A Novel Adjuvant, Mixture of Alum And Naltrexone, Elicits Humoral Immune Responses for excreted/secreted Antigens of *Toxoplasma gondii* Tachyzoites Vaccine In Balb/c Murine Model

Balb/c Fare Modelinde Alum ve Naltrexone Adjuvan Karışımı Kullanılarak, *Toxoplasma gondii* Takizoitlerinin ekskratuvar/sekretuvar Antijenleriyle Hazırlanmış Aşının Humoral İmmun Yanıtı Uyarması

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ABSTRACT

Objective: The excreted-secreted antigens (ESA) from the tachyzoites seem to play a key role in immunity against *Toxoplasma gondii*. The aim of this study is to investigate whether Alum-NLT mixture, as a new adjuvant, can induce humoral immunity in response to excreted-secreted antigens (ESA) of *Toxoplasma gondii* as a model vaccine or not.
Methods: Six- to eight-week-old female Balb/c mice were divided into five groups. Mice in the experimental groups received either ESA vaccine alone or in combination with the adjuvant Alum, NLT or Alum-NLT mixture; Mice in the negative control group received phosphate buffered saline (PBS). All mice were immunised, three times subcutaneously (s.c.) with a total volume of 150µl each with a 10-day interval. Ten days after the final immunisation, immune response to *Toxoplasma gondii* was assessed.
Results: Our results revealed that Alum-NLT mixture as an adjuvant during vaccination boosts the efficacy of the ESA vaccine by means of increasing *Toxoplasma gondii*-specific IgG, IgG2a production and the ratio of IgG2a/IgG1 (P-value < 0.05). The use of this adjuvant mixture improved the protective immunity against *Toxoplasma gondii*.
Conclusion: Administration of the Alum-NLT mixture as an adjuvant in ESA vaccine enhances humoral immunity.
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Key Words: Alum, excreted-secreted, Naltrexone, *Toxoplasma gondii*

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ÖZET

Amaç: *Toxoplasma gondii* takizoitlerindeki ekskratuvar/sekretuvar antijenlerinin (ESA) etkene karşı oluşan immünitede önemli bir rol oynadığı düşünülmektedir. Bu çalışmada yeni bir adjuvant olan Alum-NLT karışımının fare modelinde, *Toxoplasma gondii* ekskratuvar/sekretuvar antijenlerine karşı humoral immuniteyi ne düzeyde uyaran bir aşı modeli olabileceğini gözlemlemeyi hedefledik.
Yöntemler: Altı-sekiz haftalık dişi Balb/c fareler 5 gruba ayrıldı. Deney grubundaki farelere ya tek başına ESA, ya da Alum ajduvantlı, NLT adjuvantlı veya da Alum/NTL adjuvantlı aşılama yapıldı. Negatif control grubundaki farelere de sadece fosfatla tamponlanmış tuz çözeltisi (PBS) verildi. Tüm farelere, 10 gün arayla, toplam 3 kez, her aşılama sırasında cilt altına (SC) 150µl aşılama yapılmıştır. Son aşılamadan 10 gün sonra *Toxoplasma gondii*'ye karşı gelişen immünite araştırılmıştır.
Bulgular: Çalışma verileri, Alum-NLT adjuvant karışımı ile hazırlanmış ESA aşısının *Toxoplasma gondii*- özgün IgG, IgG2a ve IgG2a/IgG1 oranında anlamlı bir artış sağladığını göstermiştir (P-value<0,05). Sonuç olarak, bu adjuvant kombinasyonun *Toxoplasma gondii*'ye karşı oluşan koruyucu immüniteyi arttırdığı gözlemlenmiştir.

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Sonuç: ESA aşısının Alum-NLT adjuvant karışımı ile kombine edilmesinin humoral immun yanıtı arttırdığı kanaatine ulaşılmıştır.
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Anahtar Sözcükler: Alum, ekskratuvar/sekretuvar, Naltrexone, *Toxoplasma gondii*

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that infects all mammalian cells and occurs worldwide in a variety of intermediate hosts (1). Generally, this is benign in healthy persons (2), but in cases of primary infection that occur during pregnancy, severe neonatal malformations and ocular complications in the foetus can be seen. Additionally, toxoplasmosis may cause serious clinical manifestations in immunodeficient patients, especially in patients with AIDS, or in bone-marrow or heart-transplant recipients (3).

