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ORIGINAL PAPER

A Survey on the Adjuvant Role of Naloxone Alone or Combined with Alum in Vaccination Against Fasciolosis in BALB/c Mice

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Abstract

Background Fasciolosis is a zoonotic parasitic disease imposing a heavy load of livestock losses worldwide.

Purpose We aimed to evaluate immune-stimulatory effects of naloxone (NLX), an opioid receptor antagonist, in combination with alum in mice vaccinated with excretory–secretory antigens (E/S) of *Fasciola hepatica*.

Methods 8-week-old female BALB/c mice were subcutaneously vaccinated using E/S antigens of *F. hepatica*. Experimental groups (14 mice per group) included: vaccine (E/S antigen), alum vaccine (E/S antigen plus alum), NLX vaccine (E/S antigen plus NLX), and alum–NLX vaccine (E/S antigen plus a mixture of alum–NLX). The control group was infused with PBS. Lymphocyte proliferation and the levels of IFN-γ, IL-4, IgG2a, IgG1, and total IgG were measured.

Results Mice vaccinated with NLX or alum–NLX adjuvants showed significantly higher rates of lymphocyte proliferation, IFN-γ, total IgG, and IgG2a levels. The mice that were injected with alum showed a significantly higher concentration of IL-4. Ratios of IFN-γ/Il-4 and IgG2a/IgG1 were significantly higher in the NLX and alum–NLX groups in comparison with the groups vaccinated either with alum or without any adjuvant. A significantly higher protection rate (62.5%) was seen in mice vaccinated with the alum–NLX adjuvant compared to the other groups.

Conclusion NLX can be effective in conferring cellular immunity and protection against *F. hepatica*. It is recommended to consider this agent as a potential adjuvant in vaccines against fasciolosis.

Keywords Fasciolosis · Adjuvant · Aluminum sulfate · Naloxone · Vaccine

Introduction

Fasciolosis is a zoonotic parasitic disease caused by hepatic trematodes; *Fasciola hepatica* and *Fasciola gigantica.* The disease imposes a heavy load of livestock losses worldwide with a high financial burden mainly on the food industries [6, 34]. Chemotherapy, as the only available treatment strategy against fasciolosis, is accompanied by both safety and drug resistance concerns [17, 29]. Therefore, it is essential to develop an effective vaccine against fasciolosis.

The success of helminthic parasites in hosts may be related to their capability in suppressing the cell-mediated

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immune responses [33]. In fact, the interaction of *Fasciola* species with immune cells such as dendritic cells (DCs) and macrophages could promote a non-destructive CD4 + Th2 response [10], and subsequently a boost in anti-inflammatory regulatory cytokines such as IL-4, IL-5, and IL-13. This is also characterized by an antibody response predominated with IgG1 (in mouse), IgE and IgA isotypes. On the other hand, Th1 lymphocytes known as IFN-γ secreting cells are kept suppressed during immune responses against these parasites [1, 38].

The global increase in life expectancy in the current century is partly due to the management of infectious disease by implementation of efficient vaccines. This surely has been one of the most successful and groundbreaking achievements in medical sciences. In accordance, vaccination appeared to be a potential way to fight against drug-resistant fasciolosis [20, 34]. Adjuvants are vaccine-conjugated factors promoting the immune-inductive capacities of vaccines. Adjuvants can enhance both the intensity and direction of immune responses [3, 46]. A pathogenic agent may be eradicated by the activity of both humoral and cellular immunities. However, the cellular immunity and Th1 response are capable of effectively eliminating the intracellular pathogens.

Alum is currently the only adjuvant which is approved by FDA [11], while only a few additional adjuvants have been licensed by other facilities such as the European Medicines Agency [26, 28]. Alum is effective in the induction of humoral immunity. However, when there is a need for efficient induction of cellular immunity and Th1 response, alum is not a competent adjuvant [11, 27]. One of the drawbacks in the process of commercializing the vaccine adjuvants is the safety issue. For example, Freund's adjuvant is an agent that can promote the cellular side of the immunity, but it is not appropriate for use in humans due to the intolerability of the adjuvant in human beings [9].

