Biomedicine & Pharmacotherapy 106 (2018) 599–604

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Gamma irradiation of *Toxoplasma gondii* protein extract improve immune response and protection in mice models

Andrea da Costa[a], Nahiara Esteves Zorgi[b], Nanci do Nascimento[c,1], Andres Jimenez Galisteo Júnior[d], Heitor Franco de Andrade Júnior[e,*]

[a] Laboratório de Protozoologia – Instituto de Medicina Tropical da Universidade de São Paulo, São Paulo, Brazil
[b] Departamento de Parasitologia, Instituto de Ciências Biomédica, Universidade de São Paulo, São Paulo, Brazil
[c] Centro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares, IPEN- Universidade de São Paulo, São Paulo, Brazil
[d] Hospital das Clínicas HCFMUSP, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil
[e] Departamento de Patologia, Laboratório de Protozoologia – Instituto de Medicina Tropical da Universidade de São Paulo, São Paulo, Brazil

ARTICLE INFO

Keywords:
Gamma radiation
Irradiated proteins
Vaccine
Toxoplasma gondii

ABSTRACT

Gamma radiation induces protein changes that enhance immunogenicity for venoms, used in antivenin production. *Coccidian* parasites exposed to gamma radiation elicit immune response with protection in mice and man, but without studies on the effect of gamma radiation in soluble acellular extracts or isolated proteins. Toxoplasmosis is a highly prevalent coccidian disease with only one vaccine for veterinary use but with remaining tissue cysts. Total parasite extracts or recombinant proteins used as immunogen induce usually low protection. Here, we study gamma radiation effect on *T. gondii* extracts proteins (STAG) and its induced immunity in experimental mice models. By SDS-PAGE, protein degradation is seen at high radiation doses, but at ideal dose (1500 Gy), there are preservation of the antigenicity and immunogenicity, detected by specific antibody recognition or production after mice immunization. Immunization with STAG irradiated at 1500 Gy induced significant protection in mice immunized and challenged with distinct *T. gondii* strains. In their blood, higher levels of specific $CD19^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ activated cells were found when compared to mice immunized with STAG. Irradiated *T. gondii* tachyzoites extracts induce immune response and protection in mice in addition, could be a feasible alternative for Toxoplasma vaccine.

1. Introduction

Biological effects of gamma radiation are usually related to lethal genotoxic aspects, as in nuclear accidents or sterilization [1]. Nucleic acids effects are prominent and occur in lower doses than in other biological compounds as proteins. Vaccines involving gamma radiation of intact organisms were designed for malaria caused by Plasmodium, a coccidian parasite. X-rays irradiated mosquitos previously infected with *P.berghei* induced significant protection in mice [2] and irradiated sporozoites, the infective form of the agent, has been proposed for human use [3]. *Toxoplasma* tachyzoites gamma irradiated produce a protection in mouse models [4,5].

Subcomponent proteins and not intact organisms compose vaccines for several diseases but they need adjuvants for promoting adequate immunity and protection. Ionizing gamma radiation on isolated or mixed proteins solutions were used to improve the production of antisera against snake venom [6] and isolated recombinant proteins immunity [7]. These effects were attributed to radicals produced in the radiolysis of water by gamma radiation and not a direct effect on the structure of the protein [8]. Ionizing radiation promote the formation of poorly soluble protein aggregates [6], which can be compared to some Alum-type adjuvants that act as slow delivery systems [9]. Effective subcomponent protein vaccines require the combination of strategies such as delivery systems and adjuvants for protective, adequate and efficient immune response. The adaptive immune response to protein antigens are initiated by their uptake by antigen-presenting cells which are unable to bind most soluble proteins, which explains why isolated proteins induce a weak immune response [10].

Adjuvants are compounds that promote low solubility of the antigen and inflammation at the injection site, with influx of neutrophils and APCs, thus allowing the contact of proteins with immune cells [11]. To activate the immunogenicity of the proteins present in a vaccine, adjuvants mostly induce an acute inflammation with influx of activated neutrophils [12]. Neutrophils are potent sources of oxidants such as

* Corresponding author.
E-mail address: hfandrad@usp.br (H.F. de Andrade).
[1] In memoriam.

https://doi.org/10.1016/j.biopha.2018.06.155
Received 26 May 2018; Received in revised form 27 June 2018; Accepted 27 June 2018

hypochlorous acid (HOCl) and induce the oxidation of proteins present at the site of inflammation [13].

Toxoplasmosis is a widely distributed infection caused by the intracellular parasite *Toxoplasma gondii* [14]. One-third of the world's human population has specific antibodies, which makes this parasitic infection successful, usually asymptomatic but with infrequent eye disease [15]. Acute maternal infection can cause damage or even death of the fetus and patients with defective immune response, as HIV or transplanted patients, reactivation of a latent infection may induce severe encephalitis [16]. To date, the only available vaccine for the control of toxoplasmosis is based on the attenuated live strain S48, restricted to ovine vaccination [17].

