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RESEARCH PAPER

Evaluation of survival rate using liposome containing soluble antigens (SA) against *Toxoplasma gondii* infection in BALB/c mice

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ABSTRACT

Objective(s): *Toxoplasma gondii*, an obligate intracellular protozoan parasite, is widespread across the world. It causes congenital disease and abortion in humans and domestic animals. One of the major concerns in parasitology, thus, is an effective vaccine development to control Toxoplasmosis.

Materials and Methods: In the present research, a nano-liposomal vaccine containing soluble antigens (SA) was designed to evaluate the immunity and protective efficacy against *T. gondii* infection in BALB/c mice. Soluble antigens (SA) were achieved from tachyzoites, encapsulated in the liposome, and investigated via scanning electron microscope. Three times with 2-week intervals, BALB/c mice were immunized subcutaneously with different formulations. The level of protection against infection was assessed through the percent survival survey of BALB/c mice after challenge with tachyzoites of *T. gondii* RH strain; also, the type of generated immune response was determined by evaluating the generation of cytokine (IFN-γ, IL-4) and titration of IgG isotypes.

Results: The immunization with liposome DSPC+ SA and liposome DSPC+ Imiquimod + SA induced a substantial increase in anti-Toxoplasma IgG antibody as compared to the PBS group ($P<0.05$). The IgG2a and IFN-γ secretion highest levels were seen with liposome DSPC+ Imiquimod + SA more than the control group ($P<0.01$) and ($P<0.0001$), respectively. After challenge with tachyzoites, less mortality was detected in the immunized mice by liposome DSPC + Imiquimod + SA that was meaningfully different ($P<0.01$) in comparison to other groups.

Conclusion: Vaccination with liposome DSPC + Imiquimod + SA showed more survival rate and cellular immune reaction against *T. gondii*.

Keywords: *Cationic liposome, Imiquimod, Immune response, Toxoplasma gondii*

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INTRODUCTION

Toxoplasma gondii is an intracellular protozoan parasite of the phylum *Apicomplexa*; it is known to be one of the most common diseases in humans and animals in the world [1]. Diagnosis of the acute and chronic phase of Toxoplasmosis is performed via serological methods by determining the type and titration of specific antibodies using parasitic cytoplasmic membrane antigens in the test kit, which usually have difficulties in determining the type and extent of specific antibodies [2, 3]. Toxoplasmosis is commonly absent in healthy people and causes severe and fatal complications in immunocompromised individuals, such as people with AIDS and those undergoing transplantation. Also, in pregnant women, it causes severe ocular or neuropathic complications in the fetus or even abortions [4]. *Toxoplasma* grows in the host cell, and it seems that the immune system does not

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affect it and cannot control it. Therefore, providing a good vaccine that both prevents mother-to-baby transmission and is effective in parasitic liver disease is a complete solution to Toxoplasmosis [5].

Vaccines from live Toxoplasmosis have been reduced; DNA vaccine and recombinant vaccines, are used instead. There is only a *toxoplasma* commercial Toxovax vaccine that can cause mice to die but prevent sheep from abortions and cysts [6]. Severe and deadly complications of Toxoplasmosis in humans and animals have led to extensive research on the development of effective vaccines for this disease. An effective vaccine against *Toxoplasma gondii* is the one that can prevent severe and fatal complications in the fetus and people with immune deficiencies and also reduce economic losses. Unfortunately, there is still no vaccine against *Toxoplasma gondii* [5]. Investigations show that the excretory-secretion antigens (E/SA) of parasitic tachyzoites are very important for the purpose of this study. *Toxoplasma* extract-secreted antigens are highly immunogenic in human infections [7]. On the other hand, soluble antigens of SA (Soluble Antigens) protect the rats against the *toxoplasma* RH strain. These antigens are an appropriate substitute for membrane and cytoplasmic antigens in serological tests designed to diagnose and distinguish acute forms of chronic Toxoplasmosis [8].

