Original Article

A Comparative Study of the Effects of Al(OH)3 and AlPO4 Adjuvants on the Production of Neutralizing Antibodies (NAbs) against Bovine parainfluenza Virus Type 3 (BPIV3) in Guinea Pigs

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ABSTRACT

Aluminum-containing adjuvants are extensively used in inactive human and animal vaccines owing to their favorable immunostimulatory and safe properties. Nonetheless, there is controversy over the effects of different aluminum salts as an adjuvant for the bovine parainfluenza virus type 3 (BPIV3) vaccine. In order to find a suitable adjuvant, we studied the effects of two adjuvants (i.e., aluminum hydroxide [$Al(OH)_3$] and aluminum potassium sulfate [$AlPO_4$]) on the production of neutralizing antibodies (NAbs) for an experimental BPIV3 vaccine. The animals under study (Guinea pigs) were randomly assigned to five groups of experimental vaccines containing $Al(OH)_3$ (AH), $AlPO_4$ (AP), $Al(OH)_3$-$AlPO_4$ mixture (MIX), commercial vaccine (COM), and control (NS). The treatment groups were immunized with two doses of vaccine 21 days apart (on days 0 and 21), and the control group received normal saline under the same conditions. The animals were monitored for 42 days, and blood samples were then taken. The results indicated that all vaccines were able to induce the production of NAbs at levels higher than the minimum protective titer (0.6). An increase in titer was observed throughout the monitoring period. Moreover, an increase in both the level and mean titer of NAbs obtained from the vaccine containing $Al(OH)_3$ adjuvant was significantly higher than in the other studied groups ($P \leq 0.005$). The comparison of NAbs titer in other groups did not display a significant difference. Considering the speed of rising and the optimal titer of NAbs production in the experimental vaccine, the $Al(OH)_3$ adjuvant is a suitable candidate for preparing a vaccine against BPIV3 for immunization.

Keywords: Aluminum adjuvant, Bovine Parainfluenza virus type-3, Neutralizing antibody, Vaccine

1. Introduction

The term adjuvant, which means to help or cooperate, is derived from the Latin word *adjuvare* (1, 2). The addition of adjuvants to inactivated, subunit, or recombinant vaccines increases their efficiency and duration of immunity (3, 4). It reduces the frequency of vaccination (3, 5) and antigen dose, improves the quality of the immune response, and in some cases, improves the stability of the vaccine (1, 3, 4). Adjuvants also act as a modulator of the immune system response, selectively directing it toward the response of different antibody isotypes, immunoglobulin G subclasses, major histocompatibility complex (MHC) classes, and the types of T helper cells in humans and animals (5).

Adjuvants are currently classified into nine categories, namely mineral salts, emulsions, tensioactive compounds, derivatives from microorganisms, particulate antigen delivery systems (immune-stimulating complexes, liposomes, polymer microspheres, nano-beads, antigen delivery systems, virus-like particles), toll-like receptors (TLRs), polysaccharides, cytokines, and nucleic acids (2). Aluminum-based adjuvants, emulsions, liposomes, and microparticles are the first generation of adjuvants used (3). For the first time, Glenny et al. (1926) discovered the effect of aluminum salt on stimulating the immune system (1, 4). Different aluminum salts, such as aluminum phosphate, aluminum hydroxide ($Al(OH)_3$), aluminum potassium sulfate ($AlPO_4$), and amorphous aluminum hydroxyphosphate sulfate, are widely used in approximately one-third of licensed human vaccines (3). Due to the favorable safety and immunity properties of aluminum adjuvants, some believe that this category of adjuvants should be considered the gold standard for evaluating all new adjuvants (6, 7).

