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Journal of Cellular Biochemistry 112:2189–2199 (2011)

Journal of Cellular Biochemistry

DNA Repair BER Pathway Inhibition Increases Cell Death Caused by Oxidative DNA Damage in *Trypanosoma cruzi*

G. Cabrera,[1*] C. Barría,[1] C. Fernández,[1] S. Sepúlveda,[1] L. Valenzuela,[1] U. Kemmerling,[2] and N. Galanti[1*]

[1] *Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*

[2] *Programa de Anatomía y Biología del Desarrollo, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*

ABSTRACT

Trypanosoma cruzi, a parasitic protozoan, is the etiological agent of Chagas disease, an endemic and neglected pathology in Latin America. It presents a life cycle that involves a hematophagous insect and man as well as domestic and wild mammals. The parasitic infection is not eliminated by the immune system of mammals; thus, the vertebrate host serves as a parasite reservoir. Additionally, chronic processes leading to dysfunction of the cardiac and digestive systems are observed. To establish a chronic infection some parasites should resist the oxidative damage to its DNA exerted by oxygen and nitrogen free radicals (ROS/RNS) generated in host cells. Till date there are no reports directly showing oxidative DNA damage and repair in *T. cruzi*. We establish that ROS/RNS generate nuclear and kinetoplastid DNA damage in *T. cruzi* that may be partially repaired by the parasite. Furthermore, we determined that both oxidative agents diminish *T. cruzi* cell viability. This effect is significantly augmented in parasites subsequently incubated with methoxyamine, a DNA base excision repair (BER) pathway inhibitor, strongly suggesting that the maintenance of *T. cruzi* viability is a consequence of DNA repair mechanisms. J. Cell. Biochem. 112: 2189–2199, 2011.

KEY WORDS: *T. cruzi*; DNA DAMAGE/REPAIR; BER PATHWAY

Trypanosoma cruzi, a parasitic protozoan member of the Kinetoplastida order, is the etiological agent of Chagas disease, an endemic and neglected pathology in Latin America affecting 15–16 million people, with a high cost to public health [Coura, 2007; Yun et al., 2009]. Chagas disease mortality and morbidity are mainly due to chronic processes that lead to dysfunction of the cardiac and digestive systems. Then, it is crucial to understand the mechanisms leading to this pathology.

T. cruzi presents a life cycle that involves a hematophagous insect (triatomine) and man as well as domestic and wild mammals [De Souza, 2002]. The transmission of the disease is produced by an infected triatomine that upon feeding on mammalian blood, deposits feces with infective parasites (trypomastigotes), which enter the mammalian body mainly through the skin wound produced by the insect [Amino et al., 2002; Coura, 2007]. Upon entering the body, the parasites invade macrophages taking a round, replicative form, the amastigote [Andrade and Andrews, 2005]. After replication, the amastigotes transform back to trypomastigotes that enter the circulatory system invading heart, ganglia, and other tissues. When feeding on an infected mammalian the triatomine ingest trypomastigotes with the blood. In the insect midgut the trypomastigote differentiates to epimastigotes, the extracellular, replicative form of the parasite [Tyler and Engman, 2001].

The three cellular forms of the parasite are under attack from reactive species generated by their different hosts. Although all cellular macromolecules are subject to damage by ROS/RNS, the primary deleterious consequences of oxidative stress probably arise from damage to DNA [Riley, 1994; Wang et al., 1998]. In order to establish a chronic infection, *T. cruzi* parasites must resist oxidative DNA damage due to ROS/RNS generated by host cells [Piacenza

Grant sponsor: Fondecyt (to N.G.); Grant number: 1090124; Grant sponsor: CONICYT (to N.G) Advanced Center for Training and Research in the Design of Pharmacological and Immunological Strategies for the Control of Parasitic and Neoplasic Aggressions. Proyecto de Anillos de Investigación en Ciencias y en Tecnología; Grant number: ACT112; Grant sponsor: Fondecyt (to U.K.); Grant number: 11080166.

*Correspondence to: G. Cabrera and N. Galanti, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Casilla 70061, Santiago, Chile.
E-mail: gcabrera@med.uchile.cl; ngalanti@med.uchile.cl

Received 23 February 2011; Accepted 31 March 2011 • DOI 10.1002/jcb.23138 • © 2011 Wiley-Liss, Inc.
Published online 7 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

et al., 2009]. However, till date we are not aware about reports showing oxidative DNA damage in *T. cruzi*. Additionally, the mechanisms responsible for the repair of the DNA damage by ROS/RNS in *T. cruzi* are also unknown.

In other eukaryotes, the base excision repair (BER) system, an evolutionarily conserved process for maintaining genomic integrity, is the most important repair mechanism of base DNA damage caused by oxidative agents [Mandavilli et al., 2002; Hegde et al., 2008; Zhang et al., 2008]. This repair pathway is initiated by spontaneous or enzymatic N-glycosidic bond cleavage creating an abasic or apurinic apyrimidinic (AP) site in double-stranded DNA. A protein complex constituted of an AP endonuclease, a DNA polymerase, and a DNA ligase complete the repair [Hoeijmakers, 2001; Ide and Kotera, 2004]. AP sites that are not repaired determine DNA breaks and an increase in cytotoxicity [Loeb and Preston, 1986]. Till date, the presence and activity of several enzymes of the BER pathway have been studied in *T. cruzi*, such as UDGase (Uracil-DNA glycosylase), PARP [Fárez-Vidal et al., 2001; Fernández Villamil et al., 2008], and DNA repair polymerase β [Venegas et al., 2009]. Furthermore, an endonuclease AP gene (TcAP) has been described in this parasite; expression of this gene confers resistance to oxidant and alkylating agents when probed in hypersensitive *E. coli*, deficient in exonuclease III enzyme [Pérez et al., 1999].

In this work, we establish that hydrogen peroxide and peroxynitrite generate oxidative nuclear and kinetoplastid DNA damage in *T. cruzi* that may be partially repaired by the parasite. Furthermore, we determined that both oxidative agents diminish cell viability of *T. cruzi* epimastigotes and trypomastigotes. This effect is significantly augmented in parasites subsequently incubated with the drug methoxyamine (MX), a known BER pathway inhibitor [Liuzzi and Talpaert-Borlé, 1985] that also increases *T. cruzi* DNA fragmentation. The diminution of cell viability after ROS/RNS attack is most probably due to a population of parasites unable to repair their DNA. On the contrary, the maintenance of *T. cruzi* viability is a consequence of DNA repair mechanisms, most probable sustained by the BER pathway.

