



DNA polymerase beta from *Trypanosoma cruzi* is involved in kinetoplast DNA replication and repair of oxidative lesions

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ABSTRACT

Specific DNA repair pathways from *Trypanosoma cruzi* are believed to protect genomic DNA and kinetoplast DNA (kDNA) from mutations. Particular pathways are supposed to operate in order to repair nucleotides oxidized by reactive oxygen species (ROS) during parasite infection, being 7,8-dihydro-8-oxoguanine (8oxoG) a frequent and highly mutagenic base alteration. If unrepaired, 8oxoG can lead to cytotoxic base transversions during DNA replication. In mammals, DNA polymerase beta (Pol β) is mainly involved in base excision repair (BER) of oxidative damage. However its biological role in *T. cruzi* is still unknown. We show, by immunofluorescence localization, that *T. cruzi* DNA polymerase beta (TcPol β) is restricted to the antipodal sites of kDNA in replicative epimastigote and amastigote developmental stages, being strictly localized to kDNA antipodal sites between G1/S and early G2 phase in replicative epimastigotes. Nevertheless, this polymerase was detected inside the mitochondrial matrix of trypanomastigote forms, which are not able to replicate in culture. Parasites over expressing TcPol β showed reduced levels of 8oxoG in kDNA and an increased survival after treatment with hydrogen peroxide when compared to control cells. However, this resistance was lost after treating TcPol β overexpressors with methoxiamine, a potent BER inhibitor. Curiously, a presumed DNA repair focus containing TcPol β was identified in the vicinity of kDNA of cultured wild type epimastigotes after treatment with hydrogen peroxide. Taken together our data suggest participation of TcPol β during kDNA replication and repair of oxidative DNA damage induced by genotoxic stress in this organelle.

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1. Introduction

Knowledge regarding mitochondrial DNA repair pathways in protozoan parasites of medical relevance like *Trypanosoma cruzi*, the causative agent of Chagas Disease, is scarce and still poorly understood. *T. cruzi* possesses one single mitochondrion with a condensed genome in a catenated network of topologically interlocked minicircles and maxicircles, known as kinetoplast DNA (kDNA) [1,2]. While thousands of minicircles, comprising 90% of the

network, encode small guide RNAs used in RNA editing [3], dozens of maxicircles encode ribosomal RNAs and proteins involved in mitochondrial energy transduction as cytochrome *b*, subunits of cytochrome oxidase and subunits of NADH dehydrogenase [4]. The maintenance of kDNA integrity is vital for parasite survival during its life cycle inside mammalian cells, where they have to deal with cellular defense responses that usually involve oxygen burst. Therefore, understanding how kDNA integrity is maintained during the parasite life cycle is crucial to identify potential therapeutical targets against this disease.

In mammalian cells, reactive oxygen species (ROS) are mostly produced endogenously by normal cell respiration, and the mitochondrion is believed to be the main intracellular site of ROS generation [5]. Mitochondrial electron transport chain activity is responsible for the generation of more than 95% of superoxide

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anions produced inside cells, being considered an abundant and highly reactive intracellular ROS in mammals [6]. In order to reduce potential downstream damage, superoxides are converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. Hydrogen peroxide is freely diffusible across membranes and can oxidize nucleosides and damage bases in nuclear and mitochondrial DNA [7]. A common site of oxidative damage is carbon 8 of guanine, which leads to formation of 7,8-dihydro-8-oxoguanine (8oxoG). If unrepaired, 8oxoG can lead to potentially mutagenic and cytotoxic GC-TA transversions during cell replication [8]. Studies in mammals have shown that the frequency of 8oxoG on mitochondrial DNA is 20-fold higher than that observed on nuclear DNA [9], with a 8oxoG/dG relation between 0.5 to 4.24×10^{-6} [10–12]. In addition, the majority of mutations introduced in mitochondrial DNA can be directly correlated with quantified 8oxoG in mtDNA [13]. It is reasonable to consider that mitochondrial oxidative burden may also target kDNA in trypanosomes. In order to minimize the deleterious action of ROS, trypanosomes evolved a unique biochemical system to deal with redox metabolism. Although they lack catalase and glutathione peroxidase [14], a thiol dependent antioxidant system is active to eliminate hydrogen peroxide and associated byproducts [15,16]. Anti-oxidative protection provided by peculiar detoxifying enzymes is the primary defense against oxidative stress in trypanosomes. Cytosolic and mitochondrial trypanothione peroxidases, involved primarily in metabolizing H_2O_2 and short-chain organic hydroperoxides by using trypanothione as an electron donor *via* trypanothione, have been described in *T. cruzi* [17] and *T. brucei* [18]. However, these systems are prone to saturation and residual oxidative molecules can damage nuclear and kinetoplast DNA. DNA repair mechanisms probably evolved to specifically circumvent pre-mutagenic alterations in the genome.

Several DNA repair putative genes were predicted in *T. cruzi* genome [19], including those encoding elements involved in base excision repair (BER) pathway. BER is one of the most important pathways implicated in correcting oxidative damage in DNA [20,21], and can be reproduced *in vitro* with few purified proteins from human and *Escherichia coli* origin [22–24]. DNA repair operated by BER can occur *via* two sub-pathways. In short-patch BER, a monofunctional glycosylase catalyzes the removal of the damaged base, leaving an apurinic/apyrimidinic site (AP site). This substrate is recognized by an AP-endonuclease, that cleaves the phosphodiester bond 5' to the AP site [22]. This step is followed by insertion of the correct nucleotide to the 3'-OH end of the incised AP site and posterior excision of the remaining sugar-phosphate moiety (lyase activity), being both processes catalyzed by DNA polymerase beta. DNA polymerase beta (Pol β) belongs to the X family of DNA polymerases and acts as the main dRP lyase effector involved in repair of oxidative base lesions [25–27].

On the other hand, long-patch BER is initiated with the displacement of a short single-strand DNA segment containing the lesion (usually in the range of 2–6 nucleotides) carried on either by Pol β or Pol δ [28]. This “flap” oligonucleotide is then released by FEN1 endonuclease [29–31]. In both short- and long-patch BER, the remaining nick is sealed by a DNA ligase. Catalytic measurements using reconstituted enzymatic systems *in vitro* suggested that the rate-limiting step during BER is the removal of dRP catalyzed by the 8 kDa domain of Pol β [32]. This way, Pol β plays an essential role in orienting the lesion to be repaired through short- or long-patch BER, depending on the type of the lesion involved.

