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Research paper

Avian antibodies (IgY) against *Trypanosoma cruzi*: Purification and characterization studies



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ABSTRACT

Trypanosoma cruzi is a flagellated protozoan belonging to the Trypanosomatidae family, the etiologic agent of Chagas disease. Currently, there is neither a licensed vaccine nor effective treatment, characterizing an unmet clinical need. The IgY refers to the egg yolk immunoglobulin (Y = yolk) and its production and use are subjects of many studies due to the diversity of its diagnostic and therapeutic applications. Several researchers have shown that the use of specific IgY may prevent and/or control infectious and parasitic diseases. Based on these evidences, the aim of this study was to immunize chickens with trypomastigotes of *T. cruzi* in order to produce highly effective and pure antibodies (IgY), as well as extract, characterize, quantify, and verify cytotoxic effects of IgY anti-*T. cruzi*. After the induction of IgY production by chickens, the eggs were collected and the IgY was extracted by method of precipitation of polyethylene glycol 6000. The IgY anti-*T. cruzi* characterization was performed using polyacrylamide gel electrophoresis (SDS-PAGE), western-blot and enzyme-linked immunosorbent assay (ELISA). Moreover, the cytotoxic or proliferative effects of IgY anti-*T. cruzi* was verified by MTT assay. The concentration of IgY in yolk was 8.41 ± 1.47 mg/mL. The characterization of IgY revealed bands of stained peptides with molecular weight between 75 and 50 kDa and 37 and 25 kDa. In the ELISA test was observed that there was antigen-antibody reaction throughout the sample period. The concentrations of 1, 5 and 10 mg/mL of IgY anti-*T. cruzi* presented no cytotoxic or proliferative effects in mononuclear and VERO cells in vitro. The results indicated that *T. cruzi* is able to generate a high production of specific immunoglobulins in chickens, it did not cause damage to the cell membrane and no proliferative effect.

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1. Introduction

Chagas disease is a neglected tropical disease caused by *Trypanosoma cruzi*, an obligatory intracellular parasite widely spread in Latin America (Basso and Marini, 2015). This disease constitutes a serious worldwide public health issue (Coura and Dias, 2009), that causes a chronic parasitic condition characterized by a life-long infection of the host (Martin et al., 2015). Clinically, this disease may have an acute phase that lasts for 1 to 4 months characterized by patent parasitemia, and a chronic phase with a life-long phase including a subpatent parasitemia and tissue parasitism (Dias, 1997; Rassi et al., 2010). The diagnosis of *T. cruzi* infection during the acute phase is carried out by the

direct detection of circulating parasites. However, during the chronic phase, the diagnosis is performed by conventional serologic assays (WHO, 2002; Otani et al., 2009; Carlier and Torrico, 2003).

Chemical drugs, such as benzimidazole and nifurtimox, are still used as the method of choice in controlling this disease (Urbina and Docampo, 2003). However, the treatment with benzimidazole has many side effects, prolonged treatment time, and low and variable efficacy in the chronic phase of this infection, which is the most prevalent form of the disease (Urbina and Docampo, 2003; Viotti et al., 2009). Thus, the search for more effective medications with less adverse effects is one of the main focuses of many studies (Coura, 2007). In this context, the search for alternative models for the treatment and diagnosis of *T. cruzi* is essential, mainly because it is a disease considered neglected. Specific antibodies for immunotherapy against this parasite might be a therapeutic alternative in controlling and/or prevention infections. Immunoglobulin Y (IgY) are polyclonal antibodies obtained from egg yolk (yolk = y) of immunized chickens. Such molecules have high

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specificity for binding and inactivating foreign substances, such as antigenic molecules that are able to invade the body (Karlsson et al., 2004). For this reason, IgY has been used for diagnosis and therapy (Reilly et al., 1997).

The IgY technology allows to obtain antibodies with high affinity and avidity (Karlsson et al., 2004). In addition, IgY shows numerous advantages when compared to mammalian antibodies, such as: similar functions of the mammalian IgG and IgE, no cross reaction with mammalian IgG, do not attack red blood cells, do not activate the complement system and the coagulation cascade. Furthermore, chickens are more able to produce antibodies than rabbits, goats, horses or rodents (Sampaio et al., 2014b; Chacana et al., 2004; Contreras et al., 2005).