Opioid are immune-modulating factors attenuating immune-inductive effects of vaccines [19]. Naloxone (NLX) is an FDA-approved opioid receptor antagonist administrated for individuals with respiratory toxicity induced by opioid peptides [5, 21, 47]. NLX has been reported to boost the vaccine-induced immune responses in vaccination experiments for a number of human infections [22, 26–28, 42, 47]. NLX's capability in induction of Th1 response and cell-mediated cytotoxicity potentiates the agent as a suitable adjuvant for vaccination against Fasciola species [21, 35]. Based on various developmental stages, excretory–secretory (E/S) antigens of *F. hepatica* contain key molecules leading to pathogenicity [24]. An effective vaccine against hepatic flukes such as Fasciola should be able to reduce fluke burden in liver. As E/S antigen of *F. hepatica* constitutes key pathogenesis molecules, E/S antigen-based vaccines can be potential targets for developing effective vaccines against this disease [24]. In the present study, the effects of NLX have been evaluated as an individual or alum-associated adjuvant in stimulation of cellular and Th1 immune responses in mice vaccinated against *F. hepatica* using E/S antigen vaccine. Furthermore, the protection rate was also assessed in immunized mice infected with *F. hepatica.*

Materials and Methods

Mice

Seventy female BALB/c mice with age of 6–8 weeks were purchased from Razi Vaccine and Serum Research Institute, Hesark, Iran. The animals were maintained under standard specific pathogen-free conditions. All experiments were performed according to the Animal Care and Use Protocol of Zabol University of Medical Sciences. The study was approved by Ethical Committee of Zabol University of Medical Sciences.

Preparation of Metacercariae

Metacercariae for vaccination experiment were prepared by infecting the laboratory-cultured snail host, *Lymnaea truncatula*, with miracidia derived from *F. hepatica* eggs. The eggs were obtained from bile ducts and gall bladders of naturally infected sheep slaughtered in a local abattoir. The eggs were further incubated in 0.85% dechlorated water in a dark place for 2 weeks. After the incubation period, the eggs were stimulated with light for 2 h for hatching of miracidia. At 45 days post-infection, cercariae were shed from the snails, then settled on the floating cellophane papers, and immediately transformed into metacercariae. The metacercariae were collected and kept at 4 °C until use.

Preparation of Excretory and Secretory (E/S) Antigens

To prepare excretory–secretory antigens (E/S), adult *F. hepatica* were collected from bile ducts and gall bladders of naturally infected sheep, and washed six times with 0.85% dechlorated water to remove any trace of bile, blood, or contamination. The flukes were then moved into RPMI1640 cell culture medium (1 fluke per 5 ml), supplemented with 100 IU penicillin, and 100 µg/ml streptomycin and incubated at 37 °C. The culture medium was collected and centrifuged at 15,000*g* at 37 °C for 30 min every 6 h for 24 h [31]. The supernatant containing E/S