Adjuvants play a key role in the efficacy of the vaccine. They increase the immune response against the antigens inoculated with it and determine the type of immune response. The adjuvants can have more than one mechanism of action. The structure of the antigens improves the quality and also increases the useful life of the vaccine. These adjuvants in humans, for immunity to *toxoplasma*, should stimulate a cellular immune response that, unfortunately, is very low [5, 9]. We used the Imiquimod Adjuvant, an immune regulating agent that has anti-tumor and antiviral properties, and also stimulates interferon-alpha, interleukin 1, and alpha-tumor necrosis factor in peripheral blood and human keratinocytes [10]. Today, the nano technique for adjuvant or an effective drug delivery system for vaccination against *Toxoplasma gondii* is used, the most important goal of which is targeting the drug delivery system through higher efficacy and fewer side effects. In other words, the drug delivery system prevents drug destruction and sustains drug compounds, such as peptides, proteins, and oligonucleotides. Thus, we use a more effective drug delivery system for vaccination against *Toxoplasma gondii* from liposomes [11].

Liposomes are microscopic vesicles composed of two layers of phospholipids with phases of the water. Drugs (water- and fat-soluble) are encapsulated in more specific liposomes. Liposomes are also more effective immunoglobulins for protein antigens [12]. These vesicles can stimulate the hemorrhage or the cellular immune responses selectively. The most important features of the liposomes, such as immuno-adjuvant, are their ability to stimulate the immune system specifically [11]. Additionally, liposomes are approved as an adjuvant by the US Food and Drug Administration, well documented in clinical trials, and well tolerated by volunteers [13].

In the present study, DSPC liposome with Imiquimod Adjuvant encapsulated in the liposome with tactile antigens of *Toxoplasma gondii* is used, and the immunity level obtained from vaccination and resistance to Toxoplasmosis is evaluated.

MATERIALS AND METHODS

Animals, Ethics statement

This study was performed on female BALB/c mice 6 to 8 weeks old. Mice are kept at the Animal Research Center of Zahedan University of Medical Sciences in a special room at 21° C and 12 hours of darkness and 12 hours of light in accordance with the ethical committee's laboratory standards. The protocol has been issued by the Deputy Director of Research and Technology of the Ministry of Health and Medical Education (MOHME), based on the National Ethical Guidelines for Biomedical Research.

Parasites, Imiquimod and soluble antigen (SA)

The employed *Toxoplasma gondii* (RH strain) in the current research was provided from the Toxoplasmosis Research Center, Medical Sciences University of Mazandaran, Sari, Iran. Invivogen Company prepared imiquimod (R837). The established protocol with certain modifications was applied to prepare SA. In brief, the active and fresh tachyzoites multiplied in the BALB/c mice contaminated with intraperitoneal injection were rinsed thrice with the HEPES buffer (10 mM + sucrose 10%, pH 7.4). Through the freeze-thaw process along with probe sonication in a bath of ice, harvested tachyzoites were lysed. The centrifuged lysate parasite supernatant was collected, dialyzed facing HS buffer solution, sterilized over a 0.22 µm membrane, and stored at -70 °C [14]. BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, USA) showed the SA protein concentration (1mg/ml) [15].

Liposome preparation

Liposomes are prepared using a lipid film technique. To make the fat phase from (20 mM Avanti polar lipids, USA DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and lipids liposomal, USA) (10 mM; Avanti polar in a sterile tube of 2:1 and chloroform as a solvent. The solvent was removed from the Rotary Evaporation Unit (Hettich, Germany), followed by the formation of a thin lipid layer on the tube wall. To remove the freeze dryer (TAITEC, Japan) the remaining solvent is used for thin lipid films all night long.

The hydrated lipid film is dispersed in sterile buffer (HEPES buffer 10mM pH 7.4) and antigen SA (mg/ml). Lipid layers in the ultrasonic bath (Bandelin, Germany) at 45°C for 15 minutes become Multilamellar Vesicle (MLV). The liposomes are then extruded 13 times (Avestin, Canada) to reach 400 nm polycarbonate membrane concentrations. The zeta potential and the size of the liposome particles produced by the dynamic light scattering device (Nano-ZS, Malvern, UK) were obtained. The particle size was reported by the PDI and mean ± standard deviation (n = 3) [12].

