



## Naltrexone; as an efficient adjuvant in induction of Th1 immunity and protection against *Fasciola hepatica* infection

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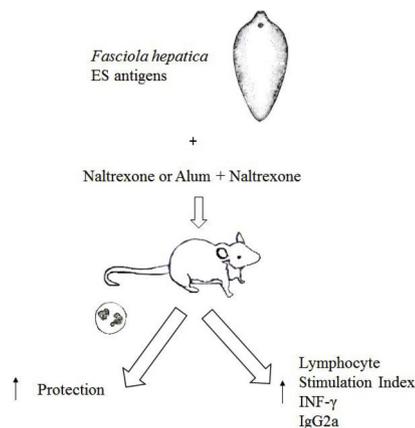
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### HIGHLIGHTS

- Naltrexone adjuvant promotes IFN- $\gamma$  and Th1 immune response against fasciolosis.
- Naltrexone renders higher stimulation index of lymphocytes in immunized mice.
- Naltrexone-adjuvanted vaccine leads to protection against fasciolosis in mouse.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Toxic effects of available therapeutics are major drawbacks for conventional management approaches in parasitic infections. Vaccines have provided a promising opportunity to obviate such unwanted complications. In present study, we examined immune augmenting capacities of an emerging adjuvant, Naltrexone, against *Fasciola hepatica* infection in BALB/c mice. Seventy BALB/c mice were divided into five experimental groups (14 mice per group) including 1- control (received PBS), 2- vaccine (immunized with *F. hepatica* E/S antigens), 3- Alum-vaccine (immunized with Alum adjuvant and E/S antigens), 4- NLT-vaccine (immunized with NLT adjuvant and E/S antigens), and 5- Alum-NLT-vaccine (immunized with mixed Alum-NLT adjuvant and E/S antigens). Lymphocyte stimulation index was assessed by MTT

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assay. Production of IFN- $\gamma$ , IL-4, IgG2a and IgG1 was assessed by ELISA method. Results showed that NLT, either alone or in combination with alum, can induce immune response toward production of IFN- $\gamma$  and IgG2a as representatives of Th1 immune response. Also, using this adjuvant in immunization experiment was associated with significantly high proliferative response of splenocytes/lymphocytes. Utilization of mixed Alum-NLT adjuvant revealed the highest protection rate (73.8%) in challenge test of mice infected with *F. hepatica*. These findings suggest the potential role of NLT as an effective adjuvant in induction of protective cellular and Th1 immune responses against fasciolosis.

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## 1. Introduction

Fasciolosis is a zoonotic infection caused by *Fasciola hepatica* and *Fasciola gigantica* trematodes. Fasciolosis is an infection primarily affecting livestock with financial and nutritional importance. Due to the close contact, humans can also be infected by *Fasciola* species. According to the world health organization report, there are currently 2.4 million affected humans across the globe with 180 million at the risk of the infection (Meemon and Sobhon, 2015). Therapeutic approaches against fasciolosis have encountered increasing drug resistance (Rojas-Caraballo et al., 2014). Because of this, prevention of fasciolosis is a priority for saving funds in both health care system and food industry. In this regard, vaccination policies provide a promising strategy.

Efficiency of vaccines in promoting a potent immune response against infections is dependent on a number of features such as immunogenicity of the antigenic determinants, adjuvant properties, and administration routes (Chantree et al., 2013). Among these, the most important may be the capability of adjuvant compartment to properly modulate immune reactivity. Currently, there is a shortage in the number of approved adjuvants in clinical application mainly due to safety concerns. Alum has been used as an adjuvant for more than seven decades and is currently in used and approved by Food and Drug Administration (FDA) agency. This adjuvant, however, acts in favor of T-helper 2 (Th2) and humoral immune responses and is considered as a desirable adjuvant against extracellular pathogens (Jazani et al., 2010, 2011). Nevertheless, application of alum in vaccines against intracellular organisms has been less promising (Harandi et al., 2010). Therefore, it is advisable to provide alternative adjuvants with ability to augment the cellular immunity against intracellular organisms.