MATERIALS AND METHODS

PARASITE CULTURE

T. cruzi epimastigotes (Dm28c and Y strains) were cultivated at 28°C in a monophasic liquid medium [Diamond, 1968] (NaCl 0.106 M, KH2PO4 23 mM, tryptose 12.5 g/L, tryptone 12.5 g/L, yeast extract 12.5 g/L, pH 7.2) supplemented with 10% bovine fetal serum, 7.5 μM hemine, and antibiotics (penicillin 75 U/ml and streptomycin 75 μg/ml). Trypomastigotes were obtained from infected RAW 264.7 cells cultured in RPMI 1640 supplemented with 10% heat-inactivated bovine fetal serum and maintained at 37°C with 5% CO_2.

IN SITU OXIDATIVE DNA DAMAGE IMMUNODETECTION

DNA damage was evidenced by in situ immune detection of 8-oxoguanine (8-oxoG) considered a marker of oxidative stress and DNA damage in eukaryotes [Fraga et al., 1990; Achanta and Huang, 2004; Dorszewska and Adamczewska-Goncerzwics, 2004]. The assay was performed on *T. cruzi* epimastigotes treated with H_2O_2 or NOO^- (50, 100, or 200 μM) for 30 min at 28°C and fixed in cold 70% methanol for 30 min. 8-oxoG within DNA was detected applying the Trevigen method as described (http://www.trevigen.com/protocols/pdf/4354-MC-050.pdf). Briefly, the parasites were incubated in the presence of a monoclonal antibody against 8-oxoG (Abcam). As a detection immune probe, affinity purified polyclonal rabbit immunoglobulins, conjugated with Alexa fluor 488 (for H_2O_2) or Alexa fluor 546 (for NOO^-), were used. Nuclear and kinetoplast DNA were detected by DAPI. Cells were analyzed by fluorescence microscopy in a Nikon Eclipse E400.

DNA ISOLATION

Epimastigote nuclear (nDNA) and kinetoplast DNA (kDNA) was isolated from 12×10^7 cells using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's protocol.

QUANTITATIVE MEASUREMENT OF *T. CRUZI* DNA DAMAGE AND REPAIR BY ARP ASSAY

Samples of parasite DNA (0.5 μg) were subjected to an AP site quantification assay using the DNA Damage Colorimetric Assay Kit (Abcam) according to the manufacturer's instructions. This kit utilizes the ARP (Aldehyde Reactive Probe) reagent that reacts specifically with an aldehyde group in the open ring form of AP sites. The data, expressed as the number of AP sites per 10^5 nucleotides, were calculated based on the linear calibration curve generated using ARP-DNA standard solutions supplied by the manufacturer.

QUANTITATIVE MEASUREMENT OF *T. CRUZI* GENOMIC AND KINETOPLAST DNA DAMAGE AND REPAIR BY LONG AMPLIFICATION QUANTITATIVE POLYMERASE CHAIN REACTION (LA-QPCR)

LA-QPCR was performed according to a protocol modified from Santos et al. [2006]. This assay is based on the principle that many kinds of oxidative DNA lesions such as strand breaks, base modifications, and abasic sites, can slow down or block the progression of DNA polymerase. Briefly, purified DNA from parasites was quantified using the Qubit Fluorometer (Invitrogen), with the Quant-iT dsDNA HS Assay Kit following the manufacturer's instructions. The DNA samples were adjusted to a concentration of 5 ng/μl and then subjected to PCR under quantitative conditions using Crimson LongAmp Taq DNA Polymerase (New England Biolabs). The primer nucleotide specific sequences were as follows: 5′-TGCTACAATTGCGGTCGTATGG-3′ (sense) and 5′-CACCA-CAATTTGATCCAGGATAG-3′ (antisense) for a *T. cruzi* nDNA 9.9 kb genomic fragment (GenBank accession: AY728266) and 5′-GCCGTTGCGGTAATTTCTATGC-3′ (sense) and 5′-GGGACTGT-TACTGTTTTGATTGG-3′ (antisense) for a kDNA 9 kb maxi-circle fragment (GenBank: FJ203996). Small nDNA (257 bp) and kDNA (248 bp) targets were amplified for normalization/verification of DNA concentration and to account for the maxi-circle copy number, respectively. For these amplifications, the primer nucleotide specific sequences were as follows: 5′-TGCTACAATTGCGGTCGTATGG-3′ (sense); 5′-GTCTGACCGCAATTGTAGCATG-3′ (antisense) for nDNA and 5′-TGTGTTTCATCGTCTACTTATTGC-3′ (sense); 5′-TAACAAA-TAATTGATCCTTGCGTAC-3′ (antisense) for kDNA.

For both sequences (nDNA and kDNA) LA-QPCR was performed with an initial denaturation for 5 min at 95°C followed by 30 cycles

of 95°C denaturation for 30 s and 65°C annealing and extension for 10 min was applied. A final extension at 65°C was performed for 10 min. To ensure quantitative conditions, reactions containing 50% control templates were included in each set of PCR reactions. Amplifications without template DNA were also performed. The reaction products were separated by electrophoresis on a 0.8% agarose/TBE (Tris-borate-EDTA) gel containing ethidium bromide at 95 V for 60 min. Experimental samples were compared with controls and the relative amplification was calculated using the Gel-Pro 3.1 Program.

EPIMASTIGOTE AND TRYPOMASTIGOTE VIABILITY ASSAY

The effect of the BER inhibitor MX on *T. cruzi* viability exposed to oxidative DNA damage agents was evaluated through the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [Muelas-Serrano et al., 2000]. Briefly, 12×10^6 parasites/ml were incubated with different concentrations of hydrogen peroxide (Sigma) or sodium peroxynitrite (Cayman), alone or combined with 10 mM MX as BER inhibitor, in Diamond's culture medium at 28°C (epimastigotes) or in fetal bovine serum-RPMI culture medium without phenol red at 37°C (typomastigotes). After 30 min incubation, the parasites were washed twice with PBS and re-incubated with fresh medium, alone or combined with 10 mM MX, for 4 h. Subsequently, 10 μl of 5 mg/ml tetrazolium dye (MTT; 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) plus 0.22 mg/ml Phenazine metosulfate (electron carrier), were added to each well containing parasites in 100 μl of culture medium. After additional incubation for 4 h, the generated water insoluble formazan dye was dissolved using 100 μl of 10% w/v SDS in 0.01 M HCl. The plates were further incubated overnight at 37°C, and optical density (OD) in each well was determined using a microplate reader (Labsystems Multiskan MS, Finlandia) at 570 nm. All experiments were performed at least three times and data are shown as the means and their standard deviations from triplicate cultures. The effect on parasite viability of MX alone was also evaluated by the same assay.