The exact mechanism of action of aluminum adjuvants is still unknown; nonetheless, the proposed mechanisms are the formation of antigen depots, induction of inflammatory response, and reduction of antigen degradation (4, 8). They also act as an antigen delivery system which provides continuous stimulation of the immune system (3). Aluminum adjuvants do not use classical TLRs and myeloid differentiation factor 88 or Toll/IL-1R domain-containing adapter inducing interferon-β signaling pathways to activate innate immunity. Instead, they act by using nucleotide oligomerization domain (NOD) receptors, such as NOD-like receptors, through direct activation of the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing inflammasome complex or by releasing uric acid (4). The injection of these kinds of adjuvants causes cell damage and release of uric acid, adenosine triphosphate, and cellular DNA. These factors activate dendritic cells (DCs) by binding to their pattern recognition receptors, leading to inflammation. This phenomenon causes the attraction of antigen-presenting cells, increased phagocytosis, and activation of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 inflammasome (3, 9, 10), resulting in the release of interleukin (IL)-1 and IL-18 through caspase 1 (3, 11). DNAs of the dying cells cause DCs to bind more tightly to T helper cells (12).

Studies have demonstrated that aluminum adjuvants increase antigen accumulation (100 times) and antigen presentation (10 times) by DCs, compared to soluble protein without adjuvants. This process is achieved by reducing the protein degradation in DCs and increasing the amount and duration of expression of peptide-MHC complexes on the DCs surface (13). Aluminum adjuvant also activates the complement system via the breakdown of the C3, the activation of the membrane attack complex, and the release of the pro-inflammatory peptides of anaphylatoxins (C5a, C3a) (14). Neutrophils, monocytes, and to a lesser extent, macrophages and eosinophils increase 24 hours after intramuscular injection of aluminum adjuvant (9, 12). Aluminum rearranges certain lipids by binding to the cell membrane. DCs uptake the antigens and

take them to the lymph nodes. They attach tightly to Th2 cells and induce a strong immune response, eventually triggering the release of antibodies from B cells (1, 3, 10). Although the significance of adding aluminum to vaccines has been generally described, the effects of different aluminum salts across different types of vaccines remain to be elucidated.

While the consequences of adding aluminum to vaccines have been discussed broadly, no systematic review has been conducted to assess the effects of aluminum adjuvants across different types of vaccines. Bovine parainfluenza virus type3 (BPIV3), with the official name "bovine respirovirus 3" belongs to the genus *Respirovirus* and the *Paramyxoviridae* family and causes asymptomatic infections to severe respiratory involvement and pneumonia, as well as abortion and mastitis. Vaccination is one of the most effective ways to prevent severe effects caused by bovine parainfluenza. In this study, the effects of $Al(OH)_3$ and $AlPO_4$ salts were investigated on the production of neutralizing antibodies (NAbs) against an inactivated BPIV3 vaccine. Two adjuvants (separately and combined) were examined, and Hiprabovis 3, a commercial vaccine, was utilized to compare.

2. Materials and Methods

2.1. Vaccines

2.1.1. Experimental vaccines

Experimental vaccines were produced from an Iranian isolate of bovine parainfluenza virus (data not published) and used as an active ingredient along with H1H9 cells. H1H9 is a sheep lymphoid suspension cell line that was established in the Razi Vaccine and Serum Research Institute (15). Bulk vaccines were inactivated with 4 mM BEI and formulated in three ways by adding 1.7% aluminum phosphate, 1.3% aluminum hydroxide, and a combination of both in equal quantities. Thereafter, quality control tests (sterility, potency, identity, and innocuity) were performed. All vaccines were produced on the basis of protocol for the production of inactivated viral vaccines at the Razi Vaccine and Serum Research Institute and according to the World Organization for Animal Health (WOAH) (30).

2.1.2. Commercial vaccine

For comparison purposes, a commercial trivalent inactivated vaccine, Hiprabovis3, containing viruses of IBR, PIV3, and BVD, manufactured by Laboratorios Hipra, S.A. Spain, Batch No. 9N90-2, and including aluminum hydroxide adjuvant was used.

