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Evaluation of Immune Responses and Histopathological Effects against Gamma Irradiated Avian Influenza (Sub type H9N2) Vaccine on Broiler Chicken

Shadi Javan[1]
https://orcid.org/0000-0001-7383-993X

Mehrouz Dezfoolian[1]
https://orcid.org/0000-0002-1003-2896

Farahnaz Motamedi-Sedeh*[2]
https:// orcid.org/0000-0003-3388-9670

Viskam. Wijewardana[3]
https://orcid.org/0000-0002-5787-8792

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Alborz, Iran; [2]Nuclear Science and Technology Research Institute, Nuclear Agriculture Research School, Veterinary and animal Science Department, Karaj, Alborz, Iran; [3]International Atomic Energy Agency (IAEA), Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, Animal Production and Health Laboratory, Vienna, Austria.

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*Correspondence: farah.motamedi@gmail.com; fmotamedi@aeoi.org.ir; Tel.: +98-26-34252322

HIGHLIGHTS

- Gamma Irradiated Avian Influenza (H9N2) Vaccine was prepared by 30 kGy irradiation.
- Broiler chickens were vaccinated by two routs; intranasal and subcutaneously.
- All the vaccinated chickens showed a significant increase in antibody titration.
- The most increasing of immune responses was in irradiated vaccine plus Trehalose.

Abstract: Vaccination is a good strategy for the prevention of avian influenza virus. In this research Gamma Irradiated Avian Influenza (Sub type H9N2) Vaccine (GAIV) was prepared by 30 kGy irradiation and used for vaccination of broiler chickens. The purpose was a comparison of immune responses in the two routes of administration for the GAIV vaccine; intranasal and subcutaneously, use of Montanide ISA70 and Trehalose accompanied with irradiated vaccine and compare with formalin vaccine. The Influenza Virus A/Chicken/IRN/Ghazvin/2001/H9N2 was irradiated and used for vaccine formulation, and formalin inactivated AIV was used as conventional vaccine. Chickens were vaccinated by GAIV with and without Trehalose, GAIV and formalin vaccines with ISA70, two routes of administration were intranasal and subcutaneously. All the vaccinated chickens showed a significant increase in antibody titration. The most significant increase of antibody titration was in irradiated vaccine plus Trehalose groups intranasal and subcutaneously. After the first and second intranasal vaccination, the amount of IFN-gamma increased in the irradiated vaccine plus Trehalose group compared to other groups. However, most of the vaccinated groups did not show any significant increase of IFN-α concentration. Histopathological examination revealed lymphocyte infiltration (++), foci dispersed of hemorrhage and edema in intranasal vaccination groups and in addition to these, thickening of alveolar septa was observed in the injection groups. GAIV vaccine can be a good candidate for vaccine preparation, and Trehalose as a stabilizer protects viral antigenic proteins, also makes more absorbance of antigen by the inhalation route. In vaccinated chickens the ulcers in injected vaccines were lower than intranasal vaccines.

Keywords: avian Influenza; gamma irradiation; vaccine; immune response; histopathology.

INTRODUCTION

Influenza virus is a member of *Orthomyxoviridae* family, it has 5 genera including: *Influenza A, B, C, Thogotovirus,* and *Isavirus* [1]. Avian influenza virus (AIV) related to genus influenza A has segmented negative sense RNA, two surface glycoproteins HA and NA. There are 16 HA and 9 NA sub types [2,3].

The AIV is divided into two groups: low pathogenic avian influenza (LPAI) and high pathogenic avian influenza (HPAI). There is a possibility that LPAI mutate and changes to HPAI. The AIV sub type H9N2 distinguished as LPAI. Wild birds are the physical storage for the AIV and sometimes this virus transmits to domestic birds including hens, chickens, ducks, goose and swan [4-8]. LPAI can break species barrier and cause infection in pig, horse, and human [9]. Vaccination is a good policy for infectious diseases such as avian influenza virus because it reduces complications of AIV in bird [10,11]. The chemically inactivated influenza virus vaccines provide surface antigens or split product. These vaccines have low immunogenicity than live attenuated whole virus vaccine because surface antigens including HA and NA can be altered by chemical treatment thus immunological epitopes essential for vaccine efficacy are lost [12]. Alternatively, there are many methods for inactivation of influenza virus including heat treatment, ionizing radiation, UV irradiation and gamma irradiation [12].