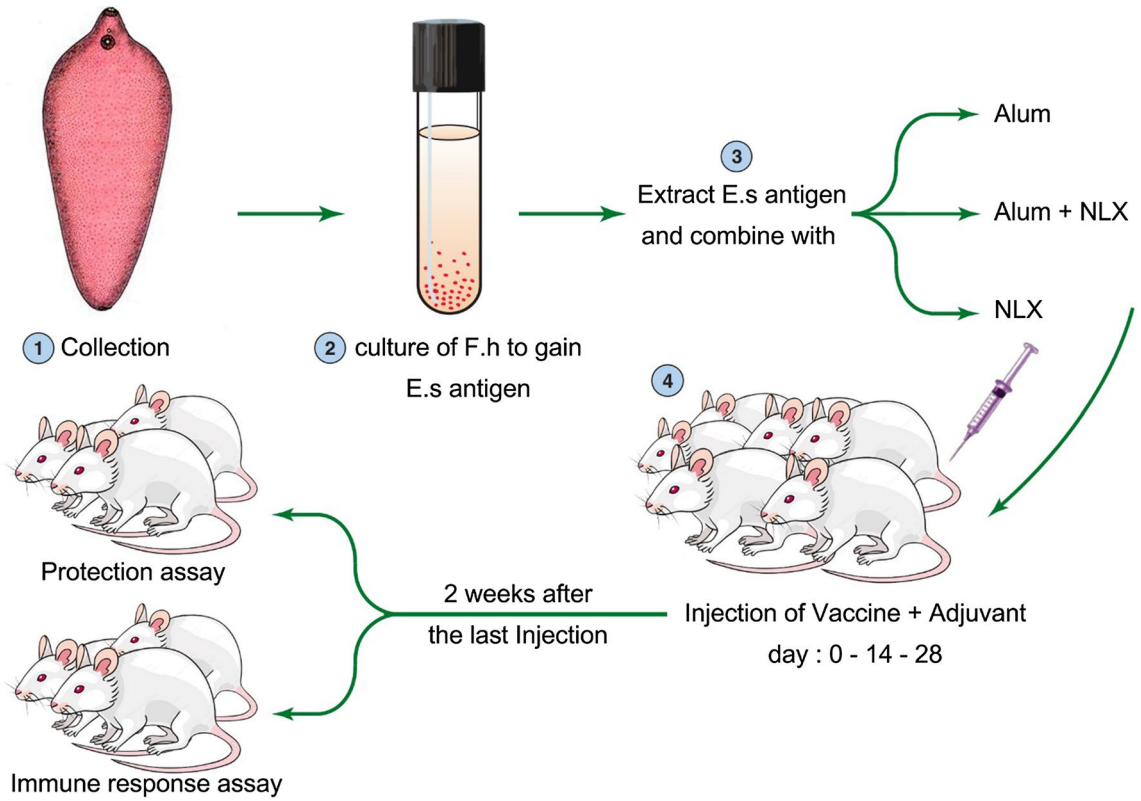

Fig. 1 The timeline of the study. After culturing adult *F. hepatica* parasites, excretory/secretory antigens were recovered each 24 h. After obtaining the intended concentrations, the antigens were incorporated with adjuvants and then injected into mice at three occasions (days 0, 14, and 28). Two weeks after the last injection, the mice in each group were divided into two groups. The mice in one group were used for cytokine assay, and the remaining were subjected to protection assay

antigens was collected, and the protein concentration was determined using Lowry's method (Bio-Rad). All samples were kept at − 80 °C until use (Fig. 1).

Immunization Protocol

The alum–NLX mixture was prepared by thoroughly mixing 50 µl of PBS containing 6 mg/ml of NLX (Sigma, Germany) with 50 µl of alum (aluminum phosphate gel, Sigma, Germany). The mixture was incubated at 4 °C in a sterile condition for 72 h [11, 27, 41].

Five groups of mice (14 per group), as described in Table 1, were subcutaneously vaccinated. The animals received booster injections at days 14 and 28.

Lymphocyte Proliferation Assay

Two weeks after the last immunization, five mice of each group were slaughtered, and spleens were recovered. Splenocyte suspensions were prepared by squishing the spleens through a wire mesh under sterile conditions. The red blood cells were eliminated using ammonium chloride,

Table 1 Experimental groups designed for vaccination against *F. hepatica*

Experimental groups	Vaccination constituents			
	PBS (volume, µl)	E/S antigens (20 µg/ml) (volume, µl)	Adjuvant (volume, µl)	
			Alum	Naloxone
Control	150	–	–	–
Vaccine	100	50	–	–
Alum vaccine[a]	50	50	50	–
Naloxone vaccine[b]	50	50	–	50
Alum–naloxone vaccine[c]	–	50	50	50

[a]100 µl of E/S antigens and PBS suspension was absorbed in alum

[b]100 µl of E/S antigens and PBS suspension was absorbed in naloxone

[c]50 µl of the E/S antigens suspension was absorbed in 100 µl of alum–naloxone mixture

and splenocytes were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco-BRL), 2 mM L-glutamine, and 25 mM HEPES. Diluted cell

suspensions (100 µl) were then seeded in 96-well flat-bottom culture plates at a density of 1×10^6 cells/ml per well. The cells were then stimulated with 10 µl of the E/S antigens (10 µg/ml). Concanavalin A (Con A, 5 µg/ml, Sigma) and culture medium served as positive and negative controls, respectively.

After 72 h, cell proliferation was measured using the MTT (3[4,5-dimethylthiazol-2-ml]-2,5-diphenyltetrazolium bromide; thiazolyl-blue, Sigma, Germany) assay. Briefly, 20 µl of MTT solution was added to each well, and the plate was incubated at 37 °C for 4 h. The supernatant was discarded from each well, and formazan crystals were solubilized by adding 100 µl of dimethyl sulfoxide (DMSO). After 20 min, the optical density (OD) of each well was read at 540 nm. The stimulation index (SI) was calculated as follows:

$$\text{Stimulation index} = \frac{\text{Mean OD of stimulated cells} - \text{Mean OD of the Blank}}{\text{Mean OD of unstimulated cells}}$$

Cytokines Assay

Two weeks after the last immunization, splenocytes were harvested and cultured for 72 h. The supernatants of the culture mediums were collected, and the levels of IFN-γ and IL-4 were measured using commercial enzyme-linked immunosorbent assay (ELISA kit, eBscience, USA) according to the manufacturer's instructions. All assays were performed in triplicate.