Characterization of the prepared formulations

SDS-PAGE was used for analysis and determination of the amount of SA encapsulated in liposome. Two gels with a thickness of 1 mm (12.5% and 4.78% w/v acrylamide, respectively) were made to rinse. Electrophoresis buffer was used from 25mM Tris, 192 mM glycine, and 0.1% SDS with a pH of 8.3. The specified amount of SA (2.5 or 5µg) was poured into each well for different formulas and the duration of the electrophoresis was 45 minutes with a constant voltage of 140 V. After electrophoresis, the gels are stained with silver for protein identification and detection [16].

Immunization and challenge

Fifty BALB/c mice (6-8 weeks old) were distributed into 5 groups at random (10 mice per group). Different mice groups were immunized intramuscularly in both thighs of the right and left foot thrice at a 2-week interval with one of these designs: PBS buffer, SA, SA + liposome DSPC, SA + Imiquimod, and Lip DSPC+SA+Imiquimod. For each injection, 100 µl of the injectable drug (consisting of 50 µg of antigen in 100 µl of sterile PBS) was first injected into the syringe, and then the place of inoculation is disinfected with alcohol and formulations were slowly injected into mice (each muscle 50 µl) in the quadriceps muscle. The immunization was carried out in 3 rounds (0, 21, and 42 days) in the same order as described above. Mice immunized were faced i.p. with 1 × 104 tachyzoites of the *T. gondii* RH strain two weeks after the last booster injection [17].

Antibody isotope assay

The level of antigen-specific serum IgG was specified over an indirect enzyme-linked immunosorbent assay (ELISA) method. Blood specimens were gained from 5 mice heads in each group from the corner of the eye six and nine weeks post-inoculation, and the sera were separated and kept at -20 °C. With 50 µl of SA (10 µg/ml) in PBS buffer (0.01 M, pH 7.3), as well as serum serial dilutions at 4 °C overnight, Microtiter plates (Nunc, Denmark) were coated. Based on the producer's protocol (Invitrogen Inc, USA), HRP-rabbit anti-mouse IgG isotype was discharged to the plates. At 450 nm by 630 nm, optical density (OD) was specified as the standard wavelength [18].

Quantitative parasitic burden after challenge

Extraction of lymphocyte from the spleen of 5 mice from each group was carried out on day 70 (four weeks after the last immunization) and after providing lymphocyte suspension from each rat, 500 µl of it was cultured in two wells from the plate. The cells of both wells were then stimulated with parasitic antigens. After 48 hours of incubation, the supernatant of the cells was centrifuged and IL-4 and IFN-γ cytokines were analyzed by Duoset ELISA Development Kit (R & D kit). After the 70th day, the challenge was performed on 5 remaining mice from each group by peritoneal *toxoplasma gondii* of RH tachyzoites of 104, and the survival rate of each group was recorded daily [19].

Statistical analysis

Statistical difference was recorded between the control and control groups and analyzed by GraphPad Prism software. One-way ANOVA was used to assess the importance of changes between different groups. A multiple comparison of Tukey-Kramer as a post-test was used for evaluation in different groups of rats. $P<0.05$ was considered as meaningful statistics.