However, as a short-lived live vaccine, it causes protection but also remaining cysts in lamb of vaccinated animals, unfit for human consumption [18]. Irradiated *T. gondii* tachyzoites induces a good protection in mice with efficient humoral and cellular immune response [4,19], but there are no studies on irradiation of protein extracts of those agents for inducing protective immunity. Protein extracts are much easier to handle than irradiated intact parasites that require more sophisticated forms of preservation and short half-life [4]. Untreated tachyzoites extracts demands adjuvants for inducing partial protection as reported [20] and isolated or associated recombinant proteins induced conflicting results, also demanding adjuvants for any protection [21]. In order to study if gamma irradiation of soluble protein extracts could have the same immunity inducing effect seen in snake venoms and recombinant proteins, we study the protection and immune response of mice immunized without adjuvants with gamma irradiated *T. gondii* tachyzoites soluble protein extracts.

2. Material and methods

2.1. Parasites and SPF isogenic mice

Parasites were *T. gondii* RH and ME-49 strains maintained as elsewhere described [5] and experimental SPF mice were bought from our colony of the Faculty of Medicine of the University of São Paulo and kept in adequate conditions according to Experimental Animal Use Committee of Tropical Medicine of the University of São Paulo (CPE-IMT 2012/115).

2.2. Soluble antigen of Toxoplasma gondii (STAG)

The soluble extract was prepared as elsewhere described [4]. Briefly, tachyzoites were filtered through in 5 µm polycarbonate filter (Millipore, Massachusetts, EUA), recovered by centrifugation, adjusted to 10^8 tachyzoites/mL in water and sonicated for complete cell lysis. After addition of one volume of 0.3 M NaCl to the lysate, the suspension was cleared by centrifugation at 10.000 *g* for 15 min at 4 °C, and supernatant defined as soluble tachyzoites antigen (STAG). The protein concentration was determined by the Bradford method and adjusted to 0.1 mg/ml and stored frozen (−70 °C) until the moment of use. STAG were irradiated at 250, 1000, 1500, 2000 and 4000 Gy from Cobalt-60, using a GammaCell™ (Atomic Energy, Canada Ltd.) at a dose rate of 1.03 Gy/h, in the presence of oxygen. For molecular weight profiles and immunogenicity, STAG proteins were separated in 12.5% SDS-PAGE and stained or transferred to nitrocellulose membranes as elsewhere reported [22,23]. Transferred proteins were reacted with sera from ME-49 strain infected animals for 18 h, 4 °C and bound antibody was detected by commercial peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, Missouri, USA) for 60 min, with detection by adding 3,3',5,5'-Tetramethylbenzidine (TMB) for membrane (Sigma-Aldrich, St. Louis).

2.3. Immunization and challenge with viable parasites

Groups of 05 BALB/c mice were immunized with 3 biweekly s.c doses of 20 µg of each STAG type in PBS. No mice were irradiated only the antigen were irradiated but non-radioactive. Blood and Serum was collected before the immunization and 15 days after the last immunization for immunological studies. The blood samples were obtained by sectioning of the tail end of the immunized and control BALB/c mice, collected on filter paper dried and, stored at −20 °C. Elution was performed by the addition of 300 µL of buffer containing 10 mM Tris/HCl pH 7.5, 150 mM NH4Cl and 10 mM NaN3 for erythrocytes lysis and conversion of hemoglobin to stabilize cyanohemoglobin [24]. After elution, sample absorbance 540 nm were determined and blood content adjusted for 1:100 dilution. For challenge, we used both models of acute or cystogenic oral infection as elsewhere reported [5]. For acute infection, 10^3 RH tachyzoites were injected intraperitoneally in mice, and symptoms and survival were observed daily until 21 days after infection, with data compared by Log-Rank test. Cystogenic ME49 *T. gondii* challenge was performed orally with 10 cysts from the brains of chronically infected mice. After 30 days of infection, the mice were euthanized, brains removed and homogenized in 10 mL of sterile saline. Cyst counts were determined in phase contrast microscopy. DNA from homogenate was extracted and the absolute number of parasites determined by real-time PCR using sense B1JW63 and antisense B1JW62 primers for the B1 gene [25] and Power SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, California, EUA) according to the manufacturer's instructions in a real-time PCR System®7500 (Applied Biosystems California, EUA)

2.4. Immune response determination in immunized mice

Total antigenic extract of *T. gondii* tachyzoites (1 µg protein/ml) was adsorbed in 96-well, high affinity ELISA plates (Costar, Washington, D.C., EUA), incubated with dilutions of serum samples from immunized mice; bound antibody detected by peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis), developed with commercial TMB solution (Sigma-Aldrich, St. Louis). After the 30 min, the reaction was stopped by addition of 4 N HCL. The absorbance readings were performed on a Multimode microplate reader Microplate Reader Filter Max FS5 (Molecular Devices®, Sunnyvale, California) in the absorbance filter 450 nm. The reactivity index of the samples was determined as the ratio of the absorbance from each samples by the mean absorbance of the negative control samples, used for intratest quality control for conjugate variation. (IR = Abs of positive samples/mean of Abs. of negative samples). IgG avidity was determined by using a 6 M Urea washing after IgG binding and before conjugate incubation. Avidity was expressed as percent of Urea resistant IgG.