Determination of IgG and Isotype Level

Levels of IgG, IgG1 and IgG2a were measured at 2 weeks after the last immunization. Total IgG, IgG1 and IgG2a levels were determined in the sera of five mice from each group [14]. Briefly, following overnight coating of microtiter plates with 100 µl of the E/S antigen (at 4 °C, with a concentration of 5 µg/ml in 0.05 M bicarbonate buffer, pH 9.6), the plates were washed two times with PBS containing 0.05% Tween 20 (PBST-20), pH 7.4. After that, non-specific binding sites were blocked with PBS containing 5% FCS for 2 h at 37 °C. After washing the plates with PBST, sera were diluted in 1% PBST-20 (1:100), and were transferred to each well. Plates were incubated for 2 h at room temperature, washed three times and incubated with HRP-conjugated rabbit anti-mouse for total IgG, IgG2a or IgG1. After sufficient washes, the reaction was developed by adding 200 ml of TMB/H_2O_2 substrate. The reaction was terminated by adding 50 µl of 2 N H2SO4. The ODs were read at 450 nm in a microplate reader (Thermo Scientific Multiskan FC, USA). All assays were performed in triplicate.

Evaluation of Fluke Burden in Biliary–Hepatic System

To evaluate the rate of protection against *F. hepatica*, the flukes were counted in the biliary–hepatic tissues of the immunized mice after challenge with the 6-week-old metacercariae. Two weeks after the last immunization, the mice were orally infected with 30 metacercariae. Six weeks later, the mice were sacrificed, and their peritoneal cavities were opened, and the parasites were collected from the livers. The parasites obtained from the livers were counted and used to calculate the percentage of protection according to the following formula [8]:

$$\text{Protection}\ (\%) = (A - B)/A \times 100$$

where "*A*" represents the mean of worms recovered from the non-immunized (control group) and "*B*" represents the mean of worms recovered from the immunized group.

Statistical Analyses

SPSS Ver. 21 (Chicago, IL, USA) was used to analyze the data. The MTT assay, cytokine levels, and the parasitic loads in livers were analyzed by one-way analysis of variance (ANOVA) and independent samples student's *t* test. A *P* value of less than 0.05 was considered as statistically significant.

Results

Lymphocyte Proliferation

Lymphocyte proliferation (represented as SI) was significantly higher in the group of mice immunized with the E/S vaccine in combination with alum–NLX mixture compared to lymphocyte proliferation of mice in the other groups

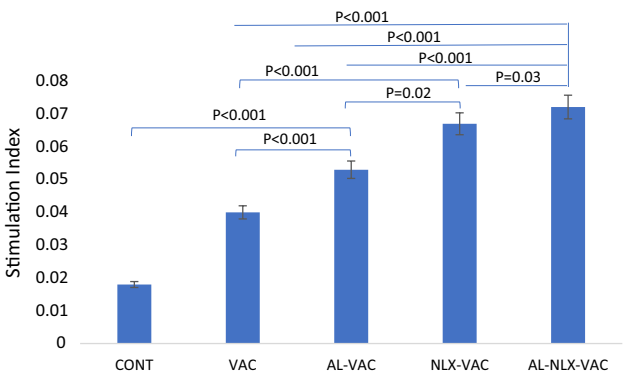

Fig. 2 Differences between SI concentrations in the studied vaccinated groups *CONT* control, *VAC* vaccine, *AL* alum, *NLX* naloxone

($P < 0.001$). Furthermore, a significantly higher SI was observed for lymphocytes derived from the mice immunized with NLX compared to the mice that received alum ($P = 0.02$), E/S vaccine ($P = 0.001$) or PBS alone ($P < 0.001$, Fig. 2).

Cytokine Pattern

The results of ELISA test have been presented in Fig. 3. The mice immunized with alum–NLX mixture showed a significantly ($P < 0.001$) higher IFN-γ level than the mice that received NLX, alum, E/S antigens alone, or the PBS alone (Fig. 3a).

The group of mice that received E/S antigens in combination with alum showed a significantly ($P = 0.001$) higher IL-4 level than the groups of mice treated with the E/S antigens plus either NLX, or alum–NLX (Fig. 3b).

As shown in Fig. 2c, IFN-γ/IL-4 ratio was significantly ($P < 0.001$) higher in the mice immunized with E/S antigens in combination with alum–NLX mixture than the ratio in the mice that received E/S antigens with either NLX, or alum.