Because of the antigenic variations in the influenza virus, designing a new vaccine that induces a broadly protective immune response is essential [13,14]. It is shown that gamma- irradiated influenza A virus (γ-Flu) as a vaccine is effective. It can induce T cell responses and provides cross-protective immunity against seasonal and pandemic influenza virus [5,13]. The gamma-irradiation dose is very important because the level of structural damage of proteins is dose and temperature dependent [5].The gamma irradiation has higher penetration, little effect on the antigenic construction if used at the right dose and it breaks RNA strand of virus, therefore it is the perfect method for virus inactivation [13,14]. Consequently, there are two mechanisms of virus inactivation by gamma irradiation: direct and indirect. Direct virus inactivation by gamma irradiation is mainly caused by radiolytic cleavage or cross linking of genetic material. Indirect effects of gamma irradiation are caused by the action of radicals such as OH, due to the radiolytic cleavage of water, and ozone which created from the radiolytic cleavage of O_2. Viral nucleic acid and viral protein can react with these molecules. Stop of viral nucleic acid replication, via both direct and indirect effects, is the main mechanism of virus inactivation by gamma irradiation [15-19]. The specific aims of this study is to compare immune responses in two routes of the administration of gamma irradiated Avian Influenza sub type H_9N_2 Vaccine (GAIV); intranasally and subcutaneously and the use of Trehalose and Montanide ISA70 accompanied with vaccine administration through prevention of virus shedding in vaccinated chicken after challenge.

MATERIAL AND METHODS

Virus and Vaccines

The virus used in our study was AIV sub type H9N2 strain, A/Chicken/IRN/Ghazvin/2001, obtained from the Razi Vaccine and Serum Research Institute of Iran. The optimum dose of gamma irradiated dose and inactivation of virus samples was carried out according to the procedure described by Salehi and coauthors [20]. Briefly; after multiplication of AIV on embryonated SPF (Specific Free Pathogen) chicken eggs, the allantoic fluids were tested using hemagglutination test for Hemagglutinin antigen (HA) titration [20] and virus titration via embryo infective dose (EID50) of infected allantoic fluid was calculated according to the Reed and Muench formula [21,23], Gamma ray dose 30 kGy was recommended for the frozen virus samples [20]. The Nordian model 220 gamma cell instruments was used for irradiation of AIV in a dose rate of 2.07 Gy/s and activity of 8677 Ci for virus inactivation. Gamma-irradiated AIV was stored as an irradiated vaccine plus 20% Trehalose (1M) (a disaccharide of glucose, as a protein protectant) under frozen conditions (-70 °C). Gamma irradiated AIV was used as irradiated vaccine. Formalin vaccine was prepared according to Razi protocol, briefly; a formalin concentration of 0.1% at 25 °C inactivated the virus for 24 h and used as formalin-vaccine [23]. In addition we used Montanide ISA 70 as an adjuvants along with irradiated vaccine and formalin vaccine. Injectable vaccine and stable Water-in-oil (W/O) emulsions were obtained by mixing Montanide ISA70 and antigenic media (H9N2) under a high shear rate. Then, 20 % of Trehalose (1 M) was added to irradiate inactivated AIV. The vaccine groups were according to table 1: sterile PBS (IN), Irradiated AIV (the two routes of administration; intranasal (IN) and subcutaneous (SC)), irradiated AIV with Trehalose (the two

routes of administration; intranasal and subcutaneous), formalin AIV (IN), formalin AIV with ISA70 (SC), irradiated AIV with ISA 70 (SC).

