

EFFECTS OF COBALT 60 IONIZING RADIATION ON THE METABOLISM AND INFECTIVITY OF A PARASITIC PROTOZOA, *Toxoplasma gondii*

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ABSTRACT

Toxoplasma gondii infection causes severe and lethal disease on fetus, AIDS patients and recipients of organ transplants. There are few reports of the use of ionizing radiation to attenuate or abolish parasite growth, without any detailed study on induced alterations or effective doses. We reported that the lower dose of ⁶⁰Co radiation that abolishes parasite growth was 200 Gy, and in this report, we described the viability, cell invasion, metabolism and immunogenicity of this parasite after irradiation. We analyze the viability (vital staining), metabolism, by MTT oxidative conversion, protein metabolism, by ³H-proline and nucleic acid synthesis, by ³H-hypoxanthine incorporation in short terms cultures. Parasite invasion was tested in LLC-MK2 cell in culture. The irradiated parasites show the same viability and invasiveness of viable parasites, without any interference of the radiation in the oxidative, proteic or nucleic acid metabolism. When mice injected with 10⁷ irradiated parasites by intraperitoneal route, were challenged 6 weeks later with viable parasites, they showed partial resistance to infection, with higher survival time. Antibody specificity was tested in sera from those animals, before challenging, and presented the same epitopic specificity of infected and treated animals, but diverse from mice injected with formaldehyde killed parasite, by Western-blot analysis against SDS-PAGE isolated *T.gondii* antigens. Those data suggests that irradiated parasites maintain its invasiveness, inducing a partial immunity and similar humoral immunogenicity than viable parasites, but without any evidence of reproductive capacity.

Keywords: Radiation, *Toxoplasma gondii*, invasion, and metabolism

I. INTRODUCTION

Toxoplasmosis, a high prevalent human infection, is caused by an intracellular obligatory parasite, *Toxoplasma gondii*. This infection is transmitted by the ingestion of raw or undercooked meat containing viable cysts, aside contamination of water or food with oocysts from cat feces [1]. Human chronic unapparent infection is common, with more than 60% infected people in Brazil [2]. Despite some acute severe or ocular cases, the immune response is efficient for acute infection control but without elimination of tissue cysts, viable for years, also resistant to chemotherapy [3]. Unfortunately, the disease is severe, deadly and destructive when this immune response is compromised by immaturity, disease or medical therapy, as in fetus of acutely infected mothers, AIDS patients or recipients of organ transplants [4]. Despite several attempts,

until now there no vaccine developed for this human infection, with some vaccines developed for veterinary use, but with low efficiency [5]. Several models were developed in mice, using different antigens and routes, all of then with conflicting results [6]. Ionizing radiation was used against *T.gondii* mostly in order to sterilize meat [7], but with few attempts of vaccine production, but with low efficiency [8]. These assays were conducted without any study of the radiation consequences in the parasite, with few ifs any quality control of the procedure. Recently, we reported that tachyzoites, the invasive form, had its reproductive ability abolished by 200Gy of ⁶⁰Co radiation, both *in vivo* and *in vitro* [9]. The radiation could destroy this ability by inducing double breaks in cell DNA, with unbalanced chromosomal distribution in daughter cells [10], or by other mechanism, as membrane peroxidation and cell lysis [11], aside other less established phenomena, as apoptosis [12].

Here, we present data on the metabolism, cell invasion, infectivity and immunogenicity of tachyzoites of *T.gondii* submitted to 200Gy ⁶⁰Co radiation.

II. MATERIAL AND METHODS

Materials and reagents: All reagents used in the experiments were purchased from commercial sources, with pro-analysis quality. The solutions were prepared with high quality water (MilliQ). The RH strain of *T.gondii* was maintained as frozen establates or by successive passages intraperitoneal (ip) in mice, in the Lab.Protozoology. Isogenic mice, C57Bl/6j or Balb/C, or out-bred Swiss mice, were obtained from the colony of FMUSP and maintained in sterilized cages and absorbent media, with food and water *ad libitum*. The animals were treated as recommended by the Animal Experimentation Brazilian College (COBEA).

Parasites purification: Parasite suspension of infected animals were recovered by phosphate buffered saline (PBS) washings of peritoneal cavity, using sterility here and thereafter. After collection, the suspension of cells and parasites were centrifuged at 2000 g, 10 min and the pellets were suspended in equal volume of PBS, passed on a needle 0.1mm for rupturing infected cells, and immediately applied on PBS stabilized SEPHADEX[®] G 50-80 column. After PBS washing, the free parasites were collected and centrifuged as above. After counting and viability control using Trypan Blue, the parasites were suspended at 10⁸ cells/ml in PBS. Preparations with more than 1% mammalian cell contamination or viability less than 95% were discarded.