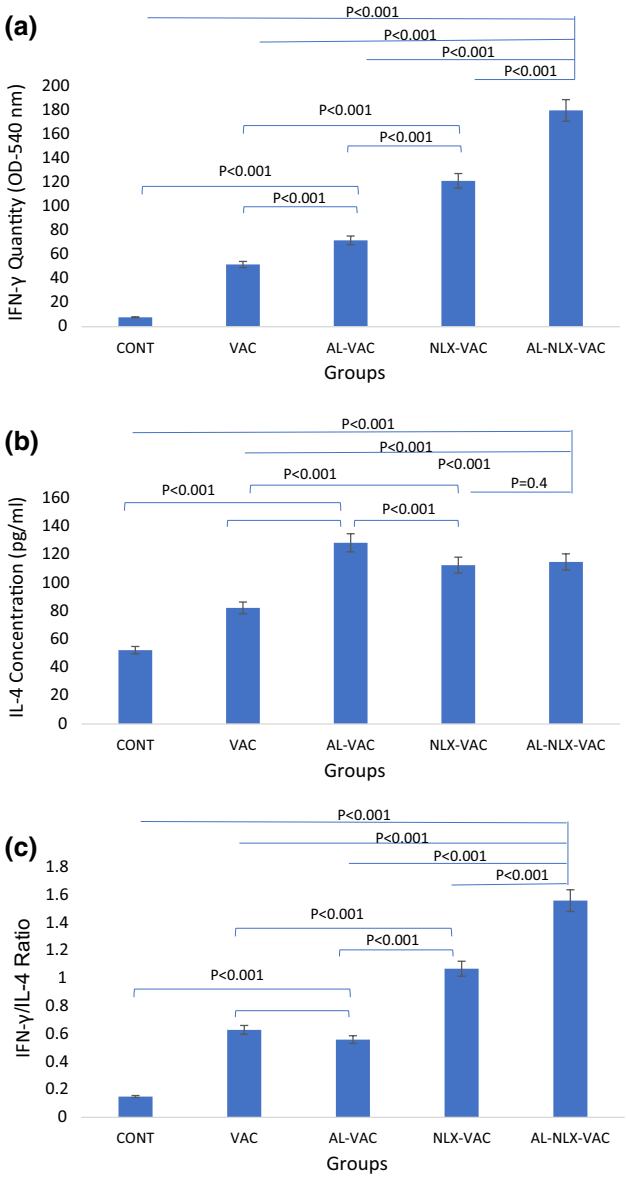

Fig. 3 Concentrations of IFN-γ and IL-4 cytokines in different vaccinated groups; **a** INF-γ, **b** IL-4, and **c** IFN- γ/IL-4 ratio concentration of INF-γ was the highest in animals that their vaccination was accompanied by alum–NLX adjuvant, while the group that was infused with alum adjuvant showed higher IL-4 concentration. *CONT* control, *VAC* vaccine, *AL* alum, *NLX* naloxone

Antibody Levels

As shown in Fig. 4a, a significantly ($P < 0.001$) higher level of anti-*F. hepatica* IgG2a was found in the group of animals infused with E/S antigens combined with the alum–NLX mixture compared with the group that received E/S antigens mixed with alum. However, the difference was not statistically significant compared with the group administrated E/S antigens plus NLX.

The IgG1 level was significantly ($P < 0.001$) higher in the mice administrated E/S antigens and either alum–NLX mixture or alum compared with the groups of mice that received E/S vaccine either with NLX or PBS (Fig. 4b).

The total IgG level showed significantly higher levels in the mice vaccinated with alum–NLX mixture compared to the groups vaccinated with either NLX, or alum ($P = 0.01$) (Fig. 4c).

As shown in Fig. 4d, IgG2a/IgG1 ratio was significantly ($P < 0.001$) higher in the group transfused with NLX compared to the groups that received either the alum–NLX mixture, or alum.

Worm Recovery

Four mice were alive at the end of week 6 in the alum–NLX vaccine group. Fluke enumeration was conducted immediately after the death of mice. The death was preceded by the mice becoming weak, and fatigued, except for the mice that survived until the end which seemed happier. The liver pathological changes in the unimmunized group included discoloring of liver to a grayish-white hue, more thickness of bile ducts, and surface scars on livers at the time of fluke recovery. Nevertheless, microscopical liver pathological changes and liver enzymes were not documented.

The highest protection rate (62.5%) was recorded in the mice infused with alum–NLX as the adjuvant (Table 2). The rate of worm recovery was expressed as mean ± SEM. The group of mice immunized with E/S antigens accompanied by alum–NLX adjuvant showed an average number of parasites as 2.4 ± 0.7 per mouse which was significantly ($P < 0.001$)