Therefore, the aim of this study was to synthesize, extract, characterize and quantify antibodies IgY anti-*T. cruzi* extracted from eggs yolk of chickens immunized with trypomastigotes of *T. cruzi*. Moreover, effects of IgY anti-*T. cruzi* in vitro against mononuclear and VERO cells were performed, in order to evaluate possible collateral effects.

2. Materials and methods

All procedures in this study were approved by the Animal Welfare Committee of Ethics in Animal Experimentation of the Universidade Federal de Santa Maria (UFSM), under protocol number 5699170915.

2.1. IgY production, extraction and purification

2.1.1. The antigen

Trypomastigotes forms of *T. cruzi* (strain Y) was used as antigen to immunize the chickens, according the method described by Sampaio et al. (2014b), with some adaptations for *T. cruzi*, as follows below: cryopreserved trypomastigotes were inoculated into mice for reactivation and multiplication of strain, and blood samples were collected at the peak of parasitemia. In order to separate the parasites, blood was diluted in DMEM medium (1:1 v/v), followed by centrifugation (400 g for 10 min). The supernatant, rich of trypomastigotes, was collected and the amount of antigen was determined, aliquoted, and cryopreserved until use.

2.1.2. Chicken immunization protocol

The chicken immunization protocol was as described by Sampaio et al. (2014b). Briefly, a solution containing trypomastigotes of *T. cruzi* was added to incomplete Freund's adjuvant (Difco®) at a 1:1 ratio, with a final volume of 1.0 mL. It was injected intramuscularly at five different sites of the pectoral muscle of two 25-week-old New Hampshire chickens. The interval between immunizations was set as 10 days (days 0, 10, 20, 30, 40, 50, 70 and 80), totaling eight inoculations, being 2.4×10^4 trypomastigotes for the first 4 inoculations and 7.2×10^3 trypomastigotes of *T. cruzi* for the other 4 inoculations. Chicken eggs were collected from the third week post-immunization and stored at 4 °C until they were processed. One chicken was not immunized and its eggs were used as controls.

2.1.3. Extraction of immunoglobulin IgY anti-*T. cruzi*

Extraction was performed by the method of precipitation of polyethylene glycol 6000 (PEG-6000) (Polson et al., 1980). Firstly, the yolk was gently separated and its fat was removed using PBS (1:2 ratio) and PEG 6000 at a final concentration of 3.5%. This mixture was held in a roller mix for 30 min and then centrifuged for 80 min at 4.000 rpm and 4 °C. The supernatant was removed, filtered in filter paper (28 µm), and the final volume measured. PEG 6000 was once again added (8.5%), and the solution kept in a roller mix for 10 min, and centrifuged for 30 min at 4000 rpm and 4 °C. In this stage the supernatant was discarded and the pellet was dissolved in 10 mL of PBS. A final step using PEG 6000 (12%) was performed as described previously. The final pellet was dissolved in 2.4 mL of PBS, and this solution was dialyzed overnight in saline solution (0.1%) at 4 °C. A final dialyzation

was performed for three more hours, the extract was collected, placed in tubes, stored at -20 °C, and lyophilized until further use.

2.1.4. IgY specific concentration

The specific concentration of IgY anti-*T. cruzi* was measured according to the Lambert-Beer's law using an extinction coefficient of 1.33 for IgY (Polson et al., 1980). The protein content of the samples was measured by the method of Bradford (1976), with Coomassie blue using bovine serum albumin as standard, and monitored by measuring the maximum absorbance of the solution at 595 nm.