2.2. Razi bovine kidney cells

Razi bovine kidney (RBK) cell line, also known as Iran Razi Khedmati Bovine Kidney, was registered in the National Cell Bank of Iran with accession number C451. This cell, which is suitable for the growth of BPIV3, was used in titration and virus neutralization tests (VNT) (17).

2.3. Immunization

A total of 25 healthy guinea pigs weighing 400±50 g were randomly assigned to four treatment groups, including experimental vaccine containing $Al(OH)_3$ (AH), $AlPO_4$ (AP), $Al(OH)_3$-$AlPO_4$ mixture (MIX), commercial vaccine (COM), and one control groups NS (5 heads each). A period of seven days was considered for the adaptation of the animals to the new environment and control of vital signs. One day before immunization (day zero), blood samples were collected from all guinea pigs, and sera were stored at -20°C. Animals in the treatment groups were vaccinated subcutaneously with two doses of relevant vaccines on days 0 and 21. The control group also received normal saline with the same dose. According to the WOAH protocol, one-fifth of the cattle dose is needed for guinea pigs (18). Blood samples were collected from all guinea pigs on days 21 and 42. The sera were immediately separated and stored at -20°C. Following that, all sera were subjected to VNT.

2.4. Virus neutralization test

After inactivating at 56°C for 30 minutes, all the

sera were serially two-fold diluted using DMEM. For each dilution, three wells were considered on microplate. A volume of 50 µl of guinea pigs serum was added to each well along with 50 µl of virus suspension (100 $CCID_{50}$/well). To evaluate the accuracy of the test, some wells were considered as control. The volumes of 100 µl of virus dilutions of 0.1, 1, 10, and 100 $CCID_{50}$/well were added to five wells. The microplates were placed in a CO_2 incubator at 37°C for an hour. Thereafter, 100 µl of the cell suspension containing 2-4 × 10^4 RBK cells was added to each well, and the microplates were placed in a CO_2 incubator at 37°C for 10 days. After the incubation period, the plates were evaluated for cytopathic effects. The virus titer (VN50) was calculated using the Kerber method (16). According to references, the acceptable titer was considered titer $Antilog_{10}$ 0.6 (19), which represents the minimum protective titer against BPIV3.

2.5. Safety test

While monitoring the vaccinated guinea pigs, twice the immunization dose (0.2 ml) was used to conduct the safety test. The number of 10 guinea pigs (200-250 g) and 30 rats (17-22 g) were used for the safety test. A volume of 0.4 ml of each vaccine was injected intramuscularly into two guinea pigs, and 0.1 ml of each vaccine was injected intraperitoneally into six rats. Two guinea pigs and six rats were considered negative controls.

2.6. Statistical analysis

The data were statistically analyzed in SAS 9.4 software in the form of "Repeated Measures Over Time" using the MIXED procedure.

3. Results

The safety test of experimental vaccines during the monitoring period did not show any local or systemic reactions in guinea pigs and rats. Moreover, temperature monitoring in the vaccinated groups was within the normal range (Table 1). Until the 21st day after the injection of the vaccines, in all treatment groups, NAbs titers increased and reached more than the protective level. The results of injecting experimental and commercial vaccines during 42 days of monitoring are displayed in table 2. The results until day 21 illustrated that increasing titer in the AH group was significantly higher than that in other groups (P≤0.05). In addition, the AH group had a

Table 1. Mean rectal temperature in guinea pigs during 14 days monitoring

		Temp.(˚°C)												
Groups	**Day**	**1**		**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
AH		38.8	38.8	38.4	38.4	38.4	38.6	38.5	38.5	38.8	38.8	38.7	38.6	38.6	38.5
AP		38.6	38.6	38.6	38.6	38.6	38.5	38.4	38.5	38.7	38.7	38.6	38.6	38.6	38.6
MIX		38.6	38.6	38.5	38.6	38.6	38.6	38.5	38.6	38.6	38.8	38.8	38.5	38.5	38.5
COM		38.5	38.5	38.4	38.7	38.5	38.5	38.4	38.5	38.5	38.6	38.4	38.7	38.7	38.6
NS		38.6	38.4	38.4	38.6	38.6	38.8	38.8	38.6	38.6	38.6	38.5	38.5	38.6	38.6

