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Original Article

IN VITRO CYTOTOXICITY AND GENOTOXICITY OF CHICKEN EGG YOLK ANTIBODIES (IGY) AGAINST *TRYPANOSOMA EVANSI* IN HUMAN LYMPHOCYTES

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ABSTRACT

Objective: The present study aims to evaluate the cytotoxic and genotoxic effects of polyclonal antibodies (IgY), produced from the immunization of chickens against protozoan *Trypanosoma evansi (T. evansi)*.

Methods: For the tests a culture of human lymphocytes and IgY samples (1, 2.5, 5 and 10 mg/mL) were used. Cell viability was assessed by employing MTT assay, and the formation of reactive oxygen species (ROS) and lipid peroxidation were measured by testing the 2-thiobarbituric acid (TBARS). Genotoxicity was evaluated by chromosomal instability test.

Results: At the concentrations tested, the study revealed that IgY anti-*T.evansi* showed no significant cytotoxic and genotoxic effects.

Conclusion: These findings demonstrate the safety of these antibodies to mammalian cells.

Keywords: MTT assay; Chromosomal instability; Avian immunoglobulin; Passive immunization.

INTRODUCTION

Trypanosoma evansi (*T. evansi*) is a protozoan that affects most of the domestic animals, as well as some wild species [1, 2].In 2005, it was reported the first case of human infection in a farmer in India [3]. The disease has a worldwide distribution, affecting mainly tropical and subtropical regions [4, 5, 6, 7].

The transmission of tripanosomosis occurs through inoculation of *T. evansi* by the saliva of flies of Tabanidae and Muscidae families, or artificially by fomites, such as needles, contaminated with infected blood [8].In South America, vampire bats (*Desmodus rotundus*) can also transmit it[9].

Following the infection is initiated the multiplication of the parasite (in the bite site) and its migration into the bloodstream and lymphatic system. As the parasitemia increases, the inflammatory response is activated and peaks of fever occur.The infection usually tends to become chronic, with periods without blood parasites and/or fever [8, 10]. At this stage, a hemolytic anemia occurs due to the release of hemolysins and enzymes or directly by the trypanosomes, which are able to induce lesions in the membrane and increase the fragility of these cells [11, 8].By the other hand, acute infections can cause rapid death in untreated horses and dogs[12, 13, 14]. Chronic infections can last for years [15] and at this stage the worsening of clinical signs and cachexia usually occurs [16, 9, 17].Neurological signs have been described in the terminal phase of the disease, especially in horses and cattle [18, 17, 19].

Chemical therapy is still the method of choice in controlling the disease in domestic animals. The major problems observed in the treatment are the high host toxicity of some drugs and the emergence of resistant strains, since most of these compounds have been used in the field for over 40 years[9].Specific avian antibodies immunotherapy against this parasite may be a therapeutic alternative in controlling and/or prevention of these infections.

Immunoglobulin Y (IgY) are polyclonal antibodies obtained from egg yolk (yolk=y) of hens immunized. The production and uses of these immunoglobulins has drawing the attention of the scientific community, mainly because of the diagnostic diversity and therapeutic applications in biomedical research. Furthermore, it is considered an alternative technique to reduce the suffering of animals used in researches [20], since the antibodies are derived from egg yolk. Since 1980 there was an increase in IgY application in laboratorial tests, mainly due to the availability of commercial reagents for purification, availability of standard IgY and specific antibodies anti-IgY labeled with fluorescein, alkaline phosphatase or peroxidase [21].

A considerable number of advantages to the use of avian antibodies, rather than mammalian antibodies are recognized, such as: avian IgY aggregates the functions of the mammalian IgG and IgE; hens antibodies do not have cross reactions with mammalian IgG, red blood cells are not attacked by it, not activating the complement system, as well as the coagulation cascade.IgY technology allows to obtain antibodies with high affinity and avidity. Individually, a hen is able to produce more antibodies than rabbits, goats, horses or rodents [22, 23].

The production and evaluation of the therapeutic activity of avian antibodies in parasitic diseases[22, 24, 25], bacterins [26, 27, 28]and viral[29]are widely reported in the scientific literature. The application of these immunoglobulins consists in an important alternative for the prevention or treatment of several acute, chronic or recurrent diseases. There is now a concern, not only with therapeutic efficacy, but also with pharmacology safety, determined by cytotoxicity and genotoxicity tests. Toxic stimuli threaten the cellular metabolic functions, and thus, the response of cells depends on the toxicity generated, may allowing the cells to adapt to the environment in which they were submitted[30]. The toxicity tests are performed to determine the potential risks that can be generate by new products on health and environment [31]. Thus, the cytotoxicity reflects the effects on cellular structures. Most of the cells should show a similar response, and respond similarly when the toxicity is measured by several criteria of viability [32].