2.2. Characterization of IgY

The IgY was characterize and evaluate regarding its specificity and sensibility to the antigen, as follow below:

2.2.1. The polyacrylamide gel electrophoresis (SDS-PAGE)

The IgY samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% (Bernardo, 2009). For this, 20 µL of the IgY samples collected from chickens on the week 3, 5, 7, 9, 11 and 13 post-immunization and a control sample of non-immunized chicken were mixed with 20 µL of the buffer (Laemmli Sample Buffer - BioRad®), boiled in a water bath at 95 °C for 5 min, and applied to the stacking gel wells in a final volume of 5 µL. The electrophoresis was submitted to a current of 70 V for 30 min and 120 V for 120 min. Finally, the gel was colored with Coomassie Blue R (Sigma-Aldrich®) for at least 1 h, and treated with a decolorizing solution (glacial acetic acid and methanol).

2.2.2. Western blot

Western blot was performed as described by Bernardo (2009). Initially, electrophoresis was performed under the same conditions as described above. Two gels were prepared: one for IgY samples (the same used in Section 2.2.1) and a second gel with samples of the antigen used to immunize chickens (trypomastigotes of *T. cruzi*) and samples of trypomastigotes of *Trypanosoma evansi*. After protein separation in polyacrylamide gels, they were electrophoretically transferred to a nitrocellulose membrane of 0.45 µm (BioRad) using a current of 140 V for 120 min. Finally, the membranes were removed carefully, and blocked overnight in a PBS-Tween solution with 5% skimmed milk powder. The membrane containing the IgY was washed three times with PBS-Tween, and incubated for 1 h with rabbit anti-chicken IgY peroxidase conjugate, diluted in PBS-Tween (1:2000) (Sigma-Aldrich®). The nitrocellulose membrane containing the antigens of *T. evansi* and *T. cruzi* was washed three times with PBS-Tween and incubated for one hour with the newly synthesized IgY anti-*T. cruzi* and the primary antibody control IgY. The membrane was washed again three times with PBS-Tween, incubated for one hour with rabbit anti-chicken antibody peroxidase conjugated. Membranes were washed three times with PBS-Tween, and finally incubated with a developing solution (DAB-di-aminobenzidine, Tris HCl, Nickel sulfate 0.3%, and H₂O₂) for the visualization of any reactive band.

2.2.3. ELISA

The Enzyme Linked Immune Sorbent Assay (ELISA) was performed as described by Sampaio et al. (2014b). A 96-well ELISA plate was coated with *T. cruzi* antigen (1 µg/well) previously diluted in 0.05 M of carbonate buffer (pH 9.6). The plates were incubated overnight at 4 °C. After incubation, the plates were washed four times with saline buffer (SB - pH 7.2). Firstly, it was blocked with 100 µL of fetal bovine serum (FBS)-(Cripion®, SP, Brazil) (SB and FBS) per well, and incubated at 37 °C. After this, the plates were washed four times with SB-Tween (SB pH 7.4 with 0.05% Tween 20). IgY samples extracted at different weeks post-immunization (3, 5, 7, 9, 10, 11, 13) were diluted (1:1000, 1:2000, and 1:5000) in FBS and SB, distributed over the plates (100 µL/well), and incubated for 1 h at 37 °C. Then, plates were washed

four times. Each sample tested was submitted to three repetitions per plate and two repetitions inter-plates. After incubation with IgY (primary antibody), the plates were washed four times with SBS-Tween and submitted to an incubation with a secondary antibody (rabbit anti-chicken IgY peroxidase conjugate, Sigma-Aldrich®) diluted at 1:3000 for 1 h at 37 °C. Finally, the plates were washed again, and 100 µL of the chromogenic substrate (orthophenylenediamine, OPD) was added. After 15 min, the reaction was blocked with 10 µL of H₂SO₄ and the reading was performed at 490 nm using a spectrophotometer.

2.3. Evaluation of IgY cytotoxicity

In order to evaluate whether IgY may cause a cytotoxic effects, we used the MTT assay in two cell lines: Peripheral blood mononuclear cells (non-adherent cells) and VERO cells (adherent cells).