Cell phenotyping was determined in spleen and blood cells (2×10^6 cells/well) stimulated by total extract antigen of *T. gondii* tachyzoites (20 µg/mL), cultured for 6 days in 24-well plates, as elsewhere reported [26]. Splenic and blood cells were purified by Ficoll-Paque™ Premium 1084 (GE Healthcare, Little Chalfont, UK) and labeled with Carboxyfluorescein succinimidyl ester (CFSE, BD Biosciences, Franklin Lakes, New Jersey, EUA), according to the manufacturer's instructions. Phenotypes were determined by incubation with adequate anti-mouse monoclonal antibodies: anti-CD3-Pacific Blue (BD Biosciences, New Jersey, EUA), anti-CD4 V500 Horizon (BD Biosciences, New Jersey, EUA), anti-CD8 APC-H7 (BD Biosciences, New Jersey, EUA), and anti-CD19 PE-Cy7 (BD Biosciences, New Jersey, EUA), according to the manufacturer's instructions, with adequate controls and submitted to a Fortessa Flow cytometer. The data were collected (50.000 events) and analyzed both by the BD FACSDIVA® software for acquisition and by the FlowJoX® software for analysis. All samples were compensated using cells from non-immunized animals. After selecting the lymphocyte population, we defined the singlet population to exclude debris and doublets. From the singlet population we separated the proliferated $CD19^+$ and $CD19^-$ populations (B cells). Of the population $CD19^-$, we determined the populations of T cells ($CD3CD4^+$ and $CD3CD8^-$).

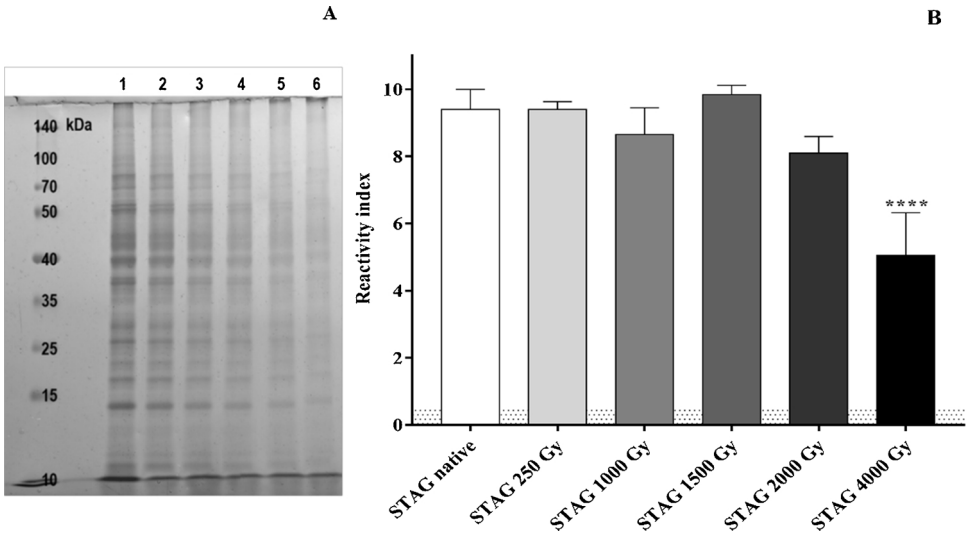

Fig. 1. SDS-PAGE **(A)** and Recognition of specific IgG antibodies by ELISA **(B)** of total antigenic extracts of *T. gondii* tachyzoites (STAG), native and irradiated at 250, 1000, 1500, 2000 and 4000 Gy of 60Co. Dotted block represents basal recognition of IgG.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant difference between immunized and control groups. Survival after challenge with the virulent RH strain, was expressed used the Kaplan–Meier curve, and the statistical difference between the immunized and control groups was calculated by the Log-Rank test, using GraphPad Prism Software 7.

3. Results

Samples of total antigen extracts of *T. gondii* tachyzoites (STAG) were submitted to different doses of ionizing gamma radiation per 60Co source. The action of radiation on the extract appears to be dose dependent. STAG 250 Gy maintained the electrophoretic profile observed in the native non-irradiated extract. Extracts irradiated at 1000, 1500 and 2000 Gy showed discrete degradation of proteins of high molecular weight and this degradation was greater in the sample irradiated at 4000 Gy. After the characterization by SDS-PAGE (Fig. 1A), we evaluated the action of ionizing gamma radiation on the antigenic properties of the extracts by ELISA. In Fig. 1B, we show that all irradiated extracts maintained their antigenic characteristics, recognizing IgG anti-*T. gondii* present in sera from mice infected with ME-49 strain. The 4000 Gy dose was the only that affected both the structural and antigenic characteristics of STAG.