In vitro cytotoxicity assay is the first test for evaluating the biocompatibility of any material for uses in biomedicine. In vitro methods, for assessing the toxicity of biomaterials, were standardized using cell cultures. In these tests the material evaluated is directly or indirectly when placed in contact with mammalian culture cells, in order to verify cellular changes may

produced by different mechanisms [33].Cell viability is a general term, and it may be assessed by assays that determine one or more cellular parameters such as: 3-(4,5)-dimetiltialzolil-2,5-diphenyltetrazolium bromide (MTT) (mitochondrial activity), lactate dehydrogenase enzyme (LDH), Trypan Blue, among others. These assays are generally suitable for measuring acute toxic effects of cultured cells [34]. Peripheral blood mononuclear cells (PBMC) have been applied for decades as biomarkers of genotoxic and cytotoxic effects. Abundant in the bloodstream, these cells are exposed to any mutagenic agent, being able to reflect recent damage. Cultured PBMC have become the promising in vitro model for many studies, highlighting the usefulness of this kind of cell line in studies of cyto-genotoxicity[35]. When a new agent is incorporated into the cellular environment, an important aspect that deserves to be evaluated is whether this agent are able to cause genotoxicity. Damage to genetic material can result in the induction or promotion of carcinogenesis, influencing cell reproduction if the DNA of germ cells was compromised[36].These compounds can react with several structures and cellular organelles, including DNA, proteins and lipids, affecting multiple cellular processes. The DNA, when exposed to certain agents, can mutate, affecting the stability of the genome and leading to the development of genetic diseases and cancer. It may also results in the formation of reactive species, which involve cell dysfunction, mutations and aging[37].Although all cellular components are susceptible to the action of reactive oxygen species (ROS), the membrane structure is generally the most affected by the lipid peroxidation, leading to changes in the structure and permeability. Consequently, there is selectivity loss in ion exchange and release of organelle contents, such as hydrolytic enzymes of lysosomes, and formation of cytotoxic products (e.g., malondealdeído), culminating in cell death [38].Therefore, the present study aims to assess the cytotoxic and genotoxic effects produced by antibodies IgY anti-*T. evansi* in cell-cultured human lymphocytes.

MATERIAL AND METHODS

IgY obtainment and characterization

Three New Hampshire hens, at 25 weeks of age, were immunized with trypomastigotes of *T. evansi*. Extraction of IgY from egg yolk was carried out according to the method previous described [39, 40].After extraction, the immunoglobulins were analyzed by electrophoresis on 10% polyacrylamide gel (SDS-PAGE), under reducing conditions [41].The antigen antibody reaction was detected by Western blot and ELISA avidity. The concentration of specific IgY anti-*T.evansi* extracted was assessed by *Coomassieblue* method [42] using bovine serum albumin as standard.

Obtention of samples for cytotoxicity and genotoxicity tests

Peripheral blood samples were drawn (Vacutainer® - BD Diagnostics, Plymouth, UK), from a healthy female volunteer, 21 years old, non-smoker, without drinking or under chronic medication and under 12-h overnight fasting. Blood samples were stored in heparin tubes and 5 mL were used to obtain cultured lymphocytes in culture media composed by 1 mL of RPMI 1640 (with 10% of fetal calf serum - FCS), 1% of penicillin/streptomycin and phytohemagglutinin (PHA). The cells were maintained in suspension culture at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 growth medium during 72 hours. Further, the cells were exposed to 72 hours treatment and the cell viability, mitotic index and chromosomal instability were assessed. All the treatments were carried out in triplicate.

Treatments: In order to test the damage effects of IgY on cell viability, as well as its protective effects on chromosomes damage in human lymphocytes, a similar protocol as described by Wilms et al. (2005)[43] was performed. The concentrations of 1, 2.5, 5 and 10 µg/mL were tested. The medium culture was used as a negative control, while the medium added of 25 µM of hydrogen peroxide was used a positive control group.

Evaluation of IgY cytotoxicity: MTT assay was based on the metabolism of MTT reagent (yellow color) in formazan crystals (violet color). The reaction occurs through the mitochondrial enzyme succinate dehydrogenase, which remains active only in viable cells. Cells were treated at the given concentrations (1, 2.5, 5 and10 mg/mL) and, after 72 hours of incubation (at 37°C, CO_2/5%), 10µL of MTT was added to each well (5 mg/mL), diluted in phosphate buffered saline (PBS-1X).Then, the plate was homogenized (150 rpm/5 minutes) and still maintained at the same conditions (37°C and CO_2/5%) for 4 hours. Later, the supernatant was removed and 150µL of dimethylsulfoxide (DMSO) was added. Finally, the plate was shaken at 150 rpm/5 minutes and the reading was performed in an ELISA reader (wavelength of 570 nm). The results were expressed as "%" of cell viability compared with the controls.