2.3.1. Peripheral blood mononuclear cells (PBMC)

Samples of peripheral blood were supplied by the Clinical Analysis Laboratory of the Franciscan University Center. The project was approved by the Research Ethics Committee of the Franciscan University Center, CAAE (31211214.4.0000.5306). Peripheral blood samples were collected by venipuncture using a top Vacutainer® (BD Diagnostics, Plymouth, UK) and heparin tubes. These samples were drawn after 12 h of overnight fasting from three apparently healthy human volunteers (22–25 years old), nonsmokers that were not taking prescription drugs, with drinking habits of no more than two alcoholic beverages a week. Lymphocyte cells were separated by Histopaque-1077 (Sigma-Aldrich Co., St Louis, USA) under density gradient centrifugation using 4 mL of blood samples. After further centrifugation for 15 min at 2500g, cells were transferred to culture media containing 5 mL of RPMI 1640 supplemented with 10% of FBS, 1% penicillin and streptomycin. The cells were cultured in a 96-well microplate at an initial density of 2×10^5 for 24 and 72 h at 37 °C in a 5% humidified CO₂ atmosphere (Wilms et al., 2005).

2.3.2. VERO cell culture

Green monkey kidney (VERO) cells (Adolpho Lutz Institute, São Paulo, Brazil) (20–30 passages) were cultured in 96-well plates (Corning, USA) with E-MEM supplemented with 10% (v/v) FBS (Gibco BRL), 1% (v/v) L-glutamine (200 mmol L⁻¹), 10 mg/mL ciprofloxacin (Baytril, Bayer), and 0.025 g/mL amphotericin B (Gibco BRL). The cells were maintained at 37 °C in a humidified incubator with a 5% CO₂ atmosphere.

2.3.3. Cell viability

The cytotoxicity of IgY against lymphocytes (non-adherent) and VERO cells (adherent) was evaluated as described by Sagrillo et al. (2015), using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric method based on the cleavage of the reagent by dehydrogenases in viable cells (Mosman, 1983). In microplates, cells were incubated at 37 °C in a humid atmosphere with 5% of CO₂ for 24 and 72 h. At the end of incubation, 50 µL of MTT solution was added to each well and the cells were incubated for three more hours. The supernatant was removed and 200 µL of dimethyl sulfoxide (DMSO) was added to each well. The microplate was analyzed using an ELISA reader at a wavelength of 560 nm. As a control, cells were grown in a medium lacking the constituents. The experiment was performed in triplicate and treatments used as concentrations of 1, 5 and 10 mg/mL of IgY anti-*T. cruzi*. Finally, cell viability was determined and expressed as a percentage of the control value.

2.4. Statistical analyses

Normality and homoscedasticity were analyzed through the Shapiro-Wilk and Levene test, respectively. Significant differences between groups were analyzed and detected by two-way analyses of variance

(ANOVA) for independent samples, followed by Tukey post-hoc test. The differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Concentration of IgY antibody

The production of IgY by immunized chickens was observed during the study period. The average concentration of IgY antibody purified between the 3rd and 13th week after immunization was 8.41 ± 1.47 mg/mL.

3.2. Gel electrophoresis SDS-PAGE

The polyacrylamide gel showed bands of stained peptides with molecular weight between 75 and 50 kDa (IgY heavy chain), 37 and 25 kDa (IgY light chain) (Fig. 1). These findings were obtained for all samples, which demonstrates that the extraction protocol was effective in separating the IgY.

3.3. Western blot assay

The results from the first Western blot, where IgY control samples and IgY from immunized chickens after 3, 5, 7, 9, 11 and 13 weeks of immunization can be seen in Fig. 2A. This figure shows bands with molecular weight between 75 and 50 kDa and other bands between 37 and 25 kDa, corresponding to the heavy and light chains of IgY. Demonstrating that the bands found on the electrophoresis gel having the same molecular weight refer to IgY.

The second membrane electrophoretically transferred (Fig. 2B) with antigen samples shows that IgY newly synthesized anti-*T. cruzi* reacted with proteins of both species of *Trypanosoma*, *T. cruzi* and *T. evansi*, which indicates a cross-reactivity. The immunoglobulin produced was able to recognize in *T. cruzi* several proteins, in this some homologous proteins in *T. evansi* were also recognized, demonstrating that IgY anti-*T. cruzi* can provide cross-reaction to other trypanosomatids and cannot be used in tests diagnoses specific. Also, results for IgY control were negative, indicating that the IgY produced has specificity for the target antigen.