Irradiation: Tachyzoites suspension, maintained in ice cold baths, were irradiated at 20, 50, 100 and 200 Gy, in a uniform source of ⁶⁰Co gamma rays in a GammaCell (Atomic Energy of Canada, Ltd.) at a dose rate of 370 Gy/h, in the presence of oxygen and room temperature. Adequate controls were maintained outside the source for use as controls.

Cell invasion assays: After irradiation the viability was determined in all samples by the Trypan Blue staining. Briefly, the parasite suspension was diluted in one volume of Trypan Blue 0,4% in Hanks' Balanced Salt Solution pH 7,2, and the total cell and stained (dead) cell count was performed by phase contrast microscopy. LLC-MK2 cells maintained in plastic ware with RPMI 1640 medium containing 10 % fetal bovine serum and gentamycin (1µg/ml). Trypsin separated cell suspensions were grown in sterile slide chambers (Lab-Tek[®]) in the same medium until semi confluence, when 10⁵ parasites irradiated with 200 Gy or viable was added. After 4h, the monolayer were submitted to three careful washings with PBS and immediately fixed with methanol and stained with Giemsa. Representative fields were photographed in an Axiophot planapochromatic microscope, and reprints scanned for reproduction.

Metabolic assays. Oxidative assay: Purified parasite suspension, 10⁷/ml, were submitted to short term culture, with RPMI 1640 medium without phenol red and supplemented with fetal bovine serum and gentamycin as described. After centrifugation to 3000 g/10min, the cells were suspended in the same medium and volume, and distributed on 24 multwell sterile plates, with MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Tyazolyl blue) (1mg/ml), and 10% samples were collected at 1, 2, 4 and 18 hr of 37°C incubation in CO₂ 5%/humid atmosphere. The samples were fixed with 1 volume of 4% formaldehyde/sodium phosphate 0,02M pH 7.2 solution and centrifuged by 800g/10min. After discarding the supernatant, the mitochondrial oxidative blue produced by cell layer was extracted with methanol (100 µl) and the absorbance at 570 nm determined in a microplate reader. **Protein and nucleic acid synthesis assays:** Short term culture of 10⁷/ml purified parasites suspensions irradiated or not were used, with Dulbecco's Modified Minimum Essential Medium, containing fetal bovine serum and gentamycin as described, in 24 multwell plates. Protein synthesis was determined by adding ³H-proline(2µCi/well) with incubation in the same above described conditions. After 1, 2, 4 e 18 hr, 100 µl aliquots were added to 2 sq cm pieces of filter paper, dried and dispensed in icecold 5% TCA for 1 hour. After two washes in ice cold 5% TCA and 1 time in ethanol, the papers were dried and dispensed on 10 ml Liquifluor solution. The TCA precipitable counts were determined in a Wallach Cintillation counter 1209, at 66% efficiency, and expressed in cpm (counts per minute). For total acid nucleic synthesis, both RNA and DNA, were used a similar model, except that the radioactive precursor added was ³H-Hypoxanthine, 1 µCi/well, collected at the same time, schedule and procedures.

Immunization schedules: Suspensions of purified parasites irradiated with 200 Gy, 10⁸ parasites/ml, were injected i.p on groups of C57Bl/6j mice. No deaths occurred. Viable parasites were injected at similar schedules, except that the mice received pyrimethamine (5 mg/Kg)/sulphadiazine (100 mg/Kg) as treatment *per os*, during 1 week, with 10% deaths due to treatment failure. Suspensions of viable parasites, at same concentration, were also fixed with a 4% formaldehyde solution for 24 hr and injected similarly. All parasite counts were adequately controlled in order to secure that the equal numbers of parasites were used. Weekly, blood (5µl) was collected from tail in standardized filter papers, dried and stored at -20°C until use. Before use, the soluble extract containing antibodies was obtained by adding 100 µl PBS to the paper, for 18hs at 4°C, in a microcentrifuge tube. After extraction, the tube was centrifuged and the supernatant used as a 1/20 dilution of blood. **Western blotting:** Purified parasites (10⁸/ml) were sonicated in PBS and immediately centrifuged for 10000g/3 min. The supernatant was collected, resulting in a 400 µg/ml protein, that was submitted to SDS-PAGE in a 12,5% running gel, as elsewhere described [13], with subsequent electrophoretic transfer to nitrocellulose membranes. The membranes were blocked with a 2,5%

bovine sera albumin in PBS containing 0.02% Tween 20(PBST), cuted in 2 mm strips and stored dried until use. Sera from animals were reacted with strips with *T.gondii* antigens re-hydrated with PBST, for 18 h at 4°C. After careful washing with PBST, the antibodies were detected by sequential incubation with peroxidase-conjugated anti-mouse IgG (Sigma), 60 min, washings, and addition of PBS containing 0,6mg/ml 4-cloro-1-naphtol and 0.03% hydrogen peroxide. After development, the strips were dried and scanned for documentation.