Chicken Experiment

A total of 160 one day-old broiler chicks (Ross 308) were purchased from the Hen & Chicken of Alborz Company (Iran), individually weighed and randomly distributed for treatments. The Light was provided continuously for the first three days post hatch, after that 23 hours light and 1 hour dark (23L:1D) was provided. At one day of age, the room temperature was set at 33 °C and subsequently reduced by 2 °C/week. Birds had free access to water and feed a corn-soybean meal-based diet. Diets were fed during the 3-phase feeding schedule: starter (0 to 14 d), grower (14-22 d), and finisher (24-48 d). All diets were mashed and formulated to meet or exceed the minimum requirements for broiler chickens according to Ross 308 catalogue (2014) [22]. The chickens were divided into 16 groups, one group was negative control, and 8 groups received one dose of vaccine at 11 days age, and sampling was done two weeks after the first vaccination. The other 8 groups received two doses of vaccines at 11 days (prime) and at 25 days (booster) and sampling was done two weeks after the second vaccination. The route of administration was either intranasal (IN); drop on the nose or injection; subcutaneously (SC) in the back of the neck. The negative control and vaccinated groups were housed at the same condition in two separate places. The chickens in all of the groups were weighed, bled and humanely sacrificed and necropsy of the lung was performed for histopathological studies. The blood samples were used for neutralizing antibody titration via the *hemagglutination* inhibition (HI) test [23].

Specific Neutralizing Antibody Response by HI test

Briefly, the chicken sera were serially twofold diluted with sterile PBS in 96-well Microplates. The diluted sera were mixed with 4 hemagglutinin units of virus antigen (the infected allantoic fluids) and incubated at room temperature for 30 min. Chicken red blood cells (1%) were added to the antigen-serum dilution mixtures and incubate at room temperature for 30 min. The HI titers are expressed as reciprocals of the highest serum dilutions that showed complete hemagglutination inhibition (HI).

Cytokine assay and Spleen Lymphocyte Proliferation Response

The spleens of the vaccinated chicken were removed aseptically two weeks after the first vaccination and two weeks after boost immunization. The single splenic lymphocyte suspensions were prepared and incubated in 96-well plates at 105 cells/well by RPMI 1640 plus 10% fetal calf serum at 37° in 5% CO2. The cells were stimulated with 3 µL of irradiated inactivated AIV (with EID50 equal 108 /mL) in triplicates. At 48 h post stimulation, supernatants of splenic cells were collected to assess interferon (IFN-gamma and IFN-alpha) production using a chicken IFNα Biotek. Inc ELISA (Cat No: DIY0908c-003) and chicken IFNγ-Cytoset ELISA kit, Invitrogen (Cat No: CAC1233) following manufacturer's instructions [20,23]. Also the spleen lymphocyte proliferation assay was carried out with Cell Proliferation ELISA, BrdU kit (colorimetric) Roche Cat. No. 11647229001 according to the manufacturer's instruction. Briefly; BrdU labeling reagent was diluted 1:100, added 10 µL/well and incubated the cells 2-24 h at 37 °C. Labeling medium was removed by tapping off, added 200 µL/well FixDenat to the cells and after 30 min at 25 °C, then 100 µL/well anti-BrdU-POD working solution was added and incubated 90 min at 25 °C. The conjugated antibody was removed by flicking off and washed wells with 200 µL/well washing solution 3 times. 100 µL/well substrate solution was added, incubated at 25 °C until color development (30 min), then stop solution was added (25 µL/well 1 M H2SO4). The absorbance was measured with an ELISA reader at 450 nm. SI was calculated for each sample (Stimulating Index= Mean of OD for stimulated well/ Mean of OD un-stimulated well).

Histopathology examination

The lung samples of vaccinated chicken were collected for histopathology studies at 2 weeks after the first vaccination and two weeks after the second vaccination [24,25]. The tissues were fixed in formalin solution 10%, embedded in paraffin and cut in to 4 µm thick sections and fixed on slides. With the staining were done by H&E (hematoxylin and eosin) method. The prepared slides were evaluated by a qualified veterinary pathologist [26-28].

Statistical analysis

The analysis of variance (oneway ANOVA) followed by Duncan's multiple range test was used for statistical analysis. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Virus titration and neutralizing antibody responses

The virus titration for the vaccine was 108.5 /mL EID50 and hemagglutinin antigen assay for irradiated and non-irradiated avian influenza A sub type H9N2 virus samples were about 10 Log2. Thus the antigenicity of the HA antigen did not change in irradiated AIV. Also the safety test was done for irradiated AIV H9N2 (at 30 kGy) on SPF eggs and did not show any virus multiplication during four blind passages. These data provide information that virus inactivation was done completely. Neutralizing antibody responses were measured by the HI technique in serum samples of vaccinated chickens and reported in table 1. According to statistical analysis, the most significant increase for antibody titration in IVT intranasal and subcutaneously at 2 weeks after the second vaccination, also in IVS and FVS groups at 2 weeks after second vaccination ($P<0.05$). All of the vaccinated groups showed a significant increase in antibody titration against preimmune and PBS groups.

