



Research paper

Characterization of *Trypanosoma cruzi* MutY DNA glycosylase ortholog and its role in oxidative stress response



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ABSTRACT

Trypanosoma cruzi is a protozoan parasite and the causative agent of Chagas disease. Like most living organisms, it is susceptible to oxidative stress, and must adapt to distinct environments. Hence, DNA repair is essential for its survival and the persistence of infection. Therefore, we studied whether *T. cruzi* has a homolog counterpart of the MutY enzyme (*TcMYH*), important in the DNA Base Excision Repair (BER) mechanism. Analysis of *T. cruzi* genome database showed that this parasite has a putative MutY DNA glycosylase sequence. We performed heterologous complementation assays using this genomic sequence. *TcMYH* complemented the *Escherichia coli* *MutY*- strain, reducing the mutation rate to a level similar to wild type. In *in vitro* assays, *TcMYH* was able to remove an adenine that was opposite to 8-oxoguanine. We have also constructed a *T. cruzi* lineage that over-expresses MYH. Although in standard conditions this lineage has similar growth to control cells, the over-expressor is more sensitive to hydrogen peroxide and glucose oxidase than the control, probably due to accumulation of AP sites in its DNA. Localization experiments with GFP-fused *TcMYH* showed this enzyme is present in both nucleus and mitochondrion. QPCR and MtOX results reinforce the presence and function of *TcMYH* in these two organelles. Our data suggest *T. cruzi* has a functional MYH DNA glycosylase, which participates in nuclear and mitochondrial DNA Base Excision Repair.

1. Introduction

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease, a condition that currently affects around 5.7 million people in Latin America, causing negative social effects and healthcare burden (Bern, 2015; WHO, 2015; Rassi et al., 2010). This parasite belongs to Kinetoplastida, a class that comprises unicellular flagellated protists, characterized by the presence of the kinetoplast, a DNA-containing granule localized inside their single mitochondrion (Rodrigues et al., 2014).

The life cycle of *T. cruzi* requires both vertebrate and invertebrate hosts. In the insect (invertebrate) vector, the protozoan undergoes extracellular replication, whereas in the mammalian (vertebrate) host it

replicates by obligate intracellular multiplication (Rodrigues et al., 2014). Hence, the parasite has to handle oxidative burst from the immune systems of its hosts, and from oxidative species inside mammalian cells. This oxidative burst generates superoxide anions (O₂^{·-}) and other reactive oxygen species (ROS), such as hydrogen peroxide (Gupta et al., 2009).

Several molecules, including carbohydrates, proteins, lipids and nucleic acids can be oxidized by ROS, leading to deleterious effects in cells (Nakabepu, 2014). ROS cause a variety of damage at the DNA level, from single- and double-strand breaks (SSBs and DSBs, respectively) to base oxidation. One of the most abundant and best-characterized oxidative lesions is 8-oxoguanine (8-oxoG). This modified base has a great biological importance, since it can mispair with

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adenine residues, increasing the frequency of spontaneous G:C to T:A mutations. It is estimated that there are approximately 10,000 8-oxoG residues per human cell nucleus (Nakabeppu, 2014; Ohno et al., 2006).

Given the importance and the abundance of 8-oxoG in eukaryotic genomes, eukaryotes have evolved a multi-defense mechanism, the so-called GO system, which is able to counteract this type of lesion. Three enzymes comprise the GO system: MTH (MutT Homolog), which hydrolyses 8-oxodGTP to 8-oxoGMP in the nucleotide pool, preventing the incorporation of 8-oxoG into DNA; OGG1, a DNA glycosylase that removes 8-oxoG from 8-oxoG:C base pairs; and MYH (MutY Homolog), also a glycosylase, which excises adenine from 8-oxoG:A pairs (Tchou and Grollman, 1993; Michaels and Miller, 1992). Our research group has previously described TcMTH and TcOGG1 activities in *T. cruzi* oxidative response (Furtado et al., 2012; Aguiar et al., 2013). Overexpression of TcMTH led to improved growth of parasites in macrophage cultures when compared to wild type cells and overexpression of TcOGG1 increased DNA lesions of parasites treated with hydrogen peroxide (Furtado et al., 2012; Aguiar et al., 2013).

OGG and MYH are involved in DNA Base Excision Repair (BER), a repair pathway that usually resolves oxidative DNA lesions, in which a set of enzymes act sequentially. First, a specific DNA glycosylase recognizes and removes the damaged base, resulting in an abasic site (AP site) that can be lethal if unrepaired. After that, an AP endonuclease cleaves the DNA backbone, leaving room for a phosphodiesterase to act. Finally, a DNA polymerase and a DNA ligase complete the DNA repair (Kim and Wilson, 2012).

MutY and its eukaryotic counterpart MYH have been characterized in several organisms, including *Escherichia coli*, *Mus musculus* and *Homo sapiens*, among others (Nghiem et al., 1988; Ichinoe et al., 2004; McGoldrick et al., 1995). This enzyme is a monofunctional glycosylase, that is, it cleaves the base from DNA, but it does not possess lyase activity (Mazzei et al., 2013; de Oliveira et al., 2014). This protein can be localized both to the nucleus and mitochondria (Ohtsubo et al., 2000). Various MYH mutations have been linked to diseases, especially colorectal cancer (Al-Tassan et al., 2002; Nielsen et al., 2011; Mazzei et al., 2013). *In silico* analysis of the *T. cruzi* genome showed that this protozoan possesses one putative copy of MYH gene (El-Sayed et al., 2005a, 2005b). Since MYH is an important protein involved in the reduction of the mutagenic events derived from oxidative stress, we decided to characterize this enzyme in *T. cruzi*, an organism that is subject to numerous and varied oxidative stress sources during its life cycle. Our results show that the *T. cruzi* enzyme is able to complement bacteria deficient in MutY gene, as well as recognize 8-oxoG paired with A. In addition, the overexpression of TcMYH in epimastigotes indicates that it could participate in responses to oxidative stress in the nuclear and mitochondrial genomes.

2. Materials and methods

2.1. Alignments

Sequence alignments and motif analyses were performed using the Multalin (Corpet, 1988) and Boxshade 3.21 (Hofmann and Baron, 1992) interfaces. Protein-targeting signals were predicted using MI-TOPROT (Claros and Vincens, 1996) and NucPred (Brameier et al., 2007). Domains were determined by Pfam (Punta et al., 2012) and CDD (Marchler-Bauer et al., 2013). GenBank accession numbers of the MutY sequences used in this work are: *Trypanosoma cruzi* (TcMYH [GenBank:XP_07715.1]); *Trypanosoma brucei* [GenBank:CAD59974.1], *Leishmania major* [GenBank:XP_001684497.1], *Arabidopsis thaliana* [GenBank:NP_193010.2], *Mus musculus* [GenBank:NP_001153053.1], *Homo sapiens* [GenBank:Q9UIF7.1] and *Escherichia coli* [GenBank:YP_491160.1].

2.2. *Escherichia coli*

2.2.1. Strains and plasmids

E. coli strains used in this work were AB1157 (*F-thr-1*, *leuB6*, *thi-1*, *argE3*, *his-G4*, Δ (*gpt-proA*), *62lacY1*, *galK2*, *xyl-5*, *ara-14*, *rpsL31*, *kdgK51* *mtl-1*, *txs-33*, *supE44*(str^R) *rfbD1*) (Bachmann, 1972) and BH980 (a derivative of CC104 (*ara* Δ (*gpt-lac*)5 (F' *lacI378lacZ461 proA*⁺ B⁺)), but *mutY::kan^R*) (Cupples and Miller, 1989). Bacteria were grown at 37 °C, with agitation (180 rpm), in 2xYT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.0) (Sambrook and Russell, 2001) containing ampicillin (100 μ g·mL⁻¹) and supplemented with kanamycin (10 μ g·mL⁻¹) or streptomycin (10 μ g·mL⁻¹), depending on the bacterial strain. TcMYH was amplified from the genomic DNA of CL Brener strain using the primers TcMutY.Xba-F (5'-TCTAGAATGAATGAGCTT-TACCGGGC-3') and TcMutY.Hind-R (5'-AAGCTTTTACTTATCACCGG-TTTTCTTAAGTT-3'). The amplification product was cloned into pMAL-c2G (NE BioLabs Inc.) using XbaI and HindIII restriction sites in order to produce pMAL_TcMYH. Bacteria were transformed by electroporation (Sambrook and Russell, 2001).