Several *Toxoplasma* antigens, such as immunodominant surface antigen (SAG1) and excreted/secreted antigens (ESA) have been identified as potential vaccine candidates (4, 5). ESA of *Toxoplasma gondii* play key role in the stimulation of the host immune system in both acute and chronic infections (6). At present, there are no chemotherapeutic agents to prevent or cure *Toxoplasmosis* completely in humans (7). Therefore, a vaccine would be very beneficial to prevent this disease. A novel vaccine against *Toxoplasma gondii* infection in humans would include antigens that can elicit humoral immune response. Adjuvants are used in designing vaccines due to their immunoadjuvant effect and the possible use for both animals and humans (8).

Aluminium compounds are the only vaccine adjuvants that are approved by the United States Food and Drug Administration (FDA). In contrast, Alum has been used as an adjuvant in human vaccines for more than 70 years (9). It is clear that it would be interesting to include an adjuvant like Alum in the development of a vaccine against *Toxoplasmosis* (10).

Naltrexone (NLT) is a drug that is synthesised in laboratories. These opioids are considered an antagonist at these receptors, and can occupy opiate receptors but not activate receptors. NLT was approved by the FDA for the treatment of heroin addiction and alcoholism, but nausea or vomiting can occur in patients who actively use naltrexone.

METHODS

Animals

Inbred Balb/c mice were purchased from Razi Institute of Iran. All mice were female, aged six to eight weeks old, documented to be specific-pathogen-free and had free access to food and water. All experiments were conducted with the approval of the Institutional Animal Care and Use protocol at the Urmia University of Medical Sciences (Urmia-Iran).

Parasite

The *Toxoplasma gondii* strain RH was used for this study. *Toxoplasma gondii* RH strain was maintained in our laboratory by intraperitoneal passage in Balb/c mice. Mice were infected peritoneally and three days following inoculation, tachyzoites were harvested from the peritoneal cavity by injecting 1 mL of phosphate buffered saline (PBS) PH 7.2. Peritoneal exudates were passed 10 times through a 27-gauge needle to release the intracellular tachyzoites. The peritoneal exudates were centrifuged in low speed (100g for 5 min at 4°C) to remove the cellular debris. The parasites were washed twice in RPMI-1640 medium (Sigma, Germany) that contained 100 IU/mL penicillin and 100 µg/mL streptomycin. The concentrations of tachyzoites were determined by counting them in a Neubauer chamber at 400× magnification (11).

Preparation of Excreted/secreted Ags (ESA)

The obtained tachyzoites of RH strain of 2×10^9 were washed with PBS and centrifuged (750g for 15 min at 4°C) three times. The pellet was solubilised by adding the distilled water, and was supplemented with protease inhibitor, 5mM phenylmethylsulphonyl fluoride (PMSF). In preparing ESA in cell-free incubation media, each sample containing 1.5×10^8 tachyzoites of filtered RH strain per millilitre was divided into 10 tubes and incubated at 37°C for 3 h under mild agitation. Tubes were centrifuged at 1000× g for 10 min and their supernatants were filtered by passing them through 0.22 µm Millipore membrane filter (Millipore Corp., Bedford, MA, USA), and stored at -20°C until use (12).

Immunisation protocol

In immunisation experiments, 25 female Balb/c mice (6-8 weeks-old) were divided into five groups each containing 5 mice.

Vaccination of mice was performed with either ESA alone, or in combination with adjuvants [Alum (50µL aluminium phosphate gel, Sigma, Germany), NLT (0.5 mg/kg, Sigma, Germany), Alum-NLT]. Each mouse received 50µL of ESA containing 20µg/mL of protein mixed (1:1) with adjuvant. The first group were selected as a control group (non-immunised) and received 150µl PBS.

All mice were immunised subcutaneously (s.c.), three times at 10-day intervals with a total volume of 150µL.