Normal temp:38-39.5 (˚°C)

Table 2. NAbs titer on day 0, 21 and 42, following administration of experimental and commercial vaccines

Day	**Experimental groups**					**SEM**
	AH	**AP**	**MIX**	**COM**	**NS**	
0	0.24^a	0.22^a	0.23^a	0.22^a	ND^b	0.02
21	1.47^a	0.93^b	0.74^b	0.75^b	ND^c	0.08
42	1.61^a	1.73^a	1.56^a	1.54^a	ND^b	0.10

COM = commercial vaccine, AH = vaccine formulated with Al(OH)3 adjuvant, AlPO4 = vaccine formulated with AlPO4 adjuvant, MIX = vaccine formulated with Al(OH)3 and AlPO4 adjuvants, NS = normal saline. SEM: Standard Error of the Mean.
In each row, the numbers marked with a, b, c were significantly different (P<0.05).

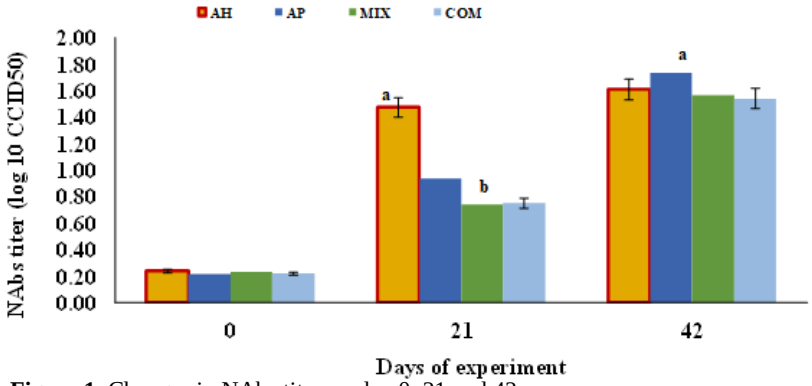

Figure 1. Changes in NAbs titer on day 0, 21 and 42.

higher level than other groups on the 21st day, and this difference was significant (P≤0.05). During days 21 and 42, the NAbs titers of MIX, COM, and AP groups demonstrated a significant increase (P≤0.05).

On day 42, there was no significant difference between NAbs titers (P≤0.05). The titer increase in the commercial and MIX group exhibited a similar trend. A more than four-fold increase in the titer of NAbs (protective titer) was observed in the AH group before day 21, in the AP group on day 21, and in the other two groups shortly after (Table 2, Figure 1). Both MIX and COM groups showed relatively similar effects in inducing antibody response, and no significant difference was observed between these two groups (P≤0.05). The mean titer of NAbs obtained from the vaccine containing $Al(OH)_3$ adjuvant was significantly higher than other vaccines, except the vaccine containing $AlPO_4$ (P≤0.05). Nonetheless, there was no significant difference in the titer of NAbs produced in other groups (Table 3).

Table 3. Mean titer of NAbs after administration of experimental and commercial vaccines

Experimental groups	NAbs titer(Antlog10)
AH	1.06^{a}
AP	0.96^{ab}
MIX	0.84^{b}
COM	0.83^{b}
NS	ND^{c}
SEM	0.05
P value	P<0.05
Treatment	<0.01
Time	<0.01
Treatment × Time	<0.01

4. Discussion

BPIV3, as one of the most important agents of the bovine respiratory disease complex, causes high economic losses in the cattle population across the globe; therefore, it is necessary to use a vaccine to prevent this disease. Due to the lack of a local vaccine (Homologues) against this disease in Iran, we examined two aluminum salt adjuvants (i.e., hydroxide and aluminum phosphate) to develop the most appropriate adjuvant for an experimental vaccine. It is noteworthy that the effectiveness of vaccination depends on various factors, such as vaccine formulation, aluminum dose, aluminum absorption, antigen dose and purity, administration method, and vaccination program.