It is generally accepted that Pol β is implicated during the repair of 8oxoG and other oxidized pyrimidines in nuclear DNA [27,33,34]. *T. cruzi* genome codes for two DNA polymerase beta genes (Tcpol β and Tcpol β -PAK) that show identity with mammalian Pol β [35–38]. Previous findings published by our group have localized both polymerases inside the mitochondrion of *T. cruzi* [38]. We have shown polymerase and lyase activity for both

proteins *in vitro* [38]. However, only Tcpol β was unable to perform translesion synthesis (TLS) across 8oxoG [38]. For this reason, we decided to investigate if Tcpol β was the main DNA polymerase operating during BER. In this work we provide strong evidence that support a role of Tcpol β in repairing DNA oxidative damage *via* BER and kDNA replication. We report that clones over expressing Tcpol β are more sensitive to the chain-terminator 3'-azido-3'-deoxythymidine (AZT), which can be incorporated in the course of DNA replication. At the same time, over expressing cells are more resistant to oxidative stress and can repair 8oxoG in kDNA with a higher efficiency when compared to control cells. In addition, epimastigotes treated with hydrogen peroxide presented a Tcpol β focus localized at the kinetoflagellar zone. Furthermore, we show that Tcpol β is associated with kDNA antipodal sites only in those developmental stages found in *T. cruzi* life cycle that are capable to replicate.

2. Materials and methods

2.1. Plasmid constructions

The vector used to generate Tcpol β over expressing parasites was constructed as follows. First, the complete DNA polymerase beta ORF was retrieved (<http://www.genedb.org>, accession number Tc00.1047053503955.20) and PCR-amplified from genomic DNA extracted from *T. cruzi* CL Brener strain using primers containing *Xba*I (forward 5'-TCT AGA ATG TTT CGT CGC ACA) and *Eco*RI (reverse 5'-GAA TTC GGG GTC GCG GTT TTC CGG) linkers. The product was cloned in pGEM-T Vector (Promega) following manufacturer's instructions. This construct was digested and the fragment liberated was cloned in plasmid pROCK [39] between *Xba*I and *Xho*I sites. Two oligonucleotides (5'AAT TCG GCC AAA AAG GCC and 5'TCG AGG CCT TTT TGG CCG) were annealed together to form a DNA adaptor that allowed ligation of 3' end of pol β amplicon with pROCK *Xho*I restriction site. To clone a truncated version of Tcpol β containing only the 8 kDa domain, amplicons were obtained by PCR (primers forward 5'AAG CTT ATG TTT CGT CGC ACG T and reverse 5'TGA ATT CCT TCT CAAG TTC CTC) and cloned into pROCK plasmid following the same strategy described above. All plasmid constructs were confirmed by DNA sequencing.

2.2. Production of Tcpol β *in vitro*

For expression and purification of Tcpol β protein, amplicons containing 5'-*Eco*RI and 3'-*Hind*III digested ends were cloned into pGEM[®]-T Easy vector and subcloned into pMAL-g2 (New England Biolabs, USA) downstream from the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP). Subsequent protein production in *E. coli* and purification was performed as described [38].

2.3. *In vitro* AZT incorporation by recombinant Tcpol β

Inhibition of DNA synthesis by recombinant Tcpol β by 3'-azido-2',3'-dideoxythymidine triphosphate (AZT) was evaluated by using a plasmid treated with DNase as substrate in a reaction that contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 μ M dNTPs and [3 H]-dTTP (100 μ Ci) and 0.2–4.0 μ g of recombinant purified Tcpol β . After 2 h at 37 °C, the DNA was spotted onto a membrane that was then washed with 0.5% trichloroacetic acid. The incorporated radioactivity was measure in a Beckman LS 6500 scintillation counter.

2.4. Parasite growth and transfection

Epimastigotes from *T. cruzi* CL Brener strain were cultured at 28 °C in liver infusion tryptose (LIT) [40] supplemented with 10%

of fetal bovine serum (Cultilab, Brazil), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, USA). Trypomastigotes were obtained from supernatant of infected LLCMK2 cells (ATCC-CCL7) and maintained in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. Amastigotes were obtained by incubating trypomastigotes in LIT medium at 28 °C for 48 h. Parasites were collected and centrifuged at 2000 × g for 10 min and the resulting pellet was washed with phosphate-buffered saline (PBS) and immediately used. Hydroxyurea (HU) synchronization was performed as described previously [41].

For stable transfections, a total of 1 × 10⁸ cells in exponential growth were washed and resuspended in 5 mL of buffer composed of 75% EPB (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose and 21 mM HEPES pH 7.5) and 25% cytomix [42] containing 50 µg of plasmid that had been linearized with *NotI* (Promega) for targeting to β-tubulin loci present in the parasite genome. A plasmid without the resistance cassette was used as negative control for selection. After incubation at room temperature for 15 min, transfections were carried out in 2-mm cuvettes using a Gene Pulser System (Bio-Rad) submitted to two pulses (10 s apart) with peak discharge of 0.3 kV and 500 µF. After electroporation, cells were transferred into 5 mL of LIT medium supplemented with 10% fetal bovine serum. Selection was applied after 24 h by addition of G418 (200 µg/mL) (Gibco). Selection of transfectants was confirmed when no living parasites were seen in negative transfection control. Parasites were seeded in agar-blood plates containing G418 and clones were isolated 30 days after incubation at 28 °C.

2.5. Northern and Western blots

To perform Northern blots, total RNA (10 µg) was fractionated in 1.2% agarose gels containing 5% formaldehyde, blotted onto a Hybond-N+ membrane (GE Healthcare) by capillary transfer, and cross-linked by UV irradiation. A DNA probe of 184pb was amplified from *Tcpolβ* gene by PCR using primers forward 5'GGC TCA CCT CCG ATT ATG AA and reverse 5'GTT GCC TCG AGG TAG TTT CG. This probe was gel purified, and labeled with [³²P]-dCTP using the Megaprime™ DNA labeling protocol from GE Healthcare. The membrane was hybridized in a 50% formamide buffer for 18 h at 42 °C and washed with 2% SSC/0.1% SDS at 60 °C, as previously described [43]. *T. cruzi* chromosomal bands were separated by pulse field gel electrophoresis (PFGE) according to protocol previously described [44] followed by transfer to a Hybond-N+ membrane.

For Western blots, total cellular lysates from 1 × 10⁹ exponential-phase epimastigote cells were prepared by washing cell pellet in phosphate saline buffer (PBS), and then dissolving washed pellets in one volume of 1 × SDS gel-loading buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and one volume of water. Cells were mechanically lysed by passage through a syringe needle and boiled for 10 min. Proteins were separated in 8% polyacrylamide gels containing 0.1% SDS using standard methods for SDS-PAGE, transferred to nitrocellulose membranes and incubated with primary (rabbit polyclonal anti-*Tcpolβ*) diluted 1:2000 and secondary peroxidase conjugated antibodies (anti-rabbit IgG diluted 1:2000, Amersham Biosciences) as described elsewhere. Proteins were detected using ECL-Plus reagents (Amersham Biosciences) according to manufacturer instructions and developed with NBT-BCIP.

