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Effect of Posaconazole in an *in vitro* model of cardiac fibrosis induced by *Trypanosoma cruzi*

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ABSTRACT

Posaconazole (POS) is an inhibitor of ergosterol biosynthesis in clinical use for treating invasive fungal infections. POS has potent and selective anti-*Trypanosoma cruzi* activity and has been evaluated as a possible treatment for Chagas disease. Microtissues are a 3D culture system that has been shown to reproduce better tissue architecture and functionality than cell cultures in monolayer (2D). It has been used to evaluate chemotropic response as *in vitro* disease models. We previously developed an *in vitro* model that reproduces aspects of cardiac fibrosis observed in Chagas cardiomyopathy, using microtissues formed by primary cardiac cells infected by the *T. cruzi*, here called *T. cruzi* fibrotic cardiac microtissue (TCFCM). We also showed that the treatment of TCFCM with a TGF-β pathway inhibitor reduces fibrosis. Here, we aimed to evaluate the effect of POS in TCFCM, observing parasite load and molecules involved in fibrosis. To choose the concentration of POS to be used in TCFCM we first performed experiments in a monolayer of primary cardiac cell cultures and, based on the results, TCFCM was treated with 5 nM of POS for 96 h, starting at 144 h post-infection. Our previous studies showed that at this time the TCFCM had established fibrosis, resulting from *T. cruzi* infection. Treatment with POS of TCFCM reduced 50 % of parasite load as observed by real-time PCR and reduced markedly the fibrosis as observed by western blot and immunofluorescence, associated with a strong reduction in the expression of fibronectin and laminin (45 % and 54 %, respectively). POS treatment also changed the expression of proteins involved in the regulation of extracellular matrix proteins (TGF-β and TIMP-4, increased by 50 % and decreased by 58 %, respectively) in TCFCM. In conclusion, POS presented a potent trypanocidal effect both in 2D and in TCFCM, and the reduction of the parasite load was associated with a reduction of fibrosis in the absence of external immunological effectors.

1. Introduction

Chagas disease (CD) is a chronic systemic parasitosis caused by the Kinetoplastid protozoan *Trypanosoma cruzi* and is the main cause of cardiomyopathy in endemic areas of Latin America [1]. The disease afflicts 6 to 7 million people worldwide and approximately 30 % of infected people develop cardiac alterations [2]. These alterations include fibrosis and hypertrophy culminating in heart electrical abnormalities and failure [2].

Currently, available chemotherapy for CD is based on two nitroheterocyclic drugs benznidazole (BZ) and nifurtimox (NF). Treatment with BZ can result in 80 % of cure when started during the acute phase and may also be indicated to chronic asymptomatic patients during the chronic phase [3]. However, the treatment of patients presenting severe chronic Chagas cardiomyopathy (CCC) is not mandatory due to the low rates of cure, despite its contribution to the reduction of parasite detection [4].

Posaconazole (POS) is an inhibitor of ergosterol biosynthesis and has been used for treating fungal infections in humans [5]. Ergosterol and other 24 alkyl-sterols are the main endogenous sterols in fungi and

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Kinetoplastid protozoan parasites such as *T. cruzi* [6]. Studies with POS demonstrated potent activity of this compound against *T. cruzi* both *in vitro* and *in vivo*, in experimental models of the acute and chronic CD [7,8]. In humans, the trypanocidal effect of POS was reported [9,10], however, a treatment protocol with POS that results in Chagas disease parasitological cure in the chronic phase in humans still needs to be elaborated [3].

The persistence of *T. cruzi* contributes to chronic inflammation and consequently fibrosis in heart tissue, cardiac hypertrophy and heart failure [11]. In this context, the treatment of CCC patients should consider a therapeutic strategy focused not only on the parasite elimination but also on the inhibition and reversion of heart tissue damage. Studies in experimental models of acute and chronic CD using trypanocidal and anti-inflammatory drugs emerge as a new strategy to eliminate the parasite and to reverse the heart damage [12–14].

The fibrosis in CCC is characterized by the deposition of extracellular matrix proteins including fibronectin, laminin and collagen in the heart tissue [15,16]. Cytokines, matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) are regulators of extracellular matrix (ECM) and consequently of fibrosis in many tissues [17]. MMPs 2 and 9 are differentially expressed in patients with indeterminate and clinical cardiac forms of Chagas disease [18]. Interestingly, it was previously demonstrated that POS inhibited the production of fibronectin in the heart tissue of *T. cruzi*-infected mice [19]. However, the effect of this compound in the reduction of ECM proteins in cardiac tissue infected by *T.cruzi* is unknown.

Two-dimensional (2D) culture systems (or monolayers) are limited in reproducing aspects observed *in vivo*. In which, tissues are organized in a three-dimensional (3D) network constituted by cells and extracellular matrix associated with signaling molecules. Cell-cell and cell-ECM interactions regulate the biochemical and molecular cell responses and are directly influenced by 3D tissue architecture. For that reason, 3D cell culture is an advanced *in vitro* method, since it reproduces with more accuracy the tissues' architecture and the responses observed *in vivo* [20].

Our group developed a functional cardiac microtissue model, also called cardiac microtissues, based on a 3D culture system, that when infected by *T. cruzi*, presents fibrosis and hypertrophy, thus mimicking aspects of CCC [21,22]. Here we will call it *T. cruzi* fibrotic cardiac microtissue (TCFCM). Recently, we also demonstrated that the sera from patients with chronic CD induced ECM components increase in TCFCM [18]. These results using a 3D culture system showed that both *T. cruzi* alone or soluble immunological regulators contribute to cardiac fibrosis in the context of CD [18,21]. Moreover, we also recently demonstrated that the treatment of TCFCM with a TGF-β inhibitor reduced fibrosis [23].

In the present study, we evaluated the trypanocidal effect of POS and also the impact of treatment on the expression of ECM in TCFCM.