RESULTS

The characterization of liposome

Liposome + SA + Imiquimod, liposome + SA, and empty liposome compositions' average

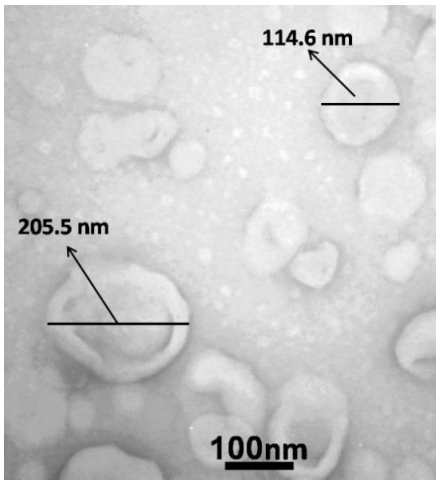

Fig 1. Transmission Electron Microscopy (TEM) photography of liposomal formulations

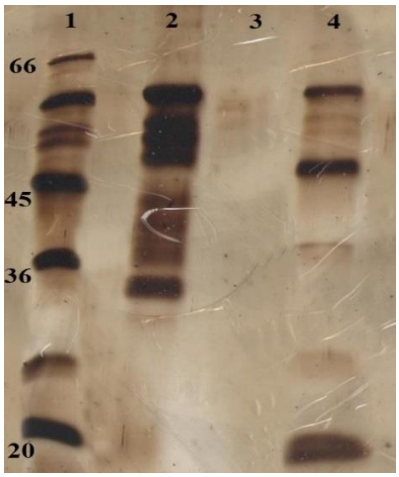

Fig 2. SDS-PAGE analysis of soluble antigens (SA) alone and liposomal SA. Lane 1: low-range protein standard, Lane 2: Soluble Antigens (SA), Lane 3: Empty liposome, Lane 4: liposome containing SA. Results band (2) and band (4) shows that the antigen is encapsulated within the liposome

diameter values were 267±12, 245±15, and 228±19nm; the zeta potentials were -14.4±6, -7.5±3, and 18.5±2mV, respectively, indicating that all various formulations were almost homogenous. The entrapment of SA in liposomes was estimated at 45±9% (n = 3) (Table 1). The TEM investigation confirmed the un-agglomerated spherical liposome nanoparticles (Fig 1). Before injection, the concentration value of SA in the compositions was set at 50 µg/50 µl. Liposomal SA and SA were characterized using SDS-PAGE electrophoresis. The analysis of soluble antigen SDS-PAGE showed various protein bands with a number of ranges. The liposomal SA analysis revealed that nearly every band was similar to the free SA, indicating that SA proteins got captured in the composition following the liposome preparation (Fig 2).

Challenge results

The percent survival of BALB/c mice was measured for 9 days (Fig 3). The percent survival decreased abruptly in the mice that were SA or buffer immunized contrary to those immunized using liposome DSPC + SA, liposome DSPC + Imiquimod + SA, and SA + Imiquimod following the challenge. On day 9, following the challenge, the percent survival in the mice immunized

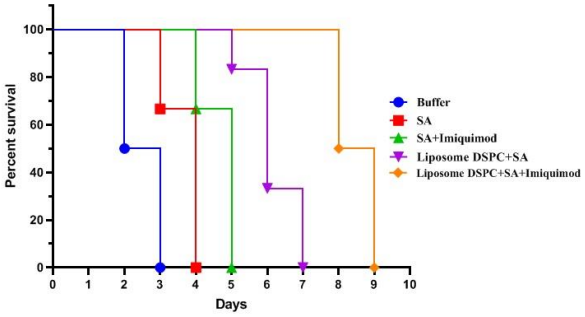

Fig 3. Percent survival in BALB/c mice immunized after challenge with *T. gondii*, with SA, liposome DSPC + SA, liposome DSPC + imiquimod + SA, SA + imiquimod or buffer alone. According to test results, the percent survival in mice immunized liposome + imiquimod + SA was significantly (P<0.05) higher compared with the other groups

Table 1. Particle size, Surface charge, Polydispersity index (PDI) and antigen entrapment of various liposomal formulations. Results denote mean ± SD (n = 3)

Formulation	Size(nm)	Zeta Potential (mv)	PDI	Entrapment Efficiency
Empty liposome	228±19	18.5±2	0.248±0.01	-
Liposome+SA	245±15	-7.5±3	0.328±0.01	37±10%
Liposome+SA+Imiquimod	267±12	-14.4±6	0.402±0.01	45±9%