Vaccination experiments against fasciolosis have been performed using different antigenic and adjuvant determinants. However, exploiting conventional adjuvants such as Freund's and alum has been associated with non-protective Th2 immune responses (Sansri et al., 2015; Changklungmoa et al., 2013). Naltrexone (NLT) is an opioid antagonist currently used by drug addicted (Woody, 2014). This prescription drug has a good safety history and is approved by the FDA for using in humans. In previous studies, the role of opioid antagonists including NLT has been noted as a proficient adjuvant in induction of both cellular and humoral immunities against multiple infections. Specially, NLT has been highlighted as an appropriate adjuvant against parasitic infections such as *Toxoplasma* and *Plasmodium* species (Khorshidvand et al., 2016; Tappeh et al., 2013; Shahabi et al., 2014). Nevertheless, there was no study on the potential role of NLT as an adjuvant in vaccination against fasciolosis. In present study, excretory-secretory (E/S) antigens derived from *F. hepatica* were used to immunize BALB/c mice along with NLT adjuvant.

## 2. Materials and methods

### 2.1. Animals

Seventy female BALB/c mice aged 6–8 weeks were prepared from Razi Vaccine and Serum Research Institute, Karaj, Iran. They were kept according to the ethical requirements and animal care instructions announced by Ethical Committee of Zabol University of Medical Sciences. These mice were assigned to one of five immunization groups in our study (14 mice per group). Five selected mice per each group were used for determination of lymphocyte stimulation index, as well as cytokines, and antibody measurements. Seven mice per group were further subjected to the challenge test. Two mice per group that were weaker than others were spared for being used in experiments.

### 2.2. Preparation of metacercariae

*F. hepatica* eggs were obtained from bile ducts and gallbladder of naturally infected sheep that were killed with standard procedure at an abattoir. Before harvesting the eggs, *F. hepatica* parasites were characterized based on morphological features (i.e. shorter length, smaller ventral sucker, and larger cephalic cone) to avoid misidentification as *F. gigantica*. The eggs were then stimulated to shed miracidia by dark incubation in 0.85% normal saline for two weeks, and subsequently illuminated for 2 h. The hatched miracidia were then transferred to *Lymnaea truncatula* with two miracidia per *L. truncatula* snail. After 45 days of the infection, cercariae began to shed from the snails. These were then collected using floating cellophane papers. Cercariae then immediately started to transform to metacercariae which were stored at 4 °C until use.

### 2.3. Obtaining excretory and secretory (E/S) antigens

For this purpose, adult *F. hepatica* parasites were gathered from gallbladder of naturally infected sheep. After collection, the organisms were rinsed in 0.85% normal saline six times to take out remnants of bile ducts and surrounding tissues. These were then cultured in RPMI1640 medium containing antibiotics (penicillin and streptomycin) at 37 °C. For obtaining E/S antigens, the medium was periodically centrifuged every 6 h (15,000 g, 30 min, 37 °C) for 24 h (Kueakhai et al., 2013). E/S antigens enriched in supernatant medium were collected. The supernatants were detected by Lowry's method (Bio-Rad) to determine the protein concentration. These antigens were stored at –80 °C until use.

### 2.4. Immunization

Mice were randomly assigned to one of five immunization groups (14 per group). These groups included: 1- Control (non-

immunized group that received 150  $\mu$ l Phosphate buffered saline (PBS)), 2- Vaccine (mice immunized with 50  $\mu$ l E/S antigens along with 100  $\mu$ l PBS without any adjuvant), 3- Alum-vaccine (immunized with 50  $\mu$ l volume of each of alum, E/S antigens, and PBS), 4- NLT-vaccine (immunized with 50  $\mu$ l volume of each of NLT, E/S antigens, and PBS), and 5- Alum-NLT-vaccine (immunized with 50  $\mu$ l of E/S antigens along with 100  $\mu$ l of Alum-NLT mixed adjuvant). NLT was administrated at the net concentration of 6 mg/kg. For the preparation of the mixed Alum-NLT adjuvant, we initially prepared 6 mg/ml solution of NLT in 50  $\mu$ l of PBS, and then the Alum–NLT mixture was formed by addition of 50  $\mu$ l alum (aluminum phosphate gel, Sigma, Germany) to the mixture. (Jazani et al., 2011; Shahabi et al., 2014; De Gregorio et al., 2008). Immunization was done by intraperitoneally injections at day 0 and 7.