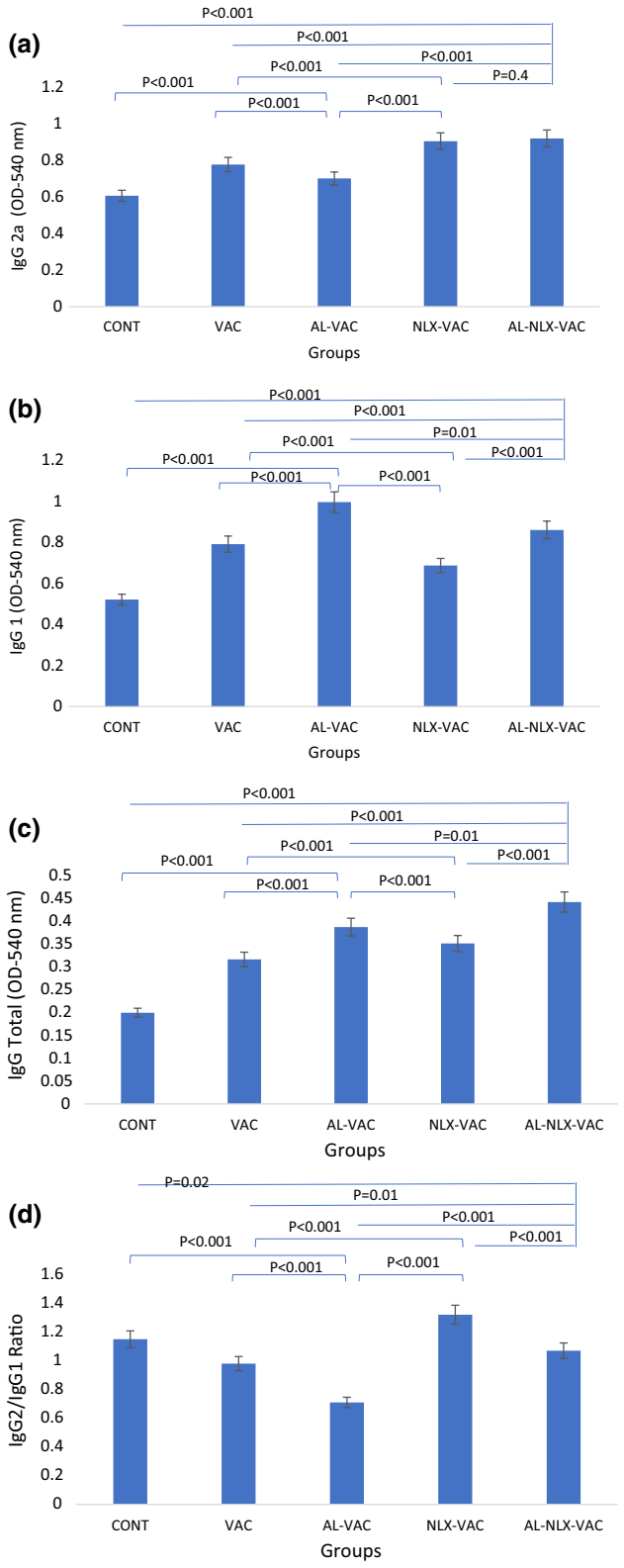

Fig. 4 Concentrations of total IgG2a (**a**), IgG1 (**b**), total IgG (**c**), and IgG2a/IgG1 ratio (**d**) in different groups of vaccinated mice. For all these immunoglobulins, the group that were administrated alum–NLX adjuvant showed significantly higher concentrations compared to the groups that were infused with either NLX or alum adjuvants, or the group that was only infused with vaccine. *CONT* control, *VAC* vaccine, *AL* alum, *NLX* naloxone

lower than the number of worms recovered from animals that received E/S antigens along with either NLX or alum (Fig. 5).

Discussion

Adjuvant is an inevitable part of an effective vaccine. In the present study, the adjuvancity of NLX was assessed in mice vaccinated with E/S antigens of *F. hepatica*. This was the first report on the role of NLX as an adjuvant in a vaccination attempt against fasciolosis. We observed significantly higher lymphocytic proliferation, higher IFN-γ production, and higher IFN-γ/IL-4 ratio in the mice injected with alum–NLX compared to the mice that were vaccinated with alum or without any adjuvant. In addition, higher contents of IgG2a with increased ratio of IgG2a/IgG1 were found in the mice administrated alum–NLX, and NLX adjuvants compared to the mice that received either alum or the vaccine alone. These observations were in accordance with a higher protection rate (62.5%) in the alum–NLX group.

Following *Fasciola* sp. infection, IFN-γ is temporary elevated in the acute phase of infection, however, the levels of IFN-γ decline in chronic fasciolosis [16]. On the other hand, IL-4 is elevated during the acute phase, and remains at high levels during the chronic phase of the disease [17]. This pattern is known as alternative activation of immune system characterized by higher production of immunomodulatory cytokines such as IL-10, IL-4, IL-5, and TGF-β [7, 18]. Interaction of parasite-derived molecules with receptors of immune cells is thought to trigger the immunomodulation process [12, 13, 15, 17, 38]. Researches have been dedicated to avoid non-protective Th2 immune response using opioid receptor antagonists [4, 28, 41].