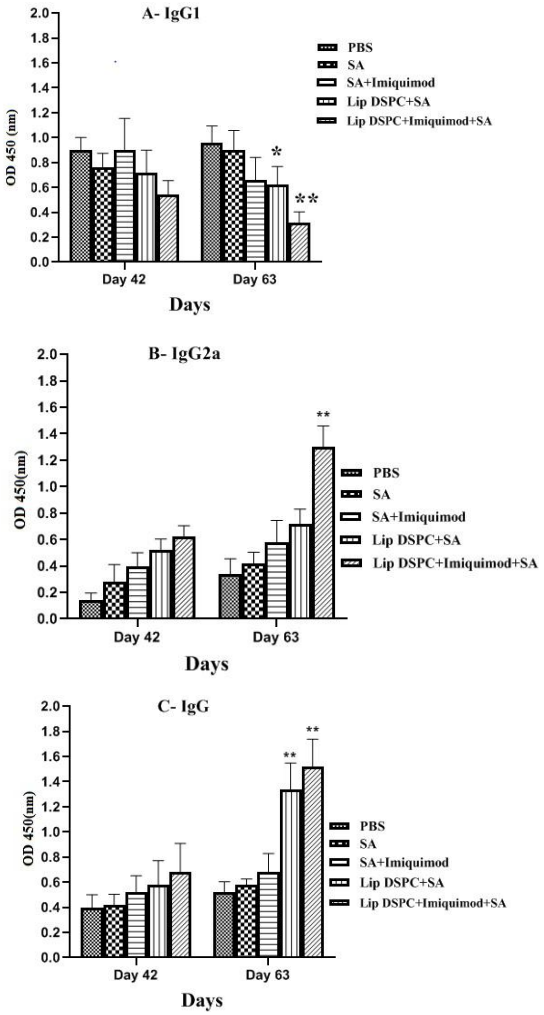

Fig 4. The levels of anti-SA specific IgG1 (A), IgG2a (B), and IgG (C) antibodies was evaluated in sera of BALB/c mice after the last booster injection. Mice immunized intramuscularly, three times in 3-week intervals, with SA, SA + imiquimod, liposome DSPC + SA, liposome DSPC + imiquimod + SA or buffer alone. Blood samples were collected from the mice on days 42 and 63 after the beginning of inoculation. The anti-Soluble Toxoplasma Antigen IgG1, IgG2a, and IgG levels were assessed using ELISA method. Values are represented as mean ± SD. * indicates significant difference between the groups; * P<0.05; ** P<0.01

with liposome DSPC + SA + Imiquimod was definitely (P<0.05) higher than the buffer group on day 9 after the challenge.

Antibody assay

To determine the type of produced immune reaction, the levels of anti-SA specific IgG1, IgG2a, and total IgG antibodies were evaluated in sera of BALB/c mice on days 42 and 63 after the beginning of inoculation (Fig 4A-C). The serum of mice immunized by liposome DSPC+ SA and liposome DSPC+ Imiquimod + SA showed meaningfully, (P<0.05) and (P<0.01), lower IgG1 amounts on day 63 compared to 42 (Fig 4A). Also, the significant difference (P<0.01) was detected in the rate of IgG2a between the liposome DSPC + Imiquimod + SA group with all immunized mice on day 63 after inoculation (Fig 4B).

Cytokine assay

To assess the effectiveness of compositions in the induction of the cellular immune reaction, the generation of IL-4 and IFN-γ triggered through various liposomal constructs was also calculated using the ELISA method. The findings implied that splenocytes removed from the group of mice immunized by liposome DSPC + SA and liposome DSPC + Imiquimod + SA secreted meaningfully greater IFN-γ amounts as compared with the mice immunized with the PBS buffer (P<0.01) and (P<0.0001), respectively (Fig 5A). On the order hands, IL-4 production was identifiable for all mice groups; however, the amounts of IL-4 in the group of mice immunized by liposome DSPC + Imiquimod + SA was meaningfully lower compared to the group received the PBS buffer (P<0.05)(Fig 5B).