DNA FRAGMENTATION (TUNEL ASSAY)

T. cruzi epimastigotes were incubated for 30 min with 200 μM H_2O_2 or 200 μM NOO^- and subsequently treated or untreated with 10 mM MX for 8 h. The parasites were fixed in cold 70% methanol for 15 min at 4°C and subjected to the TUNEL assay according to the manufacturer's instructions (DeadEndTM Fluorometric TUNEL System, Promega). In all cases DAPI nuclear staining was also conducted. The images were obtained with a fluorescence microscope Nikon Eclipse E400.

STATISTICAL ANALYSIS

All statistical analyses were performed using the GraphPad Prism 5.0 program. All experiments were done in triplicate and results correspond to means ± SEM from at least three independent experiments. Significant data differences were analyzed applying the one-way ANOVA with Turkey or the Dunnett post-test or the two-way ANOVA with Bonferroni post-test. Paired conditions were analyzed by Student's *t*-test.

RESULTS

H_2O_2 AND NOO^- INDUCE OXIDATIVE DNA DAMAGE IN *T. CRUZI* EPIMASTIGOTES

Both H_2O_2 and NOO^- induced DNA damage in *T. cruzi* epimastigotes in a concentration dependent manner as evidenced by the presence of 8-oxoG (Fig. 1A and B, respectively). Non-treated epimastigotes or parasites incubated without 8-oxoG antibody after treatment with 200 μM H_2O_2 (Fig. 1A) or NOO^- (Fig. 1B) did not show reactivity. The base modification after oxidative treatment with H_2O_2 or $NOO^-$was observed both in the nucleus and in the kinetoplast of the parasites (Fig. 1C).

To quantify the level of oxidative DNA damage the formation of AP sites was evaluated after parasite exposure to increasing concentrations of H_2O_2 (50–500 μM) for 30 min at 28°C using the ARP assay. As shown in Figure 2A, the number of AP sites in total DNA raises as a function of H_2O_2 concentration indicating an increase in the amount of abasic sites.

Additionally, DNA damage was also measured in *T. cruzi* epimastigotes using the LA-QPCR technique. Clearly the oxidative agent induces DNA lesions that are evidenced by a lower amount of amplification as function of H_2O_2 concentration due to a slowing down or a blocking of the DNA polymerase progression, both in nuclei (Fig. 2B) and in kinetoplast (Fig. 2C).

Thus, *T. cruzi* DNA damage induced by oxidative agents was evidenced directly through the immune detection of 8-oxoG, and indirectly by the quantification of AP sites in the DNA and by the LA-QPCR technique.

REPAIR OF OXIDATIVE DNA DAMAGE IN *T. CRUZI* EPIMASTIGOTES AFTER H_2O_2 TREATMENT

To evaluate oxidative DNA repair capacity of *T. cruzi* epimastigotes, parasites were exposed to 200 μM H_2O_2 for 30 min, washed, and afterwards maintained in fresh Diamond medium supplemented with 10% fetal calf serum for 1, 2, or 4 h. The amount of AP-sites in the DNA was detected as described above. As expected, a clear increase in DNA damage was observed after treatment with 200 μM H_2O_2 for 30 min (Fig. 3A). After removal of the oxidative agent, the number of AP sites remained at the same level for 2 h decreasing to approximately 40% after 4 h of incubation in fresh medium (Fig. 3A), indicating the presence of a DNA repair process in *T. cruzi*.

The same result was obtained when the LA-QPCR technique was applied. Thus, the relative amplification of the genomic fragment used diminished abruptly 30 min after oxidative treatment with 200 μM H_2O_2 (Fig. 3B). Again, after removal of the oxidative agent the relative amplification of the DNA fragment increased approximately three-fold in 4 h clearly showing DNA repair.

THE BER INHIBITOR METHOXYAMINE DECREASES THE VIABILITY OF *T. CRUZI* EPIMASTIGOTES AND TRYPOMASTIGOTES TREATED WITH OXIDATIVE AGENTS

Oxidative DNA damage in eukaryotes is repaired primarily via the DNA base excision repair (BER) pathway [Mandavilli et al., 2002; Hegde et al., 2008; Zhang et al., 2008]. MX is a drug that blocks the