3.4. ELISA assay

Positive results were obtained for all dilutions tested. There was an antigen-antibody reaction with an optical density (OD) between 0.196 ± 0.005 and 1.83 ± 0.002 (Fig. 3). It was also observed a decrease in the antigen-antibody reaction after the week 8, which refers to the reduction of trypomastigotes and the concentration of the antigen used to immunize chickens, thus, indicating a reduction of specific IgY anti-*T. cruzi* production.

3.5. MTT assay

In order to determine whether IgY led to increased cellular toxicity, the MTT assay for cell viability was used. As shown in Figs. 4 and 5, IgY did not cause cytotoxicity effects at concentrations of 1, 5 and 10 mg/mL after 24 and 72 h post-incubation in PBMC and VERO cell cultures ($p > 0.05$).

4. Discussion

Identification of alternative treatments that are more efficient and less toxic against *T. cruzi* are necessary, since several resistance and toxic effects are reported using the conventional drugs (Viotti et al., 2009). Therefore, therapeutic immunization/immunotherapy using *T. cruzi* antigens can also be used to control the infection and reduce disease progression. Our results demonstrated that it is possible to produce

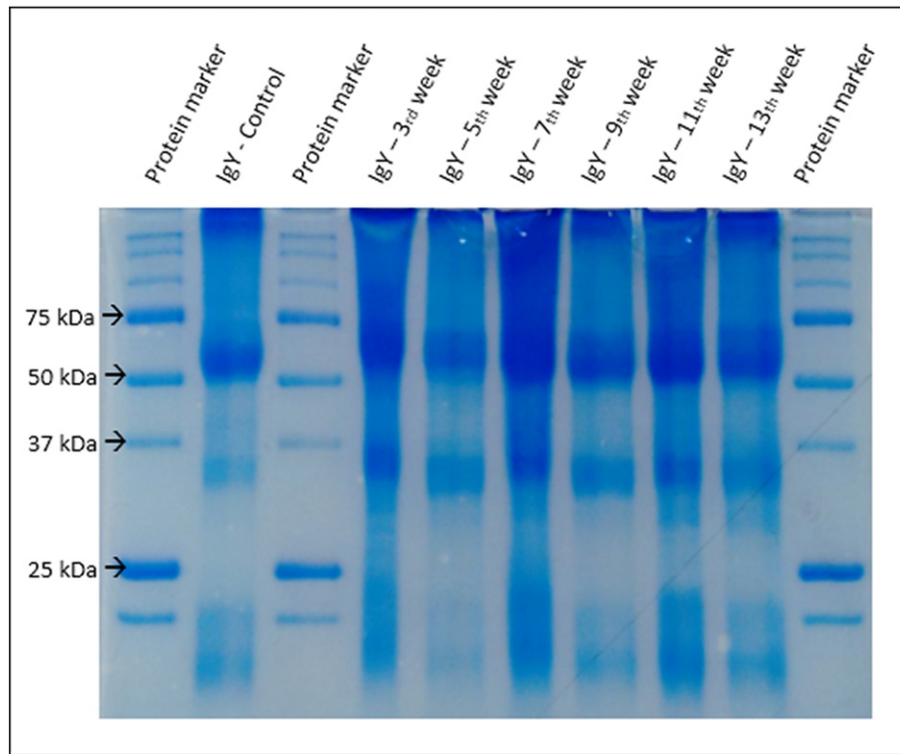


Fig. 1. IgY electrophoresis in polyacrylamide gel (10%) under reducing conditions, stained with Coomassie Blue R250 reagent (Bio-Rad) from samples of three immunized chickens. Protein marker - bands (250, 150, 100, 75, 50, 37, 25 and 20 kDa). The arrows indicates the intervals of the bands being, 75 and 50 kDa refers to heavy chain and 37 and 25 kDa refers to light chain of the IgY produced at 3, 5, 7, 9, 11 and 13 week post-immunization.