Protection assays of viable parasites challenge: Mice previously immunized 1 or two months before with one or two ip injections of 10^7 tachyzoites irradiated with 200 Gy, were challenged with ip injection of 10^3 viable parasites. The survival time of each animal was followed daily. Adequate controls non-immunized were added in each experiment. The mean survival time was compared with controls by the Mann Whitney test [14], using a significance level of 0.05.

III. RESULTS

The viability of the parasites was not affected by irradiation, maintaining more than 95% of viable unstained parasites, as detected by Trypan Blue dye test, in none of our several preparations (quantitative data not shown)

For cell invasion ability, we tested irradiated parasites on LLC-MK2 cells. In Fig 1A, representative field of this assay showed that non irradiated parasites presented the three stages of the invasion, adhesion, and orientation and penetration [15], clearly identified in the preparation.

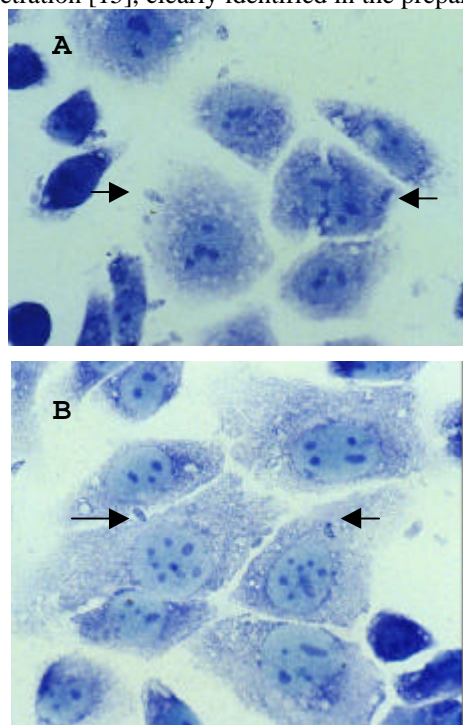


Figure 1. Scanned micrograph of LLC-MK2 cells challenged with viable or irradiated tachyzoites, stained by Giemsa. A – Viable *T.gondii* tachyzoite challenge (→). B – 200 Gy irradiated tachyzoites challenge (→).

When 200 Gy irradiated parasites were tested, the same process, with nearly equal efficiency, could be demonstrated (Fig. 1B)

Metabolic assays: The oxidative metabolism of irradiated parasites with 20, 100 and 200Gy could be seen in Fig. 2, showing that the energetic consumption of the parasites was not affected by radiation, at any doses, and also there are some dose dependent increment at longer times, demonstrated by the increase of oxidated formazan accumulation in the cells.

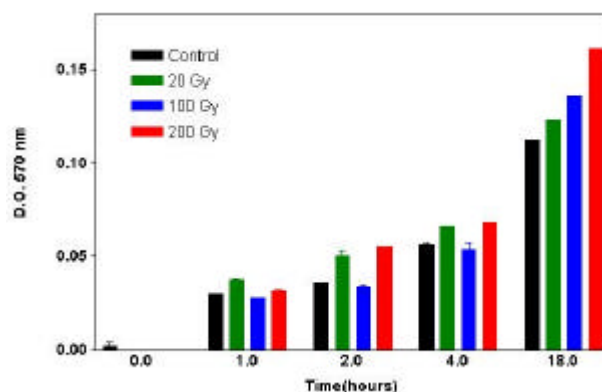


Figure 2. Oxidated formazan of MTT (respiratory burst) by tachyzoites of *T.gondii*, non-irradiated or irradiated at 20, 100 or 200Gy.

Protein synthesis was evaluated by TCA precipitable ^3H proline incorporation, using short-term cultures of irradiated tachyzoites. In the Fig. 3, is clearly seen the increment of labeled TCA product, protein, by the cells in viable parasites. The irradiated parasites (20,100 and 200 Gy) presented similar levels, showing that protein synthesis was not affected by radiation.