Cytokine assay results and Splenic Lymphocyte Proliferation Response

The results of cytokine assay in groups of vaccinated chickens two weeks after the first vaccination and two weeks after boost vaccination and shown as the concentration of IFNα and IFNγ shown in table 1. In this study, the concentration of IFN-gamma in IVT (Irradiated Vaccine plus Trehalose) intranasal group two weeks after the first vaccination was increased significantly and other groups at 2 weeks after the first vaccination were not increased for this item. Also 2 weeks after the second vaccination, concentration of IFN-gamma in IV (Irradiated Vaccine)-IN, IV-SC, IVT (Irradiated Vaccine plus Trehalose)-IN, IVT-SC and IVS (Irradiated Vaccine plus ISA70) groups were increased significantly ($p<0.05$). Therefore, the most concentration of IFN-gamma was shown in IVT two weeks after boost immunization intranasal and subcutaneously. Also, the concentration of IFN-gamma in the formalin vaccine is less than the irradiated vaccine by two routes of administration (IN and SC).

The concentration of IFN-alpha was increased in IVT.SC and FV groups two weeks after the first vaccination and in IV.SC group two weeks after the second vaccination significantly ($p<0.05$).

According to the stimulation index there is a significant increase in IV and IVT groups 2 weeks after first vaccination (wafv) and 2 weeks after second vaccination (wasv) intranasal and subcutaneously ($p<0.05$). Therefore, splenic Lymphocyte Proliferation was increased in IVT groups against other groups. So, splenic lymphocyte proliferation was increased up to the most stimulation index in IVT intranasal group 2 wasv.

Table1. The groups of vaccinated chicken and the concentration of IFNα and IFNγ

Vaccinated groups	Rout of Admins	Time of sampling	Mean of Concentration (pg/mL) IFN-γ±SD	Mean of Concentration (pg/mL) IFN-α±SD	Ab Titration±SD	SI±SD
Preimmune	--		30.17±1.49	3.91±0.72	1±0.81	0.96±0.01
PBS	IN	2wafv	19.56±2.78	5.9±2.37	0.5±0.57	0.97±0.16
IV	IN	2wafv	75.56±3.52	18.21±4.6	3±0.81	1.32±0.71
IV	SC	2wafv	47.01±16.73	47.50±11.50	2.5±0.7	1.51±0.33
IVT	IN	2wafv	188.65±24.05	23.48±2.61	3.37±0.47	1.50±0.04
IVT	SC	2wafv	78.19±19.10	73.7±3.49	2.5±0.40	1.47±0.10
FV	IN	2wafv	49.77±3.99	88.77±27.38	2.5±0.40	0.45±0.20
IVS	SC	2wafv	42.77±10.54	54.61±9.98	4.25±0.50	1.03±0.05
FVS	SC	2wafv	39.18±6.52	10.54±0.76	4±0.81	0.99±0.04
PBS	IN	3wasv	17.08±3.11	3.91±1.71	0.50±0.57	0.97±0.09
IV	IN	3wasv	268.37±11.73	38.56±16.48	3.37±0.95	1.51±0.12
IV	SC	3wasv	681.83±14.08	70.41±23.26	4±0.81	1.58±0.11
IVT	IN	3wasv	971.83±74.76	44.65±5.06	5±0.81	1.65±0.07
IVT	SC	3wasv	589.82±87.24	38.86±2.40	4.00±0.81	1.28±0.07
FV	IN	3wasv	63.20±15.91	48.48±23.87	3.37±0.47	1.09±0.05
IVS	SC	3wasv	243.06±29.71	40.27±9.84	4.50±0.57	108±0.05
FVS	SC	3wasv	76.64±30.36	14.30±3.59	5±0.81	1.09±0.06

BV= before vaccination; 2wafv= Two weeks after first vaccination; 2wasv= Two weeks after second vaccination; IV= Irradiated Vaccine; IVT= Irradiated Vaccine +Trehalose; IVS= Irradiated Vaccine+ISA70; FV= Formalin Vaccine; FVS= Formalin Vaccine+ISA70; SI= Stimulation Index for splenic lymphocyte proliferation