### 2.5. Lymphocyte stimulation index

To assess proliferation response of splenocytes/lymphocytes of mice immunized with different adjuvants, these cells were harvested two weeks following the last immunization. For this purpose, spleens from five mice per group were recovered after killing them, and splenocytes were retrieved in a sterile condition using a wire mesh. The cells were then cultured in RPMI 1640 medium containing 10% fetal calf serum<sup>1</sup> (FCS) (Gibco-BRL), 2 mM L-glutamine, and 25 mM HEPES.

For stimulation experiment, 100  $\mu$ l of splenocytes/lymphocytes suspension was transferred into 96-well plates at a density of  $1 \times 10^6$  cells/ml per well. These cells were subsequently encountered with 10  $\mu$ l of the E/S antigens (10  $\mu$ g/ml) for assessing their proliferation capacity using MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue, Sigma, Germany) assay. The procedure was performed at 72 h post antigen exposure. Stimulation index of the cells was judged by optical density (OD) of the wells at 540 nm, and calculated as follows:

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

For positive and negative controls, the cells were exposed to either 5  $\mu$ g/ml concanavalin A (Sigma), or 10  $\mu$ l of the culture medium respectively.

### 2.6. Determination of IFN- $\gamma$ and IL-4 levels

To assess Th1 and Th2 immune responses, IFN- $\gamma$  and IL-4 cytokines were measured respectively. This was carried out at two weeks after the second immunization. Splenocytes and lymphocytes were obtained from five mice (the same mice used for stimulation index determination) and were cultured as mentioned in the previous section. Medium supernatants were subjected to the cytokine measurements 72 h following the cell culture. IFN- $\gamma$  and IL-4 levels were determined by enzyme-linked immunosorbent assay (ELISA kit, eBscience, USA) according to the manufacturer's instructions. All experiments were performed in triplicate.

### 2.7. Determination of IgG2a and IgG1 levels

Specific ELISA kits for IgG2a (Mouse IgG2a ELISA Ready-Set-Go, 88-50420) and IgG1 (Mouse IgG1 ELISA Ready-Set-Go, 88-50410) were purchased from eBioscience. The antibodies levels in the sera of the mice (the same mice used for stimulation index and cytokine measurements) were determined at two weeks following the last immunization.

### 2.8. The protection rate of immunization experiments

Seven mice immunized in each group were orally transfected with 30 metacercariae after two weeks from the last immunization. Metacercariae were six-weeks old. After six weeks, challenged mice were killed and the parasites were enumerated within their livers. Livers were thoroughly cut into thin tissue segments, and each segment was carefully sought to identify the parasites. The existence of the parasites was also inspected in the peritoneal cavity. Protection rate was calculated as (Changklungmoa et al., 2016):

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

with "A" denotes the mean number of the fluke obtained from the mice of non-immunized (control group), and "B" denotes the mean number of fluke identified in the mice of immunized groups.

### 2.9. Statistical analyses

Statistical procedures were carried out in SPSS software Ver. 21 (Chicago, IL, USA). Normality of data was checked by the Shapiro-Wilk test. Statistical tests were independent sample student t-test, and one-way analysis of variance<sup>2</sup> (ANOVA). *P*-value of less than 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Lymphocyte proliferation