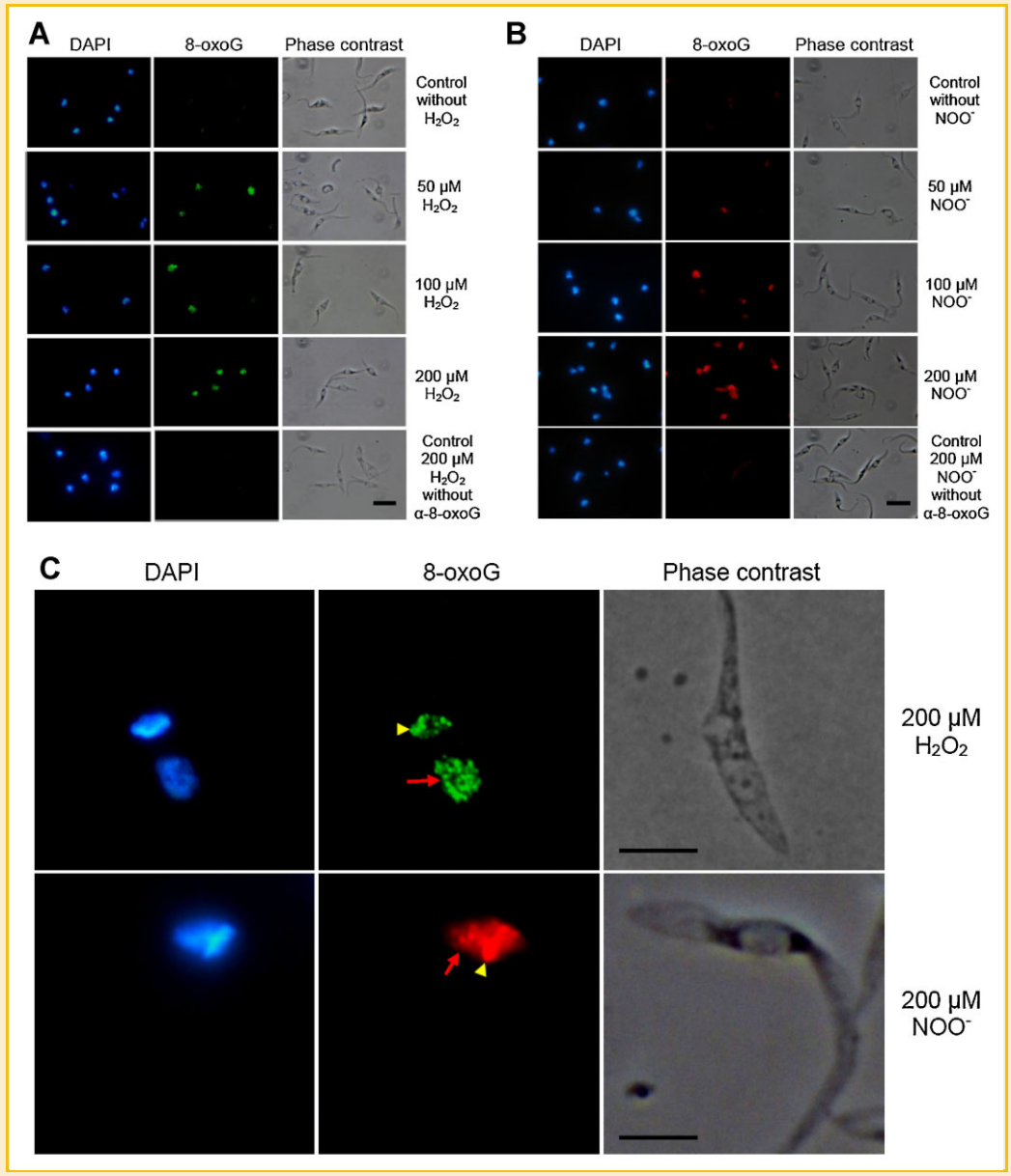

Fig. 1. Oxidative DNA damage as detected by presence of 8-oxoG in *T. cruzi* epimastigotes treated with different H_2O_2 (A) or NOO^- (B) concentrations. Y strain epimastigotes were treated for 30 min in the presence or absence of H_2O_2 (A) or NOO^- (B) in different concentrations at 28°C. The parasites were incubated in the presence of a monoclonal antibody against 8-oxoG (Abcam). As detection immune probe, polyclonal rabbit immunoglobulins conjugated with Alexa fluor 488 (H_2O_2) or Alexa fluor 546 (NOO^-) were used. Nuclear and kinetoplast DNA were detected by DAPI. Cells were analyzed by fluorescence microscopy. An enlargement of these reactions is shown (C). Nucleus (arrow); kinetoplast (arrowhead). (Bars = 10 μm).

DNA BER repair system due to binding and modification of AP sites [Liuzzi and Talpaert-Borlé, 1985]. Then, the viability of epimastigotes and trypomastigotes from two different *T. cruzi* strains was determined after treatment with the oxidative agents H_2O_2 or NOO^-, in the presence or absence of MX. Firstly, the effect of different MX concentrations (5–30 mM) on *T. cruzi* epimastigotes and trypomastigotes viability was studied. This drug did not affect parasite viability up to 20 mM (Fig. 4A). As expected, both oxidative agents decreased *T. cruzi* viability in the two epimastigote strains tested (Fig. 4B–E) as well as in Dm28c trypomastigotes (Fig. 4F). Treatment of the parasites with 10 mM MX clearly increases this effect (Fig. 3B–F). These results strongly suggest that H_2O_2 and NOO^- diminish cell viability through DNA damage as it is shown in Figures 1 and 2. The increase in this effect induced by MX may be explained by the inhibition of a DNA repair BER pathway in *T. cruzi* as it was observed in mammal cells [Taverna et al., 2001; Liu et al., 2002; Rinne et al., 2004; She et al., 2005; Fishel et al., 2007]. Additionally, these results suggest that the BER system may be used by the parasite as a mechanism to avoid the oxidative damage produced by the host.