Groups of 05 BALB/c mice were immunized with 03 bi-weekly doses of each extract subcutaneously, to evaluate the effects of ionizing gamma radiation on the immunogenic properties of extracts. All groups analyzed had significant levels of antibodies IgG anti-*T. gondii*. BALB/c mice immunized with native STAG and STAG irradiated at 250 Gy had lower levels of specific IgG antibody (Fig. 2A) compared to immunized groups with STAG irradiated at 1000, 1500 and 2000 Gy. STAG 4000 Gy, although still inducing production of specific IgG antibodies, showed that higher doses of irradiation affected both the antigenic and immunogenic characteristics of the extract. The same samples were used to detect the avidity of IgG antibodies (Fig. 2B). After the three immunizations, all groups presented significant indices in the maturation of high avidity antibodies, except the group immunized with STAG irradiated at 4000 Gy. When we compared the maturation of antibodies detected in the animals immunized with STAG native and those immunized with irradiated STAG, we observed no significant difference between the groups immunized with STAG irradiated at 250 and 2000 Gy. The groups that presented higher levels of maturation were

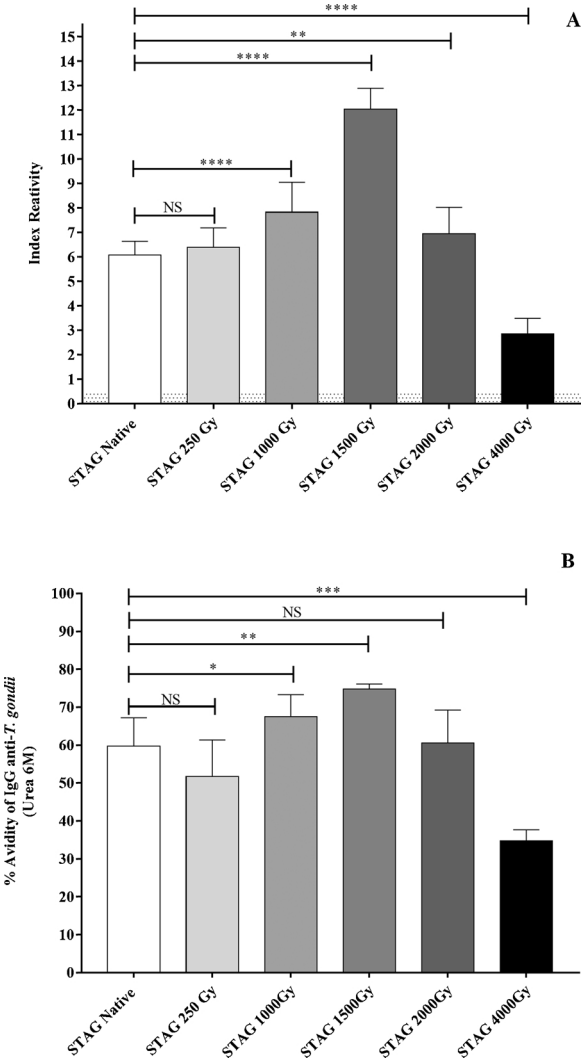

Fig. 2. Detection of total antibodies **(A)** and avidity of IgG antibodies **(B)** in the serum from BALB/c mice immunized subcutaneously with 03 doses of STAG native, STAG 250 Gy, STAG 1000 Gy, STAG 1500 Gy, STAG 2000 and STAG 4000 Gy. Bars represent the mean and standard deviation, and the presence of asterisk indicates difference ($p < 0.001$) between groups immunized with STAG native and irradiated STAG. Dotted block represents basal production of IgG.

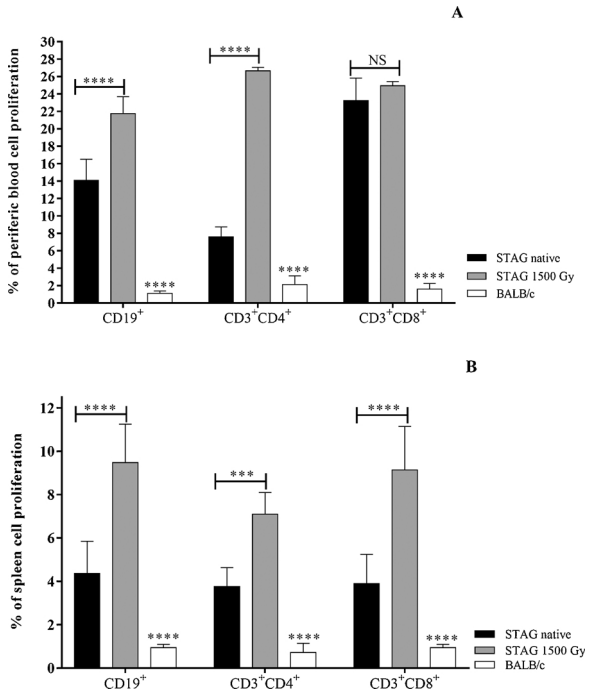

Fig. 3. Proliferation of TCD4 cells (TCD3$^+$CD4$^+$), TCD8 cells (TCD3$^+$CD8$^+$) and B cells (CD19$^+$) from peripheral blood **(A)** and spleens **(B)** of mice subcutaneously immunized with STAG native, STAG 1500 Gy and not immunized at 15 days after the immunization following stimulation with *T. gondii* antigen for 6 days. The bars represent the means and standard error of the mean of five animal, and the presence of asterisks indicates significant difference ($p < 0.001$) between control group and immunized group and between STAG native and STAG 1500 Gy immunized group.

the immunized with STAG irradiated at 1000 and 1500 Gy.