2. Materials and methods

2.1. Ethics statement

All procedures were approved by the Oswaldo Cruz Foundation Animal Welfare Committee (License number LW-40/13) and were consistent with the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

2.2. Drug

Posaconazole (SCH56592; (−)-4-[4-[4-[4-[[(2R-cis)-5-(2,4-difluorophenyl)-tetrahydro-5-(1H-1,2,4-triazol-1-ylmethyl)-3-furanyl] methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(*S*)-1-ethyl-2(*S*)-hydroxypropyl]-3H-1,2,4-triazol-3-one) (Noxafil®, Merck, Sharp & Dohme Corp., USA) was suspended in DMSO and utilized at 1.25, 2.5, 5 and 10 nM final concentrations in 2D cardiac cell cultures and at 5 nM in 3D cultures.

2.3. Parasites

The Y strain (MHOM/BR/1950/Y) of *T. cruzi* was used in this study. Trypomastigote forms of *T. cruzi* were obtained from the supernatant of infected heart muscle cells grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1 mM CaCl2, 1 mM L-glutamine, 2% chick embryo extract, 1000 U/mL penicillin and 50 μg/mL streptomycin. After 96 h of infection, the parasites were collected, centrifuged and resuspended in supplemented DMEM. Handling of live *T. cruzi* was performed according to established guidelines [24].

2.4. Cardiac cell cultures

Hearts of 18-day old Swiss Webster mouse fetuses were submitted to mechanical and enzymatic dissociation as previously described [25]. Briefly, cells were harvested using 0.05 % trypsin and 0.01 % collagenase in phosphate buffered saline (PBS) at 37 °C. Ventricular heart muscle cells (HMCs) were plated at a density of 1.5×10^5 cells/well on 0.02 % gelatin-coated glass coverslips in 24-well plates (monolayer). Cardiac microtissues (3D culture) were produced plating cardiac cells at a density of 2.5×10^4 cells/well on 1% (w/v) agarose coated 96-well plates. Cells were maintained at 37 °C in a 5% CO_2 atmosphere in supplemented DMEM.

2.5. Infection of cardiac microtissues and treatment with Posaconazole

Cardiac monolayers were infected with culture-derived trypomastigotes (20:1, parasites to host cell ratio) and the treatment of cultures was performed following the protocols: (I) addition of 1.25 − 10 nM POS after 2 h or 48 h of parasites interaction in 2D cultures. At specific times, coverslips (in triplicate) with cardiac cells in monolayers were collected, fixed with Bouin's fixative solution and stained in Giemsa solution. The percentage of infection was quantified by counting randomly at least 300 cells in light microscope.

Based in our results in monolayers, the treatment of non-infected cardiac microtissues (NICM) and TCFCM previously infected with *T. cruzi* for 144 h [21] was performed using 5 nM POS for 96 h. In TCFCM a huge infection level and fibrosis are observed at 144 h post-infection but the microtissues are not yet destroyed; for that reason, we chose this condition to start the antiparasitic treatment. TCFCM and NICM treated or not were collected in a pool of sixty spheroids for each condition for processing and qPCR, immunofluorescence and western blotting experiments.

2.6. Quantitation of parasite load by real-time quantitative PCR

Total DNA was extracted from a pool of sixty cardiac microtissues for each experimental condition, using the High Pure PCR Template Preparation Kit according manufacturer's instructions (Roche, USA) and DNA from samples was analyzed by real-time quantitative PCR (qPCR). The qPCR experiments were performed using ABI Prism 7500 Fast (Life Technologies, USA), in a final volume of 20 μL containing 750 nM of primers Cruzi 1 (5′-AST CGG CTG ATC GTT TTC GA-3′), Cruzi 2 (5′-AAT TCC TCC AAG CAG CGG ATA-3′) for targeting *T. cruzi* nuclear satellite DNA and probe (FAM- CACACACTGGACACCAA-NFQ-MGB), 5 μL DNA samples and 10 μl TaqMan Master Mix 2x (Promega, USA) [26,27]. The GAPDH gene (TaqMan probe with a FAM dye label from Applied Biosystems) was used as target to quantify the number of mouse cardiomyocytes. Thus, *T. cruzi* and cardiomyocytes were estimated by absolute quantification, results were normalized and parasite load was expressed as Par. Eq./10^6 cells. PCR assays were performed in duplicate for each sample. Three independent experiments were

