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Original Article

Effect of the alcoholic extract of the grape (*Vitis Vinifera L.)* and pomegranate (*Punica Granatum L.*) seed on the immunogenicity of the inactivated avian influenza virus subtype H9N2 in broilers

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Summary
Mineral oil-adjuvants remain in chicken meat and may be carcinogenic. Plant-based oils comprise different components, but they have suitable safety to use as an adjuvant. In this study, we investigated the adjuvant activity of pomegranate seed oil (PSO) and grape seed oil (GSO) on the immunogenicity of the inactivated avian influenza virus subtype H9N2. For this purpose, the hexanic extract of PS and red GS was separately emulsified with inactivated H9N2 virus. One hundred broiler chickens were assigned into five groups (n = 20). Group A received PSO+H9N2 emulsion, group B received GSO+H9N2 emulsion, group C received PBS+inactivated H9N2 virus, group D received a commercial vaccine, and group E did not receive anything. The chickens were treated at 14 days of age. At 21 days post-vaccination, all chickens were challenged by the H9N2 virus. Hemagglutination inhibition test, anamnestic response, and clinical signs were used to assess immunogenicity. The results showed that although the PSO and GSO have adjuvant activity and significantly increase antibody titers to the H9N2 virus after two weeks of vaccination, but the antibody titers significantly decrease after three weeks of vaccination. In conclusion, PSO and red GSO seem to have biologically active molecules and potentially adjuvant properties, but they alone cannot induce protective immunity against the avian influenza H9N2 virus.
***Keywords*:** Adjuvant, Avian Influenza, Grape seed, Immunity, Pomegranate seed

Introduction

Avian influenza virus (AIV) subtype H9N2 has spread worldwide in poultry and poses a real threat to both the global poultry industry and humans through high rates of zoonotic infection and the potential for a pandemic (David et al., 2020). The H9N2 virus, with low pathogenicity, is endemic in many countries including Iran (Peacock et al., 2019). Mineral oils as adjuvants allow antigens to be gradually and continuously released from the injection site. These oils might be a risk as carcinogens (Singh, 2007), and studies indicate that they are not entirely retained at the injection site; 2-15% and 25-35% of the mineral oils leave the injection site one week and one month after injection, respectively (O'hagan, 2000). Therefore, their use in broiler chickens is inappropriate, because production cycle of broiler chickens is short.

Plant-based oils are one of the ingredients that can be used as adjuvants in the preparation of vaccines (Tengerdy and Lacetera, 1991; Vajdi, 2011). These

oils comprise different components, but they have suitable safety use as an adjuvant (Hardy et al., 2009; Fox et al., 2011). Grape and pomegranate seed extracts are safe. Studies have shown that grape seed extract with a maximum concentration of 1% was not a sensitizer (Fiume et al., 2014). Also, various studies have shown that different forms of pomegranate have no risk of toxic reactions in human (Viuda-Martos, et al., 2010). Pomegranate seed (PS) and grape seed (GS) contain 6.3-12.2% and 8-20% oil, respectively (Topkafa et al., 2015; Shinagawa et al., 2017). Punicic acid is an important polyunsaturated fatty acid (PUFA) in pomegranate seed oil (PSO), (Eikani et al., 2012). PSOs also are a good source of tocopherols (Jing et al., 2012). In grape seed oils (GSO), the predominant fatty acid is linoleic acid and like PSO, it also has tocopherols (Sabir et al., 2012; Al Juhaimi et al., 2017).

Pomegranate has been used as a folk medicine for many years (Schubert 1999; Schubert et al., 1999); PSO has different therapeutic properties. It has nephroprotective and cardioprotective properties and has anti-cancer activity against prostate cancer, breast cancer, and colon cancer (Bhandari, 2012). PSO has components that improve immune function (Yamasaki et al., 2006). GSO has reactive oxygen species (ROS) that assist the immune system (Garavaglia et al., 2016). In this study, we investigated the adjuvant activity of PSO and GSO on the immunogenicity of the inactivated avian influenza virus subtype H9N2.

Materials and methods

Preparation of hexanic extract of pomegranate seed

Pomegranate seeds (red seed ardestani strain) were dried in the shade at room temperature. The dried seeds were crushed, and then macerated into hexane (Merck, Germany), for 1 day; during this time the mixture was stirred every three hours. After that, the solution was filtered. This process, maceration, and filtration, was repeated three times. The filtrate was concentrated in soxhlet, and then it evaporated to expel the alcohol (González-Trujano et al., 2017).