Evaluation of IgY genotoxicity

After exposure treatments, one replicate of each treatment was used to investigate the mitotic index, as well as the chromosomal instability, using the G-band cytogenetic analysis. At least 50 mitoses were analyzed in each sample.

Lipid peroxidation of IgY

Lipid peroxidation was assessed by TBARS (thiobarbituric acid reactive species) method [44]. After treatment, the cells were centrifuged for 10 minutes at 2000 rpm, in order to remove the culture medium.The supernatant was discarded and 2 additional centrifugations (10 minutes at 2000 rpm) were made using saline (0.9% NaCl).After these steps, the supernatant was discarded and 100 µL of Butylated hydroxytoluene (BHT/10 mM), plus 500 µL of trichloroacetic acid (TCA 20%) were added for a final centrifugation (5 minutes at 2000 rpm).After centrifugation, 900 µL of the supernatant was mixed with a reaction medium containing thiobarbituric acid (TBA at 0.8%), being incubated at 95°C during 1 hour. Absorbance was measured at 532 nm wavelength with results expressed in nmol MDA/10^6 cells.

Data analysis

Assay results were submitted to analysis of variance (ANOVA) and Dunnett test in order to verify the accuracy of the data. Values with $p< 0.05$ were considered statistically different.(*) $P<0.05$ when compared with the control (#) $P<0.05$ when compared with phytohemagglutinin.

RESULTS AND DISCUSSION

Our immunization protocol stimulated the production of immunoglobulins anti-*T.evansi*, since this immunoglobulin was detected and extracted from egg yolks from the fourth week post immunization. A well-defined peptide band, stained by SDS-PAGE, was observed by Western blot, revealing in the light (25-20kDa) and heavy (75-50 kDa) chains of the extracted IgY. The binding capacity of the antibody produced against the antigen was detected through the high avidity levels obtained by ELISA avidity. The average production of IgY anti-*T. evansi* was 2.64 ± 0.15 (4th week) and 2.87± 0.14(10th week) [t (3) = -4.31, p <0.05].

The IgY antibodies, when subjected to in vitro culture medium containing human lymphocytes, did not produce damage in the cellular structures. The percentage of cellular viability did not decrease in any of the tested concentrations. The cytotoxicity test showed that the IgY produced did not cause damage when in direct contact with the blood cell, especially evidenced by preservation of mitochondrial physiology. It was also observed a slight increase for cell viability as the concentration of the samples increased, although it was not statistically significant. However, when the concentration of 10 mg/mL was reached, the increase in the percentage of viability was statistically significant compared to the control group, H_2O_2 group, as well as with the lowest concentration groups (Figure 1); it did not differ from group treated with phytohemagglutinin (PHA), a substance that stimulates mitosis of lymphocytes.

These findings suggest that the concentration increase of IgY can enhance the proliferation of lymphocytes. Lymphocytosis is a late cellular response and is related to the production of specific and lasting antibodies. Antibodies are a major opsonins produced by the host defense system and they are essential for the stimulation of antigen phagocytosis [38].On *T. evansi* infection, it is desirable that

the host has an immediate cellular immune response, in order to neutralize or minimize the action of the parasite. Our finding suggests that IgY binds on *T. evansi* and, additionally, it also stimulates the proliferation of lymphocytes, inducing an immune response against this specific and long-lasting parasite. However, only in vivo tests, preferably parenterally, may evaluate the effectiveness, or not, of these cells on the protozoan infection.

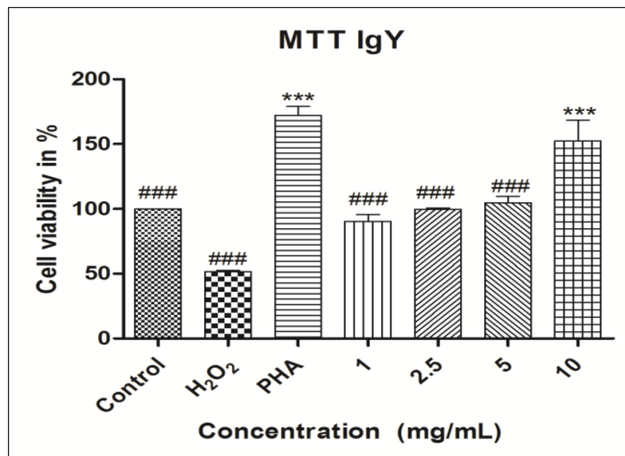

Fig. 1: IgY MTT after 72 hours of incubation.