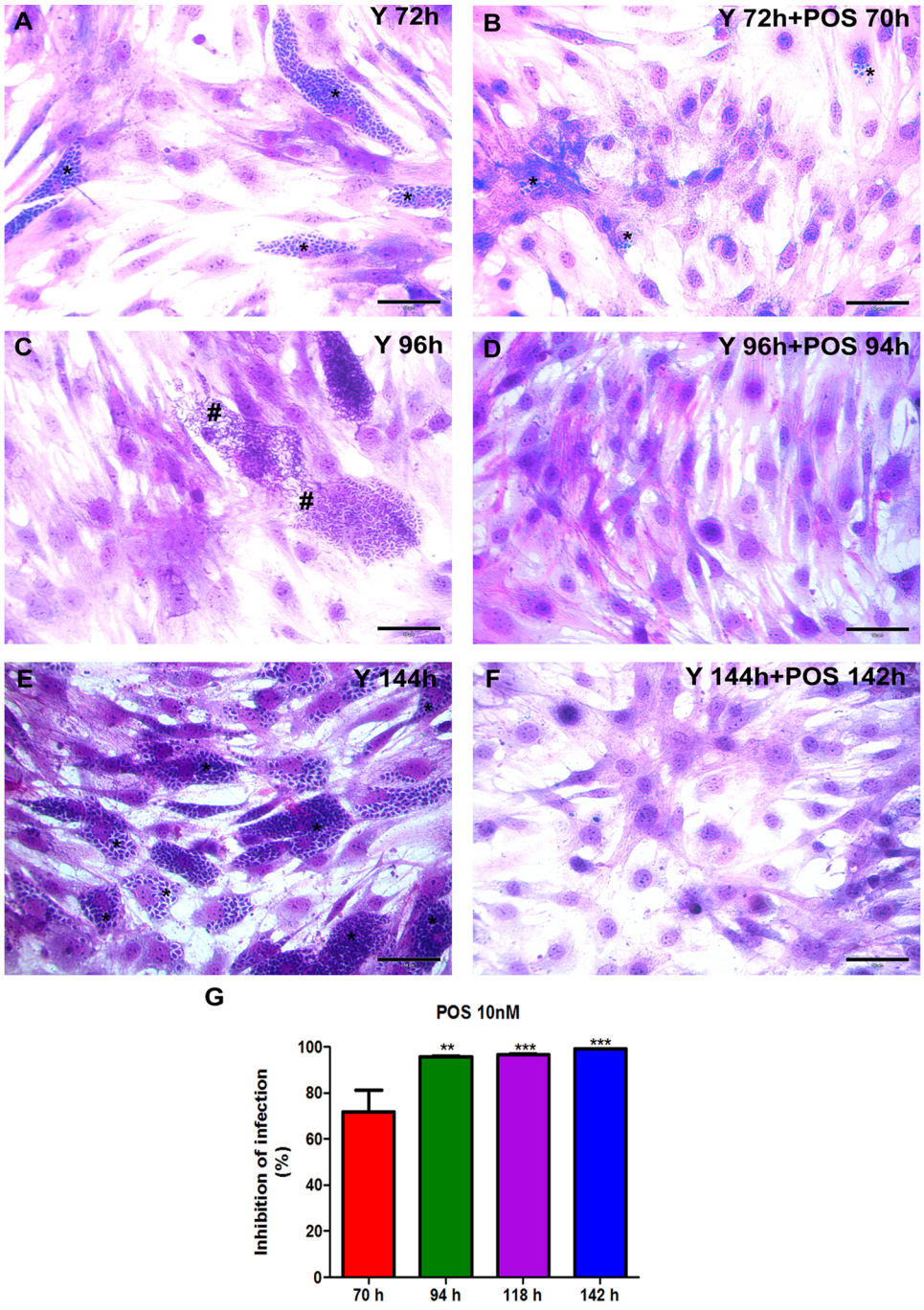

Fig. 1. Effect of 10 nM POS on the intracellular cycle of *T. cruzi*. Primary cardiomyocytes were cultured in monolayers and infected with trypomastigotes of *T. cruzi* (Y strain). After 2 h of infection, cultures were treated with 10 nM of POS. At 72 h post infection, not treated cultures (A) showed intracellular amastigotes (*). Not treated cultures exhibit trypomastigote forms (#) after 96 h of infection (C). After 144 h of infection, intense culture parasitism was observed. At 96 h post infection (D), treatment with 10 nM of POS drastically reduced the parasitism of the monolayers cultures. After 144 h of *T. cruzi* infection and 142 h of POS treatment (F), the compound promoted parasite clearance. Evaluation of treatment on inhibition of *T. cruzi* infection by light microscopy. An important effect of the POS in the reduction of the percentage of infected cells compared to infected and not treated cultures (G). Bar = 50 μm. ** $p < 0.01$; ***$p < 0.001$. One-way ANOVA test.

analyzed. *T. cruzi* DNA extracted from epimastigotes (Y strain) ranging from 10^6 to 10° parasite equivalents were utilized to build the standard curve. To cardiomyocytes, DNA extracted from cells, ranging from 1.5×10^6 to 1.5×10^1 cells equivalents was used.

2.7. Immunofluorescence and confocal microscopy

TCFCM and NICM treated or not, were fixed in 4% paraformaldehyde (PFA) for 5 min at 4 °C and permeabilized with 0.5 % (v/v) Triton X-100. The blockage was performed in PBS 3% (w/v) bovine serum albumin and cardiac spheroids were incubated overnight at 4 °C with primary antibody against fibronectin and laminin both from Sigma-Aldrich. The samples were then washed with PBS and were incubated with a secondary AlexaFluor 488 conjugated antibody at 25 °C for 5 h. DNA was stained with DAPI diluted in PBS. Three independent experiments were performed and analyzed in Zeiss 510 Meta laser scanning confocal microscope.

2.8. Immunoblotting

Proteins were extracted from a pool of sixty TCFCM or NICM for each experimental condition (treated or not) in three independent experiments. Protein extraction was performed using 300 μL of lysis buffer (2 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM $NaHCO_3$ and Roche Protease Inhibitor Cocktail) and sonicated. Samples were frozen at −80 °C until used and protein concentration was measured using the BCA Protein Assay Reagent. 20 μg of protein was loaded and resolved in 10 % or 12 % SDS polyacrylamide gels. After resolving, proteins were transferred to nitrocellulose membranes (Bio-Rad) and incubated with primary antibodies rabbit polyclonal anti-fibronectin (220 kDa), rabbit

polyclonal anti-laminin (220 kDa), mouse monoclonal anti-alpha smooth muscle actin (α-SMA) (42 kDa), rabbit polyclonal anti TIMP-4 (23 kDa) or rabbit polyclonal anti MMP-9 (92 kDa) – all from Sigma-Aldrich diluted in TBST with 5% skin milk overnight at 4 °C. For loading controls, mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (36 kDa) – from Fitzgerald monoclonal antibody and was used as loading control. Membranes were incubated with secondary goat anti-rabbit IgG or goat anti-mouse IgG HRP-labeled antibody for 1 h at 25 °C, followed by incubation with chemoluminescent substrate kit [SuperSignal West Pico PLUS Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity (both fromThermo Scientific)] and exposition to X-ray film. Biological triplicates were analyzed in a minimum. Densitometric analysis of the X-ray film bands was performed with the software Image Studio Lite version 4.0 and standardized by the respective GAPDH density. The results were showed as index of variation (IV) of the control non-infected that was a normalization of the data by the non-infected mean.

IV_{group} = group density / non-infected and non-treated density mean

2.9. ELISA TGF-β

Measurement of TGF-β in the supernatants of TCFCM or NICM for each experimental condition (treated or not) was performed using TGFβ1 Emax® ImmunoAssay System (Promega) according to manufacturer's protocol. Measurement of total TGF-β (active + latent) was performed by heat activation (80 °C) of latent TGF-β. Three independent experiments were analyzed. The results were showed as index of variation (IV) of the control non-infected that was a normalization of the data by the non-infected mean.