2.6. Survival curves

Epimastigote transfected cells over expressing *Tcpolβ* and control were maintained in logarithmic phase (5 × 10⁶ cells/mL) during 5 days. After this period, cells were treated with three different doses of 3'-azido-3'-deoxythymidine monophosphate (AZT-MP) (50, 100 and 150 µM) and hydrogen peroxide (200, 300 and

400 µM). A dual treatment was realized with 200 µM of H₂O₂ and 10 mM of methoxyamine hydrochloride (MX, Sigma-Aldrich). Cells were appropriately diluted in PBS 1 × + erythrosine 0.4% and surviving cells were counted in a Neubauer chamber after 48 h of incubation at 37 °C.

2.7. Assessment of 8oxoG accumulation in kinetoplast and nuclear DNA

To measure 8oxoG accumulation in *T. cruzi* DNA cells, we used a protocol adapted from Struthers et al. [45]. Epimastigotes were incubated in the presence of 200 µM hydrogen peroxide for 20 min at 28 °C, washed twice with PBS and fixed with 4% paraformaldehyde. Aliquots (20 µL) of the cell suspension were distributed into wells of 8-well chambered-slides. After 1 h of incubation at 4 °C, cells were permeabilized with 0.2% Triton X-100, treated with 100 µg/mL RNase A and incubated with FITC-conjugated avidin (5 µg/mL final concentration) for 1 h at room temperature protected from light. After washing with PBS 1 × and mounted with a solution of 9:1 glycerol:Tris-HCl, pH 9.0, the slides were visualized under a fluorescence microscope in a 100x oil immersion. Pre-incubation of FITC-conjugated avidin with 0.5 mM of 8oxoG results in 80% decrease in the fluorescence signal, whereas pre-incubation with dGTP has no significant effect on parasite labeling. Fluorescence intensities from manually delimited kinetoplast and nuclear DNA were averaged with the ImageJ program and plotted as fluorescence arbitrary units (average fluorescence intensity measured in 100 cells after subtracting the average background intensity). Background signals were measured in 100 fields, randomly chosen on the slides.

2.8. Immunofluorescence assays

Indirect immunofluorescence experiments were performed by attaching 2 × 10⁶ pre-washed parasites to glass slides, following fixation with 2% *p*-formaldehyde in PBS for 20 min. Fixed cells were washed three times with PBS, treated with 0.1% Triton X-100 in PBS for 5 min, blocked 30 min with PBS containing 1% BSA and incubated with the different affinity purified anti-*Tcpolβ* [38] diluted 1:2000 in PBS containing 1% BSA. Anti-dihydro lipoamide dehydrogenase (anti-LipDH), which is present in the mitochondrion matrix, was raised in rabbit [46] and was a gift of Dr. Kevin Tyler and used at 1:1000 dilution. The monoclonal antibody 2F6 (mAb 2F6) was used at 1:2 dilution and is specific for *T. cruzi* flagellar calcium-binding protein [47]. After 1 h, slides were washed three times with PBS and bound antibodies revealed with anti-IgG fluorescent conjugates in the presence of 0.01 mM 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclear and kinetoplast DNA. Slides were mounted with Vectashield (Vector Laboratories) and observed in Nikon E600 microscope or mounted in N-propyl galate and visualized by confocal laser scanning microscope (Zeiss LSM510 META). Alternatively, images were collected by using a BX61 Olympus microscope, 100× 1.4 NA objective with the CellM software, and processed blind deconvolution using the Autoquant 2.1 (Media Cybernetics). Pre-immune serum or samples incubated without the primary antibody was used as a control.

2.9. Transmission electron microscopy

Protozoa were fixed in 2.5% glutaraldehyde diluted in 0.1 M cacodylate buffer, pH 7.2, for 2 h at room temperature and post-fixed in 0.1 M cacodylate buffer containing 1% OsO₄, 5 mM calcium chloride and 0.8% potassium ferricyanide for 1 h. Then, cells were dehydrated in a graded series of acetone (30%, 50%, 70%, 90% and 2 × 100%) and embedded in Epoxy resin. Ultrathin sections were

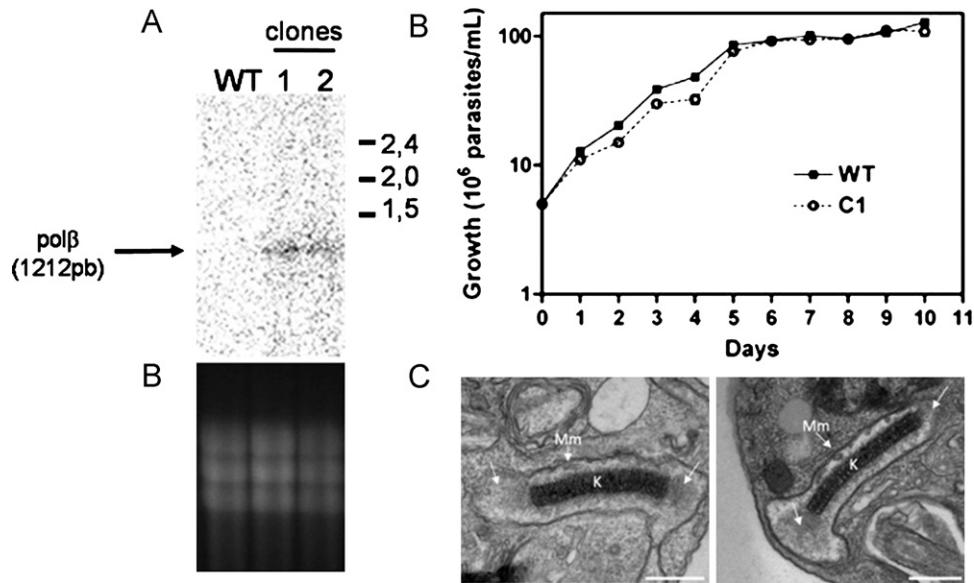


Fig. 1. Over expression of Tcpol β . Cells were transfected with pROCKpol β and stable transfectants selected in presence of G418 after 30 days. (A) Northern blot analysis using total RNA extracted from epimastigotes in logarithmic phase derived from two clones (indicated by 1 and 2) and non-transfected cells from CL Brener strain (WT). The lower part shows the corresponding RNA gel stained with ethidium bromide as loading control. (B) Growth curves for control cells (WT) and cells over expressing Tcpol β clone 1 (C1). Parasite growth was followed during 11 days in LIT medium and cells were counted in a cytometric chamber. Data are representative of two independent experiments. (C) Kinoplast ultra structural analysis of cultured epimastigote cells (day 2 from a culture initially set at 5×10^6 cells/mL LIT medium) over expressing Tcpol β (left) and control cells (right). Arrows point to position of kDNA antipodal sites. (K) Kinoplast; (Bb) Basal bodies; (Mm) mitochondrial membrane with cristae. Bars = 0.5 μ m.

stained with uranyl acetate and lead citrate and observed in a Zeiss 900 transmission electron microscope.