2.2.2. Spontaneous mutation assay

The spontaneous mutation assay using rifampicin (rif) was conducted as previously described (Augusto-Pinto et al., 2001). *E. coli* BH980 strain (*mutY*⁻) transformed with empty pMAL-c2g or with pMAL_TcMYH was used to determine the rif-resistant mutant (rif^R) frequency in this heterologous complementation test. Bacterial cells were diluted and plated on 2xYT medium complemented with ampicillin (100 μ g· μ L⁻¹), kanamycin (10 μ g· μ L⁻¹) and IPTG (0.1 mM), in order to measure cell viability. The resistance assay was performed using the cultures undiluted, and plates complemented with rifampicin (100 μ g· μ L⁻¹) and the antibiotics previously mentioned. Plates were incubated at 37 °C for 18 h. After this period, bacterial colonies were counted, and at least 15 plates per group were used. The mutation rate was calculated according to Lea and Coulson, 1949.

2.2.3. Protein expression and purification

Expression of TcMYH fused to Maltose Binding Protein (MBP) was accomplished by using pMAL™ Protein Fusion and Purification System (New England Biolabs). *E. coli* BH980 (*mutY*^{-/-}) was transformed with pMAL_TcMYH and the expression and purification of the resulting protein was conducted according to manufacturer instructions. MBP-TcMYH fusion protein was purified using affinity chromatography on an amylose column with column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA). The enzyme was stored at -70 °C.

2.2.4. *In vitro* assay for MutY DNA glycosylase activity

DNA substrates used in this work were purchased from AlphaDNA and Sigma-Aldrich. The DNA duplexes consisted of a strand containing 8-oxoguanine (GO; 5'-GAT CCT CTA GAG TC(GO) ACC TGC AGG CAT GCA-3') and a fluorescein-labelled strand, which was complementary to the previous strand and had an adenine or a cytosine in position 16 (AdenineFluor: 5'-6TGC ATG CCT GCA GGT AGA CTC TAG AGG ATC-3'; CytosineFluor: 5'-6TGC ATG CCT GCA GGT CGA CTC TAG AGG ATC-3'; 6 = fluorescein label). Annealing of oligonucleotides was performed in 100 mM Tris-HCl, 100 mM EDTA, 1 M NaCl and 20 μ M of each strand, by heating the oligonucleotide mixture for 5 min at 80 °C followed by slow cooling to 25 °C.

The MutY DNA glycosylase activity assay was performed following protocols previously described (Lopes Dde et al., 2008; Schamber-Reis et al., 2012). 1 μ L of each oligonucleotide duplex (32 nM) was added to 0.5 μ g· μ L⁻¹ of protein and 10 \times RECTM Buffer 4 (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA; R & D Systems, Inc.). Proteins used were EcMutY (R & D Systems, Inc.), MBP-TcMYH and MBP (purified with the same protocol as MBP-TcMYH). After incubation at 37 °C for 18 h, 1 M NaOH was added to the reactions, in order to complete the strand cleavage, at 90 °C for 30 min. Reactions were inactivated using

4 μL stop solution (80% deionized formamide, 10 mM EDTA, pH 8.0, 1 $\text{mg}\cdot\text{mL}^{-1}$ xylene cyanol, 1 $\text{mg}\cdot\text{mL}^{-1}$ bromophenol blue), heated to 90 °C for 2 min and then placed on ice. Samples were resolved on denaturing polyacrylamide sequencing gels in an automated ALF DNA Sequencer (GE Healthcare). Analyses were carried out using AlleleLocator software, version 1.0.3 (GE Healthcare). This experiment was repeated three times.

2.3. *Trypanosoma cruzi*

2.3.1. Parasite growth and transfection

Epimastigote forms of *T. cruzi* CL Brener strain were grown at 28 °C in liver infusion tryptose (LIT) medium, pH 7.3, supplemented with 10% heat-inactivated fetal bovine serum, 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin as previously described (Camargo, 1964).

The vector pROCK_TcMYH was constructed by amplifying TcMYH with the primers TcMYH.Xba-F (the same used for pMAL) and TcMYH.Xho-R (5'-CTCGAGTTACTTATCACCGGTTT-3') and cloning the amplification product into XbaI and XhoI restriction sites of pROCK_HYGRO (DaRocha et al., 2004). The vector pTREX_TcMYH-GFP was constructed by amplifying TcMYH with the primers TcMYH.Xba-F and TcMYH.Eco-R (5'-GAA TTC CTT ATC ACCGGTTTTCTTAAGTTT-TTCC-3') and inserting the amplification product into XbaI and EcoRI sites of pTREX-GFP (Vazquez and Levin, 1999). The parasite transfection was performed by electroporation according to a previously described protocol (DaRocha et al., 2004). Transfected parasites containing stably incorporated pROCK_TcMYH expression vector were selected after 6 weeks of culturing in the presence of Hygromycin (200 $\text{mg}\cdot\text{mL}^{-1}$).

2.3.2. RNA extraction and cDNA synthesis

Total RNA was isolated from *T. cruzi* epimastigote forms using TRIzol reagent (Invitrogen, Life technologies, CA, USA) and treated with DNase (Invitrogen) for DNA contaminant removal, according to manufacturer's instructions. cDNA was synthesized using High Capacity RNA-cDNA master mix (Applied Biosystems). The negative control was processed in the same conditions as the other samples, but without reverse transcriptase.

2.3.3. Real-time quantitative polymerase chain reaction

The reactions were conducted using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Each reaction was run in triplicate and contained 1 ng of cDNA, 300 nM of each primer, 7.5 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems) and deionized H₂O, totaling 15 μL per reaction. cDNA was amplified at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A reverse transcription negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. The primers sequences used were: TcMutYRealTimeForw: 5'-GCG AAG TTA TGT CGC AGC AG -3' (20 bp) and TcMutYRealTimeRev: 5'-CAG AGG CCT CGG CTA AAA CA-3' (20 bp), selected from the complete nucleotide sequence of TcMYH [GenBank: NW_001849441.1] (fragment size: 100 bp); RT_gGAPDH2_for 5'-CGGTGGACGGTGTGTCGGTG-3' (20 bp) and RT_gGAPDH2_rev 5'-CCGTCAGCTTGCCTGGGTG-3' (20 bp), from *T. cruzi* housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase; [GenBank: XM_807045.1]; fragment size: 129 bp). To ensure that only a single product was amplified, a melting curve analysis was performed. The relative amount of gene product in each sample was determined using the established $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001) using GAPDH as a reference gene. The real-time PCR efficiency was determined for each gene using the slope of a linear regression model (Pfaffl, 2001), which was determined by measuring the CT for a specific threshold for a range of serial dilutions.