NLX and naltrexone (NLT), another opioid antagonist, have been used in multiple studies as Th1 immune response-promoting adjuvants [4, 30, 36]. Khorshidvand et al. [30] assessed the efficacy of alum–NLX adjuvant in augmenting cellular immunity in the context of *Toxoplasma gondii* vaccination, and showed a significantly higher proliferative rate of lymphocytes in mice vaccinated with alum–NLX combination which is similar to our results. In addition, vaccine administration with either NLX or a combination of alum–NLX conferred higher production of IFN-γ indicating activation of Th1 response in the recent study [30]. In line with this, the alum–NLX adjuvant applied in vaccination against *Salmonella typhimurium* was associated with expansion of cytotoxic lymphocytes and Th1 response [27, 28]. The alum–NLX mixture showed similar immune-inductive properties as observed in our study (e.g., Th1 response, lymphocytic

Table 2 Protection rates rendered by different vaccination approaches against *F. hepatica*

Groups	Number of parasites per mouse	Number of parasites (mean ± SD)	Protection (%)
Control	5, 8, 6, 5, 6, 7, 8	6.4 ± 1.2	–
Vaccine	7, 6, 7, 7, 5, 5, 6	6.1 ± 0.8	4.6
Alum vaccine	4, 4, 4, 5, 4, 3, 5	4.1 ± 0.6	35.9
Naloxone vaccine	5, 5, 5, 4, 4, 6, 7	5.1 ± 1	20.3
Alum–naloxone vaccine	2, 3, 3, 3, 1, 3, 2	2.4 ± 0.7	62.5

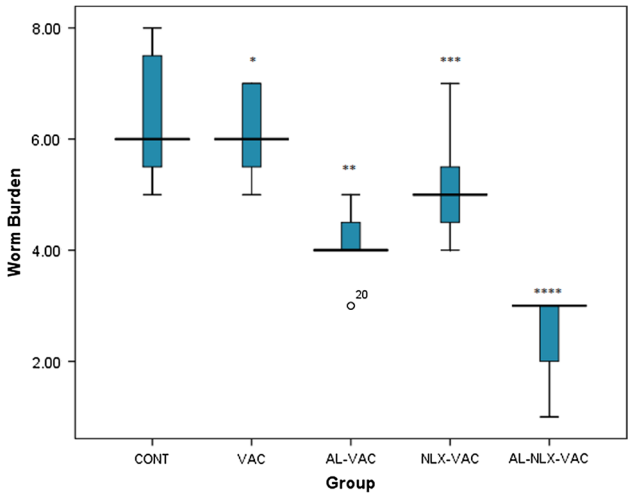

Fig. 5 Worm recovery results in different vaccinated groups compared to control (significant level, ****$P < 0.0001$ for all comparisons, except compared to group AL-VAC with $P = 0.001$), ***$P = 0.06$ (comparison with the groups of CONT and AL-VAC), $P = 0.08$ (comparison with the group of VAC), **$P = 0.001$ (comparison with the groups of CONT, and VAC), *$P = 0.6$ (comparison with the group of CONT). *CONT* control, *VAC* vaccine, *AL* alum, *NLX* naloxone

proliferation and increased IgG immunoglobulin response) when used in vaccinations against HIV [42], human papillomaviruses [47], herpes simplex virus [22], and simplex virus type 1 [21]. Using the combined alum–NLX adjuvant resulted in significantly decreased ratio of cells infected with *Brucella* that was accompanied by higher production of IFN-γ, IL-4, and IgG [36]. NLX was also effective in exploiting a cell-mediated immunity characterized by lymphocytic proliferation and Th1 response against *Listeria monocytogenes* [26]. Similar to our observations, the combination of alum and NLT adjuvant boosted the efficiency of vaccines against *Fasciola hepatica* [4], *Salmonella typhimurium* [28], and *Plasmodium berghei* [41] by induction of lymphocytic proliferation, Th1 response, and production of organism-specific antibodies of IgG2a and IgG1 isotypes with dominance of IgG2a component.