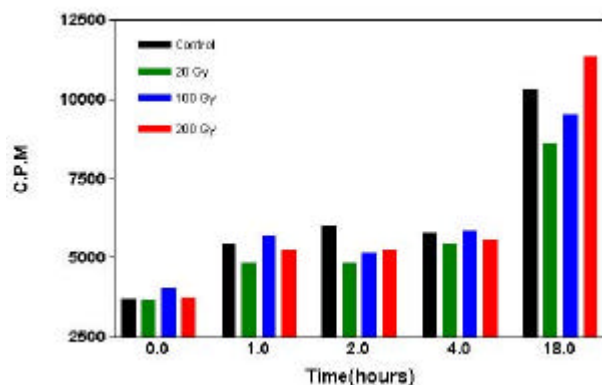


Figure 3. Incorporation of ^3H proline on TCA precipitable protein by viable or 20, 100 and 200Gy irradiated tachyzoites of *T.gondii*.

In order to observe the nucleic acid synthesis in four preparations we tried to use ^3H thymidine as a precursor of DNA synthesis, but in two experiments, no incorporation was found in viable parasites. This fact could be explained by the non-dividing stage used. For this reason, we studied the ^3H hypoxanthine incorporation, a precursor that is involved both in DNA or RNA synthesis in those parasites. The experiment was performed as described, and the TCA precipitable counts in viable and irradiated parasites could be seen in Fig. 4. As shown, there is a clear time dependent increase in nucleic acid

incorporation in viable parasites, which is similar to observe with 20, 100 and 200Gy irradiated parasites.

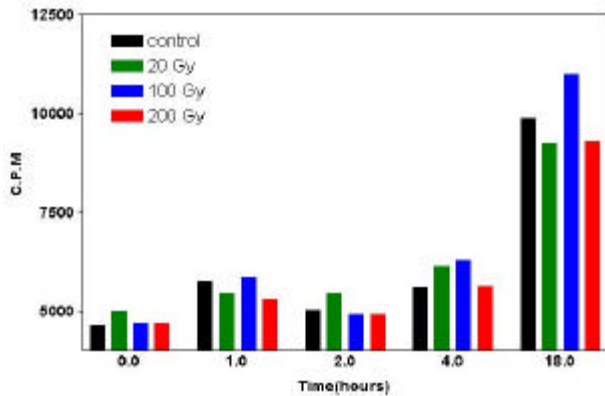


Figure 4. Incorporation of ^3H hypoxanthine in TCA precipitable nucleic acids in short term culture of purified tachyzoites of *T.gondii*, viable or irradiated with 20, 100 or 200 Gy.

Specificity of antibodies induced by irradiated parasites: We tested the antibody specificity induced by irradiated parasites, in Western Blot analysis. A scanned reprography is show in Fig.5. As clearly seen, the antibodies induced by irradiated parasites are clearly similar to the natural infection with viable parasites, unlike formaldehyde treated parasites.

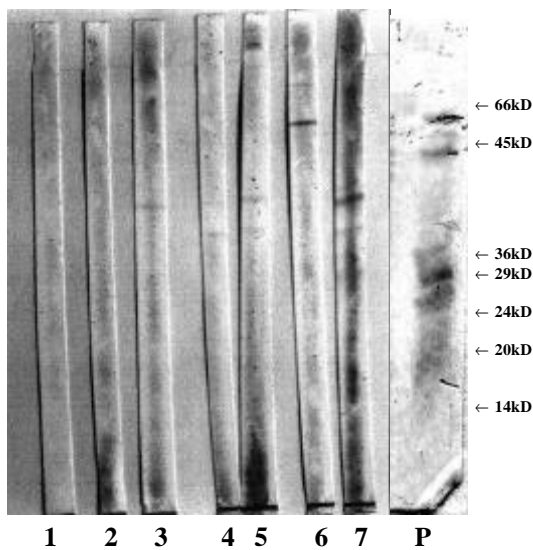


Figure 5. Antibody specificity of sera from mice challenged with *T.gondii* antigens: 1 - No serum; 2 - Normal mice serum; 3 - Mice serum inoculated with irradiated parasites at 200Gy, one dose; 4 -, two doses; 5 - Mice serum inoculated with irradiated parasites at 200Gy, three doses; 6 - Mice serum inoculated with formaldehyde treated parasites, three doses; 7 - Treated mice serum with pyrimethamine/sulphadiazine; P - Molecular weight markers.