2.10. Statistical analysis

Unpaired two-sample *t*-test or One-way ANOVA and Tukey´s post-test was used to analyze statistical significance ($p < 0.05$) in GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Effect of Posaconazole in monolayers of cardiac cells infected by *T.cruzi*

We started our experiments treating 2D cultures of cardiomyocytes infected by *T. cruzi* with 10 nM of POS. The treatment was initiated 2 h post-infection and a time-dependent response was evaluated until 144 h post-infection. Morphological alterations of intracellular amastigotes were observed at 72 h of infection in POS treated cultures (Fig. 1B). At 96 h of infection, no trypomastigote forms were observed in treated cultures, showing that POS impaired the differentiation of amastigotes to trypomastigote forms (Fig. 1D). At 72 h of infection, we observed 70 % of infection inhibition, while inhibition levels reached 95 % and > 99 % after 96 and 144 h of infection in treated cultures, respectively (Fig. 1F, G).

Starting the treatment with POS at 48 h post-infection, when invasive trypomastigote forms already differentiated to amastigote forms, and the proliferation process occurs, we also observed a dose and time-dependent reduction in the percentage of infected cells (Fig. 2). At 120 h of infection and 72 h of treatment, the lowest tested dose of POS (1.25 nM) inhibited 43 % ($p < 0.01$) and the highest (5.0 nM) inhibited 63 % of infection ($p < 0.001$). In the last time point of infection and treatment (144h and 96h respectively), 2.5 nM of POS inhibited 75 % ($p < 0.05$) and 5 nM inhibited 84 % ($p < 0.01$) of infection (Fig. 2).

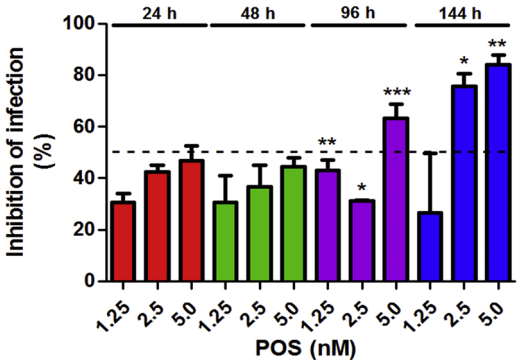

Fig. 2. Time and dose response analysis in *T. cruzi.* infected cardiomyocytes treated with POS after 48 h of infection. Quantification of the percentage of *T. cruzi* infection inhibition in cardiac monolayer culture was evaluated after 24, 48, 72 and 96 h of POS treatment. POS induced significant inhibition of *T. cruzi* infection. IC_{50} after 144 h post infection was 1.58 nM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. One-way ANOVA test.

3.2. Effect of Posaconazole in T. cruzi fibrotic cardiac microtissues

Based on our previous results, showing that at 144 h post *T. cruzi* infection most cardiac cells of TCFCM were infected by the parasite [21], we choose this time point to start the treatment with POS and used 5 nM POS and 96 h of treatment.

Fluorescence images using the DNA intercalator DAPI showed the distribution of cardiac microtissue cells and parasites DNA in cardiac microtissues. Cardiac microtissue presented a mean of 68 % of cardiomyocytes (Supplementary Fig. S1) and nuclear staining (Fig. 3A). In infected microtissue, the parasites showed nuclei and kinetoplast stained (Fig. 3B and D) and *T. cruzi* infection was not only in the microtissue surface as showed in the confocal reconstruction image (Supplementary Fig. S2). In infected and treated microtissues it was possible to visualize fragments of *T. cruzi* DNA (Fig. 3C and E), while the nuclei of cardiac cells were intact, indicating selective parasite cell death. This data was confirmed by the cell count of slices from not treated and treated cardiac microtissue, which revealed no significant difference in the number of cells per field [(106 ± 44 and 107 ± 61, respectively) Supplementary Fig. S3].

To confirm the trypanocidal effect of POS in this 3D culture system we evaluated the *T. cruzi* load by quantitative real-time PCR. It was found that 5 nM POS treatment reduced significantly (51.6 %, $p < 0.05$) the parasite load when compared to non-treated TCFCM (Fig. 3F), showing that even in a complex multilayer 3D structure, the compound was able to reach the parasites maintaining the trypanocidal activity observed in monolayer.

3.3. Posaconazole treatment reduces ECM proteins expression in T. cruzi fibrotic cardiac microtissues

Since we previously found fibrosis and hypertrophy in TCFCM at 144 h post-infection [21], we evaluated the alteration of ECM proteins associated with the reduction of the parasite load after 96 h of POS treatment that was initiated 144 h post-infection. Immunofluorescence revealed that non-treated TCFCM presented increased laminin (Fig. 4B) and fibronectin (Fig. 4E) staining compared to POS treated ones.

After 96 h of treatment with 5 nM of POS, we observed a marked reduction of staining for both proteins in TCFCM (Fig. 4C and F). We also performed western blotting and the results confirmed our observations in immunofluorescence, showing a reduction in the expression levels of laminin (53.8 %, $p < 0.05$) (Fig. 5A) and fibronectin (45 %, $p < 0.05$) (Fig. 5B) after treatment with POS. These results show that POS treatment was able to revert fibrosis induced by *T. cruzi* in cardiac microtissues.

