, Fig. 2B); the *T. cruzi* CH_2/CH_3 ratio (Fig. 4B) and PUFA-signals were not modified by nifurtimox (not shown). Meanwhile, the 'choline region' suffered a clear modification showing that the peak of PTC disappeared as a result of the development of the necrotic process (Fig. 4C).

^{1}H NMR-VML spectroscopy analysis of Trypanosoma cruzi *epimastigotes under autophagic conditions*

T. cruzi epimastigotes were starved for 24 h in PBS. When autophagy was reached, as detected by transmission electron microscopy (Fig. 5A), the cells were analysed by ^{1}H NMR. Surprisingly, no significant difference in the CH_2/CH_3 ratios was observed between untreated and starving-parasites, 1·0 ± 0·1 and 0·7 ± 0·2, respectively. Similarly, no differences were either observed between both conditions in the Cho and PUFA regions (Fig. 5B).

DISCUSSION

Our interest focused on studying the use of a non-invasive technique, such as ^{1}H NMR spectroscopy to study programmed cell death in *T. cruzi* as a target for drug control. This process was carefully studied in higher eukaryotes considering its relevance at the time of choosing a drug candidate (Blankenberg *et al.* 1996; Hakumäki and Kauppinen, 2000; Mikhailenko *et al.* 2005; Milkevitch *et al.* 2005).