3. Results

3.1. Tcpol β over expression

Due to the impossibility to obtain functional cells with genes knocked-down by RNAi in *T. cruzi*, we decided to establish epimastigotes over expressing Tcpol β . We transfected epimastigotes from CL Brener strain with a plasmid derived from pROCKNeo that carried the entire pol β gene under control of TcP2 β 5'UTR and gGAPDH II 3'UTR flanking sequences and a constitutive rRNA promoter [39]. After selection of stable transfectant clones resistant to G418, we randomly chose two clones for subsequent experimentation. Northern blot analysis showed an increased mRNA level in these over expressing cells compared to control parasites transfected with an empty plasmid (Fig. 1A). To further evaluate *T. cruzi* DNA polymerase beta protein levels after transfection, protein expression was assessed by Western blot in whole-cell lysates from cells transfected with empty vector and supposedly Tcpol β over expressing parasites (clone 1). Our results show that Tcpol β levels were elevated in clone 1 cells compared to those cells transfected with empty plasmid (Supplementary Fig. S1).

Similar growth curves were seen between control and clone 1 cells (Fig. 1B). In addition, we compared kinoplast ultrastructural organization of non-treated Tcpol β over expressing and control cells by transmission electron microscopy. Our observations showed that the kinoplast of over expressing cells were similar to those observed for control protozoa, with no detectable alterations in kDNA orientation or condensation. Alterations of mitochondrial membranes or repositioning of antipodal sites were not observed (Fig. 1C), indicating that Tcpol β over expression did not modify mitochondrial DNA organization during the cell cycle of transfected cells.

3.2. Tcpol β over expression decrease survival in presence of AZT

Several purine (e.g. allopurinol) and pyrimidine (e.g. 2',3'-dideoxyinosine and 2',3'-dideoxyadenosine) analogs can inhibit *T. cruzi* replication [48]. This is particularly true for AZT, which strongly inhibited *T. cruzi* amastigote proliferation cells [48]. By performing cellular survival experiments with control and over expressing cells treated with increasing concentrations of AZT, we found that excess Tcpol β sensitized the cells at high doses of AZT (Fig. 2A), suggesting that Tcpol β could be the main polymerase involved in incorporation of AZT *in vivo*, as it was reported for the human pol β [49,50]. To demonstrate the importance of Tcpol β in this process, we designed *in vitro* experiment with purified protein. We showed that AZT can indeed inhibit incorporation of radioactive thymine in a nicked plasmid by recombinant Tcpol β in a dose-dependent manner (Fig. 2B). Interestingly, the AZT dose that blocked the activity of Tcpol β *in vitro* (100 μ M) is similar to the dose that is lethal to *T. cruzi* *in vivo*. Collectively, these results indicate that Tcpol β can efficiently incorporate AZT during kDNA replication.

3.3. Subcellular localization of Tcpol β is restrict to antipodal sites in replicating cells

Two replicating cellular forms can be found in *T. cruzi* life cycle. Epimastigotes replicate inside the gut of the reduviidae bug, while amastigotes multiply intracellularly in mammalian host [51]. Trypomastigotes are infective, non-replicative forms that are released in the bloodstream of infected host after amastigote differentiation and cell rupture [51]. We performed immunolocalization of Tcpol β in culture-derived CL Brener *wild type* cells epimastigotes, intracellular amastigotes and trypomastigotes using polyclonal antibodies against the purified recombinant protein. We found that Tcpol β is mainly localized in antipodal sites of replicative epimastigotes (Fig. 3I–L) and in extracellular amastigotes (Fig. 3E–H) while in non-replicative cultured trypomastigotes Tcpol β is dispersed inside its single ramified mitochondrion, which extends throughout the

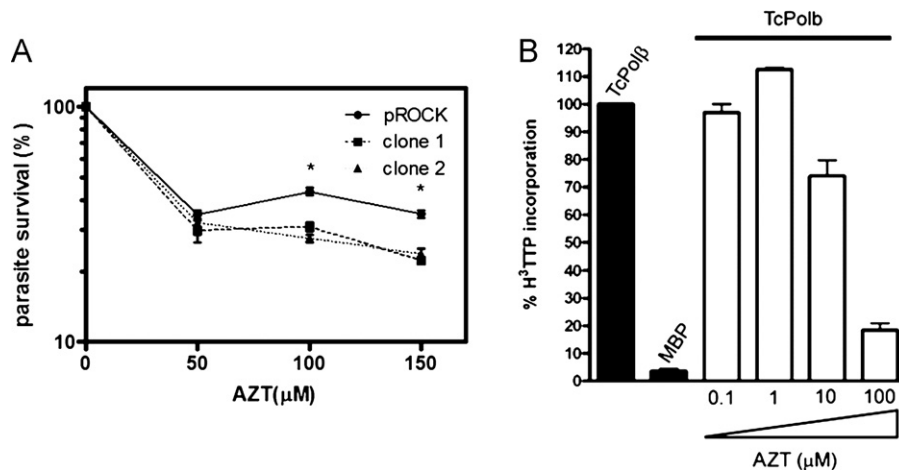


Fig. 2. DNA elongation by TcPol β is inhibited by AZT. (A) *T. cruzi* survival after treatment with AZT. Cells transfected with empty plasmid and TcPol β over expressing epimastigotes (clone 1 and clone 2) were treated with 50, 100 and 150 μ g/mL of AZT. Cells were counted in a cytometric chamber after 72 h. Data represent the mean \pm SD of three independent experiments. Significance was assessed using ANOVA with Bonferroni's post test. * $P < 0.01$. (B) Purified recombinant TcPol β was incubated with a digested plasmid in the presence of 5 μ M dNTPs containing 3 H-dTTP and different doses of AZT. Radioactivity levels were measured and compared with control reactions. Black bars indicate control reactions with TcPol β or MBP in the absence of AZT. White bars are reactions performed by TcPol β with the indicated doses of AZT.

cytoplasm (Fig. 3A–D). TcPol β mitochondrial localization in trypanomastigotes was further evidenced as it colocalized with the lipoamide dehydrogenase (LipDH) protein (Supplementary Fig. S2), a mitochondrial matrix marker [52]. Cellular localization patterns for TcPol β in the three parasite forms were consistent in more than 95% of the cells in each experiment. These results suggest participation of TcPol β during kDNA replication, due to its association to antipodal sites in replicative forms of *T. cruzi*, as it was previously observed for *T. brucei* and *C. fasciculata* [35,53].

