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Levamisole Enhances Cell-Mediated Immune Responses and Reduces Shedding of H9N2 Avian Influenza Virus in Japanese Quails (*Coturnix coturnix japonica*)

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Abstract: Problem statement: Regarding the role of Japanese quails (*Coturnix coturnix japonica*) in reassortment and spreading of avian influenza (AI) viruses and inadequate protection of vaccination in this species, the present study aimed to evaluate the effect of levamisole as an immunomodulatory agent on cell-mediated immunity (CMI), antibody responses and shedding of H9N2 AI virus in experimentally infected quails. **Approach:** On day 20 of age, 100 quails randomly allocated into 4 equal groups. Birds in groups 2, 3 and 4 were inoculated with virus where group 1 kept as control. Groups 3 and 4 orally received 15 mg kg^{-1} levamisole for three consecutive days just before virus inoculation which was repeated 10 days post inoculation (PI) only in group 4. Antibody titers and CMI of all birds were assayed by HI and delayed type hypersensitivity (DTH) test respectively and virus detection in fecal and tracheal samples performed by RT-PCR method. Data analyzed by one-way ANOVA and Tukey's test. **Results:** Levamisole in both regimens had no appreciable effect on antibody titers ($p>0.05$) while repeated regimen resulted in higher CMI response than group 2 at 48 and 72 h post DTH test ($p = 0.011$ and $p = 0.031$ respectively). Total fecal samples positive for virus from birds in group 3 and 4 were 34.4 and 40% lower than group 2 respectively. For trachea, the positive samples were 33.3% (group 3) and 46.7% (group 4) lower than group 2. Moreover; fecal and tracheal samples from levamisole treated birds (especially from group 4) became void of virus earlier than group 2. **Conclusion/Recommendations:** Levamisole administration in a repeated regimen enhances CMI response against H9N2 AI virus and reduces virus shedding in quails. This may pave the road for further investigations on potential positive effects of this agent on prevention and management of H9N2 AI infections in quail industry.

Key words: Avian influenza, immunomodulation, quails

INTRODUCTION

H9N2 avian influenza (AI) viruses are among the most commonly occurring infections in domestic poultry populations and several epidemics of this subtype have been reported in Asia and North America since 1990 (Alexander, 2000; Peiris *et al*., 2001; Tang *et al*., 1998). In Iran, an outbreak of H9N2 AI viruses occurred in broiler chickens during 1998-2001 with a mortality rate of 20% to 60% in affected farms (Nili and Asasi, 2003). Although this subtype is considered as a low-pathogenicity AI virus, it can infect a wide variety of species including Japanese quails *(Coturnix coturnix japonica)*. Ebrahimi *et al*. observed that a field-isolated H9N2 AI virus can infect all unvaccinated quails while infects 30% to 40% of birds vaccinated with inactivated vaccine, which indicates incomplete protection of this vaccine against AI viruses in quails. Interestingly, it has been shown that quails can provide an environment for the reassortments between avian and human influenza viruses and act as a potential intermediate host by carrying sialic acid receptors compatible with binding to avian and human influenza viruses (Wan and Perez, 2006).

Recently, manipulation of immune responses by various agents in order to improve efficacy of vaccination has been practiced. Immunostimulant agents such as levamisole have been used in an attempt to enhance protective immune responses of chickens for prevention or control of infectious diseases including Newcastle disease (Yin *et al*., 2007), infectious bursal disease (Singh and Dhawedkar, 1993), Marek's disease

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(Kodama *et al*., 1980) and coccidiosis (Onaga *et al*., 1984; Giambrone and Klesius, 1985) with various rates of success; however, to our knowledge the effect of this drug on immune responses and virus shedding period of H9N2 AI viruses in quails has not been addressed yet. Regarding to the growing interests in quail industry in Iran, inadequate efficiency of vaccination and the role of this species in reassortment and spreading of AI viruses, the present study was conducted to evaluate the effect of levamisole administered in two regimens on cell-mediated immunity (CMI), antibody responses and virus shedding period of H9N2 AI viruses in experimentally infected quails.

MATERIALS AND METHODS

H9N2 AI virus: The virus used for this study, A/chicken/Iran/772/1998 (H9N2), was obtained from Razi serum and vaccine research institute, Tehran, Iran. Virus was propagated in 10-day-old embryonated chicken eggs and stored at -70°C. Avian influenza a virus was titrated to determine the 50% Egg Infectious Dose (EID_{50}) by the method of Reed and Muench (1937).

Animals and experimental design: One hundred one-week-old Japanese quails from both sexes were purchased and randomly allocated into 4 equal groups (n = 25 each). Birds had free access to feed and water and reared under bio security conditions. Maternal antibody titer against AI was assayed on the entrance day of birds by HI method. On day 20 of age, birds in groups 2, 3 and 4 were inoculated through the nares with a concentration of $10^{6.5}$ EID_{50}/bird H9N2 AI viruses; while birds in group 1 kept as control and received normal saline. Birds in groups 3 and 4 received 15 mg/kg levamisole (Razak Pharmaceutical Laboratories, Tehran, Iran) for three consecutive days just before virus inoculation (days 17, 18 and 19 of age) by oral gavages. This treatment was repeated 10 days post inoculation (PI) (days 30, 31 and 32 of age) only in birds of group 4.