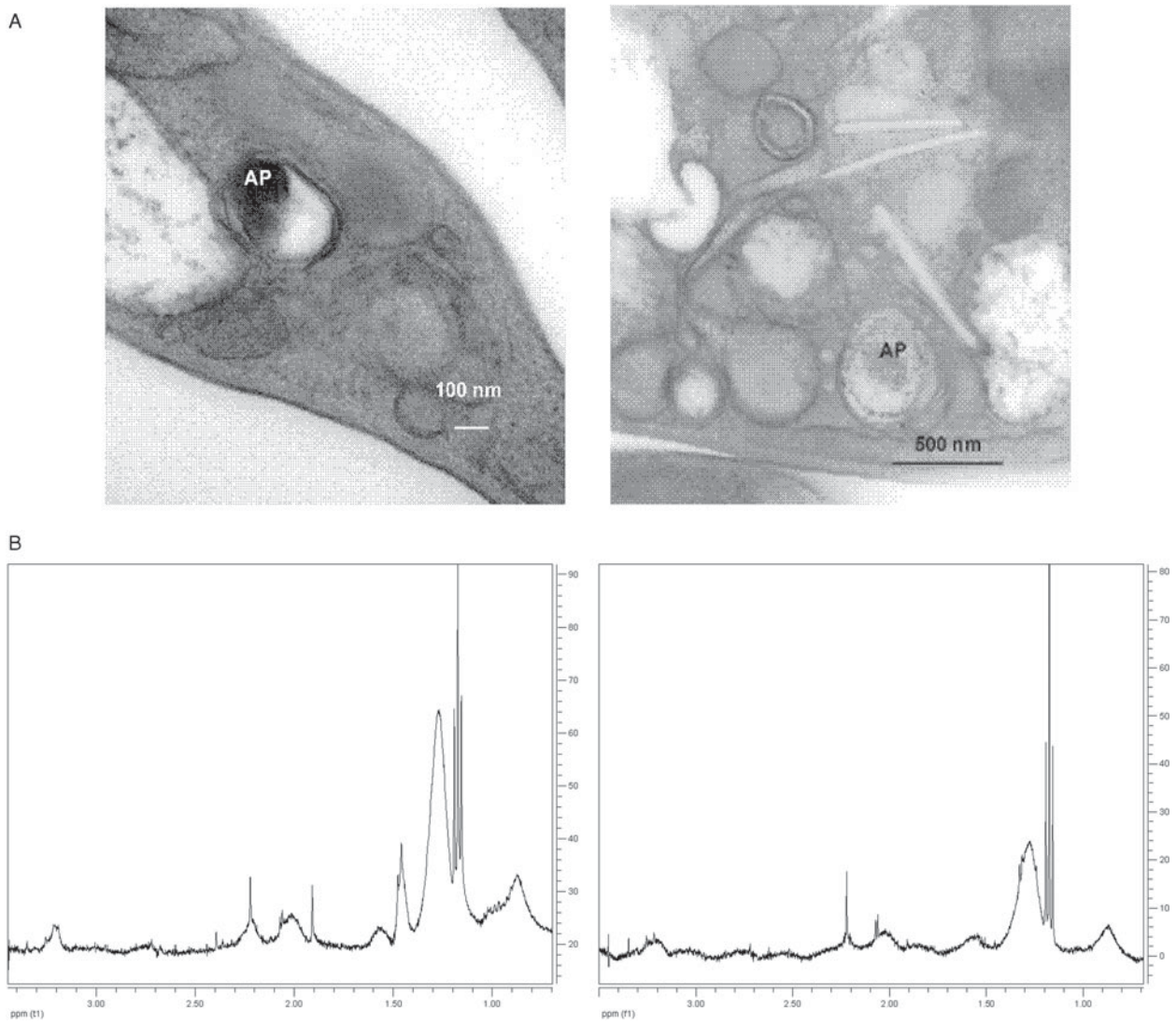


Fig. 5. *Trypanosoma cruzi* epimastigotes under autophagic conditions. (A) Transmission electron micrographs of parasites exposed for 24 h to PBS. AP, autophagosome. (B) ^{1}H NMR spectra of whole parasites, control- (left) and starving-parasites (right).

Our results, analysing VMLs, showed that the CH_2/CH_3 ratio increased under typical apoptotic conditions for epimastigotes of *T. cruzi*. These enhancements in the methylene signals are, also in the case of apoptotic eukaryotic cells, demonstrating the significance of the membrane turnover perturbations (Hakumäki and Kauppinen, 2000). Furthermore, an increment in the choline-containing compound peaks was detected by ^{1}H NMR under these conditions (Fig. 6). The choline region shows a disclosure in the signals with an enhancement of signals near to 3·18 and 3·21 ppm that could be assigned to Cho and PC, respectively (Bollard *et al.* 2002). The PTC signal was confirmed by PTC-standard addition (Fig. 6D). The appearance of peaks for PTC, PC, and Cho in apoptotic conditions (Fig. 6B) convinced us to assume that they are signals from membrane-lipids. This observation was also apparent in other eukaryotic apoptotic cells (Milkevitch *et al.* 2005; Kettunen and Brindle, 2005). All these facts are in agreement with information related to lipid modifications upon induction of apoptosis (Al-Saffar *et al.* 2002; Jimenez *et al.* 2008; Vilchez Larrea *et al.* 2011). However, under necrotic conditions (Fig. 6C), the appearance of the same signals (PTC, PC, and Cho) was more defined than in the apoptotic situation, i.e. narrow and broad, respectively (see also these appearances in Fig. 3C). It is possible that during necrosis and at the end stage of apoptosis, the cell membrane disruption and leakage of Cho-containing metabolites into the extracellular fluid explaining the presence of well-defined methylcholine resonances, $N^{+}(CH_3)_3$, were from associated membrane metabolites (Griffin *et al.* 2001).

In order to study necrosis in *T. cruzi,* treatments with high concentrations of hydrogen peroxide or nifurtimox were applied. In both conditions, no modifications in the CH_2/CH_3 ratio and in PUFA-signals were observed; however dissimilar results were obtained for choline-containing compounds,

Table 1. Summary of results

	Changes in the ^{1}H NMR signals[a]		
Type of parasite cell death	CH_2/CH_3ratio	'Choline region'	'PUFAs regions'
Apoptosis	Increment	Appearance of PTC, PC, and Cho	Little modifications
Necrosis	n. m.[b]	Depended on the necrosis-condition	n. m.
Autophagy	n. m.	n. m.	n. m.

[a] With respect to untreated parasites.
[b] n. m., no detected modifications.