The results of titration of NAbs in all the animals tested on day zero confirmed the absence of exposure to the BPIV3 virus and the absence of specific antibodies. On the other hand, after vaccination, the antibody titer increased, and its level was higher than the protective level at the end of the monitoring period. This signifies that vaccines are effective and successful in inducing suitable and acceptable titers of NAbs and indicates the suitable potency of vaccines. The high titer of NAbs (more than acceptable criteria) and the induction of protective immunity in a short period after vaccination were caused by the proper stimulation and immune response by the adjuvants in the used vaccines. These adjuvants have stimulated the humoral immune

system and antibody secretion by releasing danger signals, inducing an inflammatory response, and gradually releasing antigens. Furthermore, the NAbs titer of the vaccine adjuvanted with $Al(OH)_3$ increased faster than others. This is especially important in infected areas where it is necessary to induce effective immunity quickly. This is in line with the results of the application of aluminum hydroxide gel and its effect on increasing the production of neutralizing antibodies in goats and pigs (22).

Researchers obtained favorable results using similar adjuvants. It has been demonstrated that the use of $AlPO_4$ adjuvant in DNA vaccine induces a much higher NAbs titer (23). Issa et al. (2014) indicated that $AlPO_4$ adjuvant was more effective than calcium phosphate (24). In a similar vein, Mahboubi et al. and Liang et al. pointed out that the $AlPO_4$ adjuvant was more effective than aluminum hydroxide in the induction of NAbs in the hepatitis B recombinant vaccine (25, 26). Moreover, Akbarian et al. (2021) reported that the use of peste des petits ruminants vaccine containing $Al(OH)_3$ and $AlPO_4$ adjuvants, each separately, induced sufficient protection in rats, guinea pigs, sheep, and goats (27). Considering the upward trend of titer rise in all vaccines, the increase and duration of immunity could be expected until the end of the monitoring period.

Regarding the difference in the antibody induction process by studied adjuvants, the following can be mentioned: different physical and chemical properties of $Al(OH)_3$ and $AlPO_4$ have diverse functions on the innate immune system and regulate the processes related to the body's immune system differently. For instance, intramuscular $Al(OH)_3$ adjuvant injection induces neutrophil recruitment, while $AlPO_4$ adjuvant attracts monocytes/macrophages to the injection site (28). Moreover, different particle sizes of adjuvants can determine the level of antigen absorption. This can affect antigen conformation and the presentation of functional epitopes to the immune system (29). On the other hand, different properties of combining adjuvants with antigens cause the antigen to be absorbed on the surface of the adjuvants to a different extent and may even undergo structural changes; as a result, this combination affects the stability of the antigen (29).

As evidenced by the obtained results, all investigated vaccines induced favorable NAbs titer. Nevertheless, the rapid titer increase in the vaccine adjuvanted with $Al(OH)_3$ and the higher titer during the monitoring period indicated that $Al(OH)_3$ could be a suitable candidate for the production of an inactive vaccine against BPIV3.

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Authors' Contribution

Study concept and design: Lotfi.

Acquisition of data: Heidary.

Analysis and interpretation of data: Lotfi. and Heidary.

Drafting of the manuscript: Heidary.

Critical revision of the manuscript for important intellectual content: Lotfi., Nikbakht., Hajizadeh.

Statistical analysis: Yousefi

Administrative, technical, and material support: Lotfi, Nikbakht, Hajizadeh.

Ethics

All the procedures were approved by Medical Ethics Committee of the Veterinary Faculty of Tehran University, Tehran, Iran (Certificate No:IR.UT. VETMED.REC.1401.008).

Conflict of Interest

The authors declare that they have no conflict of interest.

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