Determination of total IgG titre and IgG isotyping

Ten days after the final immunisation, the levels of IgG antibodies were measured in the sera of all mice in all groups by ELISA using 96-well microtitre plates. The optimum dilution of the sera and the optimum dose of ESA to be used in the ELISA were determined using the checkerboard assay. Then, 200µL of antigen in the coating buffer (0.1 M carbonate, PH 9.5) was added to each well of a 96-well microtitre plate. Coated plates were incubated at 4°C overnight, then washed with PBST (PBS with 0.05% Tween 20) three times and blocked with 5% bovine serum albumin in PBST for 2 h at 37°C. After washing the plates with PBST, different dilutions of sera 200 µL/well were added. Plates were incubated at 37°C for 2 h. After washing three times with PBST, the plates were incubated with horseradish peroxidase conjugated with rabbit anti-Mouse IgG (Sigma), IgG1 or IgG2a (Serotec). After washing with PBST three times, the reaction was developed by adding 200µL of a TMB/H_2O_2 substrate. The reaction was stopped by the addition of 50 µL of $2NH_2SO_4$ and the absorbance was read at a wavelength of 450 nm (13).

Statistical Analysis

The IgG, IgG1 and IgG2 levels were evaluated by using one-way analysis of variance (ANOVA) followed by Tukey's test. P-value (< 0.05) were considered significant.

RESULTS

Antibody titre

Sera obtained ten days after the final immunisation were screened for the presence of IgG against excreted/secreted antigens of *Toxoplasma gondii* tachyzoites. As shown in Fig 1, a significant increase in anti-ESA IgG titres was observed in mice vaccinated with the ESA; this also compared the mice that were administered PBS, the ESA vaccine alone, the ESA vaccine with Alum or NLT vaccine with ESA.

IgG isotyping

Blood samples that were obtained ten days after the final immunisation were evaluated for the levels of anti-ESA IgG1 and IgG2a antibody titres by ELISA. The isotype profile of antibody responses is related to the cytokine produced by antigen-specific T cell that is an indirect measure of the Th1/Th2 cytokine profile.

As shown in Fig 2, the mice that were administered ESA vaccine with the Alum-NLT mixture or ESA vaccine with Alum had significantly more IgG1, compared to the mice that received ESA vaccine, ESA vaccine with NLT and PBS alone.

According to Fig 3, the IgG2a levels were significantly higher in all mice immunised with the ESA vaccine in combination with the Alum-NLT mixture compared to those mice that received ESA vaccine with NLT, ESA vaccine with Alum or the ESA vaccine alone and PBS. Also, the mice vaccinated with ESA vaccine with NLT had more IgG2a compared to the mice that received ESA vaccine with Alum, the ESA vaccine alone or PBS alone.

Descriptives				
IgG	**N**	**Mean**	**Std. Deviation**	**Std. Error**
1	5	.06660	.011127	.004976
2	5	.20940	.039966	.017873
3	5	.67820	.042346	.018938
4	5	.79360	.127276	.056920
5	5	1.05460	.088118	.039408
Total	25	.56048	.382294	.076459

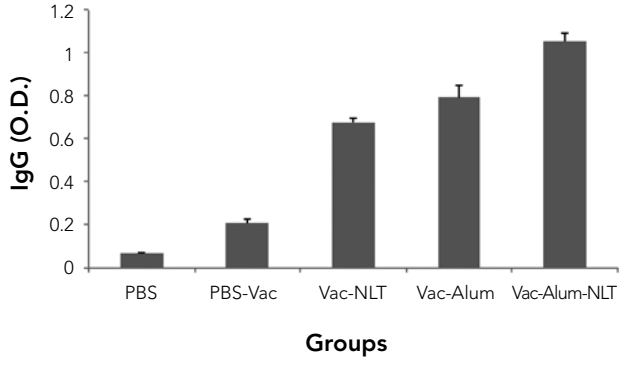

Figure 1. Effect of administering the Alum-NLT mixture on IgG isotype

Descriptives				
IgG1	**N**	**Mean**	**Std. Deviation**	**Std. Error**
1	5	.09500	.032596	.014577
2	5	.15780	.041421	.018524
3	5	.29460	.083901	.037521
4	5	.99700	.600939	.268748
5	5	1.25680	.194594	.087025
Total	25	.56024	.549995	.109999