NLX seemed to avoid such immunomodulatory interactions, and can effectively accelerate cell-mediated immunity. In fact, opioid peptides can generate their immunomodulatory effects through both direct interactions with immune cells, and also through hormonal signaling pathways [37]. These peptides are known to interfere with vaccine function against pathogens. NLX may promote its stimulatory effects on immune responses through attenuating opioid-originated effects. In addition, NLX can boost the cellular-based immunity by promoting an inflammatory state, and therefore activating cellular components of innate and acquired immunity [2]. In line with this, NLX promoted cell-mediated immunity through induction of neuropeptide inflammatory molecules. Furthermore, NLX can be associated with depression of regulatory T lymphocytes (CD4 + /CD25 + /FoxP3 +), and this reduction can be accompanied by a simultaneous increase in the number of cytotoxic lymphocytes and higher IFN-γ production [35]. Regulatory T lymphocytes can repress antigen-presenting cells such as dendritic cells (DCs), and further extinguish the cell-mediated immunity. Likewise, alum–NLX adjuvant has also been associated with cytokine profile corresponding to Th17 response which is known as another immunomodulatory process [47]. Although alum and NLX activate different immune responses (Th2 and Th1, respectively), the combined utilization of alum–NLX adjuvant significantly enhanced the Th1 response, which may indicate a synergistic effect for alum in augmentation of NLX-induced responses. This potential synergistic effect may further become of clinical importance regarding the significantly lower protection rate of mice vaccinated with NLX in comparison to the mice administrated with alum–NLX.

In addition to the nature of adjuvant component, types of the antigenic parts of vaccines may also influence the outcomes of vaccination against pathogens [40]. E/S antigen of *F. hepatica* contains different pathogenic molecules at different developmental stages with cathepsin L5, cathepsin L1g and cathepsin B being the prominent antigenic markers in adult, metacercariae, and young *F. hepatica* [24]. In the study of Jayaraj et al. [24], recombinant DNA vaccines of cathepsins B + L5 incorporated with Quil A adjuvant, an emulsion of water and non-mineral oil (Montanide) adjuvant, retrieved the lowest fluke recovery and liver damage index, as well as the highest protection rate compared to mice vaccinated with either single or other combinations of cathepsins B, L5 or L1g (representing

juvenile, adult and metacercariae stages of *F. hepatica,* respectively) in mice infected with *F. hepatica* metacercariae. Furthermore, the recombinant DNA vaccines of cathepsin B expressed in young *F. hepatica* were shown to induce parasite-specific immune responses by boosting high avidity IgG1, IgG2a and IgE immunoglobulins in BALB/c female mice [25]. Previous vaccination experiments against fasciolosis used different adjuvants along with various antigenic determinants. Sansri et al. [39] used a combination of cathepsin L1H peptide of *F. gigantica* along with Freund's adjuvant. Using this vaccination approach represented 66.1% protection in adjuvant-vaccinated mice. This is similar to the protection rate that we observed in mice that were immunized with alum–NLX adjuvant. In a recent study, Kueakhai et al. [32] used recombinant saposin-like protein-1 of *F. gigantica* along with alum adjuvant rendering 73% protection against fasciola infection. However, despite using Freund's adjuvant, the previously mentioned approach activated a Th2 immune response [39]. This phenomenon may propose a role for antigenic components in influencing the nature of immune responses. In other studies, vaccination of mice with *F. hepatica*-derived phosphoglycerate kinase (PGK) peptide resulted in no elevation in Th1 response against the parasite [23, 45]. On the other hand, using non-haemolytic Quillaja saponaria (QS) saponin as an adjuvant along with *F. hepatica* recombinant fatty acid-binding protein (FABP) inhibited production of Th2 regulatory cytokines, and promoted the production of proinflammatory cytokines (IL-1 and IL-6) and IgG2a [43, 44]. In the present study, combination of E/S antigens seemed to accommodate the Th1 inductive properties of NLX. It is recommended, however, to incorporate various antigenic determinants with NLX adjuvant to establish this finding.

Mentioning limitations, we did not determine the IgE levels in immunized mice in our study. However, in the study of Jayaraj et al. [25], it was shown than IgE levels well-correlated with IgG1 and IgG2a immunoglobulins in BALB/c mice vaccinated with *F. hepatica* DNA vaccines. Furthermore, liver pathohistological changes and fluke sizes and biomass were not recorded in this study.

Conclusively, NLX, an opioid receptor antagonist, was revealed as a potential adjuvant to be utilized in E/S vaccines against fasciolosis. Both NLX and alum–NLX adjuvants promoted lymphocytic proliferation and Th1 immune response. However, a combined alum–NLX adjuvant delivered a more potent Th1 immune response and higher protection rate. Considering that NLX is an opioid antagonist, it is recommended to further divulge the molecular determinants involved in opioid-derived signaling. A full knowledge of these pathways will assist us to develop new adjuvants addressing opioid-derived immunomodulatory mechanisms. As the safety profile of NLX has been excellent, it is recommended to consider this agent to be used in human and livestock vaccination against intracellular agents such as *F. hepatica*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experiments were performed according to the Animal Care and Use Protocol of Zabol University of Medical Sciences. The study was approved by Ethical Committee of Zabol University of Medical Sciences.

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