Preparation of hexanic extract of red grape seed

The extraction method was similar to the method used to prepare the hexanic extract of pomegranate seed (González-Trujano et al., 2017).

Propagation of the avian influenza virus subtype H9N2 antigen

A/Chicken/Iran/ZMT-101(101)/98(H9N2) was used for propagation. This virus was kindly offered by the department of poultry disease, Faculty of Veterinary Medicine, University of Tehran. The virus propagated in embryonated chicken eggs as described by Peter (2020). Formaldehyde (Merck, Darmstadt, Germany) was used for the inactivation (King, 1991). To ensure the viruses are inactivated, 0.6 mL of the stock solution inoculated to six 10-day-old embryonated chicken eggs (0.1 mL/egg).

Hemagglutination (HA) Test

The titers of AIV subtype H9N2 in-stock solution (aminoallantoic fluid) were measured after propagation and after inactivation of the virus as recommended by Mary (2020). Briefly, 50 µL of PBS was added to every well in the first row of a microplate (U-bottom). Then, 50 µL of aminoallantoic fluid was added to the first well. The contents of the first well were mixed, and two-fold dilutions of the sample continued across the entire row, and excess 50 µL was discarded from the last well. After that, 50 µL of 0.5 % chicken red blood cell was added to every well. After 30 min incubation at room temperature, the HA titer of the virus was determined and expressed as a reciprocal dilution titer.

Hemagglutination Inhibition (HI)

The titers of antibodies to AIV were measured in serums as recommended by Janice (2020). Briefly, for each serum sample, 25 µL of PBS was added to every well in the first row of a microplate (U-bottom). Then, 25 µL of a serum sample was added to the first well. The contents of the first well were mixed, and two-fold dilutions of the sample continued across the entire row, and excess 25 µL was discarded from the final well. After that, 25 µL of 4 hemagglutinating unit/25 µL of AIV antigen was added to every well, and then, after incubation for 30 min at room temperature, 25 µL of 1% chicken red blood cell was added to every well.

After 30-45 min incubation at room temperature, the HI titers of the sera were determined and expressed as reciprocal dilution titer.

Study of microbial contamination of the extracts

To ensure that the extracts are free of microbial contamination, they were cultivated on a brain-heart fusion agar (BHA) medium (Merck, Germany).

Preparation of the emulsion of the pomegranate seed extract and inactivated AIV subtype H9N2

To prepare the oil phase, 4.5 mL of the pomegranate seed extract and 0.5 mL of span®80 (Merck, Germany) were mixed in a sterile tube. To prepare the aqueous phase, 5 mL of the inactivated virus suspension (EID50=$10^{6.16}$/mL), and 5 mL of sterile PBS were mixed in a sterile tube. To prepare the emulsion solution, the oil phase was homogenized (IKA Ultra-Turax® T 18 basic; IKA, Staufen, Germany) at 11000 rpm for 5 min at 4 to 8 ˚C, then, the aqueous phase was slowly added to the oil phase in 60 seconds.

Preparation of the emulsion of the red grape seed extract and inactivated AIV subtype H9N2

The method was similar to the method used to prepare the previous emulsion.

Preparation of the mixture of PBS and inactivated AIV subtype H9N2

Five mL of the inactivated virus suspension was mixed with 5 mL of sterile PBS.

Study of microbial contamination of the emulsions and the mixture

To ensure that the emulsions and mixture are free of microbial contamination, they were cultivated on a BHA medium and incubated at 37 ˚C for 24 hours (Merck, Germany).

Experimental design for biological study

One hundred 12-day-old broiler chickens (Ross 308®) were randomly divided into five groups of 20 chickens each. Each group had two subgroups of 10 chickens. Group A received the emulsion of hexanic extract of pomegranate seed and the inactivated virus. Group B received the emulsion of hexanic extract of grape seed and the inactivated virus. Group C received the mixture of sterile PBS and the inactivated virus. Group D received a commercial inactivated avian influenza vaccine, containing mineral oil adjuvant, against subgroup H9N2, 0.1 mL per chick as recommended. Group E did not receive anything.

The emulsions and the mixture were injected subcutaneously in the dorsal cervical region (Najarri et al., 2015) at 14 days of age (0.5 mL/chicks).