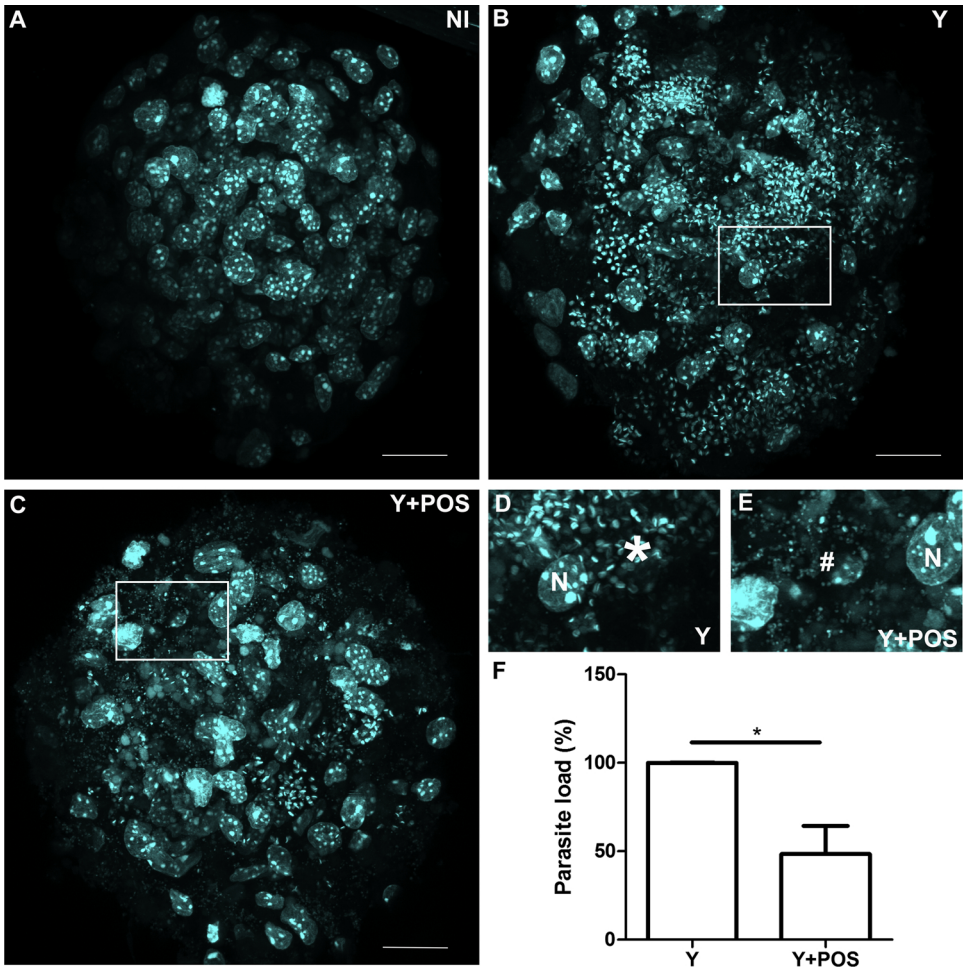

Fig. 3. Evaluation of *T. cruzi* infection in cardiac microtissues by DNA staining with DAPI and qPCR. DAPI staining shows the nuclei of cardiac cells with nucleoli (A-E) and the nuclei and kinetoplast of intracellular amastigote forms (B-E). (A) A non-infected cardiac microtissue showing a regular morphology and distribution of nucleus. (B) Cardiac microtissues after 240 h of *T. cruzi* infection presenting a high number of amastigote forms and the sparse distribution of nucleus. (C) Cardiac microtissue after 240 h of *T. cruzi* infection and 96 h of treatment with POS showing reduction of intact parasites. (D) Increased image of an insert area of microtissue presented in B, showing the kinetoplasts of intracellular amastigotes (*) and cardiomyocyte nucleus (N). (E) Increased image of an insert area of microtissue presented in C, showing remnants of parasites (#) after the treatment with POS and cardiomyocyte nucleus (N). (F) Quantification of parasite load evaluated by qPCR. Bar graph shows the reduction in parasite load after 96 h of treatment with POS. $^{*}p < 0.05$. *t* test. Bar = 20 μm.

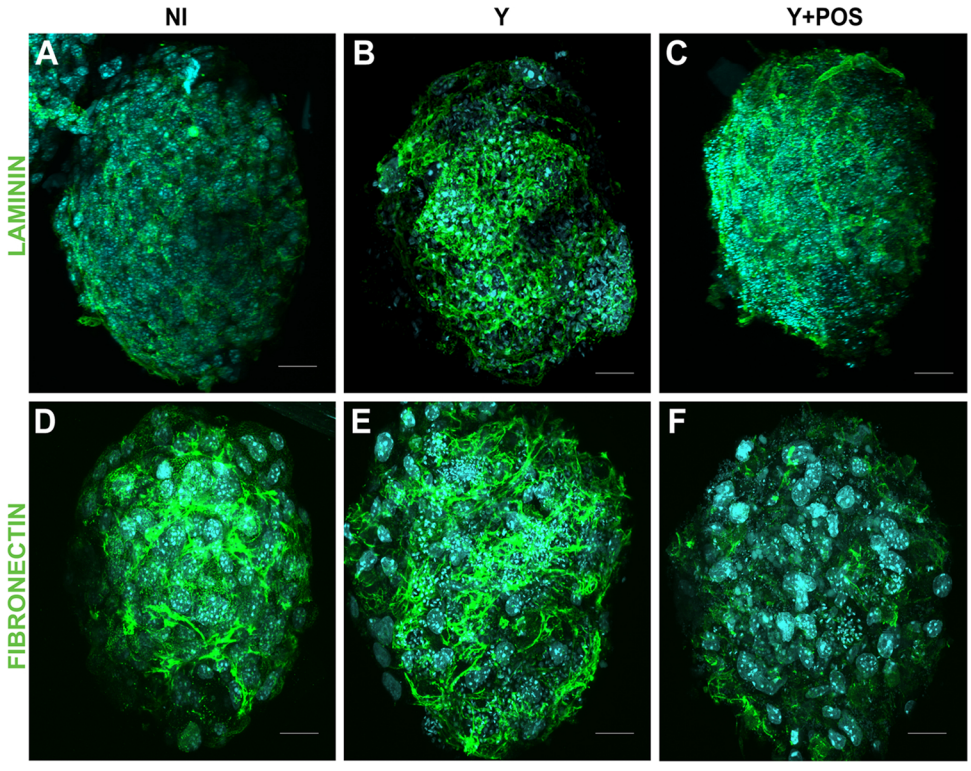

Fig. 4. Immunofluorescence analysis of laminin (A-C) and fibronectin (D-F) after 240 h of *T. cruzi* infection and 96 h of POS treatment. Protein distribution labeled with AlexaFluor 488 conjugated secondary antibody (green) and DAPI staining (blue) in control non-infected (NI), *T. cruzi* infected (Y) and *T. cruzi* infected and treated with POS (Y + POS) 3D culture. *T. cruzi* infection induces intense deposition of laminin and fibronectin after 240 h of infection. POS treatment initiated 144 h post infection and continued for 96 h inhibited fibrosis. Bar = 20 μm.