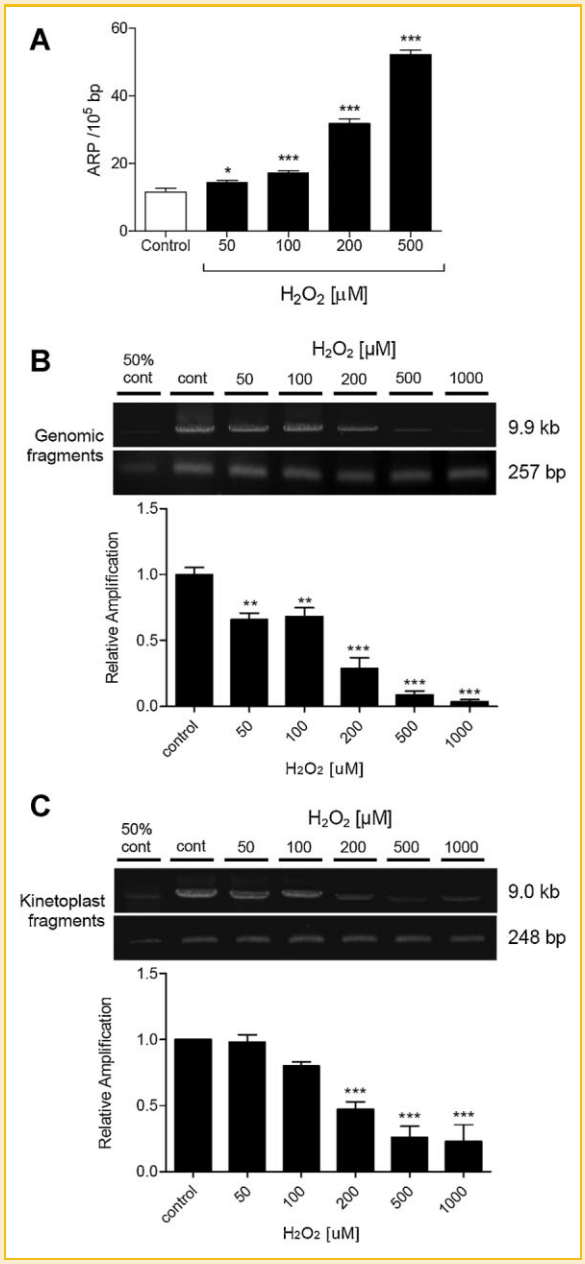

Fig. 2. Oxidative DNA damage as measured by formation of AP sites and by blocking progression of a DNA polymerase in nuclei and kinetoplast of *T. cruzi* epimastigotes treated with H_2O_2. Generation of AP sites evaluated after epimastigote Dm28c strain exposure to increasing concentrations of H_2O_2 for 30 min at 28°C using the ARP assay (A). Relative amplification of a *T. cruzi* 9.9 kb genomic DNA fragment (B) and of a 9.0 kb maxicircle DNA fragment (C) after parasite exposure to increasing concentrations of H_2O_2 for 30 min at 28°C by blocking progression of a DNA polymerase using the LA-QPCR technique. Small nDNA (257 bp) and kDNA (233 bp) targets were amplified for normalization/verification of DNA concentration and to account for the maxi-circle copy number, respectively. Data were analyzed using one-way ANOVA test with Dunnett post-test ($^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control).

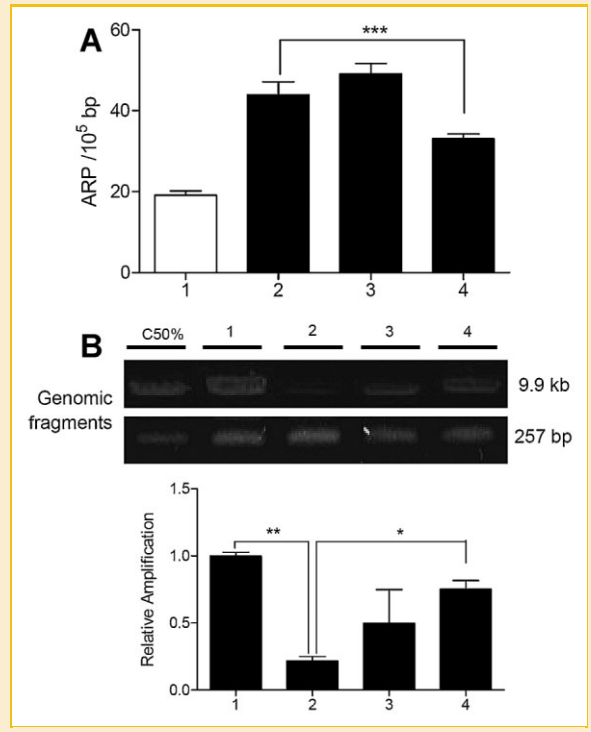

Fig. 3. Repair of oxidative DNA damage as measured by a decrease in the number of AP sites and in the blocking progression of a DNA polymerase in *T. cruzi* epimastigotes treated with 200 μM H_2O_2. (A) Generation of AP sites measured by the ARP assay in epimastigotes Dm28c strain. (B) Relative amplification of a *T. cruzi* 9.9 kb genomic DNA fragment using the LA-QPCR technique. Small nDNA (257 bp) was amplified for normalization of DNA concentration. (1) Control, non-treated epimastigotes, (2) epimastigotes treated with 200 μM H_2O_2 for 30 min, (3) epimastigotes treated with 200 μM H_2O_2 for 30 min, washed and maintained in fresh medium for 2 h, (4) epimastigotes treated with 200 μM H_2O_2 for 30 min, washed and maintained in fresh medium for 4 h. In (B), C50% is control DNA diluted to 50%. Data were analyzed using one-way ANOVA test with Turkey post-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

THE BER INHIBITOR METHOXYAMINE INCREASES NUCLEAR DNA FRAGMENTATION INDUCED BY OXIDATIVE AGENTS IN *T. CRUZI* EPIMASTIGOTES

The decrease in epimastigote viability observed when MX is combined with oxidative agents also correlates with an increase in DNA fragmentation. Figure 5A shows representative TUNEL images of *T. cruzi* epimastigotes exposed to 200 μM NOO^- for 30 min at 28°C and subsequently treated or non-treated with 10 mM MX for 8 h. Epimastigotes non-treated or treated with 10 mM MX for 8 h were used as controls. Clearly, MX combined with NOO^- increases the DNA fragmentation as detected by the TUNEL assay while MX alone does not. Same results were observed when 200 μM H_2O_2 was used (not shown). A quantitative analysis of TUNEL positive nuclei of epimastigotes treated with 200 μM NOO^- (Fig. 6A) or 200 μM H_2O_2 (Fig. 6B) alone or associated to 10 mM MX is shown. The percentage of parasites exposed to 200 μM NOO^- showing