2.3.4. Growth and survival curves

Parasite cultures containing initially 5×10^6 parasites $\cdot\text{mL}^{-1}$ were incubated at 28 °C. The cell growth was monitored by counting parasites daily, for 8 days. Cells were counted in a cytometric chamber using erythrosine vital stain to differentiate living and dead cells. For testing resistance to H₂O₂, parasite cultures containing 5×10^6 parasites $\cdot\text{mL}^{-1}$ were treated with 0, 75, 100 or 125 μM H₂O₂. After incubation for 72 h, cell number was measured by counting as described above. For testing sustained oxidative conditions, 20×10^6 parasites were incubated with 5 mM glucose plus 25, 50 or 100 mU glucose oxidase from *Aspergillus niger* (Sigma) for 24 h at 28 °C. These conditions generated a range of 45–65 μM H₂O₂ concentration (measured by Amplex Red Hydrogen/Peroxide/Peroxidase kit (Invitrogen)). Parasites were then washed and counted as previously described. The results were expressed as percentage of growth when compared to untreated cultures. Experiments were performed in triplicate.

2.3.5. AP sites analysis

Cultures of *T. cruzi* epimastigotes containing 1×10^7 cells $\cdot\text{mL}^{-1}$ were treated with 100 μM H₂O₂. DNA from these cultures were extracted using tissue protocol from QIAGEN Blood & Cell Culture DNA Mini Kit after the exposure to hydrogen peroxide, at the time points 0.5 h, 1 h, 6 h, 10 h and 24 h. DNA damage was estimated using Abcam DNA Damage – AP Sites – Assay Kit (Abcam - ab65353) standard protocol. The AP sites assay kit is based on the ARP reagent (Aldehyde Reactive Probe, which is linked to biotin) interaction with the aldehyde group present in the open ring of AP sites. After treatment of DNA samples with ARP, AP sites are tagged with biotin residues and can be quantified using an avidin-biotin assay followed by a colorimetric detection with HRP developer. The relative number of AP sites was estimated by comparison of the OD (450 nm) value from wild type CL Brener strain and TcMYH-overexpressor lineage.

2.3.6. Fluorescence microscopy

Epimastigote forms of *T. cruzi* were transfected with the pTREX vector encoding GFP or TcMYH fused to GFP. Transfected parasites were harvested 24 h after electroporation, washed once in PBS and centrifuged at 3000 g for 10 min. Cell pellets were re-suspended in PBS such that the sample was concentrated 100-fold and then fixed in 4% paraformaldehyde for 30 min at 4 °C. Paraformaldehyde-treated cells were then placed on slides, washed with PBS and incubated with DAPI (4-6-diamino-2-phenylindol, Invitrogen), diluted 1:1000 v/v, for 1 min at 37 °C. Epimastigotes were washed in PBS and anti-fading reagent (SlowfadeAntifade kit, Invitrogen) was added. Images were captured with a Nikon Eclipse Ti (Nikon Instruments Inc.) microscope using 408 and 561 nm lasers for probe excitation, with the pinhole aperture adjusted to 1 airy unit. Images were analysed with NIS Elements (Nikon Instruments Inc.) and ImageJ (Schneider et al., 2012) software. All the images were captured at CAPI (Centro de Aquisição e Processamento de Imagens - ICB/UFGM).

2.3.7. Analysis of DNA damage level after genotoxic treatment using quantitative PCR assay (QPCR)

Parasite cultures at 1×10^7 cells $\cdot\text{mL}^{-1}$ were incubated with 100 μM H₂O₂ for 0.5 h and then cells were harvested by centrifugation at 3000 g for 10 min. Immediately after, DNA of treated and untreated cells were extracted using QIAGEN QIAamp DNA Blood Mini Kit. Quantification, quantitative PCR assay (QPCR) amplification, and data analysis were conducted as reported by Rajao et al. (2014). The large nuclear fragment was amplified using the forward primer QPCRNucF (5'-GCACACGGCTGCGAGTGACCATTCAACTTT-3') and the reverse primer QPCRNuc2R (5'-CCTCGCACATTCTACCTTGCCCTCAATGCC-TGC-3'). Mitochondrial damage was measured using the same methodology, but with a specific primer set (QPCRMitF 5'-TTTTATTGGG-GGAGAACGGAGCG-3'; QPCRMitR 5'-TTGAAACTGCTTCCCCAAACG CC-3'). In order to eliminate any bias regarding the different copy

numbers of nuclear and mitochondrial genomes, an internal primer was used to amplify a small fragment (250 bp) for each one of these genomes. For the small nuclear fragment, was used the forward primer QPCRNUCint (5'-TCGAGCAAGCTGACACTCGATGCAACCAAAG-3') with the reverse primer QPCRNUC2R. For the mitochondrial genome was used the forward primer QPCRMITint (5'-CGCTCTGCCCCATAAAAAACCTT-3') with the reverse primer QPCRMITR. The normalized amplification was compared between treated and untreated samples and these values were used to estimate the average number of lesions per 10 kb of both genomes (using Poisson distribution). The results are expressed as a mean for two biological replicates.

2.3.8. Treatment with MtOX reagent

Parasite cultures containing initially 5×10^6 parasites·mL⁻¹ were incubated at 28 °C. Cell growth was monitored by counting parasites every other day, for 9 days. The resistance assay was carried by exposing 5 mL parasites cultures containing 1×10^7 parasites·mL⁻¹ to 16 µM MtOX reagent. After incubation for 1 h, the survival was measured by counting the cells (as already described) just after the end of the treatment, and after 24 h of growth. The results are presented as percentage of growth comparing each strain with their respective untreated cultures. All experiments were performed in triplicate.

2.4. Graphics and statistical analysis

All graphics and statistical analyses were performed using GraphPad Prism 5.0 and 7.0 softwares.

3. Results

3.1. *Trypanosoma cruzi* has a MYH ortholog

Analysis of *T. cruzi* genome database (El-Sayed et al., 2005a, 2005b; The Kinetoplastid Genome Resource: TriTrypDB - <http://tritrypdb.org/tritrypdb>) showed that this protozoan has a putative MYH DNA glycosylase gene (*TcMYH*) present in a single copy in CL Brener strain. The 451 amino acids sequence from *TcMYH* protein was aligned with MutY orthologs from other organisms (Fig. 1). *TcMYH* sequence exhibits high conservation of the α -helix-hairpin- α -helix-Gly/Pro-Asp motif and a conserved aspartic acid residue, both characteristic of the base excision DNA repair HhH-G/PD protein superfamily, in which MutY/MYH is included (Jansson et al., 2010; Jacobs and Schar, 2012).

Furthermore, *TcMYH* contains the residues that are responsible for linkage to DNA, for 8-oxoguanine recognition, and for substrate linkage (Brinkmeyer et al., 2012; Punta et al., 2012; Marchler-Bauer et al., 2013; Markkanen et al., 2013).

3.2. *TcMYH* complements *mutY*-bacteria

In order to characterize *TcMYH* activity *in vivo*, heterologous functional complementation assays were carried out in *Escherichia coli*. *TcMYH* gene sequence was cloned in pMAL vector, generating pMAL-*TcMYH* plasmid.

Bacterial strains AB1157 (*mutY*+) and BH980 (*mutY*-) were used in this test. Both are sensitive to the antibiotic rifampicin; thus, when plated on medium containing this antibiotic, only mutant clones grow. It is already known that the AB1157 (WT) strain has low rates of spontaneous mutations, while the BH980 strain has high mutation rates (Cupples and Miller, 1989). Cells were transformed with empty pMAL or pMAL-*TcMYH*, and plated on agar dishes containing rifampicin and ampicillin, or only ampicillin. Table 1 shows that *TcMYH* complemented BH980. The mutation rate of this strain with empty pMAL was 61.1 ± 5.23 mutants per 10^8 cells, while BH980 transformed with *TcMYH* had approximately 3 mutants every 10^8 cells, giving a reduction of about 20 times when we compare cells that express *TcMYH* and cells that do not express this gene. Wild type (AB1157 pMAL and AB1157

TcMYH) cells had even lower mutation rates, from around 0.5 to 1 mutant per 10^8 cells.

3.3. *TcMYH* has MutY DNA glycosylase activity

TcMYH fused with MBP was partially purified from transformed *E. coli* lysates and assayed for DNA glycosylase activity *in vitro*.