Histopathology observation

The histopathology results in the negative control group (PBS) were very small foci of congestion in the parenchyma and pulmonary sub epithelium as well as multiple foci of hemorrhage in the airway, perivenular edema (Figure 1A). Microscopic findings didn't vary from mild to severe in the trial groups. In the intranasal vaccination, slides were shown lymphocyte infiltration (++), foci dispersed of hemorrhage and edema (Figure 1B, Figure 1C) but in the injection vaccination (Figure 1D, Figure 1E), had intranasal vaccination symptoms addition to thickening of alveolar septa.

Table 2. The groups of vaccinated chicken and the histopathology observation

No	groups	Rout of Adminstration	Dose of vaccine	Date of sampling	Histopathological findings
1	PBS	IN	1	2wafv	Scattered very small blood foci of congestion in parenchyma and pulmonary sub epithelium as well as multiple foci of hemorrhage in the air way, perivenular edema
2	IV	IN	1	2wafv	thickening of alveolar septa, multiple foci dispersed of hemorrhage, pulmonary edema in air spaces, Lymphocytic infiltration (+)
3	IVT	IN	1	2wafv	Multiple foci dispersed of hemorrhage, edema and hemorrhage, Mild lymphocyte infiltration in para bronchial (+), desquamation of epithelium in air way, Severe thickening of alveolar septa
4	FV	IN	1	2wafv	Mild lymphocyte infiltration (+) in para bronchial, small foci of edema and hemorrhage
5	IVS	SC	1	2wafv	Mild thickening of alveolar septa
6	FVS	SC	1	2wafv	Moderate thickening of alveolar septa
7	PBS	IN	2	3wasv	Scattered very small foci of congestion in parenchyma, pulmonary sub-epithelium as well as multiple foci of hemorrhage in the air way, perivenular edema
8	IV	IN	2	3wasv	Mild lymphocyte infiltration (+), thickening of alveolar septa, extensive foci of hemorrhage and congestion.
9	IVT	IN	2	3wasv	Mild lymphocyte infiltration (+), thickening of alveolar septa, extensive foci of hemorrhage and congestion and edema
10	FV	IN	2	3wasv	Moderate thickening of alveolar septa
11	IVS	SC	2	3wasv n	Lymphocytic infiltration (+) in forming foci and limited, Mild thickening of alveolar septa
12	FVS	SC	2	3wasv	Lymphocytic infiltration (+) and foci dispersed of hemorrhage

2wafv= Two weeks after first vaccination; 3wasv= Three weeks after second vaccination; IV= Irradiated Vaccine; IVT= Irradiated Vaccine +Trehalose; IVS= Irradiated Vaccine+ISA70; FV= Formalin Vaccine; FVS= Formalin Vaccine+ISA70