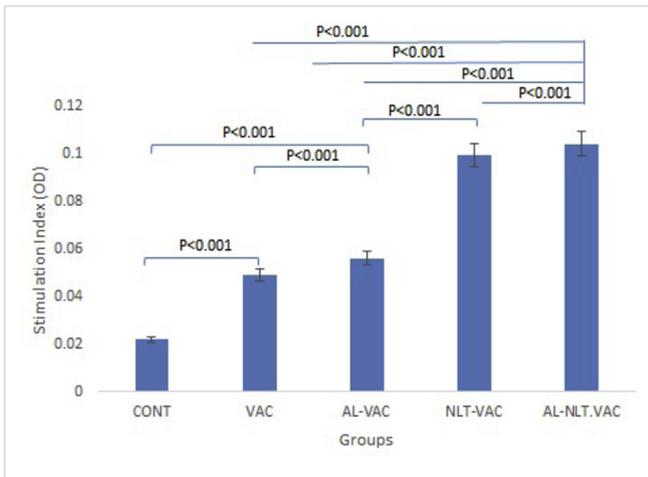
The highest stimulation index was observed in mice immunized with either NLT or Alum-NLT adjuvants. Between these two groups, using the mixed adjuvant was associated with significantly higher stimulation index than NLT alone ( $P < 0.001$ ). In addition, mice immunized with Alum adjuvant showed significantly higher stimulatory index respective to either vaccine or control groups ( $P < 0.0001$ ). Fig. 1 shows results of stimulation experiment in different immunization groups.

### 3.2. Cytokine levels

IFN- $\gamma$  and IL-4 levels were assessed as representatives of Th1 and Th2 immune responses respectively. Results indicated that using combined Alum-NLT adjuvant was the most efficient approach in induction of IFN- $\gamma$ . Following this, the highest IFN- $\gamma$

<sup>1</sup> Fetal calf serum.

<sup>2</sup> One-way analysis of variance.



**Fig. 1.** Stimulation index in different experimental groups. Mice received NLT either alone or in combination with Alum showed higher stimulatory index respective to mice that received Alum or no adjuvant. Abbreviations; Cont; Control, Vac; Vaccine, NLT; Naltrexone, AL; Alum.

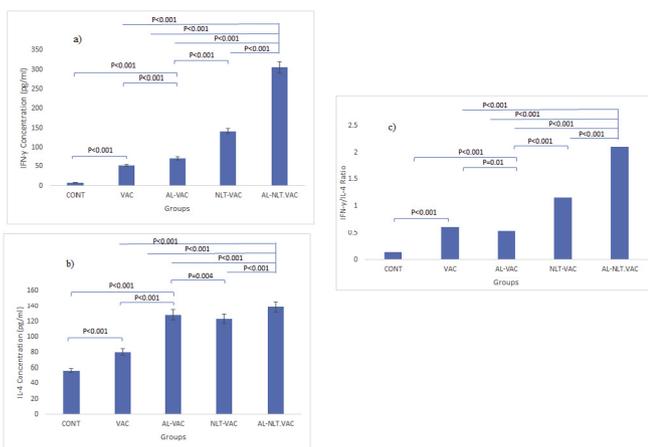
production was related to mice immunized with NLT-vaccine, Alum-vaccine, vaccine, and PBS (Fig. 2a) respectively.

IL-4 production was significantly higher in splenocytes of mice immunized with Alum-vaccine respective to mice immunized with either NLT-vaccine or Alum-NLT vaccine ( $P = 0.004$ , and  $P < 0.001$  respectively, Fig. 2b).

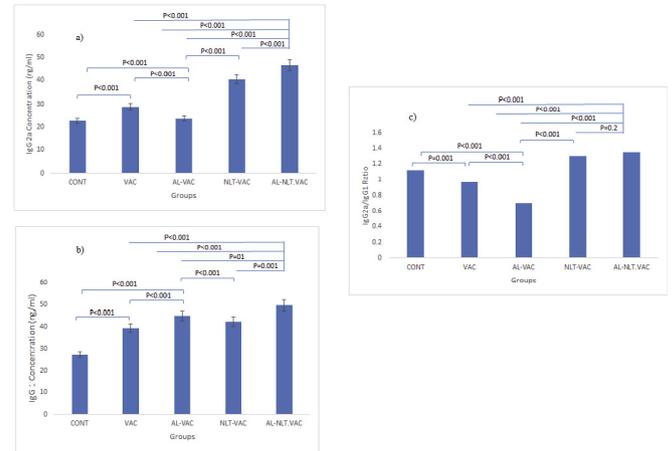
The ratio of IFN- $\gamma$ /IL-4 as an indicator of Th1/Th2 balance was significantly higher in Alum-NLT-vaccine group than either NLT-vaccine ( $P < 0.001$ ), or Alum-vaccine ( $P < 0.001$ ). Fig. 2c shows the IFN- $\gamma$ /IL-4 ratio in different immunized groups.