After induction with IPTG, followed by purification of the soluble protein extract on an amylose resin column, a strong band at the 93 KDa position could be seen on the SDS-PAGE gel (Fig. 2A, Lane 1), indicating induction of expression of the recombinant protein MBP-*TcMYH*.

The *in vitro* assay was performed using 30-mer oligonucleotide duplexes as substrates, in which one strand contained an 8-oxoguanine (GO) residue at position 15, while the other strand was labelled with fluorescein contained an adenine or a cytosine opposite GO (Fig. 2B). Such duplexes were incubated with MBP-*TcMYH*, MBP, or *EcMutY* (the last one was purchased from R & D Systems, Inc.). After 18 h of incubation, NaOH was added to the reactions in order to finalize the breakage of the strand that had undergone excision by MutY, since MutY is a monofunctional glycosylase and does not cleave the phosphodiester bond of DNA (Fig. 2B). For duplexes containing cytosine (C/GO), there was only one band, about 34 nucleotides in length, for all the enzymes, even after addition of NaOH (Fig. 2C), corresponding to the intact strand (30 nucleotides). This demonstrates that none of the enzymes tested (*EcMutY*, MBP-*TcMYH*, and MBP) were able to remove the cytosine paired with 8-oxoguanine. For duplexes with adenine (A/GO), there was a band of 30 nucleotides, and another band near the 17 nucleotides standard for *EcMutY* enzyme (positive control) and for MBP-*TcMYH*, but not for MBP (negative control), after incubation with NaOH (Fig. 2C). This ~17 nucleotides band corresponds to the cleaved strand generated by MBP-*MutY* DNA glycosylase activity of the aforementioned enzymes.

3.4. Overexpression of *TcMYH* sensitizes *T. cruzi* to hydrogen peroxide and to glucose oxidase treatment, probably due to AP site accumulation

A *T. cruzi* population stably overexpressing *TcMYH* was generated using the integrative vector pROCK carrying the *TcMYH* gene. As shown in Fig. 3A, the overexpressor lineage (pROCK-*TcMYH*) presented a relative *TcMYH* mRNA expression about ten times higher (*p* value < 0.01) than *TcMYH* mRNA expression in the control lineage (pROCK), when analysed by RT-Real Time PCR. Despite this difference in the expression of *TcMYH*, both control parasites and *TcMYH*-overexpressor showed similar growth rates (Fig. 3B).

TcMYH-overexpressing cells were subjected to hydrogen peroxide (H₂O₂) or glucose oxidase treatment. These substances cause oxidative stress, leading to oxidative DNA lesions such as 8-oxoG. *TcMYH* overexpression considerably decreased the survival of *T. cruzi* against any of the H₂O₂ concentrations used (Fig. 4A), whereas treatment with glucose oxidase reduced *TcMYH*-overexpressing cells survival only at higher concentrations (50 mU and 100 mU; Fig. 4B). This decrease in cell resistance was not due to alteration in cell growth since both control and *TcMYH*-overexpressing cells show similar doubling times in the absence of genotoxic treatment (Fig. 3B). To test whether this alteration in survival response was caused by accumulation of DNA lesions, we performed an AP site assay (using Abcam DNA Damage – AP Sites – Assay Kit). *TcMYH* overexpression led to generation of higher amounts of AP sites in *T. cruzi*, when compared to control cells, at time points: 0.5 h, 6 h and 10 h (Fig. 4C). *TcMYH*-overexpressing cells showed a faster AP site accumulation, as the peak of AP sites' number for these cells is at 0.5 h, while for wild type the peak is at 1 h. The quantity of non-repaired AP sites persisted longer in *TcMYH*-overexpressor cells than in control cells, as the former were unable to reduce the number of AP sites even after 10 h, whereas wild type cells were able to repair AP sites at 6 h (Fig. 4C).

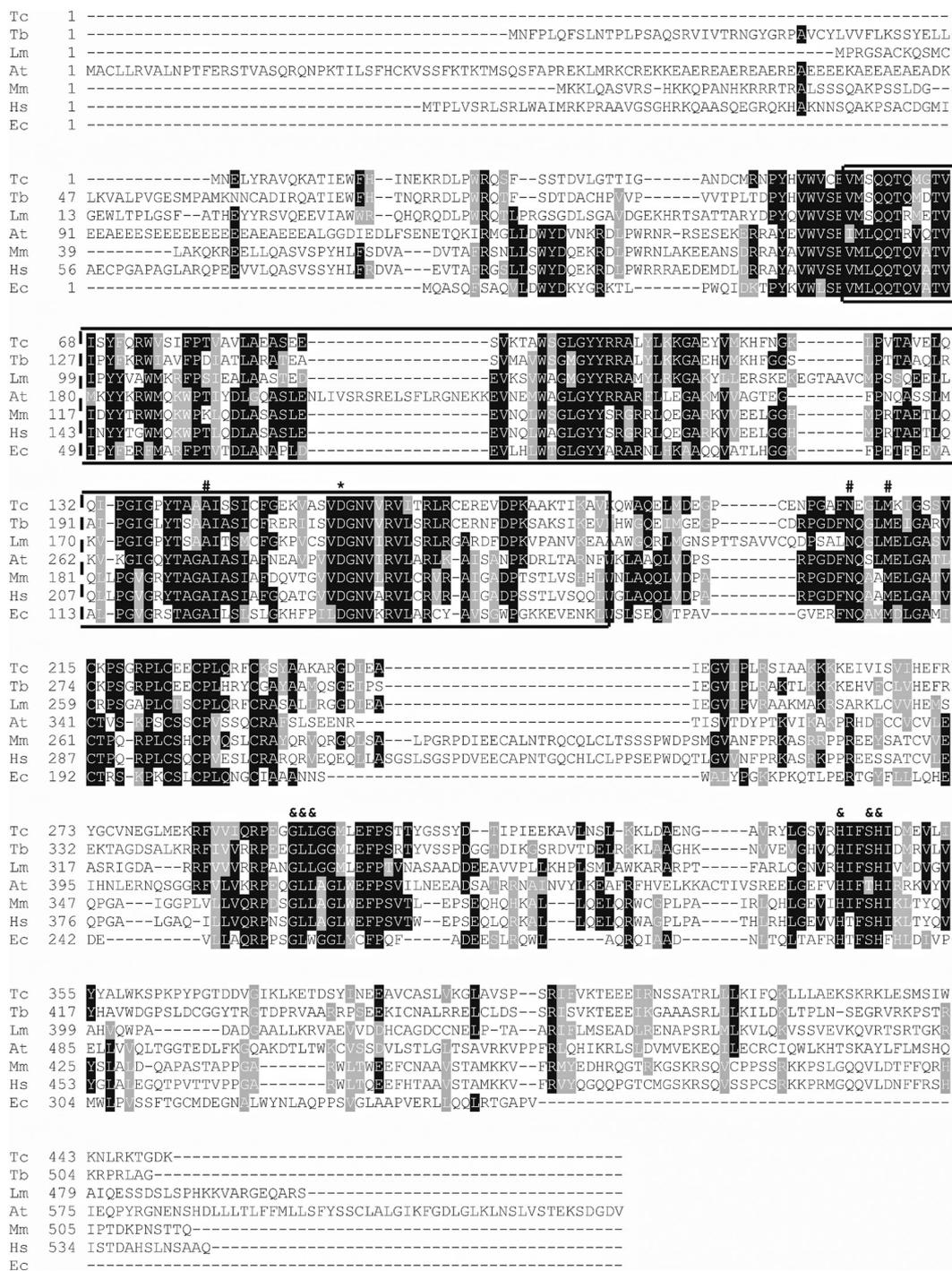


Fig. 1. Alignments with the predicted *TcMYH* products. Amino acid sequence comparison between MutY and MYH from *T. cruzi* (Tc), *T. brucei* (Tb), *L. major* (Lm), *A. thaliana* (At), *M. musculus* (Mm), *H. sapiens* (Hs) and *E. coli* (Ec). Residues shaded in black are identical amino acids. Residues shaded in gray indicate similar function. The motif α -helix-hairpin- α -helix-Gly/Pro-Asp, characteristic of HhH-GPD/K family, is enclosed by the box; the catalytic residue aspartate (D) is signaled by an asterisk (*); & corresponds to residues involved in the ligation to DNA and 8-oxoguanine recognition and # corresponds to residues that ligate to the substrate (adenine).