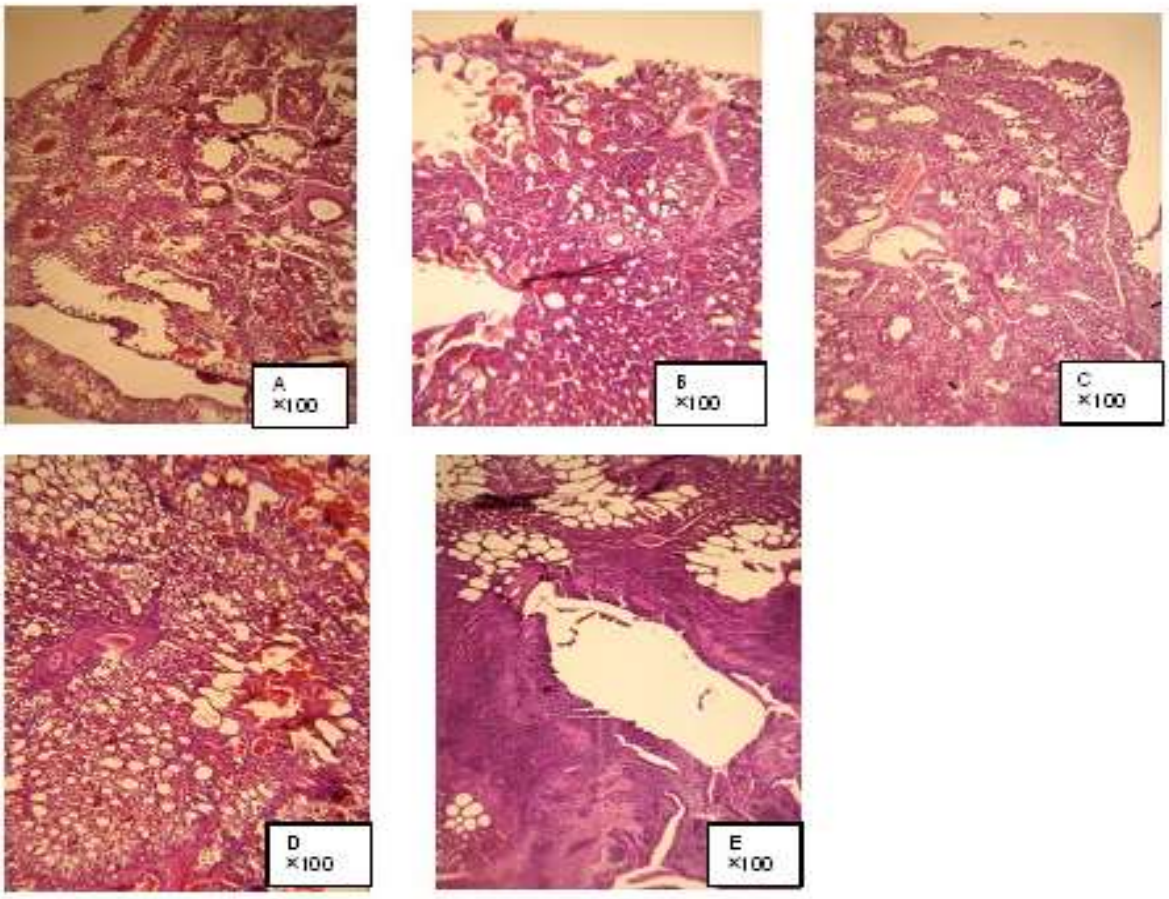

Figure 1. The histopathology results for the negative control group (A), intranasal vaccination with the irradiated vaccine (B), intranasal vaccination with irradiated vaccine plus Trehalose (C), subcutaneously vaccination of irradiated vaccine (D), subcutaneously vaccination of formalin plus ISA70

DISCUSSION

The Avian influenza virus causes a viral disease in many bird species and also one of the instances of human health risks because it can create disease in humans by contact with infected poultry or its meats. Avian Influenza sub types H5, H7 and H9 are the most important zoonotic viruses. The H9N2 virus has low pathogenicity in birds but it is a serious threat to public health and prevailing vaccines decline the incidence of disease in birds, they can't completely prevent infection and not capable of preventing the shedding of the AIV. Vasfi indicated H9N2 virus can't induce viremia in infected poultry and suggested that strengthening conditions and simultaneous viral and bacterial infections influenced sever mortality (to 80%) and egg fall (to 75%) in the respiratory outbreak [1]. It is necessary to develop safer vaccines enable of providing protection against Influenza viruses. In this study was calculated gamma ray dose 30 kGy for complete inactivation of the frozen AIV sub type H9N2 because it has been reported that frozen material during irradiation decreases free radical formation and accordingly barely the indirect damage to proteins. Shannon and coauthors used 50 kGy; their data showed the immunogenicity of highly pathogenic influenza virus irradiated at 50 kGy to induce higher antibody titration and cell-mediated immune responses and our data indicated that we could preserve maintained protein biological activity and high immune responses after exposure to 30 kGy [6].

Furuya explained gamma-irradiated, γ-APC [A/Port Chalmers/1/73(H3N2)], has major immunogenicity and its protection was 100%, a lower weight loss in mice who vaccinated with formalin or UV inactivated vaccines [29]. Their data indicated γ-ray inactivated virus induced immunity with high quantitatively and qualitatively to the inactivated virus by formalin or UV-irradiation [30]. Also, they reported γ-A/PC-vaccinated mice had reduced lung inflammation and reduced lung viral loads [24]. He indicated that disinfection by γ-ray is very capable than the other vaccine preparation methods, in providing immunogenicity of the influenza virus and infusion of cross- protective immunity [30].