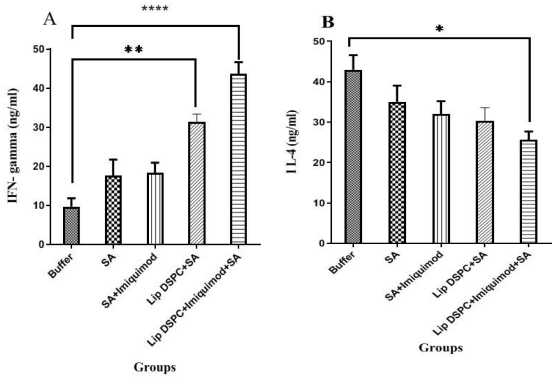

Fig 5. Cytokine level was evaluated through measuring IFN-γ and IL-4 production in immunized mice. Splenocytes were cultured in the presence of Soluble Toxoplasma Antigen (10 µg/ml), and the IFN-γ release and IL-4 release from splenocytes induced by different liposomal formulations were determined using Duoset ELISA. The data are represented as mean±SEM

DISCUSSION

In the current knowledge, limiting the parasitic infection, Toxoplasmosis, through developing new vaccines against *T. gondii* is still a major scientific challenge [20, 21]. Lack of sufficient knowledge of the parasite pathogenicity and the

complex immune responses required to protect it are important reasons for the controversy on this major challenge[5]. Therefore, the development of an optimal vaccine against Toxoplasmosis seems to be the most effective tool for controlling this type of infection. The use of drug delivery devices and systems that trigger immune responses to Toxoplasmosis is one of the best recommendations for the production of vaccines against such infections[22]. In parasitic infections, such as Toxoplasmosis, the simultaneous use of nanoliposomal systems loaded with antigen-dominant epitopes, together with adjuvant molecules that partially mimic the immune-stimulating mechanism of pathogens, can be a suitable formulation for the protective induction of T cells of the immune system[22, 23]. In the present study, DSPC liposomes containing Imiquimod adjuvant as the first generation vaccine for delivery of *T. gondii* soluble tachyzoite antigens are studied, and then the immune reactions and Toxoplasmosis immunity in the rat model are addressed.

Previous studies have shown a unique strategy for the protection and delivery of encapsulated antigens, besides specific stimulation of cellular immunity for antigens encapsulated in DSPC liposomes, although the pathway of this immune response is not yet well understood[24]. The use of the liposomal drug delivery system, due to its binding to target ligands and specific hydrophobic structure, appears to be an attractive tactic to penetrate infected tissues and target parasitic infections [11, 25]. On the other hand, innate immune responses to parasitic infections are probably via Toll-like receptors (TLRs), which is a critical phase to induce an immune response. Therefore, the use of TLR agonists as vaccine adjuvants can be a useful method for developing more efficient vaccines on the immune system [26]. Since Imiquimod is considered as an efficient TLR agonist and safe adjuvant for humans, it is used as a vaccine adjuvant in a BALB/c rat model with Toxoplasmosis in the present study. Also, the adjuvant is utilized to improve and prolong the immune response, and their mechanism of action is well known. Imiquimod adjuvants can act through mechanisms, including the forming vaccine reserves, improving antigen uptake and delivery, assisting antigen-processing cells, and improving the stimulation of cytokines and chemokines [27]. Moreover, Imiquimod increases the Th1 immune response and stimulates the release of cytokines, such as IFN-γ and IL-12, from macrophages. Although Imiquimod can reduce parasite density in the host cell by increasing the production of free radicals, it does not significantly affect the pathogenicity of the parasite [28].

The present study results revealed that Lip DSPC + Imiquimod + SA formulation in BALB/c mice had the highest increase in lifespan compared to other vaccine-injected formulations, as well as buffer group. In a study by El-Zawawy et al., Triclosan-liposomal nanoparticles significantly reduced the number of *Toxoplasma* cysts in the studied rats, which resulted in a decrease in the mortality of the rats [29]. In the study of Tanaka et al., it was found that the use of a potent antigen encapsulated in liposomes as a vaccine for infection caused by *T. gondii* increased the survival time and reduced the parasitic burden in infected rats [30].