IgY through the inoculation of trypomastigotes forms of *T. cruzi* in chickens from eggs yolks. The method of immunization recommended by Sampaio et al. (2014b) demonstrated to be an effective and easy method to be implemented for production of IgY anti-*T. cruzi*.

Our results showed the production of IgY after the inoculation of 10^4 and 10^3 trypomastigotes of *T. cruzi*, which produced an average of 8.41 mg/mL of IgY, whereas 10^7 trypomastigotes of *T. evansi* produced an average of 2.7 mg/mL (Sampaio et al., 2014b). This difference might be related to the immunization protocol, since we used only 10 days of intervals. Moreover, this difference may occur due a greater

immune response mounted by the chickens opposite to the antigenic epitopes of *T. cruzi* antigenic epitopes that may be more complex than *T. evansi*. In addition, regarding the ELISA results, higher optical densities were observed up to 8 weeks, coinciding with the inoculation of a greater number of trypomastigotes (2.4×10^4 trypomastigotes/mL), which shows that the antigen concentration is closely related to the specific production of IgY. Furthermore, there was a major reduction of IgY activity, and this can be due to a reduction in the immune responses of the animals against the antigen, since after several inoculations this antigen cannot be seen as a challenge anymore.

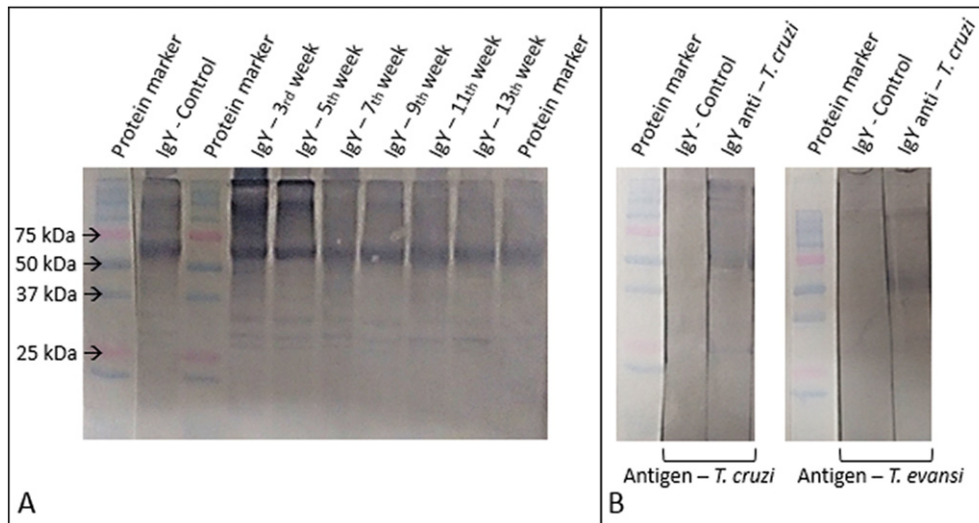


Fig. 2. A) Immunoblot showing the characterization of IgY after the addition of the secondary antibody (rabbit anti-chicken IgY peroxidase conjugate, Sigma-Aldrich®). Protein marker - bands (250, 150, 100, 75, 50, 37, 25 and 20 kDa). The arrows indicate the bands (75, 50, 37 and 25 kDa) to demonstrate the range of IgY heavy and light chain bands of IgY produced at 3, 5, 7, 9, 11 and 13 week post-immunization and Control IgY. B) Immunoblot showing the specific recognition of IgY - anti-*T. cruzi* and IgY - Control, to the protein antigens from *T. cruzi* and *T. evansi* samples after addition of the peroxidase-conjugated secondary antibody.