3.5. *TcMYH* is localized to the nucleus and to the antipodal sites of *T. cruzi*

Initially, *in silico* analysis was used to predict where *TcMYH* would be localized within the parasite. The protein has a 60% probability of being exported to the nucleus according to NucPred software (Brameier et al., 2007), and the same 60% probability of being targeted to the mitochondrion according to Mitoprot program (Claros and Vincens, 1996) (data not shown).

The subcellular localization of *TcMYH* in *T. cruzi* was analysed by confocal microscopy through the expression of this protein fused to GFP (Green Fluorescent Protein) at its C-terminus, using the construct pTREX-*TcMYH*-GFP. It was verified that *TcMYH*-GFP is localized to the nucleus and to the antipodal sites of the parasite's kinetoplast present in the mitochondrion (Fig. 5). This distribution was observed in two different combinations: in the nucleus alone (Fig. 5A), or simultaneously in the nucleus and in the antipodal sites (Fig. 5B and C).

Table 1

TcMYH complements *mutY*^{-/-} bacteria. *E. coli* AB1157 (*mutY*⁺) and BH980 (*mutY*⁻) transformed with empty pMAL or pMAL_ *TcMYH*. The expression of *TcMYH* in BH980 reduced mutation rate about 20 times, when compared to BH980 with empty pMAL.

Bacterial strain	<i>TcMYH</i> expression	Mutation rate ($\times 10^{-8}$)
AB1157 (WT)	-	0.5 \pm 0.143
AB1157 (WT)	+	0.959 \pm 0.28
BH980 (<i>mutY</i> ⁻)	-	61.1 \pm 5.23
BH980 (<i>mutY</i> ⁻)	+	3.06 \pm 1.25

3.6. *TcMYH* overexpression increases nuclear and mitochondrial DNA damage

To analyse if *TcMYH* protein is involved in nuclear and mitochondrial DNA repair, we used QPCR-based measurement of H₂O₂ induced damage on CL Brener (WT) and *TcMYH*-overexpressor strains. For nuclear damage, it was seen that at 0.5 h and 2 h after hydrogen peroxide treatment the number of DNA lesions were higher for *TcMYH*-overexpressor cells than for WT (Fig. 6A). For mitochondrial damage, an increase in DNA lesions number was observed only at the time point 0.5 h (Fig. 6B). These results corroborate the survival curves for hydrogen peroxide (Fig. 4A) and the localization of *TcMYH* protein in the parasite (Fig. 5).

Reagent MtOx is described as a highly specific agent for causing damage to mitochondrial DNA from mammalian cells (Jean et al., 2016). We used this reagent to further characterize the mitochondrial response of *TcMYH*-overexpressor cells to oxidative stress. As shown in Fig. 6C, *TcMYH*-overexpressor strain was more sensitive to the reagent, at any time point, when compared to CL Brener strain. This result, together with localization and QPCR assays (Figs. 5 and 6B), is an indicative of the role of *TcMYH* protein in mitochondrial DNA metabolism.

4. Discussion

The GO system is an important mechanism for maintaining the integrity of genetic material in many eukaryotic organisms. This is

demonstrated by the high conservation, across different evolutionary lineages, of the sequences of the proteins that compose this system (Jansson et al., 2010) and by the functional conservation of these enzymes (Takao et al., 1999; Bai et al., 2005; Ali et al., 2008).

Trypanosoma cruzi CL Brener strain's genome annotation, in 2005, by El-Sayed and collaborators, showed that this parasite has homologs of GO system proteins. Given the importance of these enzymes in the maintenance of genome integrity, as well as their possible role in generating variability in *T. cruzi*, we decided to study these proteins. *T. cruzi* OGG1 (*TcOGG1*) and MTH (*TcMTH*) were previously described and characterized elsewhere (Furtado et al., 2012; Aguiar et al., 2013). In the present study, we characterized MYH from *Trypanosoma cruzi* (*TcMYH*).

Given *MutY*/*MYH* sequence conservation between *T. cruzi* and other species (Fig. 1), heterologous complementation assays - specifically, spontaneous mutation assays - were carried out in *E. coli* to test the functions of *TcMYH*. Expression of *TcMYH* in *E. coli mutY*^{-/-} (BH980) reduced the mutation rate about twenty times, when compared to cells that do not express this gene, showing that *TcMYH* complemented BH980. This rifampicin spontaneous mutation assay is largely used and validated (Tajiri et al., 1995; Pope et al., 2008; Schaaper and Mathews, 2013), indicating that this result is strong evidence that *TcMYH* has *MutY* DNA glycosylase activity.

E. coli was also employed for large-scale expression and purification of MBP- *TcMYH*, so that *in vitro* glycosylase tests could be performed. *In vitro* assays using synthetic oligonucleotides are commonly employed to characterize MYH orthologs (Takao et al., 1999; Bai et al., 2005; Ali et al., 2008; Fig. 2B). DNA breakage only in the presence of NaOH (Fig. 2C) confirms that *TcMYH* is a monofunctional DNA glycosylase. Therefore, after *TcMYH* activity it should be necessary to have activity of an AP endonuclease. There are also extra bands where *TcMYH* was added, probably due to spurious activity of *TcMYH* fused with MBP; however, this activity does not compromise the *MutY* glycosylase function of *TcMYH*, as the fragment indicating this function is only present when GO is opposite adenine, in the presence of NaOH. In contrast, the extra bands are present with or without NaOH addition, and even when GO is present opposite cytosine. MBP was purified using

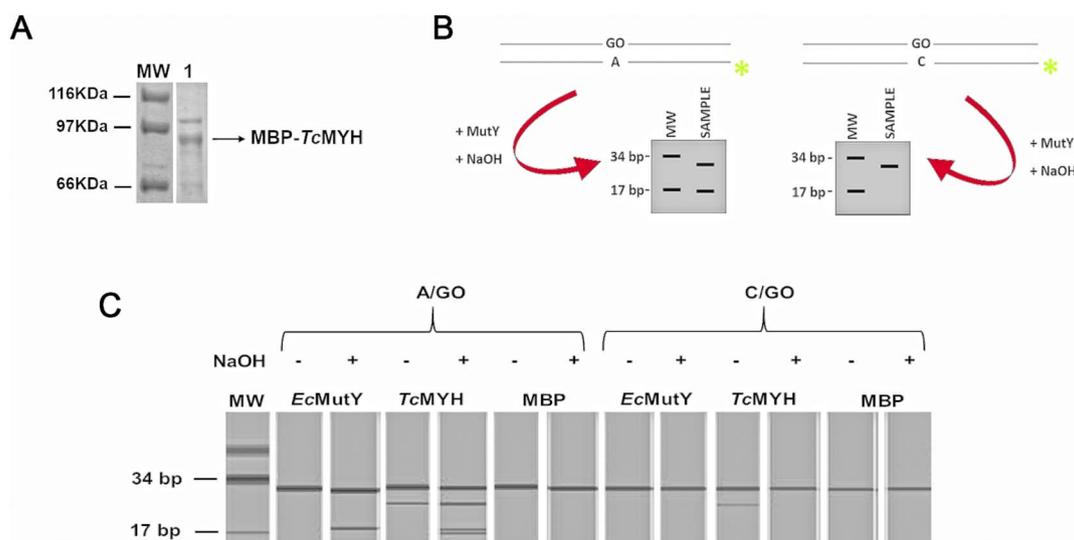


Fig. 2. Excision of adenine by *TcMYH*.