To determine antibody titers against AI- virus subgroup H9N2 by HI, blood samples were taken every week starting from 14 days of age. At 21 days post-vaccination, all of the chickens were challenged intraocular (40 µL/bird) by the AI-virus subgroup H9N2, with the EID50 of $10^{6.16}$/mL. Responses to challenge were determined by clinical signs and infection. Two weeks post-challenge, blood samples were taken from all of the chickens to evaluate the anamnestic response by determining HI titers of the sera. If HI titers increase 4 times or more, this means the emulsions could not prevent treatment chickens from infection.

Statistical analysis

One-way ANOVA and Duncan multiple range tests were used for analysis of the HI titers. The SPSS statistics, 22 version, was used for statistical analysis. Differences between the groups were significant at $p < 0.05$.

Results

Inactivation of the virus suspension

The embryos of inoculated embryonated chicken eggs were alive 6 days post-inoculation, and HA titers of chorioallantoic fluid were negative.

Study of microbial contamination

The cultivation of the extracts, emulsions, and mixture on BHA medium was negative.

Clinical signs

After the challenge, mortality was not observed, but in treatment groups, especially in the control groups, sneezing, nausea, sinusitis, anorexia, diarrhea, and conjunctivitis were observed.

HI titers

The mean HI titers of serum samples against AI-virus subgroup H9N2 at 14 and 21 days of age were 2.9 and 0.2, respectively. The mean HI titers in groups A, B, C, D, and E, at 2 weeks post-

vaccination were 0.75, 0.75, 0.05, 0.1, and 0.4, respectively. Statistically, there were significant differences between groups that had been received the two emulsions, group A and B, and the three other groups ($P < 0.05$; Table 1). The mean HI titers of serum samples in groups A, B, C, D, and E, at 3 weeks post-vaccination were 0.35, 0, 0.7, 1.7, and 0.2, respectively. There was a significant difference between the groups ($P < 0.05$). Group D had the highest HI titers and group B had the lowest HI titers (Table 1). The mean HI titers in groups A, B, C, D, and E were 5.3, 4.8, 6, 5.3, and 5.2, respectively, at 2 weeks post-challenge. There was a significant difference between the three groups A, D, and E, and the other two groups, and also between the groups B and C ($P < 0.05$; Table 1). The mean HI titers of the negative control group before the challenge were 0.2, which indicated that the groups had not been infected before the challenge.

Table 1- The mean HI titers, as a reciprocal dilution titer, against avian influenza virus subgroup H9N2 ± standard error of mean in the chickens in the groups.

Groups	Titers (14 days post-vaccination)	Titers (21 days post-vaccination)	Titers (14 days post-challenge)
A	0.75 ± 0.08^{c}*	0.35 ± 0.50^{c}	5.3 ± 0.11^{b}
B	0.75 ± 0.17^{c}	0^{a}	4.8 ± 0.08^{a}
C	0.05 ± 0.05^{a}	0.2 ± 0.06^{b}	6 ± 0.08^{c}
D	0.1 ± 0.07^{a}	1.7 ± 0.06^{d}	5.3 ± 0.08^{b}
E	0.4 ± 0.07^{b}	0.2 ± 0.07^{b}	5.2 ± 0.10^{b}
P-value	0.000	0.000	0.000

Group A: The group that received the emulsion of hexanic extract of pomegranate seed and the inactivated virus.
Group B: The group that received the emulsion of hexanic extract of red grape seed and the inactivated virus.
Group C: The group that received the mixture of sterile PBS and the inactivated virus.
Group D: The group that received a commercial avian influenza vaccine against subgroup H9N2.
Group E: The group that received anything, as a control group.
*The data with the same letter in each column have not significant differences ($p < 0.05$).

Discussion

The results indicated that the PSO and GSO as an immunological adjuvant, in comparison with other groups such as the commercial AI vaccine, significantly increase antibody to AIVs subtype H9N2 after two weeks of vaccination, but unlike the commercial vaccine, the antibody titers significantly decrease after three weeks of vaccination, especially in group B. The titers after challenge indicated that the commercial vaccine induces protective immunity, but the emulsions of H9N2 antigens with PSO or GSO, are not able to induce protective immunity against AI viruses. The results of this study showed that, unlike hexanic extracts of olive and fig fruits, the hexanic extracts of both grape and pomegranate seed better absorb the H9N2 virus antigen and stimulate the humoral immune system, so that after two weeks of vaccination, the mean HI titers increased, while fig and olive fruit extracts were not able to increase HI titers (Najarri et al., 2015). However, in this study, the HI titers decreased rapidly three weeks post-vaccination. A study shows that peanut oil, like commercial mineral oil adjuvants, can induce protective HI titers so that it can prevent morbidity and death of challenged chickens (Stone, 1993).