Evaluation of IgG2a and IgG1 antigen isotypes is considered as an indicator of Th1 and Th2 immune activity, respectively. The findings of the present study showed that the highest increase in IgG2a and IgG, as well as a decrease in IgG1 in BALB/c mice with Toxoplasmosis after vaccine injection, was related to Lip DSPC + Imiquimod + SA formulation ($P < 0.05$). According to the results, IFN-γ secretion induced by Th1 activity only increased in Lip DSPC + SA and Lip DSPC + Imiquimod + SA formulation compared to buffer group, which was maximal in Lip DSPC + Imiquimod + SA formulation ($P < 0.05$). In addition, Lip DSPC + Imiquimod + SA formulation also significantly decreased IL-4 levels compared to the buffer group ($P < 0.05$).

Since *T.gondii* is an intracellular parasite, cellular immune responses and the release of cytokines play a more significant role in protecting the body against this parasite than hemorrhagic immunity [31]. Accordingly, Th1 is a more important reaction in protection against *T.gondii* infection; thus, it seems that a vaccine formulation that can ultimately drive Th1 responses more than that of Th2 produces a more favorable outcome [32]. However, it should be borne in mind that immune responses to parasitic infection are complex to both the Th1 and Th2 phenotypes that occur at different stages of infection. Therefore, in immune responses, which response represents a type of dominance, it does not mean the complete presence or absence of one of the Th1 and Th2 variants [33]. Results of previous studies have shown that the use of Imiquimod adjuvants

can partly help modulate the production of proinflammatory and anti-inflammatory cytokines in parasitic infections [34]. In the present study, it can be stated that Lip DSPC + Imiquimod + SA formulation can induce long-term immunity to Toxoplasmosis in the rat model by Th1 stimulation. This vaccine formulation has shown the highest efficacy in previous studies [35]. Also, earlier studies have confirmed that the use of soluble antigens encapsulated within liposomes can be a powerful strategy for maximizing Th1 stimulation and immunogenicity in parasitic infections [10]. In confirmation of the present study results, Azadi et al. report that using nanoliposomal antigen-containing soluble drug as a vaccine in *Toxoplasma* infection can increase immunity [22].

Earlier studies also have shown that the use of liposomes in vaccine formulation has a high protective role in protecting vaccine contents against the body's cleansing system [21]. Among the phospholipids used in the vaccine formulation, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), due to the high transition temperature, increases membrane strength by reducing fluidity, thereby reducing the cleaning ability of MPs [36]. On the other hand, the position of cholesterol in the phospholipid bilayers increases the distance between the choline head, also it reduces the hydrogen bonding strength and electrostatic interactions, thereby enhancing the liposome membrane stability and preventing the penetration of water and molecules [37]. Furthermore, due to weaker electrostatic interactions between liposomes and antigens, the use of DSPC liposomes in comparison with other cationic liposomes reduces the deposition of antigens at the injection site, thereby providing more antigen to MHC receptors. Thus, it better induces immune responses [38-41].

CONCLUSION

In the present study, DSPC liposomes, Imiquimod adjuvants, and soluble antigens complex of *Toxoplasma gondii* were used for better influence and activation of the immune system in a BALB/ c mice model. According to the results, vaccine formulation of DSPC liposomes as a carrier of soluble antigens of Toxoplasmosis parasite with Imiquimod adjuvants indicated maximum cellular immunity compared to soluble antigens alone and using Imiquimod and antigen adjuvants. This formulation, providing persistent immunity against Toxoplasmosis infection by increasing Th1 cytokines and decreasing Th2 cytokines like IL-4, can be a good vaccine for immunization against parasites by optimally stimulating cellular immunity and providing appropriate antigens to MHC receptors.

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