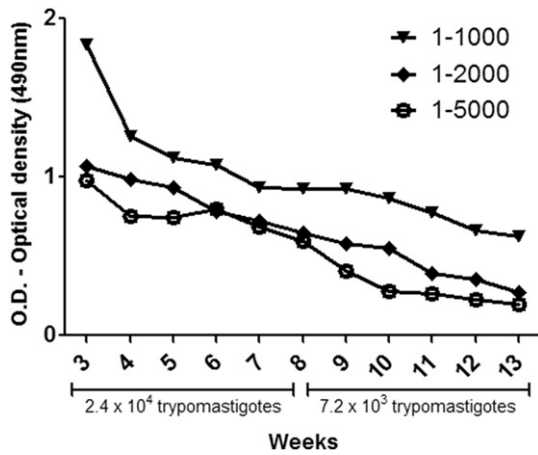


Fig. 3. Optical density values obtained in the ELISA using purified IgY from egg yolk of immunized chickens. The samples tested were IgY extracted at different weeks after immunization (3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13) at different concentrations (1:1000, 1:2000 and 1:5000). The arrow indicates the time of the last immunization.

Currently, there are several methods to isolate and purify functional active chicken IgY from egg yolk, even for large scale purification (Munhoz et al., 2014). These methods can be performed by salt precipitation (Deignan et al., 2000; Chacana et al., 2003), chromatographic techniques (Meulenaer and Huyghebaert, 2001), and ultrafiltration (Kim and Nakai, 1998). We choose to use the salt precipitation method using PEG-6000 as described by Polson et al. (1980) since it is an easy to perform. This technique is widely described in the literature as effective, high-yield antibody, besides having the advantage of possibility of

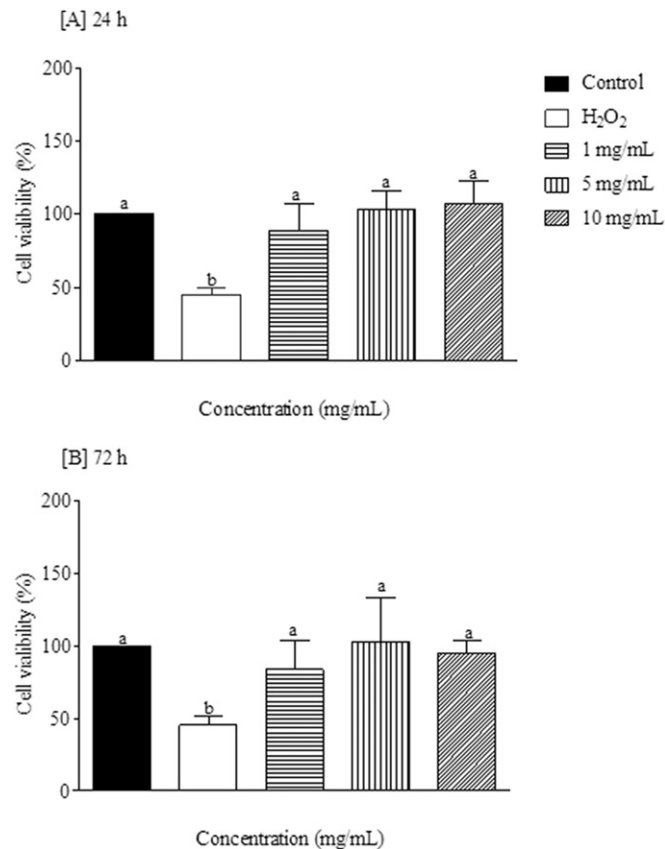


Fig. 4. Percentage of mononuclear cells (PBMC) viability after exposure to different concentrations of IgY anti-*T. cruzi* after 24 [A] and 72 h [B]. Different letters indicate statistical difference considering $p < 0.05$ using a Tukey post-hoc test.