From the results obtained by STAG 1500 Gy, we evaluated the cellular (Fig. 3) and protective (Fig. 4) immune response of mice immunized with STAG native and irradiated at 1500 Gy. Proliferation assays using the CFSE marker showed that splenic and peripheral blood cells from immunized mice were stimulated after contact with total antigens of *T. gondii* tachyzoites. Both peripheral blood cells (Fig. 3A) and splenic cells (Fig. 3B) showed a greater proportion of TCD3$^+$TCD4$^+$, TCD3$^+$TCD8$^+$ T cells and B cells (CD19$^+$) in proliferated cell counts when compared to the levels presented by mice immunized with STAG native.

BALB/c mice were immunized with 03 doses of, STAG native and 1500 Gy and challenged 15 days after the third dose of immunization. The Kaplan–Meier survival curve (Fig. 4A), shows the induced protection in BALB/c mice immunized with STAG native, STAG 1500 Gy and unimmunized control mice challenged with 10^3 tachyzoites of the RH strain. All immunizations induced significant levels of protection compared to unimmunized groups ($p < 0.0001$). Mice immunized with native STAG survived 9 days, while those immunized with STAG irradiated at 1500 Gy survived for 22 days after challenge. In the fourth day all control mice had already died. The challenge using 10 cysts of the ME-49 cystogenic strain showed a significant decrease of brain cysts in immunized mice both counting by optical microscopy (Fig. 4B) and by the quantification of DNA by Real-Time PCR (Fig. 4C). Although the decrease in the number of cysts was observed in STAG native and irradiated groups, the group immunized with STAG 1500 Gy showed at least one animal in which we did not identify the presence of cysts.

4. Discussion

Our results show that STAGs should be irradiated at an optimal dose for maintaining their integrity and antigenicity while increasing immunogenicity. SDS-PAGE and ELISA analysis showed that higher doses of radiation induced degraded epitopes or protein aggregates, similar to elsewhere described [27]. Despite these effects, we still observed some reactivity to specific antibodies confirming that higher doses of radiation cause severe structural changes and partial losses of antigenic characteristics as described in snake venoms [6]. Irradiated proteins undergo chemical changes such as fragmentation, cross-linking, aggregation and oxidation by oxygen radicals generated in the radiolysis of water, with losses in biological activity and structure [28], but those effects are more evident in higher doses, as in our data.

Mice immunized with native or irradiated STAG shown that the optimal dose for extract irradiation was 1500 Gy that induced highest specific antibody levels, with greater affinity maturation. These findings were reported in snake venoms irradiation, adjusted doses of ionizing radiation attenuates toxicities without affecting its immunogenic properties allowing better antisera production [6]. Immunization schedule was identical in all immunogen without addiction of adjuvants and most of them resulted in some antibody levels, lowest for STAG 4000 Gy. This immunogenicity is unexpectedly high in 1500 Gy STAG without adjuvants, as several reports describes the crucial necessity of adjuvants for vaccine. The use of adjuvants enhances immunogenicity of subcomponent vaccines (proteins or polysaccharides), but this effect is harmful and the number of adjuvants licensed for use in humans and animals is still scarce and sometimes with adverse effects scarce [29].

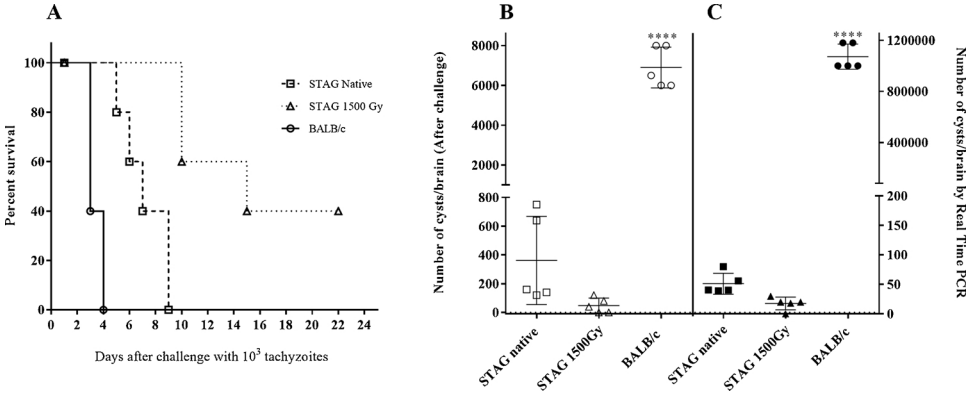

Fig. 4. Induced protection in BALB/c mice immunized with native STAG and irradiated at 1500 Gy of 60Co. **(A)** Kaplan–Meier survival curve of BALB/c immunized mice and BALB/c control non immunized, challenged with 10^3 *T. gondii* RH tachyzoites 30 days after the last immunization. A Log-rank (Mantel-Cox) test demonstrated a significant difference between the immunized and control groups ($p < 0.0001$). **(B)** Number of brain cysts from immunized BALB/c mice and BALB/c mice infected by ME-49 detected by microscopy (cast symbols), and **(C)** Real Time PCR (full symbols) challenged with 10 cysts of *T. gondii* ME-49 15 days after the last immunization. Bars represents the means and standard deviation. The presence of asterisk indicates a statistically significant difference ($p < 0.05$) compared to infection in non-immunized BALB/mice.