A Purification of *TcMYH*. *TcMYH* fused to maltose-binding protein (MBP) was analysed by SDS-PAGE and visualized by Coomassie blue staining. Lane 1: elution fraction containing partially purified MBP-*TcMYH*, with the full-length protein (~90 kDa) indicated by an arrow. MW: molecular weight marker. **B** Scheme of the *in vitro* assay. The oligonucleotide containing 8-oxoguanine (GO) was annealed to an oligo stained with fluorescein (asterisk), containing either adenine (A) or cytosine (C) in the same position as GO. The duplexes were incubated with *MutY* and NaOH. For cytosine (C/GO), only one band was expected on the gel, corresponding to the intact strand. For adenine (A/GO), two bands were expected: one of the intact strand, and other from the cleaved strand, resulting from the enzymatic activity. **C** Result of the *in vitro* assay. For C/GO, regardless of the protein used, only the intact strand was observed (~30 mer). For A/GO, when treated with *EcMutY* or MBP-*TcMYH* and NaOH, two bands were observed: the intact strand (~30 mer) and the cleaved strand (~15 mer). MBP (negative control) was not able to remove the adenine, even in the presence of NaOH. +: with NaOH; -: without NaOH. This experiment was repeated three times and this figure is from a representative assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

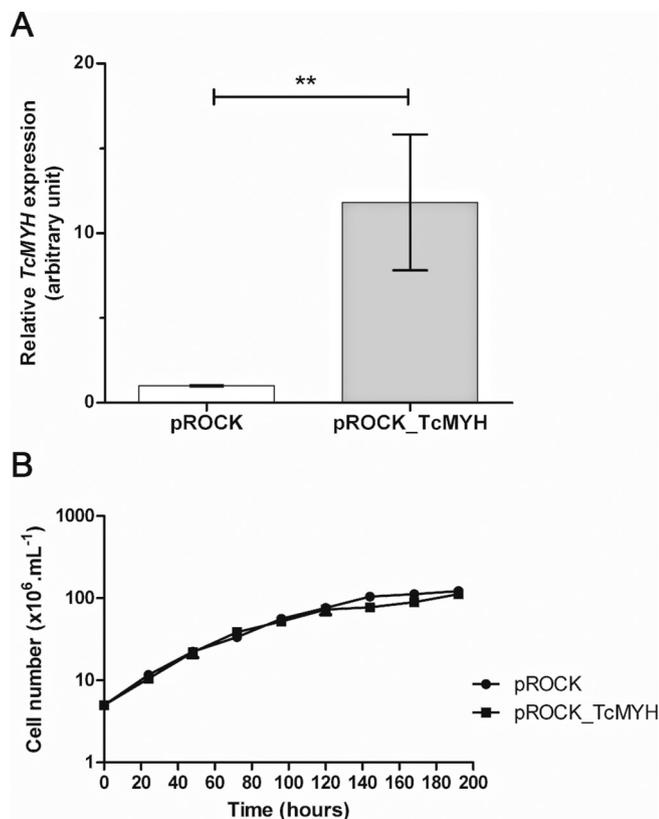


Fig. 3. Overexpression of *TcMYH* does not affect parasites' growth.

A RT-Real Time PCR analyses of *T. cruzi* CL Brener strain transfected with empty vector or with pROCK_ *TcMYH*. Relative *TcMYH* mRNA expression was determined using the established $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with GAPDH as a reference gene. The overexpressor lineage (pROCK_ *TcMYH*; gray bar) showed a relative expression about ten times higher than the control (pROCK; white bar). ** = *p* value < 0,01. B *T. cruzi* *TcMYH*-overexpressor growth curve. CL Brener transfected with pROCK (●) or with pROCK_ *TcMYH* (■) were grown in liquid media. Living parasites were counted in a Neubauer chamber, in the presence of vital dye erythrosine, every 24 h. There was no difference in growth between the two cell lineages.

the same protocol as for *TcMYH*-MBP and it did not show the band related to MutY glycosylase function, neither the extra bands.

Thereafter, *T. cruzi* epimastigotes overexpressing *TcMYH* were constructed (Fig. 3A) and *TcMYH*-overexpressor parasites showed to be more sensitive to hydrogen peroxide or glucose oxidase than control cells (Fig. 4 A and B). There are no reports in the literature of cytotoxic effects after overexpression of MutY, except in situations of high oxidative stress (Oka and Nakabeppu, 2011; Sheng et al., 2012). Cells that overexpress glycosylases are usually more sensitive to exogenous agents than control cells (Kaasen et al., 1986; Coquerelle et al., 1995; Frosina, 2000; Fishel et al., 2003; Rinne et al., 2005; Ondvcik et al., 2013). A similar result could be seen in our characterization of *TcOGG1*, where *T. cruzi* cells that overexpressed this protein were more sensitive to H_2O_2 than control cells (Furtado et al., 2012). Thus, our result indicates that a DNA glycosylase that removes oxidative lesions is being overexpressed in *T. cruzi* transfected with pROCK_ *TcMYH*. This phenotype observed in glycosylase-overexpressing cells was attributed to impairment of the DNA BER pathway (Glassner et al., 1998; Posnick and Samson, 1999; Oka and Nakabeppu, 2011). Thus, as only the DNA glycosylase is being overexpressed and the other proteins that participate on repair do not accompany this elevated expression, intermediary lesions - such as AP sites - are produced but not completely repaired, giving rise to a sensitive phenotype. Therefore, accentuated death of *TcMYH*-overexpressing epimastigotes can be explained by insufficient processing of excess intermediary lesions (e.g. AP sites, SSBs, DSBs) generated by the high levels of *TcMYH* in a situation where substrate is