Our results did not detect structural changes in metaphase of the chromosomes (chromatid breaks, centromeric, acentric and dicentric fragments, as well as the formation of tri and tetra radialfigures), which could indicate the occurrence of chromosomal aberrations (Figure 2).

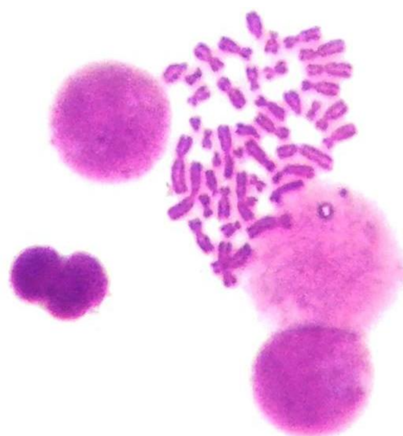

Fig. 2: Lymphocyte in metaphase by the karyotype technique, without chromosomal alteration.

The analysis of chromosomal instability test (Table 1) demonstrated, at all concentrations tested, 100% of metaphase integrity, as evidenced in the control group. Similarly, the interphase nuclei were normal and without any metanuclear changes. The dependent dose effect on the mitotic index was well evidenced (data shown in Table 1), where concomitant, as the concentration of IgY increased, also the number of metaphases enhanced. The concentration of 10 mg/mL showed a significant difference when compared with the control (p<0,05). The proliferative activity observed in the MTT assay, at the concentration of 10 mg/mL, was also observed by the mitotic index in the chromosomal instability test. Our findings suggest that the therapeutic use of avian antibodies do not promote change in cell reproduction or change the DNA structure in human lymphocytes.

Table 1: Karyotypic changes (%) in mononuclear cells of human peripheral blood exposed to different concentrations of IgY.

Concentration	Metaphases	Intact metaphases (%)	Morphology of the nucleus	Markers of Chromosomal Instability
Control	700 ± 5	100	Normal	-
H_2O_2	350 ± 4 ***	5	Karyopyknosis	Karyopyknosis
1 mg/mL	730 ± 3	100	Normal	-
2.5 mg/mL	760 ± 6	100	Normal	-
5 mg/mL	793 ± 2	100	Normal	-
10 mg/mL	810 ± 5***	100	Normal	-

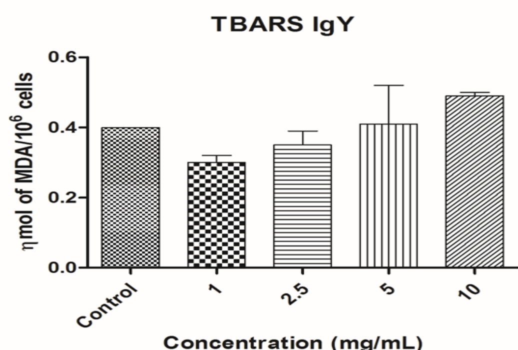

Fig. 3: TBARS assay after 72 h of incubation. Results are expressed as nmol of MDA/10^6 cells.

According to TBARS test, the MDA values found in different concentrations of IgY, did not differ statistically from the control group (p> 0,05). The dose-dependent effect, on the formation of MDA, is evidenced in figure 3, where it was observed MDA growth following the increasing concentration of IgY. However, the amount of ROS produced did not result in the peroxidation of the cell membrane in any tested concentration(p> 0.05).

Lipid peroxidation is the process in which the ROS act on phospholipids of cell membranes, causing their disintegration. Lipid peroxides formation results in the disruption of the cell membranes, DNA mutations, oxidation of unsaturated lipids and formation of chemical waste [38].No report has been found in the literature about the occurrence of lipid peroxidation in the presence of avian antibodies.

CONCLUSION

IgY antibodies anti-*T. evansi*, at concentrations of 1, 2.5, 5 and 10 mg / mL did not cause damage to the cell membrane of human lymphocytes, did not affect the cell viability, as well as did not produce DNA damage at the chromosomal level; thus, not presenting cytotoxicity and genotoxicity. At 10 mg/mL of concentration, IgY had a proliferative effect, consisting in the normal cell proliferation, as evidenced by the chromosomal instability test, a result that can be seen in cellular viability test and mitotic index.

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