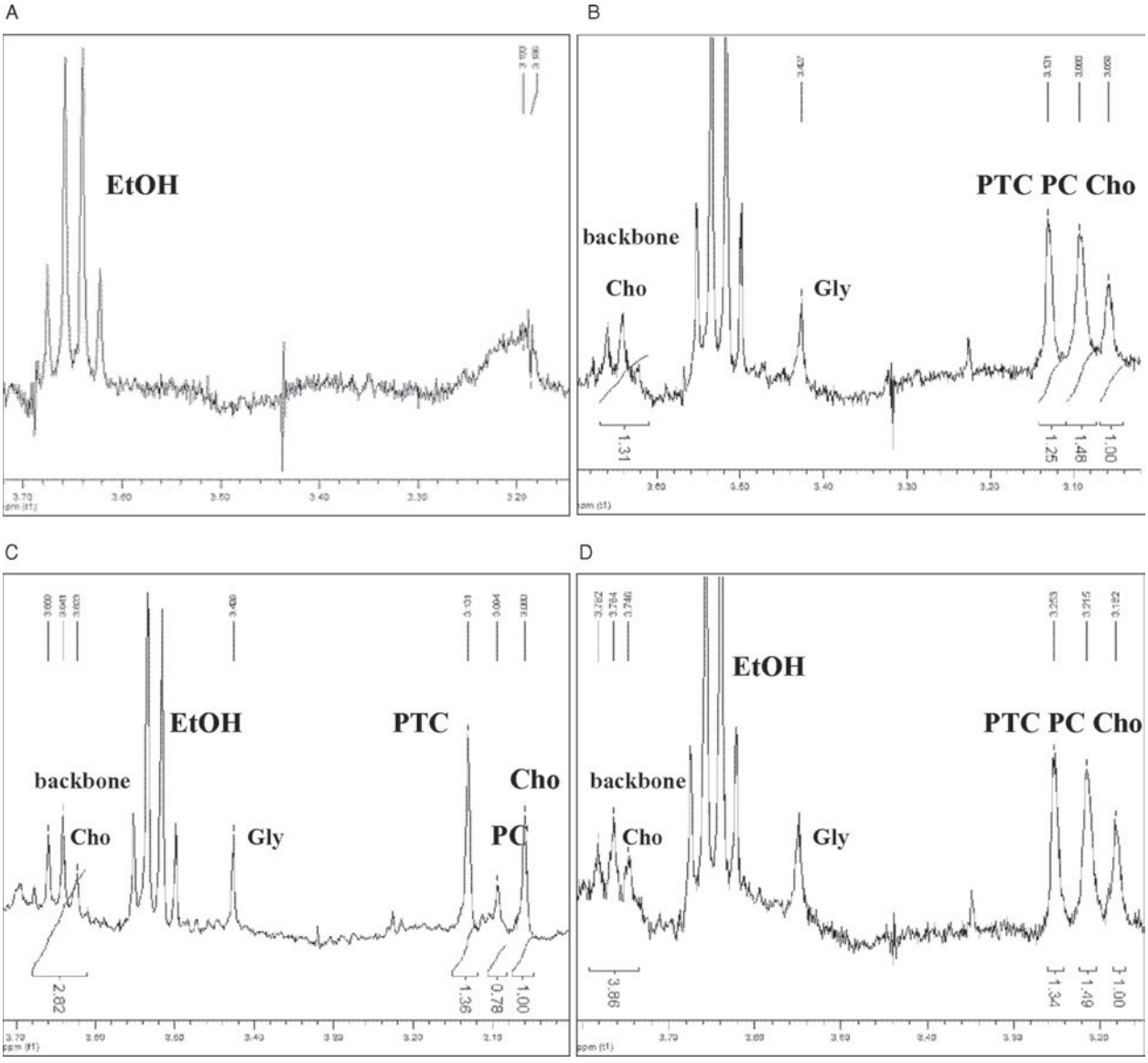


Fig. 6. 'Choline region'. (A) Control PBS-untreated parasites. (B) H_2O_2 (53 μM/10^6 parasites/ml, 0·5 h)-treated parasites. (C) H_2O_2 (530 μM/10^6 parasites/ml, 0·5 h)-treated parasites. (D) The same conditions as in (B) + PTC.

particularly an increase in backbone Cho near to 3·8 ppm (Bollard *et al*. 2002) (Fig. 6C). These data suggest possible differences in the death-process induced by each necrotic agent. In trypanosomatids, a higher proportion of PUFAs than in their eukaryote mammalian host has been described, i.e. oleate and linoleate constitute around 60% of the total fatty acids in *T. cruzi* epimastigote and trypomastigote cellular forms (Florin-Christensen *et al*. 1997), suggesting an essential role in membrane fluidity to adapt the parasite to the environmental changes in its different hosts (Alloatti *et al*. 2009; Alloatti and Uttaro, 2011). In spite of these descriptions, in our experiments PUFAs from *T. cruzi* epimastigotes seemed to be

non-VML in ^{1}H NMR maybe as a result of the effects of the large dipolar couplings produced between lipids in cell membranes (Siminovitch *et al*. 1988).

Data reported in the present study imply that ^{1}H NMR of whole-*T. cruzi* in autophagy did not show any differential aspect in the VMLs. Although epimastigotes contains reservosomes (Cunha-e-Silva *et al*. 2006) that concentrate lipids, these seemed to be not visible in the NMR spectroscopy. These invisible lipids could be immersed in vacuola that are immobilized in the cytoplasm and not exposed to relaxing protons (Hakumäki and Kauppinen, 2000).

Our work, summarized in Table 1, may contribute to the use of a new non-invasive technique to analyse *T. cruzi* death pathways. As a consequence, studies on biological or biophysical aspects on *T. cruzi* plasma membrane and/or on the effect of natural drugs, as well as of synthetic compounds on this parasite would be easily conducted. To the best of our knowledge, there has been no previous report on the use of this technique for the study of cell death processes in trypanosomatid organisms.

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