3.4. Subcellular localization of TcPol β follows parasite cell cycle

Specific morphological changes that occur during *T. cruzi* cell cycle involve a short flagellum that emerges from a flagellar pocket in G2 phase, followed by kinetoplast redistribution prior to mitosis and nuclear division. Growing of the emerging flagellum continues

until two new daughter cells are formed [41]. Information concerning localization of any DNA polymerase enzyme throughout the cell cycle of kinetoplastida members is still limited. In order to understand how DNA replication operates in *T. cruzi*, we followed the TcPol β localization by immunofluorescent approach during cell cycle progression. We tracked TcPol β in the mitochondrion of epimastigote CL Brener *wild type* cells by confocal microscopy using a polyclonal antibody raised against recombinant TcPol β . We were able to detect cells with main morphological patterns that allowed us to follow the epimastigote cell cycle (Fig. 4). Cells in G1/S phase of nuclear cell cycle are characterized by having one flagellum, visualized with a monoclonal antibody (mAb 25) that recognizes a 24 kDa flagellar calcium-binding protein, one kinetoplast and one nucleus, labeled by DNA staining by 4',6'-diamidino-2-phenylindole (DAPI). Once cells begin to proceed through G1/S, TcPol β redistributes to the antipodal sites (Fig. 4A). In early G2 phase (Fig. 4B) when the new

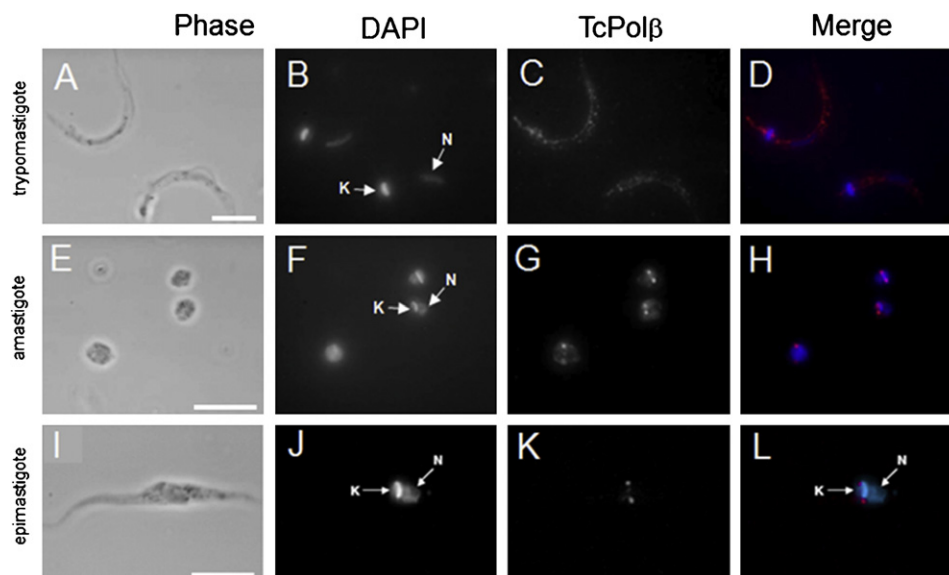


Fig. 3. Localization of TcPol β by immunofluorescence. *T. cruzi* tripomastigotes (A–D), extracellular amastigotes (E–H) and epimastigotes at logarithmic phase (I–L) were labeled with anti-TcPol β . Representative images were selected from a total of 100 cells analyzed for each parasite form. DNA staining with DAPI shows the nucleus (N) and kinetoplast (K). Note that in replicative forms (amastigotes and epimastigotes) the TcPol β labeling is seen exclusively at the antipodal sites, while in the trypanomastigote form it is observed dispersed throughout its unique ramified mitochondrion. The composed merge figure shows nuclear and kinetoplast DNA in blue and TcPol β in red. Bars = 5 μ m.

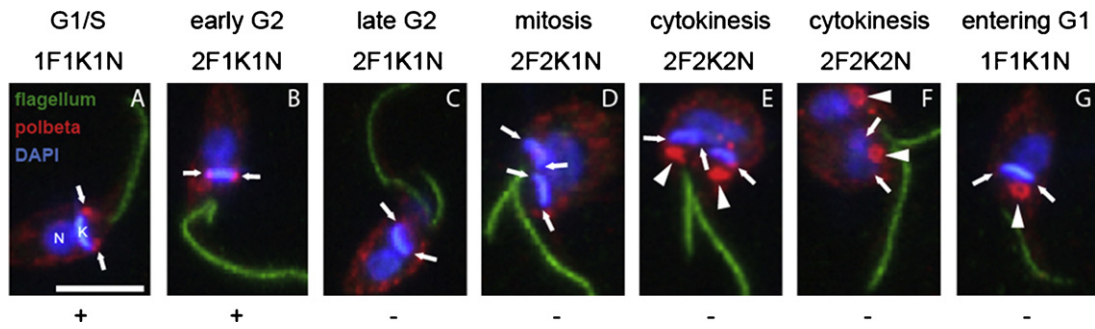


Fig. 4. Cellular morphological alterations and Tcpol β redistribution during *T. cruzi* epimastigote cell cycle. The stages of epimastigote nuclear cell cycle were identified based on the length and number of flagellae (F), number of kinetoplasts (K) and nucleus (N) indicated above each representative image. The new flagellum emerges from cellular pocket when cells are in G2 stage. During mitosis the flagellar pocket segregates leading to cytokinesis, which generates two identical daughter cells. White arrows indicate the position of antipodal sites. White arrowheads indicate the position of kinetoflagellar zone where Tcpol β seems to accumulate in a ring-shaped structure during cytokinesis and G1 phases. Positive (+) and negative (-) symbols indicate presence or absence of Tcpol β in antipodal sites, respectively. See Section 3.4 for details. Bar = 5 μ m.

flagellum has just started to grow, much of Tcpol β was still found located in antipodal sites. Curiously, cells in late G2 phase (Fig. 4C) in addition to have a longer growing flagellum compared to early G2, showed a dispersed staining for Tcpol β , that was no more found associated with both antipodal sites. This pattern persisted until the end of mitosis, when kinetoplast and nuclear DNA are completely duplicated (Fig. 4D). Cells with two kinetoplasts and one nucleus were not found considering that both structures segregate almost simultaneously [41]. Strikingly, during and immediately after cytokinesis, Tcpol β migrates to a specific point between the kinetoplast DNA and basal body forming a doughnut-like structure (Fig. 4E–G). We believe that this site corresponds to the kinetoflagellar zone. After cytokinesis, Tcpol β seems to redistribute again to antipodal sites for a subsequently round of cell replication.