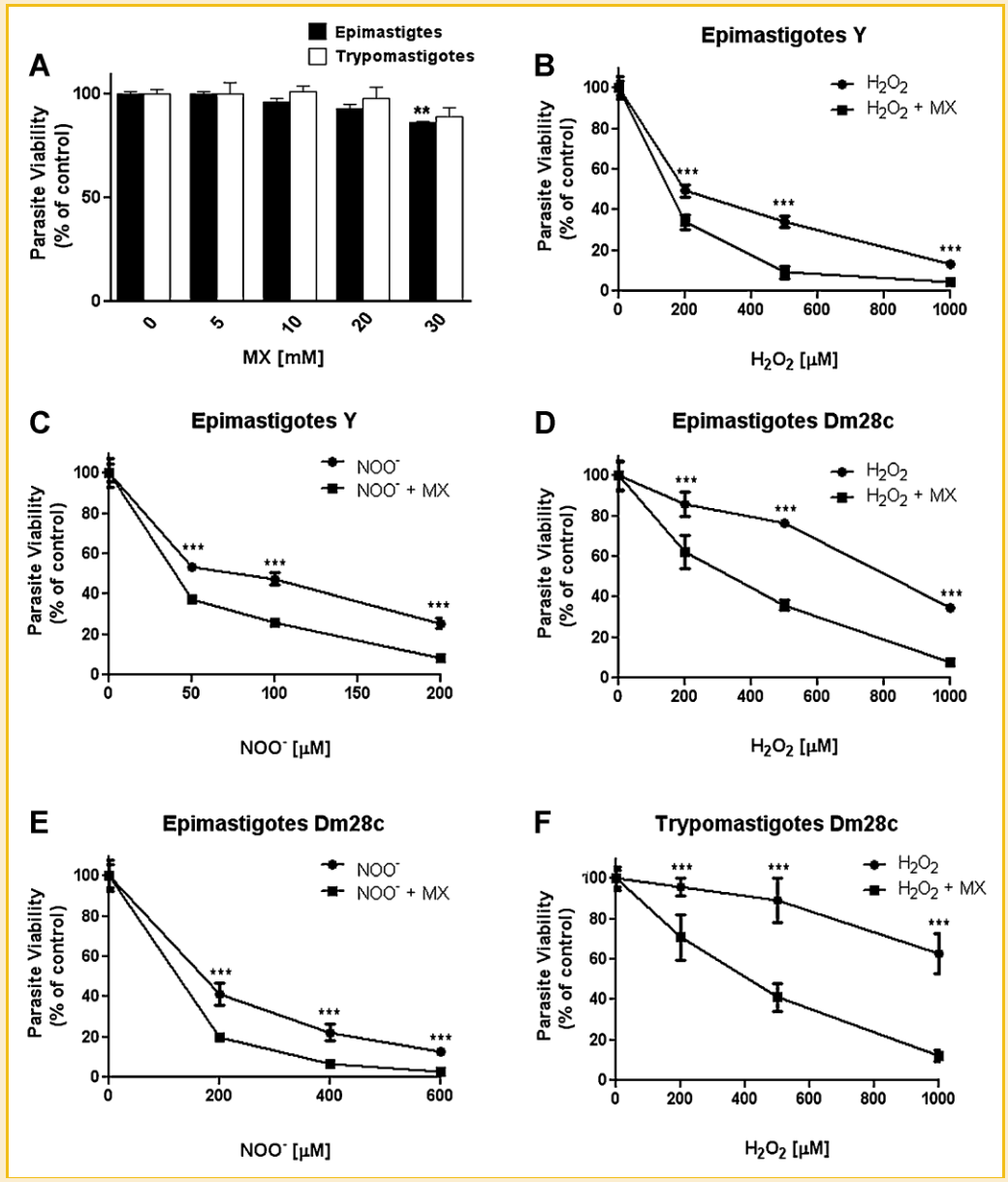

Fig. 4. Viability of *T. cruzi* epimastigotes and trypomastigotes treated with different concentrations of H_2O_2 and NOO^-, alone or combined with MX. (A) Viability of epimastigotes Dm28c strain (black squares) and trypomastigotes Dm28c strain (white squares) treated with different concentrations of methoxyamine for 8 h at 28 and 37°C, respectively. (B)–(F) Viability of parasites treated with increasing H_2O_2 or NOO^- concentrations for 30 min, washed and subsequently incubated in the presence or absence of 10 mM MX for 8 h. (B) and (C) Epimastigotes Y strain treated with H_2O_2 or NOO^-, respectively; (D) and (E) epimastigotes Dm28c strain treated with H_2O_2 or NOO^-, respectively; (F) trypomastigotes Dm28c strain treated with H_2O_2. Data were analyzed using two-way ANOVA test with Bonferroni post-test ($^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

nuclear DNA fragmentation ($12.33\% \pm 6.71$) increases three-fold after 10 mM MX addition ($32.66\% \pm 11.23$, $P<0.05$). Similarly, nuclear DNA fragmentation induced in *T. cruzi* epimastigotes by 200 μM H_2O_2 ($13.33\% \pm 5.79$) is increased approximately 3.5-fold after MX treatment ($46.83\% \pm 2.36$, $P<0.01$). Positive TUNEL cell indexes were obtained counting 200 nuclei in three independent experiments.

In exponential epimastigote cultures it was observed that some parasites show TUNEL positive in their kDNA though the nuclei in the same cells were TUNEL negative (Fig. 6C, control non-treated). We have no explanation for this result; however, it should be taken into account when examining TUNEL assay in these parasites. To avoid this artifact we consider as TUNEL positive those parasites showing reactivity in both nuclei and kDNA (Fig. 6C, 200 μM H_2O_2).