Vaccines without adjuvants are promising, since they open a new perspective for a better understanding of the immune response of the host and the effective role of the addition of adjuvants in formulations. We describe a subcomponent vaccine without adjuvants with good protection and safe for human and veterinary use.

The mode of action of adjuvants in vaccines are not completely understood but these products promote a slow release of the antigen such as alum and also attract inflammatory response to the site of injection promoting the interaction of the antigen with the immune cells [30]. Gamma irradiation of antigen can mimic these effects by generating smaller solubility protein aggregates or oxidizing the antigens similar reported for neutrophil myeloperoxidase in acute inflammation [31].

The humoral immune response together with the cellular immune response plays an important role in the control of *T. gondii* infection [32]. Specific antibodies against *T. gondii* interfere in the attachment of the parasite to host cells receptors, and to be involved in the process of killing intracellular parasites by macrophages [33]. Thus the presence of highest levels of high affinity specific IgG will result also in best protection. Affinity maturation or avidity of an antibody depends on selection of B cells in germinal centers due to cell contacts mediated by specific T cell receptors [34] and the highest affinity maturation was also found with immunization with 1500 Gy STAG, a fact that indirectly suggest the best immune cell cooperation.

Gamma radiation of antigens also induced better cellular immune response [7]. In an adjusted, the gamma radiation was able to alter immunogenic properties of the proteins, resulting in better immune recognition of antigens. Our results showed that irradiation of STAGs at an optimal dose induced a protective immune response in mice after challenge with two distinct viable strains of *T. gondii*. This immune response was mediated by in higher specific antibody blood levels and higher $CD4^+$ T cells and B memory cells in blood, as we detected in antigen driven proliferation assays. Effective vaccines induce responses to specific long-term antibodies by inducing T and B memory cells. The subsets of these phenotypes vary according to the type of vaccine [35]. Our studies shows that we induced a high proportion of activated cells in blood, responsive to non-irradiated antigens in our immunized mice, an effect essential to a vaccine, that must responded to the original natural antigen found in environmental exposition.

The challenge using the strains ME-49 and RH of *T. gondii* showed significant protection against several types of parasites with antigen present only in irradiated parasite soluble extracts without irradiation and adjuvants, were reported to fail to promote protection [4] and the use of adjuvants improves their results [36]. ME-49 Challenge was by oral route, thus irradiated STAG induced also mucosal protection, which is expected in the induced cell cooperation studies. The advent of molecular biology resulted in thousands or reports using recombinant proteins in the production of vaccine for toxoplasmosis, but all of then used with adjuvants and low protection. Vaccines produced from surface SAG1 proteins of *T. gondii* tachyzoites could be protective against acute toxoplasmosis [37], but with addition of potent adjuvants such as Freund's complete adjuvant and QuilA [38]. In the search of improving their efficiency, several associations of recombinant proteins were attempted with little protection involvement. In our model, the whole process was performed without the addition of adjuvants to the extracts, and despite its absence, we obtained humoral and cellular protective immune response for several strains of the parasite, only possible with total soluble parasite extracts.

Coccidian's vaccines produced by gamma irradiation until now uses whole parasites irradiated at lower doses, usually less than 500 Gy, for inducing mitotic death due DNA double breaks [2,4]. The view of the use of gamma radiation in those models and frequently in Medicine or Immunology is a destructive one, looking for destruction of immune response, destruction of parasites or sterilization, but gamma radiation offers much more. Water radiolysis products are similar to free radicals induced in effective immune response by neutrophils and macrophages and gamma radiation could simulate these radicals to immune cells, inducing a better recognition and immune response with adding any new radical or chemical to the vaccine. We must look for the effect of gamma radiation on proteins and vaccine production, which is complex due to the need of the smaller dose that affects proteins without destroying it and resulting in enhanced immunogenicity, as we show. These effects could result in a simple treatment for better vaccines produced by parasite extracts as in our case for toxoplasmosis. Gamma radiation must be carefully dosed for this effect but this would be a useful tool for vaccine production.

Disclosure statement

The authors reports no conflicts of interest. The authors are responsible for the content and writing of this article.

Acknowledgments

We gratefully thank for the expertise during technical assistance of R.P.A. Cardoso. This work was a part of the Master's degree program of A. da Costa in Relation of Host Pathogen Biology, USP. A. Costa and H. F. Andrade Jr were receipts of Conselho Nacional de Pesquisas fellowships and A. da Costa is fellow of Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP2014/17029-4). We gratefully thank the support of Biotechnology division of IPEN/CNEN-SP and LIMHCFMUSP.