### 3.3. Antibody levels

Production of parasite specific antibodies plays a substantial role in immunity against parasitic infections. Here we observed that mice immunized with combined Alum-NLT adjuvant showed significantly higher serum levels of IgG2a antibody compared to



**Fig. 2.** Induction of T helper immune response in mice immunized with Alum or NLT adjuvants. Combined Alum-Naltrexone adjuvant induced IFN- $\gamma$  production considerably more than other groups (a), while IL-4 production was significantly higher in splenocytes derived from mice immunized with Alum-vaccine (b). The ratio of IFN- $\gamma$ /IL-4 as an indicator of Th1/Th2 balance was significantly higher in Alum-NLT-vaccine group than other groups (c). Abbreviations; Cont; Control, Vac; Vaccine, NLT; Naltrexone, AL; Alum.



**Fig. 3.** The concentrations of IgG1 and IgG2a in mice immunized with Alum and Naltrexone adjuvants. The levels of IgG2a were significantly higher in NLT-vaccine group respective to Alum-vaccine (a). The highest levels of IgG1 were related to Alum-NLT and Alum adjuvants (b). The IgG2a/IgG1 ratio was significantly higher in mice received NLT or Alum-NLT than other groups (c). Abbreviations; Cont; Control, Vac; Vaccine, NLT; Naltrexone, AL; Alum.

either NLT ( $P < 0.001$ ) or Alum adjuvants ( $P < 0.001$ ). Furthermore, the levels of IgG2a was significantly higher in NLT-vaccine group respective to Alum-vaccine ( $P < 0.001$ , Fig. 3 a).

Fig. 3b shows the concentration of IgG1 antibodies in different immunization groups. The findings showed that IgG1 levels were significantly higher in all the immunization groups compared to the control group. Among vaccinated groups, the highest levels of the antibody were related to Alum-NLT and Alum groups.

The IgG2a/IgG1 ratio as an indicator of the extent of Th1/Th2 immune responses was significantly higher in mice received NLT or Alum-NLT in comparison with those immunized with Alum or E/S antigens alone ( $P < 0.001$ ). The difference between NLT and Alum-NLT groups was not statistically significant ( $P = 0.2$ , Fig. 3c).

### 3.4. Fluke recovery

Using Alum-NLT adjuvant rendered the highest protection rate (73.8%) in the challenge test. This is while the protection rate reached 53.3% and 15.3% in mice received either NLT or Alum adjuvants respectively. The lowest mean numbers of worms recovered from liver of mice belonged to Alum-NLT ( $1.7 \pm 1.3$ ) and NLT ( $3.1 \pm 1$ ) groups (Table 1).

## 4. Discussion

There have been multiple efforts to produce efficient vaccines against fasciolosis. In present study, the role of NLT along with Alum was assessed in mice vaccinated by E/S antigens of *F. hepatica*. Vaccination against fasciolosis has been enrolled with various antigenic determinants such as cathepsin, *Fasciola* proteases, fatty acid-binding proteins, and oxidoreductase enzymes (Sansri et al., 2015). In present study, we exploited E/S antigens of *F. hepatica*. E/S antigens consist of cathepsin proteases as the main constitute (Yap and Smooker, 2016). Cathepsin proteases participate in a wide spectrum of activities including migration, invasion, and pathogenicity of *Fasciola* species (Norbury et al., 2011). Fatty acid binding protein is another major component of E/S antigens of *F. hepatica* which has been shown to suppress the production of inflammatory cytokines by direct interaction with macrophages (Figueroa-Santiago and Espino, 2014). Therefore, E/S antigens render a potential target for development of vaccines against fasciolosis.