Protection from challenge with viable parasites: The survival of mice previously immunized with 200 Gy irradiated parasites were observed, comparing with control mice. The challenge was low but causes the death of all animals, but immunized mice presented a clear longer survival, as shown in Fig.6, with several immunization schedules, which did not presented clear differences. This fact allows us to consolidate all data from immunized

animals, as shown in Fig.7, with a significant increase in survival ($p=0.0265$, Mann-Whitney test).

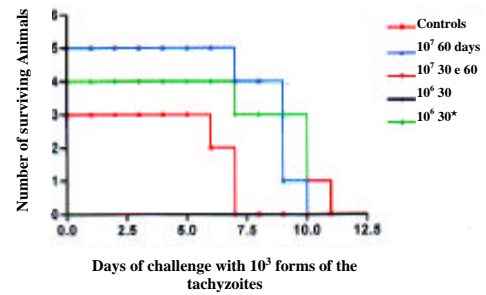


Figure 6. Daily survival of mice with several schedules of immunization with 200 Gy irradiated parasites.

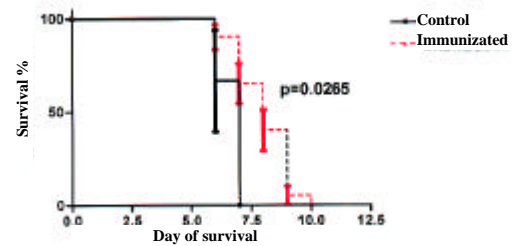


Figure 7. Consolidated survival time of immunized mice compared to non immunized controls.

IV. DISCUSSION

Our data clearly showed that the ionizing radiation modifies only the reproductive ability of tachyzoites of *T.gondii*, without affecting early viability, cell invasion, oxidative respiration and metabolism, for protein or nucleic acid synthesis.

Despite some immunological approaches, most studies dealing with the action of ionizing radiation on *T.gondii* relegate those functional aspects to a secondary level [6,7,8]. Our studies on antibody specificity show that this approach could induce a humoral response similar as comparable to that of viable agents, which is not induced by chemically treated parasites.

The technique that was used in this experiment has been used by others authors, for similar parasites [16,17], but has been not used for *Toxoplasma* tachyzoites until now.

The use of labeled proline provided a quantitative approach to the protein synthesis of this agent and at least two proteins rich in these residues were found [12,13]. As this incorporation was not affected by radiation, is quite acceptable that this metabolic path is maintained after irradiation, with consequent implications. The nucleic acid synthesis was present in the tachyzoite, judged by labeled hypoxanthine incorporation, which could be explained by mRNA or ribosomal RNA synthesis [10], due to Go stage of the parasite, that starts its cell division only after entering the host cell [3]. As our assays show that this incorporation was not affected by radiation, it is probable that these syntheses would be maintained. Double breaks in DNA strands are widely located in the cell genome, and,

probably, could exist away from the exact sequences of genes codifying RNA, usually a small part of the genome [10]. Thus, our data are compatible with this action of radiation that impedes only the correct balance of the genome between daughter cells in cell division, inducing a mitotic death of the agent. The repair of single strand breaks of DNA in *T.gondii* was reported as highly efficient, specially those induced by OH^{*}[18], which could also provided a quick repair of mRNA areas in the genome.

As antigens, irradiated tachyzoites provided the same antibody specificity of viable parasites, suggesting that its antigen processing paths are very similar, unlike chemically treated antigens. These data are conflicting with some reports that found the same antigenicity of extracts or whole parasites [6], but in our system no adjuvant was used nor higher amounts of antigen.

In our protection assays a low efficiency protection was achieved, similar to those found by others [8]. This fact could be induced by the way, which the challenge was provided, quite different from the usual contagion. New schedule of testing, using *per Os* contamination could demonstrate more clearly a usefulness of irradiated parasites in these studies. Another fact was the extreme virulence of RH strain, similar to rarely naturally found parasites, unlike cyst inducing less aggressive more frequent strain [3].

Concluding, our data show that ionizing radiation could be an excellent tool for abolish the reproductive ability of *T.gondii* tachyzoites, without affecting most of its functions, allowing the immune system to recognize the same of the viable agent without risk of progressive infection or residual cysts, with remarkable importance in vaccine production.

ACKNOWLEDGMENTS

We are grateful to Roselaine A. P. Cardoso for him help in laboratory preparations and Eng. Carlos Gaia da Silveira and Eng. Elizabeth Sebastiana Ribeiro Somessari from IPEN to technical assistance during irradiation. FAPESP no 1996/5875-8 and LIMHCFMUSP49 supported this work. R.M. Hiramoto was a fellow from CNPq.

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