Thomas showed poly I:C as adjuvant can increase both humoral and cellular immune responses, IFNγ, IFNα, IL-2 cytokines production in mice and reduce AIV replication and shedding in poultry and control H9N2

IV in ducks [31]. In our study, after the first intranasal vaccination, the amount of the IFN-gamma was increased to different levels in the samples of the irradiated vaccine with Trehalose compared to other vaccine groups. It indicates that Trehalose had a positive effect on cytokines production and enhanced the IFNγ level in poultry. In the second intranasal vaccination, compared to the first vaccination level of IFNγ was significantly increased ($P<0.05$). In this study, we showed IFNγ concentration in an intranasal routes were more than an injection way. One of the opinion for the reason to increase IFN-γ, antibody titration and lymphocyte proliferation in the IVT-intranasal group is that Trehalose not only is as a protectant for viral protein during freezing of irradiated viral stock at -70 ° C [32], but also it makes more viscosity in the vaccine solution. Therefore, when a drop of vaccine puts into the bird's nose and the most of the time, the bird shakes its head and it makes a portion of the drop throw out, however, the vaccine solution with Trehalose is used, by head shaking it doesn't throw out because of the more viscous solution.

Motamedi-Sedeh showed that radiation can keep the antigenic characteristic of inactivated virus antigen and thus it is a good way for the immunization of animals to induce neutralizing antibody titers and cytokine concentration such as IL-2 and IFNγ [33]. Meng explains IFN-α can to protect chickens against AIV [34]. In this study most of the vaccinated groups haven't shown any increase in IFN- α concentration, only the peak IFN-α level was shown in the formalin vaccine group. As we know IFN-α production after virus infection is transit, fast and nonspecific and after about 10 hours it disappeared. Therefore, after two weeks it was disappeared.

Dong-hun Lee demonstrated that the use of the VLP vaccine would help in restricting the safety effects compared to live-attenuated and inactivated whole virus vaccines and because absences of viral antigenic materials have a high safety will be a good vaccine candidate in a feature. They depicted ISA70 is a safe and impressive adjuvant in many poultry diseases and VLP with the ISA70 induce high titers of antibody compare with VLP without adjuvant [8]. Our results indicated that ISA70 induce a high titer in INFγ. But when we used of Trehalose, IFNγ titer had more increasing, therefore the magnitude of protection was in the irradiated with Trehalose vaccine group.

Gauger reported live attenuated flu vaccines with genetic modifications have steady cross-protection and decrease in shedding compared whole inactivated virus (WIV) vaccines. They demonstrated live attenuated influenza vaccines (LAIV) reduce clinical and lung lesions compared to WIV vaccine and unvaccinated animals, also was decreased Pneumonia in the LAIV-IN and WIV-IN pigs [35]. In our study suggested that ulcers in injection vaccine groups were lower than intranasal vaccine groups. We observed lymphocytic infiltration (+) in forming foci and limited, mild thickening of alveolar septa in injection vaccines but we have thickening of alveolar septa, multiple foci dispersed of hemorrhage, pulmonary edema in air spaces and Lymphocytic infiltration in intranasal vaccines. What are important, the enhanced respiratory lesions were observed in irradiated vaccine plus Trehalose in intranasal and the lowest lesions in irradiated with ISA70 in SC vaccines. The results of this study show critical advantages to the use of irradiated vaccines with Trehalose in poultry populations.

Nakayama showed formalin-inactivated whole virus particles vaccines by nasal vaccination could induce antigen-specific IgE secretion and eosinophilic infiltration and suggested not only mucosal and serum IgA but also blood IgG of B cells. They explained flu vaccine-specific IgE have a protective role but it can cause an increase in the appearance of allergy because of the gathering of antigen specific IgE in the nasal mucosa. However they did not detect any anaphylaxis or allergic symptoms in their samples [10]. In addition, we showed FV intranasal can induce antibody titration and IFN-γ concentration.

CONCLUSION

Finally this research showed irradiated AIV sub type H9N2 can be a good candidate for vaccine preparation, and Trehalose as a disaccharide makes more viscosity in vaccine solution so, it makes more absorbance of antigen by the inhalation route.

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