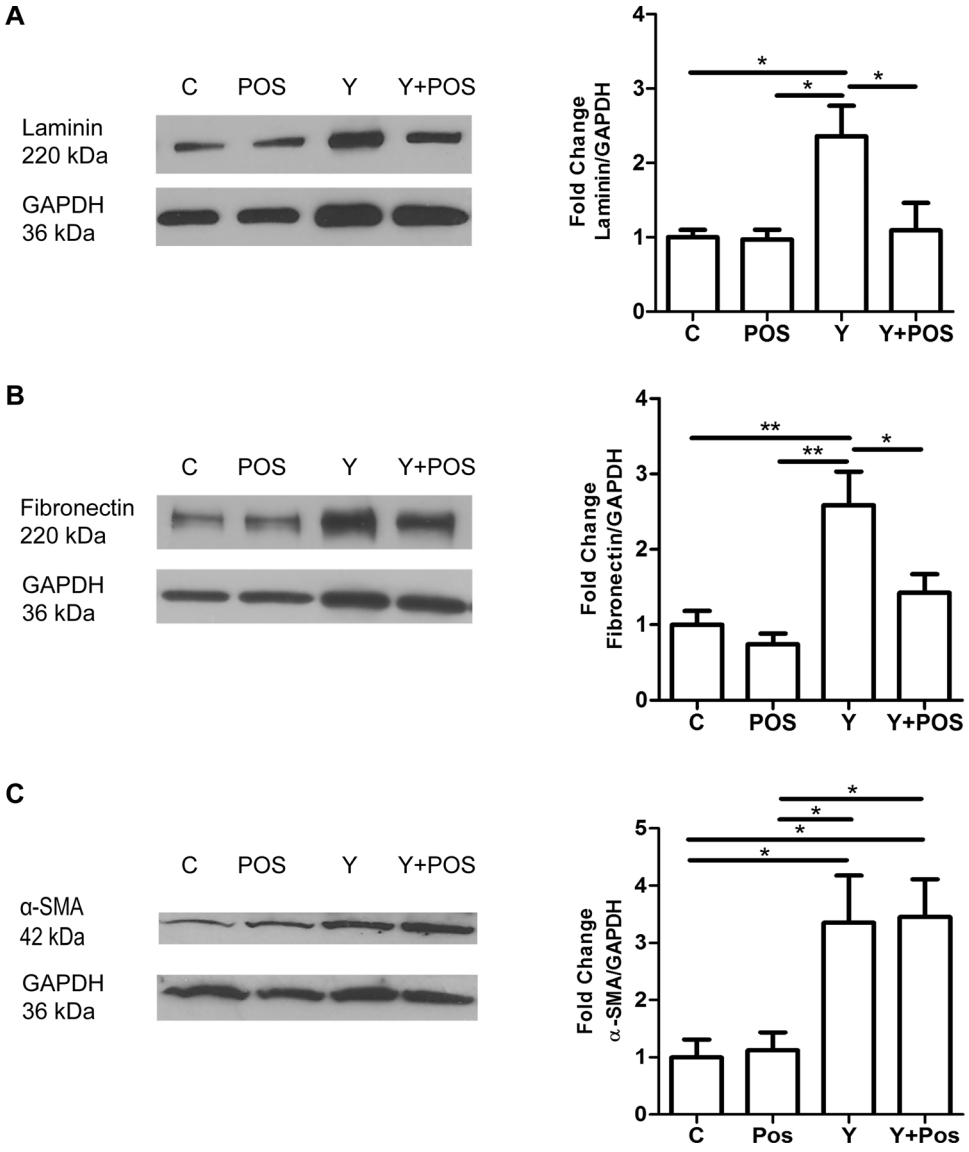

Fig. 5. Western blot analysis of laminin (A), fibronectin (B) and α-SMA (C). Anti-GAPDH (36 kDa) antibody was used as a loading control. Expression in control non-infected (NI), non-infected and treated with POS (POS), *T. cruzi* infected (Y) and infected and treated with POS (Y + POS) 3D culture. 3D cultures infected by *T. cruzi* exhibit higher laminin, fibronectin and α-SMA protein levels compared to non-infected cultures. Reduction of laminin and fibronectin was observed after 96 h of POS treatment in infected cultures. Results are shown as index of variation of the mean (IV) ± SEM. *$p < 0.05$; **$p < 0.01$. One-way ANOVA test.

Trying to understand the effect of POS treatment on mechanisms of ECM synthesis in TCFCM, we decided to investigate the expression of α-smooth muscle actin (α-SMA). This protein is expressed in activated myofibroblasts in the development of fibrosis [28]. By western blotting, we observed a 3.3-fold increase ($p < 0.05$) in α-SMA in non-treated TCFCM (Fig. 5C), suggesting that one of the mechanisms involved in the induction of ECM proteins expression in TCFCM is the differentiation of fibroblasts to myofibroblasts. We also found that the treatment with POS did not change the levels of this protein (Fig. 5C), showing that neither the reduction on parasite load nor POS *per se* are sufficient to reduce myofibroblast activation.

3.4. Posaconazole treatment alters levels of ECM regulators (TGF-β, MMP-9 and TIMP-4) in T. cruzi fibrotic cardiac microtissues

We also investigated molecules directly involved in the degradation of ECM proteins (metalloprotease MMP-9 and the inhibitor of metalloproteases TIMP-4). We did not observe statistically significant changes in MMP-9 levels in *T. cruzi* infected microtissues (Fig. 6A). In contrast, the infection induced a 6-fold increase ($p < 0.001$) in the expression of TIMP-4 (Fig. 6B), suggesting that *T. cruzi* inhibit the mechanisms involved on the degradation of ECM, while treatment with POS reduced 2.4-fold ($p < 0.05$) the levels of TIMP-4 in *T. cruzi*-infected cultures (Fig. 6B).