**Table 1**  
Protection rates rendered by different vaccination approaches against *F. hepatica*.

Groups	Number of parasites per mouse	Number of parasites (Mean $\pm$ SD) <sup>a</sup>	Protection (%)
Control	8, 7, 6, 7, 6, 6, 6	6.5 $\pm$ 0.7	–
Vaccine	6, 6, 4, 4, 6, 7, 7	5.7 $\pm$ 1.2	12.3
Alum-Vaccine	8, 6, 4, 6, 7, 3, 5	5.5 $\pm$ 1.7	15.3
Naltrexone-Vaccine	2, 3, 3, 4, 3, 5, 2	3.1 $\pm$ 1	52.3
Alum- Naltrexone -Vaccine	1, 0, 3, 3, 0, 3, 2	1.7 $\pm$ 1.3	73.8

<sup>a</sup> ; Significant difference was observed comparing Alum-Naltrexone group with either Alum-vaccine ( $P = 0.001$ ), vaccine ( $P < 0.001$ ) or control ( $P < 0.0001$ ) groups but not with Naltrexone group ( $P = 0.05$ ).

*F. hepatica* can mediate complex interactions with various immune cells including antigen presenting cells (macrophages and dendritic cells), and in this way regulate substantial functions of the immune system (Rodriguez et al., 2015). Generally, such interactions lead to a non-effective humoral Th2 immunity which help persistence of the infection (Dalton et al., 2013). In present study, we showed that using NLT adjuvant either alone or in association with Alum resulted in shifting immune response toward protective Th1 branch represented by higher levels of IFN- $\gamma$  and IgG2a. IFN- $\gamma$  and IgG2a are produced by antigen specific lymphocytes, and are considered as indirect indicators of Th1 immunity (Jamali et al., 2007, 2009). In addition, NLT also induced higher splenocytes/lymphocytes stimulation index. These changes are indicative of a pronounced cellular immunity against the infection.

In other studies, Alum-NLT adjuvant incorporated along with *Toxoplasma gondii* lysate antigen resulted in augmenting of cellular immunity, higher production of IFN- $\gamma$ , and delayed type hypersensitivity response (Khorshidvand et al., 2016). In the recent study, the highest concentrations of IFN- $\gamma$  was recorded in mice immunized with Alum-NLT adjuvant compared to the mice received either NLT or Alum adjuvants which was similar to our findings (Khorshidvand et al., 2016). Increased ratio of IFN- $\gamma$ /IL-5 was observed in the *T. gondii* experiment that highlighted the efficiency of Alum-NLT mixture in promotion of Th1 immune response (Khorshidvand et al., 2016). This was also the case in our study which revealed higher IFN- $\gamma$ /IL-4 ratio in mice received either Alum-NLT or NLT. In another study, Tappeh et al. used Alum-NLT adjuvant in a vaccine along with E/S antigens of *T. gondii* (Tappeh et al., 2013). The results of the recent experiment showed significant induction of specific IgG2a antibodies by using NLT as an adjuvant (Tappeh et al., 2013) which was in line with our findings. Immune induction properties of NLT have also been tested against *Plasmodium berghei* infection (Shahabi et al., 2014). The results of the recent study demonstrated significantly higher lymphocyte proliferation index, and higher IFN- $\gamma$  production in Alum-NLT received mice in comparison with mice treated with either individual NLT or Alum. In line with this, lymphocyte stimulation index was also the highest in NLT-Alum group in the recent study (Shahabi et al., 2014). Altogether, these findings recommend NLT as a potential and efficient adjuvant against parasitic infections including fasciolosis.