In inactivated vaccines, in addition to antigens, adjuvants also play a key role in stimulating and sustaining the immune response (Aiyer-Harini et al., 2013), because most antigens are weakly immunogenic and are not able to provide sustained protective immunity alone (Petrovsky and Aguilar, 2004; Song and Hu, 2009). Adjuvants accelerate, maintain, and enhance specific immune responses by storing antigens at the injection site and releasing them gradually, or by stimulating the immune system (Petrovsky and Aguilar, 2004). In this study, the results showed that the two treatment

groups that received the two emulsions (the mixtures of H9N2 viruses and PSO or GSO) compared with the group that received the virus-containing suspension, the emulsions induced a better immune response and produced more antibodies after two weeks of injection. This means that GSO and PSO have a positive effect on stimulating specific immunity against the H9N2 subtype of avian influenza. Grape seed contains about 8-20 percent oil and about 90% of GSO is PUFA. Linoleic acid with 72% is the dominant PUFA in GSO, followed by oleic acid with 16%. Saturated fatty acids (SFA) are present in GSO in lower quantities; palmitic acid with 7% is the most abundant SFA (Hussein and Abdrabba, 2015). Pomegranate seed contains about 12.2-23.9 percent oil, and 86.72-90.3 percent of the oil is PUSFA (Miranda et al., 2013). The predominant PUSFA are punicic acid (C18:3; 74%-88%), conjugated linolenic acid (CLnA), and linoleic acid (5%-16%) (Shaygannia et al., 2016; Fernandes et al., 2015). Unlike alum, these oils possibly do not create a depot of antigen at the injection site (Shah et al., 2015); it seems these oils and other potentially immunogenic compounds are found in the hexanic extract of pomegranate and grape seeds, and thereby directly and indirectly increase immune response. MF59 is a commercial oil-based adjuvant that works by creating an immunocompetent environment and enhancing the production of cytokines and chemokines (Shah et al., 2015). The other compositions in GSO and PSO include resveratrol, quercetin, procyanidins, flavonoids, carotenoids, phenolic acids, and gallic acid, which stimulate the immune system to respond to foreign antigens by activation of signaling pathways (Garavaglia et al., 2016; Shabbir et al., 2017; Ding et al., 2018). Antioxidant activities are also the most known properties of phenols. Antioxidants promote the immune system by destroying free radicals (Bendich, 1993; Morel et al., 2011). Tocopherols are other compounds in PSO and GSO that not only have antioxidant properties but also help ASO3 adjuvant to support the highest humoral immune response, to balance some cytokines secretion, to help antigens present to monocytes, and to recruit granulocytes in the draining lymph nodes (Morel et al., 2011; Sabir et al., 2012; Peng, 2019).

The results showed that in contrast to the commercial inactivated vaccine, GSO and PSO produce antibodies against the H9N2 virus in the second week after injection but, unlike the commercial vaccine, thery are unable to prolong and enhance antibody production against the H9N2 virus in the following weeks. Therefore, PSO and GSO used in this study as adjuvants along with the H9N2 virus are not able to provide protective immunity after the challenge. Incidence of clinical signs and anamnestic response in the two groups that received a mixture of virus and GSO and PSO confirmed infection. Various factors are involved in the proper response of the immune system to an antigen, including the long half-life of the adjuvant, no adverse effect of adjuvant on the antigen, and adjuvant formulations (Aiyer et al., 2013). Considering that unsaturated fatty acids are predominant in both PSO and GSO, it seems the oils are metabolized and readily absorbed from the injection site (Sabir et al., 2012). Therefore, they have short half-lives, which reduces the amount of antigen at the injection site and subsequently declines the antibody titers. Soybean oil also contains unsaturated fatty acids, but a study has shown that if ginseng root saponins are added to this oil, it will have an adjuvant effect (Zhang et al., 2018). The negative effect of adjuvant on antigen can decline the immunogenicity of the antigen. A study has shown that phenolic compounds in PSO and GSO have antiviral properties (Bhandari, 2012), and interfere with the replication of the influenza virus. There may be other compounds in PSO and GSO that destroy immunogenic antigens.

Conclusion

In conclusion, PSO and red GSO seem to have biologically active molecules and potentially adjuvant properties. They accelerate a specific immune response to the H9N2 virus, but the immune response is not long-lasting and therefore they alone cannot induce protective immunity against the avian influenza H9N2 virus.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethical approval

The authorization number is 1793 in the Faculty of Veterinary Medicine, University of Tabriz.

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