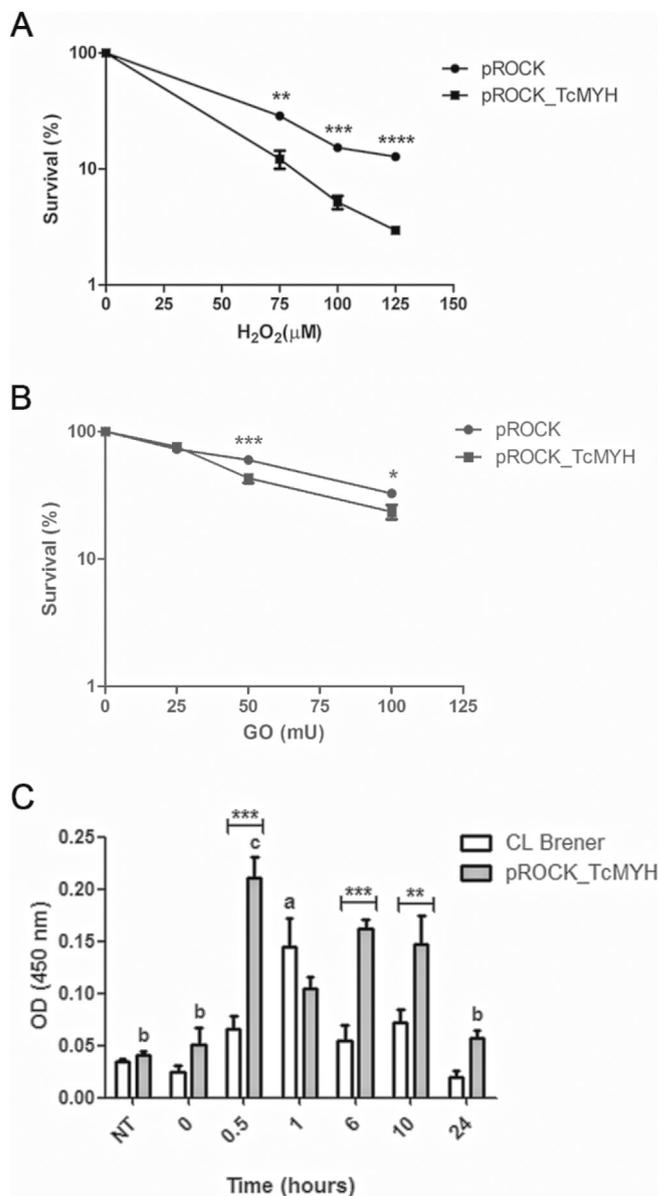


Fig. 4. *TcMYH*-overexpressor *T. cruzi* is more sensitive to hydrogen peroxide and to glucose oxidase treatment than control cells, probably due to AP site accumulation. A H_2O_2 treatment of *TcMYH*-overexpressor *T. cruzi*. Control cells (pROCK (●)) or *TcMYH*-overexpressor *T. cruzi* (■) were treated with 0, 75, 100 and 125 μM H_2O_2 . After 72 h, living parasites of each culture were counted in a Neubauer chamber. Survival percentage was measured in relation to untreated cells. *TcMYH*-overexpressor parasites were more sensitive to H_2O_2 than control parasites. B Glucose oxidase treatment of *TcMYH*-overexpressor *T. cruzi*. *T. cruzi* cells were treated with 0, 25, 50 and 100 mU glucose oxidase. After 24 h, living parasites of each culture were counted in a Neubauer chamber. Survival percentage was measured in relation to untreated cells. *TcMYH*-overexpressor parasites were more sensitive to glucose oxidase than control parasites. C AP site accumulation in *TcMYH*-overexpressor epimastigotes. Cells were treated with 100 μM H_2O_2 and had their DNA extracted 0.5, 1, 6, 10 or 24 h after treatment. After incubation with reagents from AP sites assay kit, OD 450 was measured. *TcMYH*-overexpressor has a higher peak of DNA lesions than control cells. White bars = control cells; gray bars = *TcMYH*-overexpressing cells; NT = non treated cells. Each point represents the average of three independent experiments; bars represent SEM. Statistical analysis were made using GraphPad Prism 5. Unpaired *t*-test was used for the curves (A and B) and Two-way and One-way ANOVA with Bonferroni correction were used for AP site assay (C). * = *p* value < 0,05; ** = *p* value < 0,01; *** = *p* value < 0,001; **** = *p* value < 0,0001. a = all time points for CL Brener, with exception of 10 h, statistically differ from this time point; b = 0.5, 6 and 10 h time points are statistically different from these time points, for pROCK_ *TcMYH*; c = all time points for pROCK_ *TcMYH*, with exception of 6 and 10 h, statistically differ from this time point.

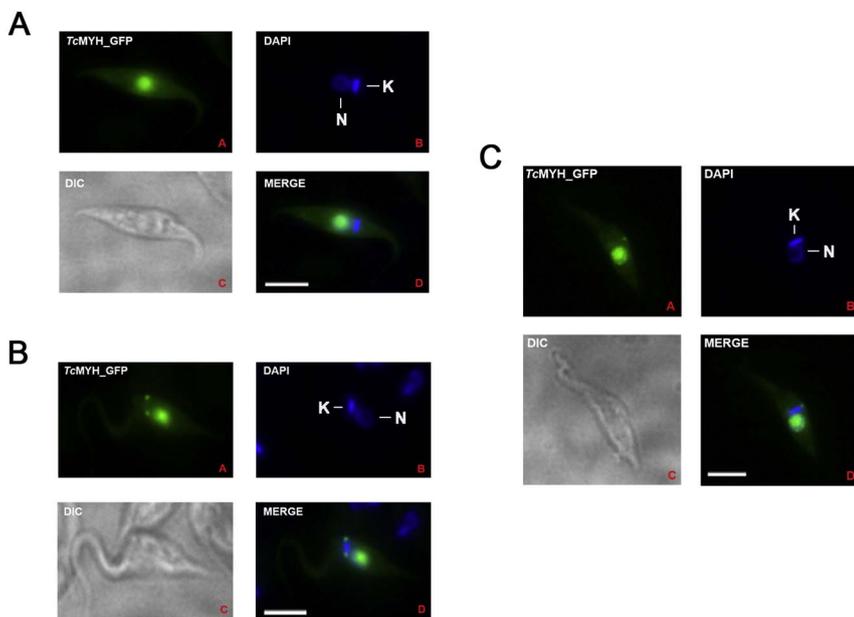


Fig. 5. Subcellular localization of *TcMYH* in *T. cruzi* epimastigotes. CL Brener strain epimastigotes were transfected with pTREX-*TcMYH*, resulting in the expression of GFP-fused *TcMYH* (*TcMYH_GFP*), which enabled the visualization of the protein under fluorescence microscopy. Nuclear DNA was stained with DAPI. **A** *TcMYH_GFP* colocalizes with *T. cruzi* nucleus. **B** *TcMYH_GFP* in the antipodal sites and in the nucleus of *T. cruzi*. **C** Same as in **B**. *TcMYH_GFP* (**A**): *TcMYH* protein in fusion with GFP; DAPI (**B**): staining of DNA; N: nucleus, K: kinetoplast (mitochondrion); DIC (**C**): differential interference contrast; Merge (**D**): GFP and DAPI images merged. 5 μ m scale bars are present in **D**.

not limiting. This hypothesis is corroborated by the AP assay result shown in Fig. 4C. *TcMYH*-overexpressor parasites showed a higher OD peak than wild type cells, and this higher amount of AP sites persisted longer in cells that overexpressed *TcMYH* than in control. This persistence of lesions in DNA was probably caused by a BER imbalance. AP sites data is in congruence with the DNA repair kinetics observed in QPCR technique (Quantitative Polymerase Chain Reaction; Fig. 6A and B). CL Brener epimastigotes had most nuclear lesions resolved between 2 and 10 h of treatment (Fig. 6A), while we observed that at 6 h AP sites in CL Brener's total DNA had already decreased to levels similar to non-treated cells (Fig. 4C). As in the quantification of AP sites, *TcMYH*-overexpressor strain also presented higher levels of DNA damage, as well as their persistence, when compared to WT (Fig. 6A and B). Additionally, MYH activity was reported as a cell death-signaling molecule in human cells (Markkanen et al., 2013). A model describes this function, in which DNA repair by MYH generates SSBs, leading to cell death by PARP or calpain signaling, making these SSBs cytotoxic specially on situations of oxidative burst. Indeed, this model explains why when there is oxidative burst the absence of MYH is beneficial for cell survival (Oka and Nakabeppu, 2011). Following this line of thinking, it has been suggested that hMYH has a role in mitochondrial dysfunction in Parkinson's disease (Fukae et al., 2007; Nakabeppu et al., 2007). However, it has not been established how MYH initiates apoptosis under some circumstances, while in other cases this protein protects cells from death.

Using expression of *TcMYH* fused to GFP, it was observed that MYH is present in the nucleus and in the mitochondrion of *T. cruzi* (Fig. 5). It is known that MYH protein from human, rat and mouse exhibits both mitochondrial and nuclear localization (Ichinoe et al., 2004; Lee et al., 2004; Nishioka et al., 1999; Takao et al., 1999; Nakabeppu, 2001). This dual localization has been described only for mammalian cells. Therefore, *TcMYH* is the first non-mammalian MYH to be characterized as present in both cell compartments. *TcMYH* has predicted nuclear and mitochondria localization; however, we could not determine the precise sequence involved in the transport of the protein to these organelles. Future investigations will be necessary to determine how *T. cruzi TcMYH* could be differently directed to the nucleus and to the mitochondrion.