Naloxone (NLX) is another opioid antagonist that has been studied as a potential adjuvant in vaccination against various infections. NLX has delivered significantly higher production of IgG2a and IgG1 specific antibodies against *Salmonella typhimurium* (Jazani et al., 2011). In a vaccination effort against HIV, higher lymphocyte proliferation index was observed in mice vaccinated along with Alum- NLX mixture (Velashjerdi Farahani et al., 2016). NLX alone or in combination with Alum also conferred efficient cellular and Th1 immunity in vaccination against human papilloma viruses (Yasaghi and Mahdavi, 2016). Using NLX as an adjuvant also demonstrated boosted cellular and humoral immunities with high production of IFN- $\gamma$  in vaccination against herpes simplex virus type 1 (Jamali

et al., 2007, 2009). Therefore, it seems that opioid antagonists, either NLT or NLX can exert beneficial effects on immune response against intracellular pathogens. This highlights the role of opioid signaling pathways as modulating mechanisms of immune system.

The mechanisms of immunomodulatory functions of opioid antagonists are not well understood. Induction of Th1 immune response is critical for activation of cytotoxic lymphocytes and efficient eradication of intracellular pathogens. NLT may in part promote its immune regulating activities by induction of the cytotoxic activity of natural killer cells (Boyadjieva and Sarkar, 2010). NLT also can promote toxic activities of macrophages (Yi et al., 2016). The effects of opioid receptors on immune responses may be partly mediated by modulation of CD4<sup>+</sup>CD25<sup>+</sup> + Foxp3<sup>+</sup> regulatory T lymphocytes (Molla Hassan et al., 2009). In a report, NLX resulted in significant elevation of IL-17 immunoregulatory cytokine (Yasaghi and Mahdavi, 2016). Using NLX has also been related to higher counts of CD8<sup>+</sup> and lower counts of CD4<sup>+</sup> lymphocytes (Molla Hassan et al., 2009). In an experiment by Meng et al., NLT induced expression of multiple stimulatory and co-stimulatory molecules including MHC II, CD40, CD80, and CD86 on antigen presenting dendritic cells (Meng et al., 2013). These immunoregulatory effects accompanied by induction of lymphocytes proliferation and secretion of IL-2 and TNF- $\alpha$  (Meng et al., 2013). Synergistic effects NLX with other adjuvants on promoting cellular immunity has been highlighted in vaccination of animal models against *Plasmodium vivax* (Someabozorg et al., 2015). Detailed mechanisms participating in immune modulating activities of opioid antagonists are to be disclosed in future.

We showed in our experiment that Alum-NLT adjuvant resulted in the highest protection rate (73.8%) against *F. hepatica* infection. This is while NLT or Alum alone only resulted in 52.3% and 15.3% protection rates respectively. In comparison, mice immunized with Alum-NLT mixture showed significantly higher survival rate in challenge test against *T. gondii* (Khorshidvand et al., 2016). Vaccination experiments using NLX rendered significant protections against herpes simplex virus type 1 in mice (Jamali et al., 2007, 2009). In challenge test against *Salmonella typhimurium*, mice received Alum-NLX adjuvant were showed significantly lower bacterial load in their spleens and livers compared to the mice treated with either NLX or Alum alone (Jazani et al., 2011). The higher protection rates may be partly related to the induction of proliferative, cytotoxic and IFN- $\gamma$  producing activities of lymphocytes by opioid antagonists (Velashjerdi Farahani et al., 2016). Evidences are emerging that accentuate the role of antigenic epitopes in influencing protection rates in mice vaccinated against fasciolosis (Rojas-Caraballo et al., 2014). Using *F. gigantica* derived Leucine aminopeptidase as antigenic determinants along with Freund's adjuvant resulted in significant protection rate (60.8%). (Changklungmoa et al., 2013). Similar results were produced by vaccination with cathepsin L as the antigenic peptide which delivered protection rate of 62.7% (Sansri et al., 2015). It seems that protection against infections is dependent on both adjuvant and antigenic peptides.

## 5. Conclusion

NLT, either individually or in combination with Alum, can be an efficient adjuvant for induction of Th1 and cellular immunity responses against *F. hepatica* infection. In accordance, NLT can dedicate significant protection against the infection in immunized mice. NLT can serve as a potential adjuvant in vaccine production efforts against fasciolosis.

## Conflicts of interest

None.

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