3.5. Tcpol β over expression improves epimastigote survival in presence of H₂O₂

A role of Pol β in DNA repair was extensively demonstrated in mammals extracts [54], mainly in oxidative damage repair [21,27,55]. To investigate whether Tcpol β could also participate in repair of oxidative damage in *T. cruzi*, we measured the survival of *T. cruzi* epimastigotes over expressing Tcpol β that were grown in liquid LIT medium supplemented with increasing concentration of hydrogen peroxide (H₂O₂), known to generate multiple oxidized bases in DNA, including 8oxoG, a strong mutagen if not corrected by BER [56]. We found that over expressing Tcpol β clones were more resistant to all concentrations of H₂O₂ tested when compared to control (Fig. 5A), supporting that Tcpol β contributes to the process of DNA repair of oxidative lesions in epimastigotes and suggesting that excess Tcpol β could enhance BER efficiency. To better clarify the role of Tcpol β during kDNA repair, we generated parasites over expressing its lyase domain (8 kDa) without the DNA polymerase domain. We treated this culture, as well as empty plasmid-transfected control parasites and Tcpol β over expressing clone 1, with hydrogen peroxide (100, 200 and 300 μ M). We verified that 8 kDa over expressing parasites showed an intermediate survival curve between control and Tcpol β over expressing parasites (Fig. 5B). This data shows that the lyase activity is important but not enough for complete oxidative DNA lesion repair. Fully functional Tcpol β activity seems to be crucial in promoting a high efficiency BER in *T. cruzi* parasites submitted to oxidative stress.

We next tested survival of Tcpol β over expressing cells in the presence of H₂O₂ and methoxyamine hydrochloride (MX). MX can react with the C1 of the deoxyribose moieties of apurinic/aprimidinic sites preventing cleavage of the phosphodiester bond adjacent to the AP site by AP endonuclease and, consequently, inhibiting BER [57,58]. Control and Tcpol β over

expressing cells were simultaneously treated with 200 μ M of H₂O₂ and 10 mM of MX, and cell survival was then evaluated after 48 h. We found that in the presence of MX, excess of Tcpol β was not beneficial for survival (Fig. 5C). This result suggests that Tcpol β over expression can stimulate BER regarding repair of oxidative damage in kDNA after H₂O₂ treatment, although the precise mechanism is unclear.

Finally, cells were treated with methyl methanesulfonate (MMS). MMS is a methylating agent that transfers alkylating adducts to DNA, mainly 3-methyladenine and 7-methylguanine [59,60]. We observed no significant differences in survival between Tcpol β over expressing and control cells (Fig. 5D). This result suggests that Tcpol β over expression is related to resistance to oxidative stress and is not involved in repair of methylating damage in *T. cruzi*.

3.6. 8oxoG is better repaired in kDNA of Tcpol β over expressing cell

Considering the possible role of Tcpol β in processing mitochondrial oxidized bases, we monitored 8oxoG levels in the kinetoplast and nuclear DNA of over expressing Pol β cells after treatment with H₂O₂. Over expressing and control cells were exposed to H₂O₂ and were then treated with a FITC conjugate of avidin, a known biomarker of oxidative DNA damage [45]. Two hours after treatment with H₂O₂, the fluorescent signal of FITC in kDNA was lower for Tcpol β over expressing cell (clone 1) compared to control (Fig. 6A and C). This difference persisted at last 24 h after treatment. In contrast, measurement of FITC signal in nuclear DNA showed similar levels of oxidative damage both in control and Tcpol β over expressing clone 2 h and 24 h after H₂O₂ treatment (Fig. 6B and C). These data support that excess Tcpol β favors the repair of 8oxoG in kDNA.

3.7. Tcpol β triple foci formation after treatment of cultured epimastigotes with H₂O₂

Several mammalian DNA repair proteins like Pol β and OGG1 can associate in discrete foci after genotoxic stress, including oxidative stress [61,62]. In these cases, the foci persist for a defined period of time and then are dismantled, which suggests formation of a repairosome that remains active only during DNA repair processes. Therefore, we decided to immunolocalize the native Pol β from *T. cruzi* after treating wild type epimastigote cells from CL Brener strain with H₂O₂. Six hours after exposure to oxidative stress, we were able to detect formation of a third focus of Tcpol β additionally to that two previously seen adjacent to kDNA antipodal sites in more than 90% of the cells analyzed (Fig. 7I–L). This induced focus is

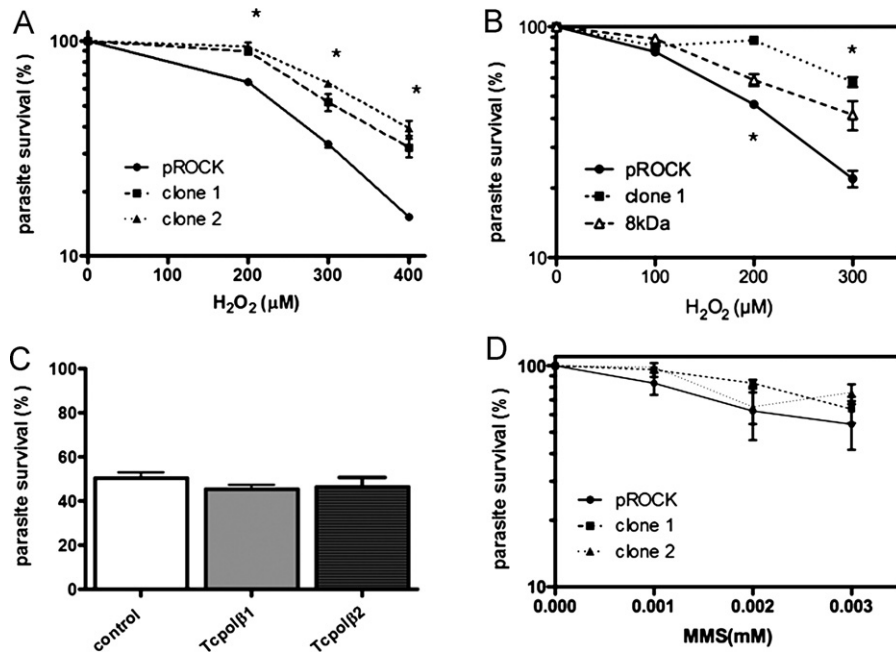


Fig. 5. Tcpolβ is involved in oxidative stress response in *T. cruzi*. (A) Cells transfected with empty pROCK plasmid and Tcpolβ over expressing clones (clone 1 and clone 2) were treated with 200, 300 and 400 μM of H₂O₂. Cells were counted in a cytometric chamber after 48 h. (B) Empty plasmid-transfected cells (pROCK), 8kDa and Tcpolβ (clone 1) over expressing parasites were treated with 100, 200 and 300 μM of H₂O₂. Cells were counted in a cytometric chamber after 48 h. (C) Simultaneous treatment of Tcpolβ over expressing and control cells with 200 μM H₂O₂ and 10 mM methoxyamine (MX). Cells were counted after 48 h in a cytometric chamber. (D) *T. cruzi* survival after treatment with MMS. Cells transfected with empty plasmid (pROCK) and Tcpolβ over expressing clones were treated with MMS ranging from 0.001 to 0.003%. Cells were counted in a cytometric chamber after 72 h. Experimental data represent the mean ± SD of three independent experiments. Significance was assessed using ANOVA with Bonferroni's posttest. **P* < 0.01.