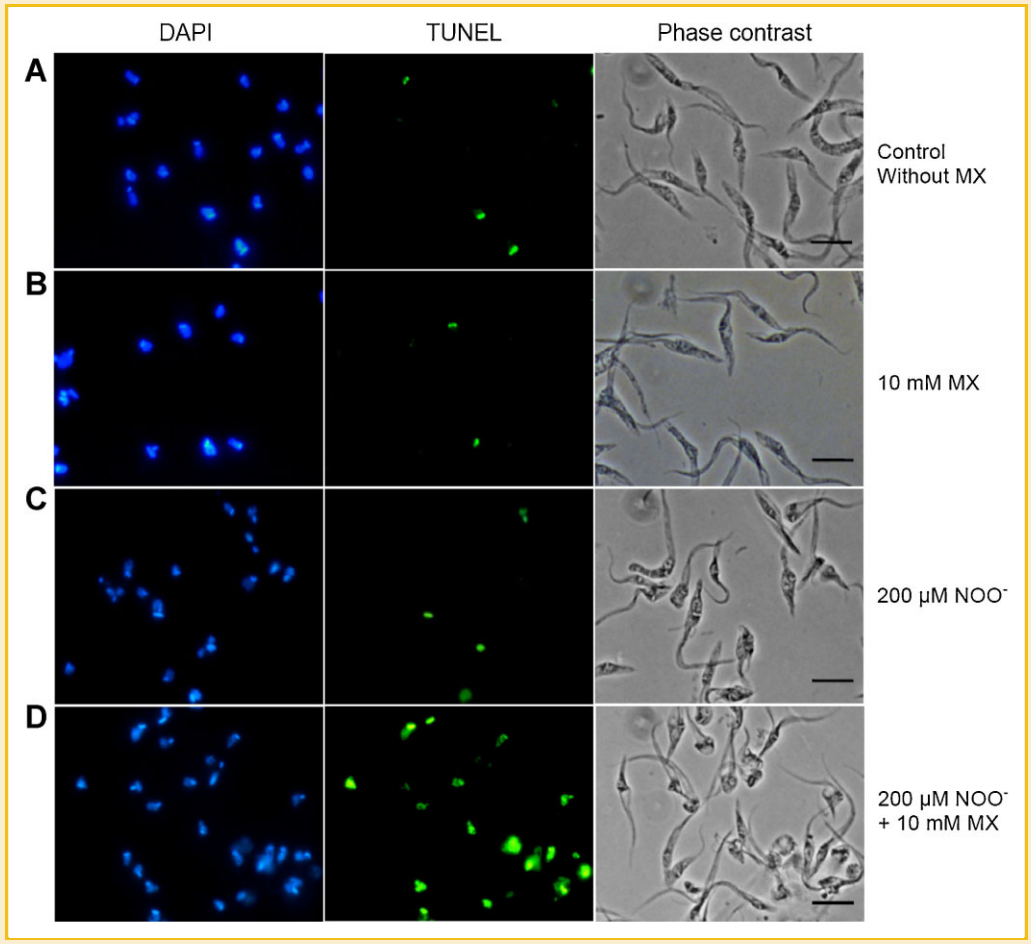

Fig. 5. The BER inhibitor methoxyamine increases DNA fragmentation induced by peroxinitrite in *T. cruzi* epimastigotes. Representative TUNEL assay images of *T. cruzi* epimastigotes Dm28c strain without treatment (A) or exposed to 10 mM MX for 8 h (B), or to 200 μM NOO^- for 30 min at 28°C, washed and subsequently non-treated (C) or treated (D) with 10 mM MX for 8 h.

Parasites showing DNA fragmentation in the nuclei only were never observed.

DISCUSSION

DNA damage induced by ROS/RNS includes simple and double strand breaks, base and sugar modifications, DNA/protein interactions, and DNA depurination or depirymidation, among others [Shackelford et al., 2000]. DNA damage affects key processes of cellular metabolism and as a consequence, several alternative pathways are activated, such as DNA repair mechanisms, stimulation of cell cycle checkpoints detention, triggering of apoptosis or other mechanisms of cell death [Norbury and Hickson, 2001; Sancar et al., 2004].It has been reported in the literature that the three cellular forms of *T. cruzi* (epimastigote in the insect, trypomastigote and amastigote in the host mammalian cells) survive oxidative stress conditions. In relation to epimastigotes, evidence for intense production of H_2O_2 after a blood meal has been provided for the triatomine *Rhodnius prolixus* due to digestion of vertebrate hemoglobin in the midgut [Paes et al., 2001]. The production of hem has been shown to act as a promoter for the formation of free radicals, leading to DNA oxidation [Aft and Mueller, 1983]. On the other hand the trypomastigotes, upon entering mammalian macrophages through the wound provoked by the insect, are attacked by oxygen free radicals (ROS) generated by the NADPH oxidase system [Ho et al., 1992; Cardoni et al., 1997; Piacenza et al., 2008] and by RNS produced by arginine metabolism [Martins et al., 1998; Peluffo et al., 2004]. Finally, the intracellular amastigote forms in the cardiac tissue must resist the oxidative damage exerted by oxygen and nitrogen free radicals (ROS/RNS) generated by infiltrating inflammatory cells from the immune innate system (macrophages and monocytes) [Machado et al., 2000; Silva et al., 2003; Zacks et al., 2005] as well as from cardiomyocytes mitochondrial dysfunction [Vyatkina et al., 2004; Wen et al., 2006; Gupta et al., 2009]. Thus, to establish parasite persistence in the heart and other host tissues, *T. cruzi* should repair its own damaged DNA activating DNA repair molecules to insure parasite survival in the host leading to the establishment of the chronic phase of Chagas disease.

Regarding *T. cruzi* DNA damage and repair Goijman et al. [1985] have shown that 3 h of incubation of epimastigotes with 10 μM