Finally, we investigated TGF-β levels, since its involvement in cardiac fibrosis during Chagas cardiomyopathy is well established [16]. We performed ELISA of cultures supernatant to evaluate the effect of POS treatment on TGF-β secretion (Fig. 6C). We found that *T. cruzi* infection increased the levels of TGF-β secretion (20 %, $p < 0.05$) in the supernatant of TCFCM when compared to non-infected cardiac microtissue. Treatment of TCFCM with POS increased by 50 % the TGF-β secretion levels ($p < 0.001$) when compared to non-treated TCFCM and 70 % ($p < 0.001$) when compared to non-infected ones. These results show that POS changes TGF-β secretion levels in infected cardiac microtissues, suggesting an immunomodulatory effect of this compound on cardiac cells.

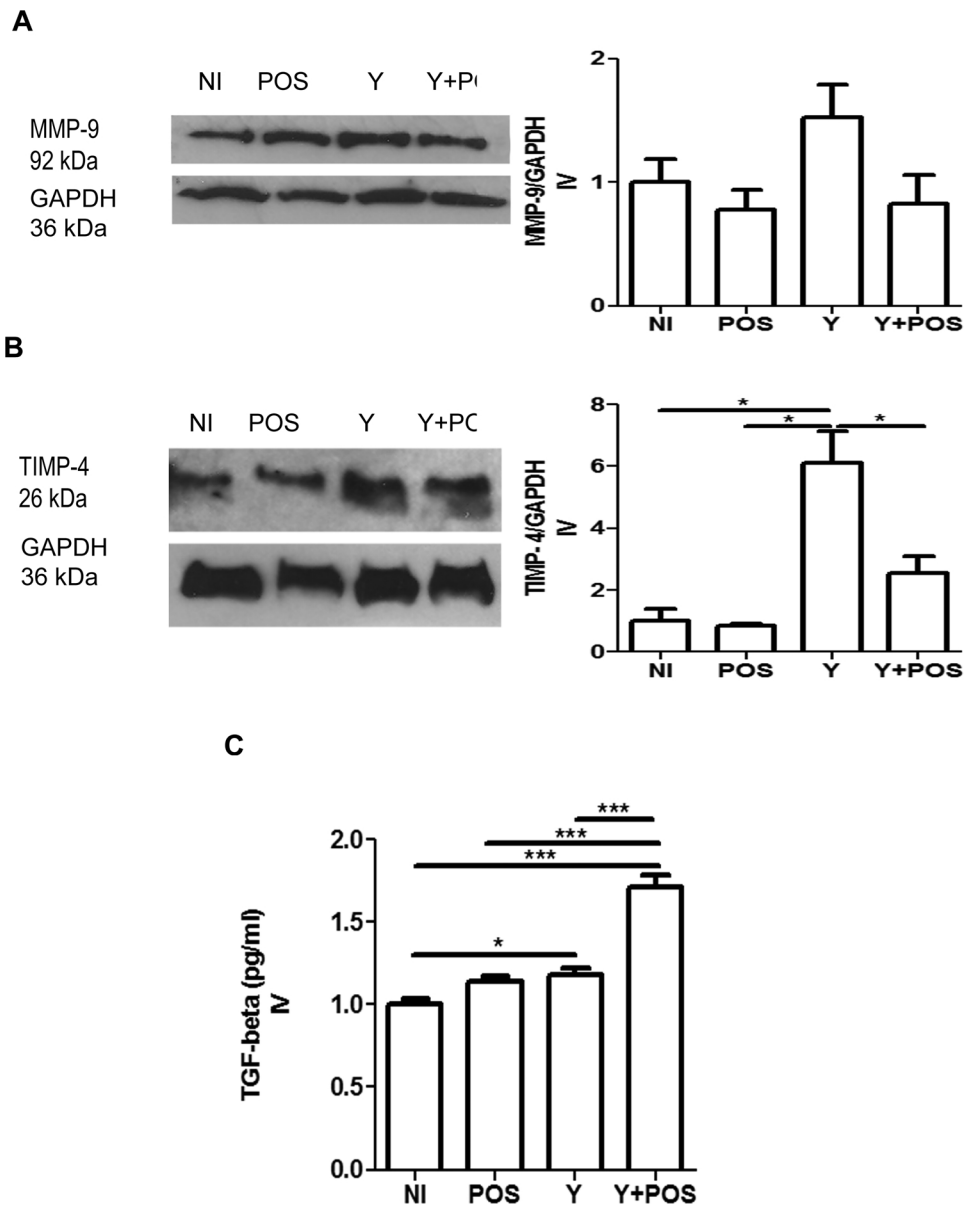

Fig. 6. Western blot analysis of MMP-9 (A) and TIMP-4 (B). Anti-GAPDH (36 kDa) antibody was used as a loading control. *T. cruzi* infected cardiomyocytes 3D culture have higher TIMP-4 levels compared to control group. POS treatment of infected culture induces reduction of this protein. TGF-β levels in the supernatant (C) of 3D cultures non-infected (NI), non-infected and treated with POS (POS), *T. cruzi* infected (Y) and 3D culture infected and treated with POS (Y + POS). *T. cruzi* infection induces TGF-β secretion when compared to non-infected cultures. In addition, infected and POS treated cultures present even higher TGF-β levels in the supernatant. Results are showed as index of variation of the mean (IV) ± SEM $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. One-way ANOVA test.

4. Discussion

CD is mostly silent, the symptoms during the acute phase are generic, making difficult the diagnosis and consequently the specific treatment with BZ or NF, which can reach 80 % of cure if started during the early stages of the disease. However, these drugs can present adverse effects and have poor efficacy in the chronic stages of infection [3,29].