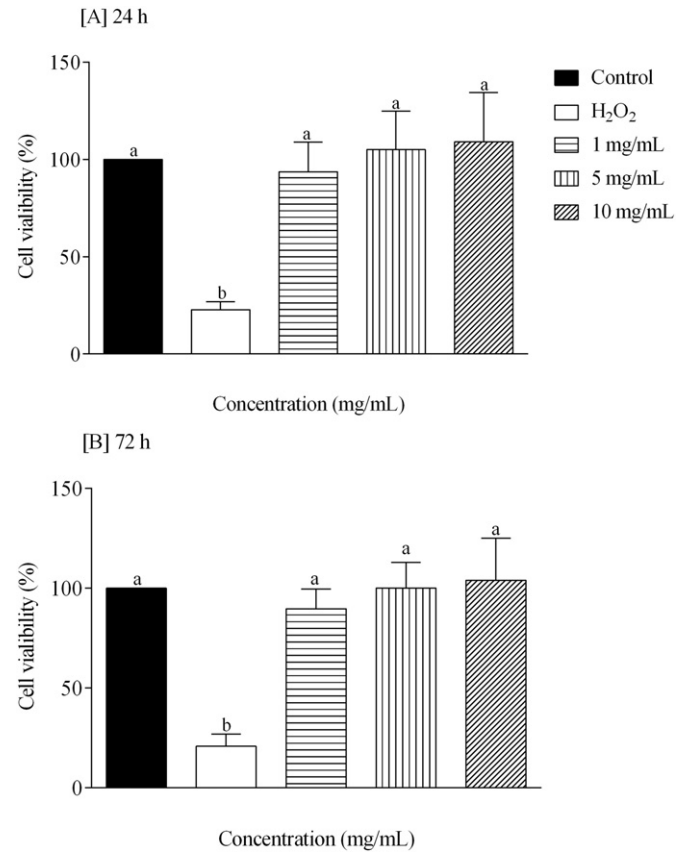


Fig. 5. Percentage of VERO cells viability after exposure to different concentrations of IgY anti-*T. cruzi* after 24 [A] and 72 h [B]. Different letters indicate statistical difference considering $p < 0.05$ using a Tukey post-hoc test.

handling at room temperature without risk of denaturation of the immunoglobulin (Akita and Nakai, 1993).

The specificity and characterization of anti-*T. cruzi* IgY were confirmed by polyacrylamide electrophoresis gel and Western blot. The molecular weight of 75 kDa (50 for the heavy chain and 25–37 for the light chain) found in our study was similar to that described by Contreras et al. (2005), where avian antibodies were produced by intradermal immunization of chickens with *T. cruzi* epimastigotes. However, the literature reports that the light chains of IgY can weight 18 kDa (Sun et al., 2001) to 21 kDa (Hatta et al., 1993), differing from our results. There are other molecular weight characterization of different IgY as the IgY anti-*Staphylococcus aureus* with light chain of 15–25 kDa and heavy chain of 55–70 kDa (Reddy et al., 2014), and IgY anti-*Schistosoma japonicum* with light and heavy chain between 25 and 68 kDa, respectively (Cai et al., 2012), demonstrating that there is a certain variability in the molecular weight of IgY produced.

IgY technology has been used in many medical areas, especially for diagnosis purposes. Specific chicken antibodies have been successfully used against a wide variety of antigens (Munhoz et al., 2014). Various authors reported the efficiency, sensitivity, and specificity of diagnostic tests using IgY from various microorganisms such as *Toxoplasma gondii*, *Schistosoma japonicum*, *Trichinella spiralis* and coronavirus (Ferreira Júnior et al., 2012; Cai et al., 2012; Wang et al., 2012; Palaniyappan et al., 2012). The results of our experiment regarding IgY anti-*T. cruzi* indicate that this antibody could not be used in a diagnostic test, since it showed cross-reactivity with antigens of *T. evansi*. But on the other hand, this finding leaves the possibility that IgY could be used as immunotherapy against many trypanosomes.

The MTT assay is widely used to evaluate the cell viability, as well as the level of mitochondrial metabolic activity of viable cells, with high degree of accuracy (Mosman, 1983). In the present study, the IgY

concentration of 10 mg/mL was able to maintain cell viability in all exposure times tested using PBMC and VERO cells, and it did not cause damage to DNA. Our results are similar to those found by Sampaio et al. (2014a), using IgY anti-*T. evansi* against PBMC cells on concentrations of 1, 5 and 10 mg/mL, other important blood protozoan belonging to the Trypanosomatidae family. Thus, we can conclude that all concentrations of IgY may be considered safe against these cells.

5. Conclusion

The results indicated that *T. cruzi* is able to generate a humoral immune response in chickens, resulting in a synthesis of high specific immunoglobulins against *T. cruzi*, that did not cause damage to the cell membrane, as well as no proliferative effect in mononuclear and VERO cells culture.

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