References

[1] J.A. Reisz, N. Bansal, J. Qian, W. Zhao, C.M. Furdui, Effects of ionizing radiation on biological molecules—mechanisms of damage and emerging methods of detection, Antioxid. Redox Signal. 21 (2014) 260–292, http://dx.doi.org/10.1089/ars.2013.5489.

[2] R.S. Nussenzweig, J. Vanderberg, H. Most, C. Orton, Protective immunity produced by the injection of x-irradiated sporozoites of Plasmodium berghei, Nature 216 (1967) 160–162.

[3] T.C. Luke, S.L. Hoffman, Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated Plasmodium falciparum sporozoite vaccine, J. Exp. Biol. 206 (2003) 3803–3808.

[4] R.M. Hiramoto, A.J. Galisteo, N. do Nascimento, H.F. de Andrade, 200 Gy sterilised Toxoplasma gondii tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice, Vaccine 20 (2002) 2072–2081.

[5] N.E. Zorgi, A. Costa, A.J. Galisteo, N. do Nascimento, H.F. de Andrade, Humoral responses and immune protection in mice immunized with irradiated T. gondii tachyzoites and challenged with three genetically distinct strains of T. gondii, Immunol. Lett. 138 (2011) 187–196, http://dx.doi.org/10.1016/j.imlet.2011.04.007.

[6] N. Do Nascimento, C.S. Seebart, B. Francis, J.R. Rogero, I.I. Kaiser, Influence of ionizing radiation on crotoxin: biochemical and immunological aspects, Toxicon 34 (1996) 123–131.

[7] J.R. Pinho, B.A. Cardi, H.F. Andrade, P.J. Barr, I.C. Bathurst, E.J. Vicente, A.C. Schenberg, Immunogenic properties of the M. leprae recombinant 18-kDa antigen purified from Saccharomyces cerevisiae; enhancement of delayed-type hypersensitivity after gamma-irradiation, Int. J. Lepr. Other Mycobact. Dis. 63 (1995) 381–390.

[8] S. Moon, K. Bin Song, Effect of γ-irradiation on the molecular properties of ovalbumin and ovomucoid and protection by ascorbic acid, Food Chem. 74 (2001) 479–483, http://dx.doi.org/10.1016/S0308-8146(01)00166-2.

[9] C.B. Fox, R.M. Kramer, V. Lucien Barnes, Q.M. Dowling, T.S. Vedvick, Working together: interactions between vaccine antigens and adjuvants, Ther. Adv. Vaccines 1 (2013) 7–20, http://dx.doi.org/10.1177/2051013613480144.

[10] B.A. Cardi, H.F. Andrade, J.R. Rogero, N. Nascimento, Differential biodistribution of native and 2 kGy 60Co irradiated crotoxin in tissues of CBA/J mice, Nat. Toxins 6 (1998) 19–25.

[11] E. De Gregorio, E. Caproni, J.B. Ulmer, Vaccine adjuvants: mode of action, Front. Immunol. 4 (2013) 214, http://dx.doi.org/10.3389/fimmu.2013.00214.

[12] C.-W. Yang, B.S.I. Strong, M.J. Miller, E.R. Unanue, Neutrophils influence the level of antigen presentation during the immune response to protein antigens in adjuvants, J. Immunol. 185 (2010) 2927–2934, http://dx.doi.org/10.4049/jimmunol.1001289.

[13] V. Papayannopoulos, K.D. Metzler, A. Hakkim, A. Zychlinsky, Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps, J. Cell Biol. 191 (2010) 677–691, http://dx.doi.org/10.1083/jcb.201006052.

[14] K. Kim, L.M. Weiss, Toxoplasma gondii: the model apicomplexan, Int. J. Parasitol. 34 (2004) 423–432, http://dx.doi.org/10.1016/j.ijpara.2003.12.009.

[15] J. Flegr, J. Prandota, M. Sovičková, Z.H. Israili, Toxoplasmosis—a global threat.

Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries, PLoS One 9 (2014) e90203, http://dx.doi.org/10.1371/journal.pone.0090203.
[16] S. Hoffmann, M.B. Batz, J.G. Morris, Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens, J. Food Prot. 75 (2012) 1292–1302, http://dx.doi.org/10.4315/0362-028X.JFP-11-417.
[17] N.-Z. Zhang, J. Chen, M. Wang, E. Petersen, X.-Q. Zhu, Vaccines against Toxoplasma gondii: new developments and perspectives, Expert Rev. Vaccines 12 (2013) 1287–1299, http://dx.doi.org/10.1586/14760584.2013.844652.
[18] D. Buxton, E.A. Innes, A commercial vaccine for ovine toxoplasmosis, Parasitology 110 (Suppl) (1995) S11–S16.
[19] N.E. Zorgi, A.J. Galisteo, M.N. Sato, N. do Nascimento, H.F. de Andrade, Immunity in the spleen and blood of mice immunized with irradiated Toxoplasma gondii tachyzoites, Med. Microbiol. Immunol. 205 (2016) 297–314, http://dx.doi.org/10.1007/s00430-015-0447-5.
[20] E. Jongert, C.W. Roberts, N. Gargano, E. Förster-Waldl, E. Petersen, Vaccines against Toxoplasma gondii: challenges and opportunities, Mem. Inst. Oswaldo Cruz 104 (2009) 252–266, http://dx.doi.org/10.1590/S0074-02762009000200019.
[21] H. Zhou, J. Min, Q. Zhao, Q. Gu, H. Cong, Y. Li, S. He, Protective immune response against Toxoplasma gondii elicited by a recombinant DNA vaccine with a novel genetic adjuvant, Vaccine 30 (2012) 1800–1806, http://dx.doi.org/10.1016/j.vaccine.2012.01.004.
[22] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979, Biotechnology 24 (1992) 145–149.
[23] J. Crawford, E. Lamb, J. Wasmuth, O. Grujic, M.E. Grigg, M.J. Boulanger, Structural and functional characterization of SporoSAG: a SAG2-related surface antigen from Toxoplasma gondii, J. Biol. Chem. 285 (2010) 12063–12070, http://dx.doi.org/10.1074/jbc.M109.054866.
[24] M.D. Frenchik, L.I. Tsonev, S.J. McFaul, A microplate assay for the determination of hemoglobin concentration, Clin. Chim. Acta 339 (2004) 199–201, http://dx.doi.org/10.1016/J.CCCN.2003.09.017.
[25] M.E. Grigg, J.C. Boothroyd, Rapid identification of virulent type I strains of the protozoan pathogen Toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene, J. Clin. Microbiol. 39 (2001) 398–400, http://dx.doi.org/10.1128/JCM.39.1.398-400.2001.
[26] N.E. Zorgi, A.J. Galisteo, M.N. Sato, N. do Nascimento, H.F. de Andrade, Immunity in the spleen and blood of mice immunized with irradiated Toxoplasma gondii tachyzoites, Med. Microbiol. Immunol. 205 (2016) 297–314, http://dx.doi.org/10.1007/s00430-015-0447-5.
[27] C. Houée-Levin, K. Bobrowski, The use of the methods of radiolysis to explore the mechanisms of free radical modifications in proteins, J. Proteom. 92 (2013) 51–62, http://dx.doi.org/10.1016/j.jprot.2013.02.014.
[28] M.J. Davies, Protein oxidation and peroxidation, Biochem. J. 473 (2016) 805–825, http://dx.doi.org/10.1042/BJ20151227.
[29] R. Rappuoli, Bridging the knowledge gaps in vaccine design, Nat. Biotechnol. 25 (2007) 3–6.
[30] S.G. Reed, S. Bertholet, R.N. Coler, M. Friede, New horizons in adjuvants for vaccine development, Trends Immunol. 30 (2009) 23–32, http://dx.doi.org/10.1016/j.it.2008.09.006.
[31] R. Biedroń, M.K. Konopiński, J. Marcinkiewicz, S. Józefowski, Oxidation by neutrophils-derived HOCl increases immunogenicity of proteins by converting them into ligands of several endocytic receptors involved in antigen uptake by dendritic cells and macrophages, PLoS One 10 (2015) e0123293, http://dx.doi.org/10.1371/journal.pone.0123293.
[32] C.D. Dupont, D.A. Christian, C.A. Hunter, Immune response and immunopathology during toxoplasmosis, Semin. Immunopathol. 34 (2012) 793–813, http://dx.doi.org/10.1007/s00281-012-0339-3.
[33] W. Freppel, P.-H. Puech, D.J.P. Ferguson, N. Azas, J.P. Dubey, A. Dumètre, Macrophages facilitate the excystation and differentiation of Toxoplasma gondii sporozoites into tachyzoites following oocyst internalisation, Sci. Rep. 6 (2016) 33654, http://dx.doi.org/10.1038/srep33654.
[34] H.N. Eisen, Affinity enhancement of antibodies: how low-affinity antibodies produced early in immune responses are followed by high-affinity antibodies later and in memory B-cell responses, Cancer Immunol. Res. 2 (2014) 381–392, http://dx.doi.org/10.1158/2326-6066.CIR-14-0029.
[35] B. Pulendran, R. Ahmed, Immunological mechanisms of vaccination, Nat. Immunol. 12 (2011) 509–517 (Accessed 10 April 2018), http://www.ncbi.nlm.nih.gov/pubmed/21739679.
[36] A. Stanley, D. Buxton, E.A. Innes, J.F. Huntley, Intranasal immunisation with Toxoplasma gondii tachyzoite antigen encapsulated into PLG microspheres induces humoral and cell-mediated immunity in sheep, Vaccine 22 (2004) 3929–3941, http://dx.doi.org/10.1016/j.vaccine.2004.04.022.
[37] E. Petersen, H.V. Nielsen, L. Christiansen, J. Spenter, Immunization with E. coli produced recombinant T. gondii SAG1 with alum as adjuvant protect mice against lethal infection with Toxoplasma gondii, Vaccine 16 (1998) 1283–1289.
[38] I.A. Khan, K.H. Ely, L.H. Kasper, A purified parasite antigen (p30) mediates CD8+ T cell immunity against fatal Toxoplasma gondii infection in mice, J. Immunol. 147 (1991) 3501–3506.