CCC results from parasite persistence and chronic inflammation [30], which cause microvascular abnormalities, myocytolysis, necrosis, fibrosis and compensatory hypertrophy. These clinical alterations contribute to cardiac dysfunction and consequently to heart failure, observed in advanced stages of CD [11].

For that reason, the treatment with trypanocidal agents, despite contributing to parasite load reduction, is in many cases not sufficient to reverse the cardiac alterations. In this context, is necessary to find treatment strategies that not only kill the parasite but also reduce cardiac injury. Since fibrosis is one of the more nefarious cardiac alterations observed in CCC, it should be considered as a target in the treatment of chronic CD.

Ergosterol biosynthesis inhibitors have been tested as new therapeutic agents for Chagas disease [31]. Here we showed that the treatment with POS has potent dose and time-dependent trypanocidal effect in *T. cruzi*-infected cardiac cells in monolayer, corroborating previous data [8,32,33]. POS reduced *T. cruzi* infection also in experimental models of acute and chronic Chagas disease in mice [7,19].

In humans, the CHAGASAZOL [10] and STOP CHAGAS [34] clinical trials showed that POS had trypanocidal and trypanostatic activity in chronic patients with Chagas disease, although less than that of BZ, most probably because the plasma exposure levels in the patients resulting from the dose of the drug used in these studies (400 mg b.i.d.) were just 10–20 % of optimal dose attained in mice (20 mg/kg.d) [3].

In an experimental model of acute CD in mice, the treatment with POS started 4 days after *T. cruzi* infection reduced the parasitemia and prevented fibronectin deposit in the heart of infected mice [19]. Since fibrosis contributes to electrophysiological changes and compensatory ventricular dilation leading up to clinical manifestations of CCC, such as heart failure [34], evaluation of POS treatment on progression of cardiac alterations in CD is necessary.

In this perspective, in this study we decided to evaluate the effect of POS *in vitro* in a model of cardiac fibrosis induced by *T. cruzi*, here called *T. cruzi* infected fibrotic cardiac microtissue (TCFCM). TCFCM

mimics aspects observed in CCC including fibrosis and hypertrophy, even in the absence of inflammatory cells [21]. Cardiac microtissues are formed without any artificial scaffold in an agarose substrate, where the isolated cardiac cells agglomerate, retaining the ECM proteins secreted by them and forming a spheroidal 3D structure. It mimics cell-cell and cell-ECM interactions, similar to tissues *in vivo*, an advantage over 2D culture in monolayer [22,35].

Moreover, similar to what is observed during chagasic cardiomyopathy [16], the fibrosis induced by *T. cruzi* in cardiac microtissues, also results in TGF-β increased levels, which are reduced by inhibitors of TGF-β signaling pathway [16,23]. Here, we evaluated in TCFCM the effect of POS treatment on parasite load and ECM proteins levels (laminin and fibronectin), including its regulators (MMP-9, TIMP-4 and TGF-β).

After the series of experiments in monolayers, we selected the concentration of 5 nM POS to perform the experiments in TCFCM. Starting the treatment at 144 h post-infection, this concentration maintained for 96 h, POS treatment reduced parasite load as evaluated by qPCR. We found that besides reducing the parasite load, POS also reduced the expression levels of fibronectin and laminin in TCFCM, two proteins strongly involved in cardiac fibrosis during the CCC [15,36]. However, if this effect was a consequence of the reduction in parasite load or a direct effect of POS over fibrosis mechanisms, is difficult to conclude.

Our results showing an increase of TIMP-4 expression in TCFCM suggests that the fibrosis observed in this system is influenced by an unbalance in the regulation of the ECM. MMPs are zinc-dependent proteases crucial for physiological tissue remodeling and TIMPs inhibit the activity of MMPs [37]. Any change in the balance of these proteins can modify the myocardial structure. TIMP-4 is a low-molecular-weight protein of 23 kDa that is highly expressed in the heart [37], and modifications in its levels may influence the myocardial matrix remodeling. TIMP-4 is a key endogenous modulator of MMP-9 and a reduction in the levels of this protein has been described in heart failure [38]. Here, the treatment of TCFCM with POS reduced the levels of TIMP-4 that had been before increased by *T. cruzi* infection.

We also observed an increase in TGF-β secretion induced by *T. cruzi* infection. This finding corroborates previous observations in cardiomyocytes monolayers, in animal experimental models of CD and in humans, which associate this molecule to the host cell-parasite invasion and cardiac fibrosis during CD [16]. Moreover, this cytokine presents an anti-inflammatory effect in CD [39] and it was previously showed that POS regulates the trypanocidal immune response by controlling unspecific splenocyte proliferation in the early acute phase of CD [19].

TGF-β is a molecule that remains attached to the ECM after its production in a latent form [40]. Since we observed a higher level of secreted TGF-β in infected and treated supernatant of TCFCM, an explanation could be lower retention of TGF-β in ECM linkage sites, that could result from the reduction in fibronectin and laminin levels induced by the drug. Fibronectin is a critical protein for the incorporation of latent TGF beta-binding protein-1 [41]. More studies involving the understanding of mechanisms of fibrosis and inflammation in the context of treatment with POS and TGF-β response during *T. cruzi* infection are necessary.

We conclude that POS, apart from its potent intrinsic anti-*T. cruzi* activity may contribute to the prevention or reversal of CCC by reducing fibrosis of the infected cardiac tissues, due to an intrinsic reorganization of the myocardial tissue resulting from a decreased parasite load.

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Data availability statement

All datasets generated for this study are included in the manuscript.

CRediT authorship contribution statement

Lindice Mitie Nisimura: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Patrícia Mello Ferrão:** Investigation, Formal analysis. **Alanderson da Rocha Nogueira:** Investigation. **Mariana Caldas Waghabi:** Supervision, Formal analysis. **Marcelo Meuser-Batista:** Investigation, Formal analysis. **Otacílio C. Moreira:** Formal analysis, Investigation. **Julio A. Urbina:** Writing - review & editing, Funding acquisition. **Luciana Ribeiro Garzoni:** Supervision, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molbiopara.2020.111283.

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