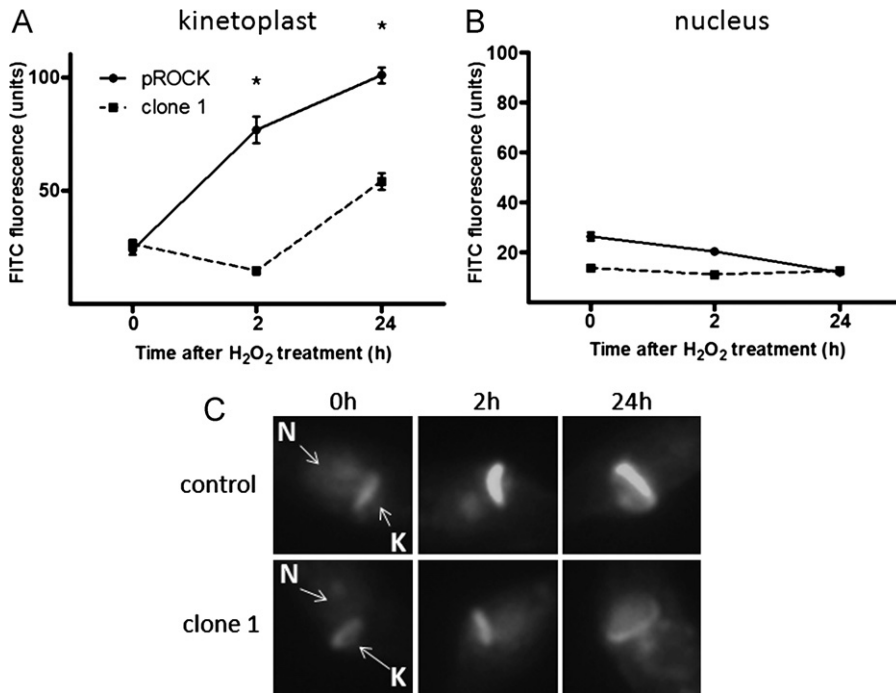


Fig. 6. Kinetics of 8oxoG accumulation in the kinetoplast DNA of wild type and Tcpolβ over expressing cell after oxidative stress. Epimastigote cells were treated with 200 μM H₂O₂ for 20 min, transferred to fresh medium and aliquots collected in different times (0 h, 2 h and 24 h after treatment) were fixed with 4% paraformaldehyde. Fixed cells were transferred to chambered glass slides, incubated with 5 μg/mL avidin-FITC for 1 h and visualized by fluorescence in a 100× oil immersion. Fluorescence intensity was analyzed with ImageJ program (<http://rsbweb.nih.gov/ij/>) both in kinetoplast (A) and nuclear DNA (B) and plotted as fluorescence arbitrary units. Significance was assessed using ANOVA with Bonferroni's posttest. **P* < 0.01. Representative images from cells in each time point are shown in (C), where the nucleus (N) and kinetoplast (K) are indicated by arrows. Data are representative of mean ± SD of three independent experiments in which 100 cells were analyzed at each time point. Bars = 5 μm.

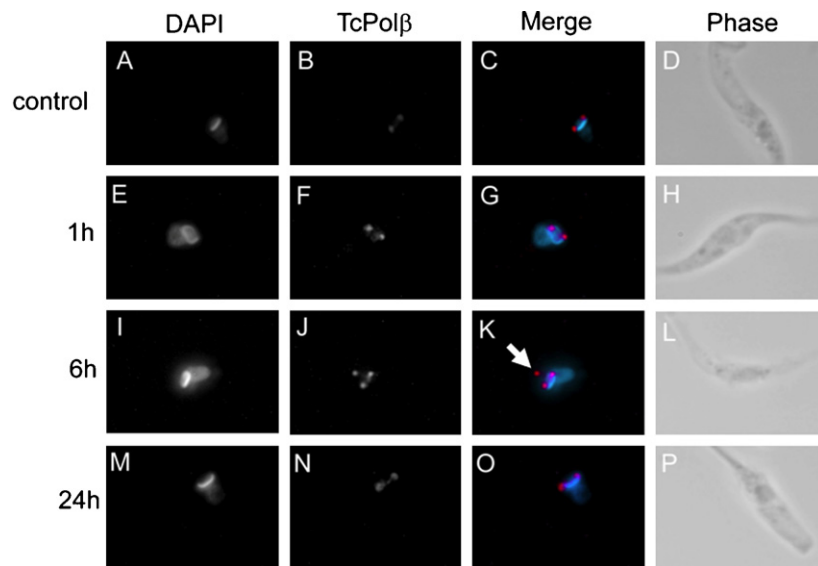


Fig. 7. Accumulation of TcPol β in epimastigote cells treated with hydrogen peroxide. Fixed cells immunostained for endogenous TcPol β in epimastigote forms of *T. cruzi* after treatment with 100 mM H₂O₂. Representative images were selected from a total of 100 cells analyzed in each time point. Localization patterns of untreated cells (A–D), and of cells 1 h (E–H), 6 h (I–L) and 24 h (M–P) after treatment are shown. Merge shows both nuclear and kinetoplast DNA in blue and TcPol β in red. The white arrow indicates a TcPol β focus formation observed after oxidative treatment. Bar = 5 μ m.

clearly positioned outside the kDNA region, and we believe that it is probably located in kinetoflagellar zone (KFZ). This focus was not detected in non-treated cells (Fig. 7A and B) and its detection was not possible at 1 h and 24 h after H₂O₂ treatment (Fig. 7E–H and M–P, respectively). This extra focus was not observed after treating cells with monofunctional alkylating agent methoxyamine (MMS) (data not shown).

4. Discussion

Maintenance of kDNA integrity relies on a complex process involving a large collection of enzymatic factors and an intricate dynamics of minicircle and maxicircle network replication. Although our knowledge about these processes has been improved considerable, little is known about how oxidized bases in kDNA of trypanosomes are recognized and repaired. Using *T. cruzi* as model, we focused on the role of TcPol β , a key component of BER repair pathway in preventing the deleterious effects of 8oxoG in the mitochondrial genome of this parasite.

Parasites over expressing TcPol β were found more sensible to AZT regimen compared to control cells. This diminished survival could be attributed to an increased incorporation of AZT by excess of TcPol β into kDNA during mitochondrion replication, as it was previously shown in mammalian cells.