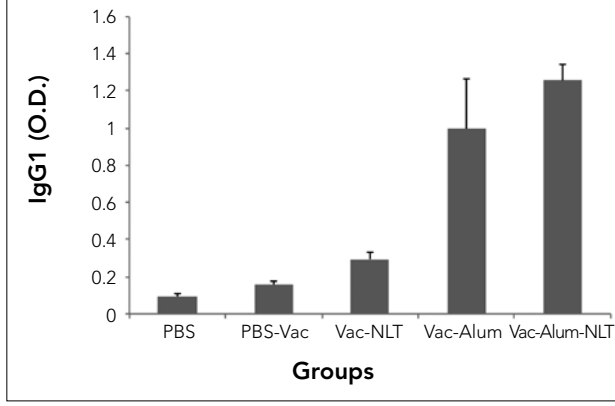

Figure 2. Effect of administering the Alum-NLT mixture on IgG1 isotype

Descriptives				
IgG2a	**N**	**Mean**	**Std. Deviation**	**Std. Error**
PBS	5	.04860	.047857	.021402
Vac	5	.20260	.046790	.020925
Vac_NLT	5	1.22780	.142305	.063641
Vac_Alum	5	.57340	.460362	.205880
Vac_Alum_NLT	5	1.40040	.200708	.089759
Total	25	.69056	.591256	.118251

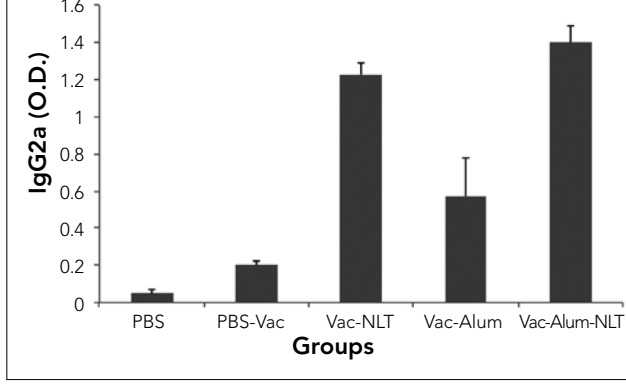

Figure 3. Effect of administering the Alum-NLT mixture on IgG2a isotype

DISCUSSION

Toxoplasmosis may lead to severe pathology in both animals and humans. With recent advances in developing vaccines using adjuvants, it appears that adjuvants play an important role in protective immunity against *T. gondii*.

Previous studies conducted on mice show that IgG_1 antibody response is primarily driven by Th_2, and IgG_2a is driven by Th1. Type 1 T helper cells mediate macrophage activation and stimulate the production of IgG_2a opsonizing and complement-fixing antibodies and produce IFN-γ, TNF and IL-2. The type 2T helper cells provide help for B cells. Several studies have previously indicated that using Alum in the immunisation of mice against *T. gondii* and *T. cruzi* have been shown to be effective (14-17). The ability of Alum to shift the immune response toward a Th_2 profile has been shown (18-20), which primarily stimulates IgG_1 isotype antibodies, and is one of the effective mechanisms induced by the Th_2 response (14, 21). As our results show, a mixture of Alum-Naltrexone is far more effective in provoking Th_1 responses than the prescribed Naltrexone alone. Therefore, it seems that Alum increases the activity of Naltrexon in shifting immune responses toward the Th1 paradigm. This finding is in agreement with the study of Su et al. (22), which showed that the co-administration of Alum and IL-12 augments the potency of IL-12 in IFN-γ production. The mechanism of adjuvant function is unknown, although the expression is stored as a source of antigen in Alum injection site works. Other proposed mechanisms include complement activation or the activation of macrophages and eosinophils; thus, Alum was effective as the cell supplier antigen and led to the production of GM-CSF, IL-8, IL-4 and TNF-α (23).

CONCLUSION

Results in this study showed that the administration of the Alum-NLT mixture as an adjuvant in combination with ESA vaccine can enhance humoral immunity.

Conflict of Interest
No conflict of interest was declared by the authors.

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Author Contributions
Concept - Z.K., K.H.T.; Design - Z.K., S.S.; Supervision - Z.K., K.H.T.; Funding - Z.K., K.H.T., S.S.; Materials - Z.K., K.H.T., S.S.; Data Collection and/or Processing - Z.K., K.H.T., S.S.; Analysis and/or Interpretation - Z.K.; Literature Review - Z.K., S.S., H.M.; Writing - Z.K.; Critical Review - K.H.T., H.M.; Other - K.H.T.

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Yazarlar herhangi bir çıkar çatışması bildirmemişlerdir.

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