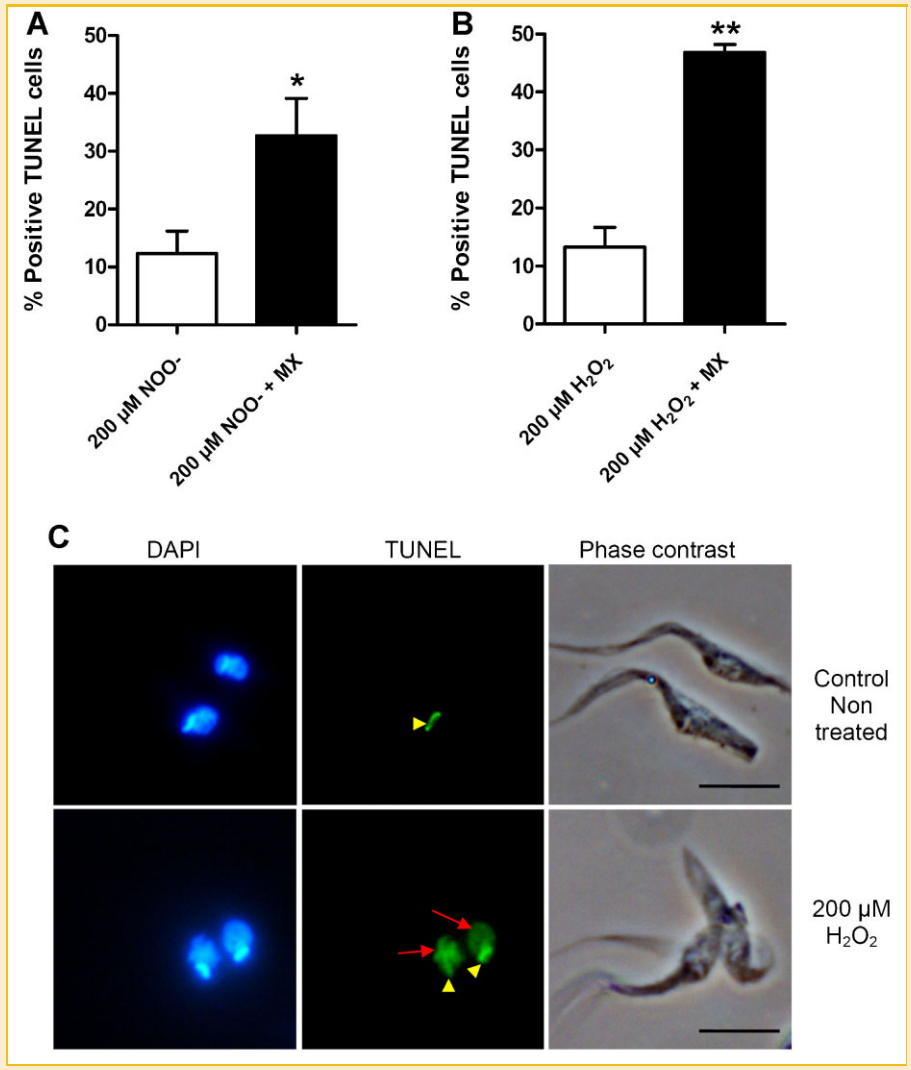

Fig. 6. Methoxyamine increases the percentage of TUNEL positive *T. cruzi* epimastigotes exposed to oxidative agents. Percentage of TUNEL positive epimastigotes Dm28c strain treated with 200 μM NOO^- (A) or to H_2O_2 (B) for 30 min, respectively, washed and subsequently incubated in the presence or absence of 10 mM MX for 8 h. Two hundred parasites were counted in each experiment in triplicate. Statistical analysis was performed by *T* student test. (C) TUNEL positive reaction in *T. cruzi* epimastigotes kDNA (arrowheads) and nDNA (arrow) treated or non-treated with 200 μM H_2O_2 for 1 h at 28°C.

Nifurtimox or 38 μM Benznidazole, drugs used for treatment of Chagas disease, induces nuclear and kinetoplastid DNA damage. This effect is reversed when the drugs are removed and the cells are incubated in fresh medium for 24 h. These results strongly suggest the presence of repair mechanisms of DNA damage in *T. cruzi*. However, the direct effect of ROS/RNS on DNA has not been yet proved in this parasite.

The most prevalent oxidative DNA damage involving the reaction of ROS with DNA is the conversion of guanine to 7,8-dihydro-8-oxoguanine (8-oxoguanine or 8-oxoG) [Slupphaug et al., 2003]. On the other hand, RNS interacts with DNA via direct oxidative reactions or via indirect, radical-mediated mechanisms [Pacher et al., 2007].

We report here for the first time a direct oxidative DNA damage produced by ROS (H_2O_2) and RNS (NOO^-) agents in *T. cruzi* as measured by the presence of 8-oxoG both in nuclei and kinetoplast, using immunocytochemistry. This technique was used for the same purpose to test in situ oxidative DNA damage in rat hepatocytes [Kemeleva et al., 2006], in TK6 human lymphoblastoid cells [Soultanakis et al., 2000], and in the nuclei of *Echinococcus granulosus* [Cabrera et al., 2008].

Additionally, *T. cruzi* DNA damage induced by ROS/RNS was also detected using indirect methods such as the ARP assay in total DNA and the LA-QPCR in nuclear and kinetoplast DNA. The ARP assay was previously used to detect DNA damage in HeLa cells [Chastain et al., 2006], in *Borrelia burgdorferi* [Boylan and Gherardini, 2008], and in *Boletus edulis* [Collin-Hansen et al., 2005]. On the other hand, the LA-QPCR was used to quantify oxidative DNA damage and the kinetics of DNA repair by several authors [Sawyer et al., 2003; Chen et al., 2007; Kovalenko and Santos, 2009]. Using these three different approaches we demonstrated that ROS/RNS provokes

oxidative DNA damage in the nucleus and in the kinetoplast of *T. cruzi*.

Base excision repair (BER) is an evolutionarily conserved process for maintaining nuclear and mitochondrial genomic integrity caused predominantly by ROS/RNS [Mandavilli et al., 2002; Hegde et al., 2008]. In *T. cruzi* enzymes proper of the BER system were identified [Pérez et al., 1999; Fárez-Vidal et al., 2001; Fernández Villamil et al., 2008; Venegas et al., 2009]. However, there is no evidence regarding the function of this DNA repair system in this parasite. Then, it was necessary to prove that *T. cruzi* is able to repair oxidative DNA damage caused by ROS/RNS. Indeed 4 h after removal of the oxidative agent DNA repair is evident as measured by both ARP and LA-QPCR methods. Accordingly, DNA repair systems are present and active in *T. cruzi* exposed to ROS/RNS.

At present there are several studies devoted to inhibit the BER pathway for the treatment of neoplasias resistant to chemotherapeutic agents that produce DNA damage. MX, a compound that impairs DNA repair through indirect inhibition of the BER pathway activity, improves the cytotoxic effects of temozolomide in cancer ovary cells [Fishel et al., 2007], in colon cancer cells [Liu et al., 2002], and in cancer cells of the mammary gland [Rinne et al., 2004]. Additionally, MX increases the effect of DNA damage via ROS as it was reported in myeloid leukemia U937 cells treated with manumycin [She et al., 2005]. The indirect inhibition of the BER pathway enzyme APE1 activity by MX derives from the capacity of this drug to react with the acyclic pentose generated after removal of the damaged base by DNA glycosilases [Liuzzi and Talpaert-Borlé, 1985]. Moreover, Taverna et al. [2001] have shown that MX potentiates single and double DNA strands fractures induced by temozolomide in colon cancer cells.

As it is reported here, MX increases the cytotoxic effect induced by H_2O_2 and NOO^- in *T. cruzi* epimastigote and trypomastigote cellular forms. These results clearly suggest the presence of an active DNA repair BER mechanism in *T. cruzi*.

Several authors have shown that oxidative species induce DNA fragmentation as measured by the TUNEL assay [Ryu et al., 2003; Thomson et al., 2009; Chen et al., 2010], which is increased by MX [Taverna et al., 2001]. Accordingly, in this report we show DNA fragmentation in parasites treated with ROS/RNS species that increases vertically when MX is added simultaneously and maintained after the removal of the oxidative species.

The increase in cell death induced by MX after treatment with the oxidative agents is probably explained by fragmentation of *T. cruzi* DNA as observed by TUNEL assay.

In summary (a) ROS/RNS induces nuclear and kinetoplastid DNA damage in *T. cruzi*, (b) this parasite is able to repair the oxidative DNA damage, (c) ROS/RNS diminishes parasite cell viability, (d) inhibition of the BER DNA repair pathway increases the cytotoxicity induced by ROS/RNS.

As a consequence, the BER DNA repair system may be considered as a pharmacological target. Considering that amastigotes persistence in cardiac host cells are responsible for the chronic phase of Chagas disease [Levin, 1996; Tarleton, 2001; Hyland et al., 2007; Gutierrez et al., 2009] and taking into account that this cytoplasmic form of the parasite is also under ROS/RNS attack [Piacenza et al., 2009], pharmacological agents directed to inhibit *T. cruzi* DNA repair mechanisms, should be important to control the chronic phase of this illness.

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