By immunolocalization assays we showed that TcPol β is directed to the kinetoplast antipodal sites in epimastigotes and amastigotes, while in trypomastigotes it is spread throughout the cell body, most likely inside its single branched mitochondrion. The biochemical processes supposed to occur at antipodal sites, mainly from studies in *T. brucei*, include degradation of RNA primers still annealed to the newly replicated minicircles by SSE1 (after migration of minicircles from the kinetoflagellar zone) and gap linking by DNA polymerase beta [35]. Although our work do not formally demonstrate the role of DNA polymerase beta in *T. cruzi*, the clear association between replicative status and pattern of subcellular localization is suggestive of a role of this polymerase in kDNA duplication, when TcPol β could fill the gaps between Okasaki fragments after replicating the minicircles. Similar patterns of localization in the antipodal sites of kDNA were observed in *T. brucei* and *C.*

fasciculata [35,53]. This result is different for *L. infantum*, which possess a nuclear Pol β [37]. Intriguingly, no TcPol β signal could be detected in the nucleus of *T. cruzi*. We believe that other polymerases, like Pol ϵ and Pol δ , could promote functional redundancy in short and long-patch nuclear BER, as demonstrated in mammalian cell extracts deficient in Pol β [34,63].

In order to elucidate the role of TcPol β in kDNA repair, hydrogen peroxide was used as genotoxic agent in survival curves by its capability to induce oxidative DNA damage [64,65]. Using different doses of H₂O₂ we verified that epimastigotes over expressing TcPol β showed increased survival when compared to control cells. However, parasites over expressing a truncated form of TcPol β (8 kDa domain only) presented an intermediate resistant phenotype between cells transfected with empty plasmid and clone 1 parasites. The results suggest that the 8 kDa domain alone could participate in BER removing the dRP and other polymerases, such pol ϵ , could substitute TcPol β to insert nucleotides, but with lower efficiency. In this sense, fully functional TcPol β enzyme is mandatory to allow BER to efficiently remove oxidative lesions in *T. cruzi* kDNA.

We believe that BER could be the main DNA repair pathway acting on detoxification of peroxides in this condition, considering that initial sanitization of H₂O₂ by trypanredoxin peroxidase is efficient in low doses ranging from 2.5 to 20 μ M of H₂O₂ and we used higher doses (200–400 μ M H₂O₂) in survival curves [66]. The ability of MX, a specific short-patch BER blocking agent, to sensitize over expressing cells to H₂O₂ suggests specific participation of TcPol β in improvement of survival when it is overexpressed inside cell. In addition, survival experiments performed with MMS did not show any significant difference between TcPol β over expressing and control cells. Mammalian BER is involved in repair of alkylating damage caused by MMS, including the cytotoxic adducts 3-methyladenine and 7-methylguanine [59,60,67,68]. Purines damaged by MMS are recognized by 3-methyladenine DNA glycosylase (AAG) in humans [69]. The observed insensitivity of *T. cruzi* to MMS can be due to inexistence of a functional homologue of AAG in *T. cruzi* mitochondria to initiate BER. MMS also induce homologous recombination by generating single and double-strand breaks [70,71]. Another possible interpretation is that MMS is incapable to sensitize *T. cruzi* cells considering the action of an efficient recombinational

process in this organism [44]. It is also possible that MMS damage is mainly repaired by long-patch BER in *T. cruzi*, considering that MMS was administered to logarithmic growth phase cells, where short-patch BER predominates. Additional experiments are being performed to elucidate resistance of *T. cruzi* to MMS treatment.

We believe that Tcpol β over expressing cells resistance to H₂O₂ is probably due to enhanced DNA repair of oxidized nucleotides in kinetoplast, as we saw depleted levels of oxoguanine in this structure compared to control cells. Depleted oxoguanine levels in over expressing clone 1 after 2 h can indicate higher activity of BER in these cells, probably stimulated by an excess of Tcpol β . After 24 h, 8oxoG levels increased in both control and over expressing cells, although less pronounced in over expressing cells. This is probably due to incorporation of oxodG deriving from oxidized nucleotide pool during cell replication. We were not able to detect differences in accumulation of 8oxoG in nuclear DNA from both cells, which could indicate strictly stimulation of mitochondrial BER by Tcpol β over expression. These results suggest that other DNA repair pathways in *T. cruzi* that can recognize oxidative damage are active in the nucleus. Taken together these evidences support a role of Tcpol β in repair of 8oxoG in kDNA of *T. cruzi*.

A striking finding is that genotoxic treatment of epimastigotes with hydrogen peroxide induces mobilization of Tcpol β to a defined focus, in addition to the two foci previously seen in the antipodal sites in the absence of treatment. It was shown in HeLa cells that BER proteins like Pol β , OGG1 and XRCC1 can be directed to repair foci after inducing local oxidative stress [61,62]. We speculate that this new focus is located in the kinetoflagellar zone where minicircle replication takes place, considering the unavailability of mitochondrial markers that could be used to refine this localization. We hypothesize that with the higher level of oxidative stress imposed by H₂O₂ treatment, Tcpol β could be directed within this area to participate of extra temporal BER events in a specialized focus outside the kinetoplast during possible asynchronous DNA repair events that not occur during G1/S.

This is the first time that a member of X family of DNA polymerases is immunolocalized in trypomastigotes of *T. cruzi*. The diffuse localization of Tcpol β seen in trypomastigote cells gave us precious insights about how a BER polymerase could promote protection against genotoxic stress in this stage. This is consistent with the fact that trypomastigotes do not replicate DNA, releasing Tcpol β to be directed to antipodal sites in this stage. Probably the stoichiometry of Tcpol β inside mitochondrion is tightly regulated. A rapid supply of Tcpol β to antipodal protein complexes during differentiation from trypomastigotes to epimastigotes and amastigotes could be efficiently achieved by internal mobilization instead of being degraded and produced when it is needed. The hypothesis of Tcpol β degradation can be also considered and additional experiments are required to better understand of this process.

Previous studies involving localization of DNA repair proteins in trypanosomatids presented static models of subcellular kinetoplast organization of the replication machinery [1]. In these models, pol β is clearly localized in antipodal sites [35,38,72]. In this article we also showed that during epimastigote cell cycle Tcpol β is mobilized inside mitochondrion, and it seems that the polymerase is only required in antipodal sites in G1 to S phase when mitochondrial DNA is being simultaneously replicated and repaired. This is the first time that redistribution of a DNA repair protein along cell cycle is shown for a kinetoplastida member. It is possible that this relocation to different subcellular domains must be an additional enzymatic control for Tcpol β that the parasite uses to improve its BER repair efficiency during genome replication. Additional approaches must be taken to better elucidate this phenomenon.

In summary, our experimental data points toward a role of Tcpol β from *T. cruzi* in conferring resistance to genotoxic oxidative stress. Acquisition of a deeper understanding regarding involvement of this DNA polymerase and BER pathway in repair of oxidative lesions will certainly allow better experimental approaches and intervention during treatment of Chagas Disease.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2012.02.007.

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