MutY/MYH acts only after the replication process, since formation of the base pair GO:A is only possible with DNA duplication (Hayashi et al., 2002; Oka and Nakabeppu, 2011). Boldogh et al. (2001) reported that the nuclear isoform of hMYH colocalized with BrdU (a thymidine

analog used for the detection of proliferative cells) and with the nuclear antigen of cellular proliferation (PCNA). Like PCNA, hMYH levels increased about three to four times during S phase, compared to G1 phase, whereas levels of hOGG1 and the mitochondrial isoform of hMYH did not change during cell cycle. These studies suggest that MYH is directed to the replication fork to ensure that its activity takes place in the newly synthesized DNA strand. It was also described that the interaction with PCNA is critical for the DNA glycosylase activity of hMYH and *Schizosaccharomyces pombe* MYH (Parker et al., 2001; Hayashi et al., 2002; Chang and Lu, 2002). These data support the association between MYH function and DNA replication. The antipodal sites are points adjacent to *T. cruzi* kDNA, where the duplication of mini circles occurs right before cells enter S phase (Schamber-Reis et al., 2012). Thus, the presence of *TcMYH* in the antipodal sites occurs during S phase. Since the parasites used in the localization experiments were not synchronized in their cell cycle, some were marked in antipodal sites, while others were not (Fig. 5), which suggests that there is a control over *TcMYH* transport to the mitochondrion, which might occur during mitochondrial DNA replication. This control could be related to the fact that in *T. cruzi* cells, the AP endonuclease is located only in the nucleus (Sepulveda et al., 2014). As *TcMYH* is a monofunctional DNA glycosylase, an AP endonuclease would be necessary to fix AP sites generated by MutY activity. Once AP endonucleases are absent from the parasite's mitochondria, the repair of MutY-related AP sites could occur in a way similar to the repair of gaps generated by mitochondrial genome replication. In the last phase of mitochondrial replication, minicircles migrate to the antipodal sites, where the final reactions of minicircle duplication, including the repair of gaps by DNA polymerase β , occur (Liu et al., 2005).

To further characterize *TcMYH* localization in *T. cruzi*, we performed QPCR assays to measure DNA lesions in nuclear and mitochondrial DNA of *TcMYH*-overexpressor and WT cells (Fig. 6A and B). Cells overexpressing *TcMYH* had higher levels of DNA lesions than the control in certain time points (Fig. 6A). The quantification of mitochondrial damage demonstrated a small increase in the level of DNA lesions in *TcMYH*-overexpressor strain (Fig. 6B), although the number of damage, and the difference between the strains are lower than those observed for the nucleus. This strain also had a lower survival, when compared to the wild-type strain, to MtOx reagent (Fig. 6C). These results suggest a role of *TcMYH* protein in mitochondrial DNA metabolism. Other BER proteins have already been described as acting in the mitochondrion of *T. cruzi* (Lopes Dde et al., 2008; Furtado et al., 2012)

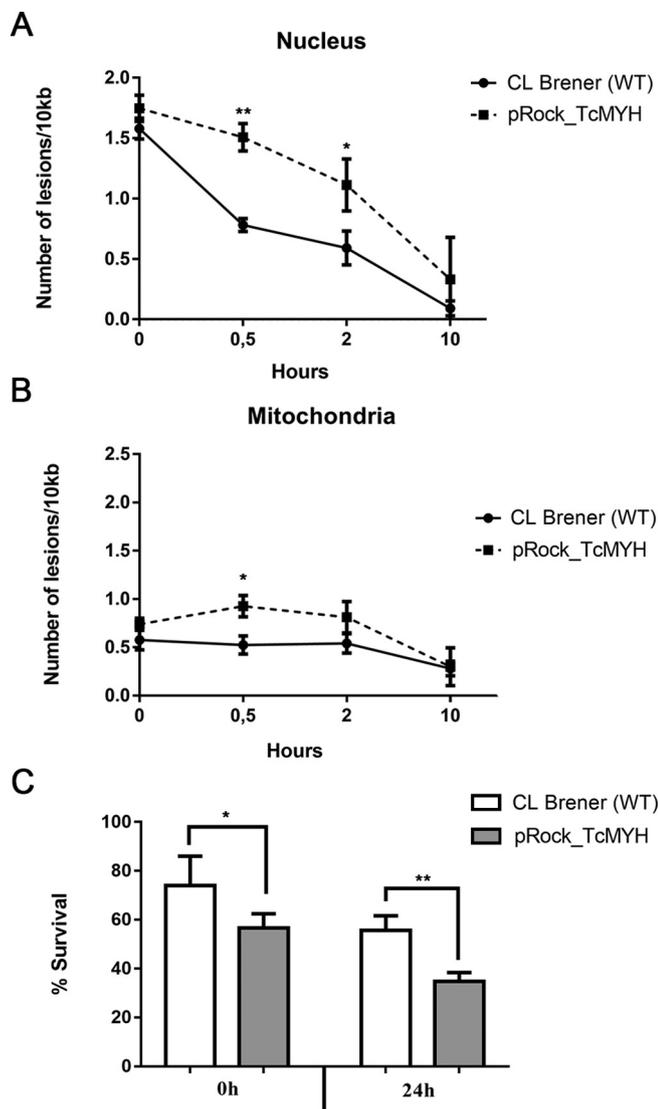


Fig. 6. *TcMYH* overexpression generates higher levels of nuclear and mitochondrial damage, and sensitizes *T. cruzi* cells to mitochondrial oxidative stress. **A** Nuclear QPCR-based measurement of H_2O_2 induced damage on CL Brener (WT;●) and *TcMYH*-overexpressor (■) strains. Cells were treated with $100 \mu M H_2O_2$ for half an hour and then the DNAs were extracted, amplified and measured as described on Materials and Methods (Section 2.3.7). The results are expressed as the mean of two experiments (with standard deviations represented as bars for each point). As show on the figure, *TcMYH*-overexpressor strain presents a higher number of lesions, in comparison to control. **B** Mitochondrial QPCR-based measurement of H_2O_2 induced damage on CL Brener (WT) and *TcMYH*-overexpressor strains. The conditions of the experiment were the same as those used for nuclear measurement. As observed for nuclear damage, the levels of mitochondrial damage are also higher for *TcMYH*-overexpressor strain when compared to CL Brener (WT) strain, although the number of damage, and the difference between the strains are lower than those observed for the nucleus. **C** MtOx treatment of CL Brener (WT) and *TcMYH*-overexpressor *T. cruzi*. The cells were treated for 1 h with $16 \mu M$ and then cells were counted just after the treatment and after 24 h of growth. Survival percentage was calculated comparing treated and untreated cells for each strain. Cells overexpressing *TcMYH* were more sensitive to MtOX than the WT strain, on both times analysed, indicating a phenotype related to mitochondrial DNA metabolism. Unpaired *t*-test was used for all the graphs, calculated on GraphPad Prism V7.0. * = *p* value < 0,05; ** = *p* value < 0,01.

and the presence of *TcMYH* could indicate the presence of a specific repair system for the damages caused by the oxidative stress in this organelle. The results for nuclear and mitochondrial DNA damage support the survival curve (Fig. 4A), AP sites (Fig. 4C) and localization (Fig. 5) results, once they demonstrate that there is accumulation of DNA damage in *TcMYH*-overexpressor cells, in both organelles that

contain DNA in a cell, and show that *TcMYH* is involved in nuclear and mitochondrial DNA metabolism.

The study of *TcMYH* protein is a step forward for the characterization of a GO system in *T. cruzi*. The complete system appears to be present and our findings demonstrate that is important to maintain DNA stability of the nuclear and mitochondrial genomes, particularly in the presence of oxidative stress (Furtado et al., 2012; Aguiar et al., 2013).

5. Conclusions

The data presented in this work indicates that *Trypanosoma cruzi* has a functional MYH DNA glycosylase, which participates in nuclear and mitochondrial DNA Base Excision Repair mechanisms of this parasite.

Conflict of interest

The authors declare that there are no conflicts of interests.

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