All methods used in the study were in compliance with the institutional ethical guidelines of School of Veterinary Medicine, Shiraz University for use of animals in research.

HI test: Antibody titers of all birds against AI were assayed on the day of virus inoculation followed by days 9, 14, 21 and 32 PI by HI method.

DTH test: Cell-mediated immune response was assayed by performing delayed type hypersensitivity (DTH) test as described by Munir *et al*. (2009) with few modifications. Fourteen days PI all birds were sensitized with 0.25 mL of 2, 4-dinitrochlorobenzene (DNCB) solution (10 mg mL^{-1}) by SC injection in the breast. After 14 days, these sensitized chickens were challenged with 0.25 mL of DNCB (1.5 mg mL^{-1}) injected with about 2 cm distances on the left side of the first injection site. Skin thickness was measured at zero (defined as immediately after DNCB challenge), 24, 48 and 72 h post DNCB challenge by a Vernier caliper with the precision of 0.02 mm.

Evaluation of virus shedding period: During 15 days PI samples from feces and trachea of 3 birds of each group were randomly collected with 3 days intervals and stored in -70°C. Virus detection was performed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method.

RNA was extracted by RNX^{TM} (-plus) (CinnaGen Co., Tehran, Iran) commercial kit according to manufacturer's instructions. For this purpose 10% suspension of fecal samples in normal saline was prepared and after centrifugation at 7500 rpm for 10 min in 4°C, 200 μL of supernatant was used. Extracted RNA was reverse transcribed using AccuPower® RT PreMix (Bioneer Co., Daejeon, South Korea) kit. H9F and H9R primer pair which yield in specific amplification of a 488 bp fragment within the *H9* gene were used with the following sequences: H9F: 5′ CTY CAC ACA GAR CAC AAT GG 3′ and H9R: 5′ GTC ACA CTT GTT GTT GTR TC 3′ as described by Lee *et al*. (2001) for cDNA synthesis. 5 μL of the cDNA was used for PCR amplification. The PCR thermocycling condition for the gene was as follows: 30 cycles with denaturation at 95°C for 60 sec, primer annealing at 53°C for 60 sec and primer extension at 72°C for 60 sec and a final extension step at 72°C for 10 min (Tajmanesh *et al*., 2006). 5 μL of PCR product was subjected to 1% agarose gel electrophoresis containing ethidium bromide and visualized under ultraviolet light.

Statistical analysis: Data were presented as mean±SD. Data analysis was carried out by using one-way ANOVA and Tukey's multiple comparison tests as the *post hoc* (SPSS 11.5 for windows software). Differences were considered significant at $p<0.05$.

RESULTS

Clinical observations: No adverse clinical manifestations or mortalities were observed in birds of all groups during the experimental period.

Humoral immune responses: Figure 1 depicts the results from the HI test. Antibody titer of birds from the control group remained very low all through the sampling period.

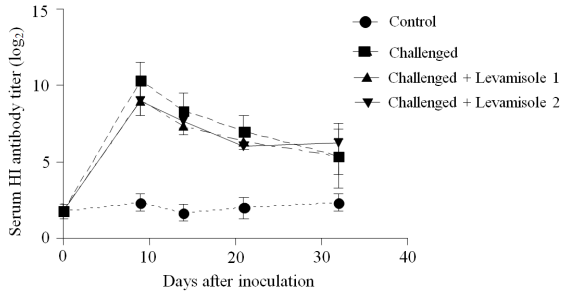

Fig. 1: HI Antibody (mean±SD) titers at 0, 9, 14, 21 and 32 days post inoculation of birds in different groups

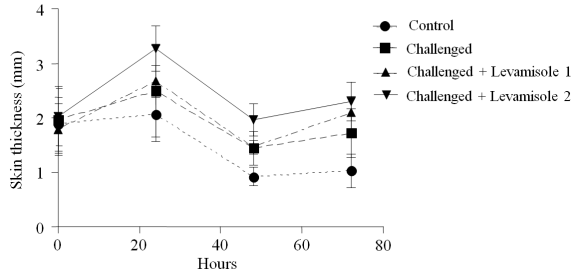

Fig. 2: Skin thickness (mean±SD) of birds in different groups, measured at 0, 24, 48 and 72 hours post delayed type hypersensitivity test

Table1: Comparison of viral detection in feces and trachea of Japanese quails in different groups during days post inoculation (PI)

Samples	Feces				Trachea			
Days PI	Co	Ch	Ch+L1	Ch+L2	Co	Ch	Ch+L1	Ch+L2
3	0/3	2/3	1/3	1/3	0/3	2/3	0/3	0/3
6	0/3	2/3	1/3	1/3	0/3	3/3	0/3	0/3
9	0/3	2/3	1/3	1/3	0/3	3/3	3/3	2/3
12	0/3	2/3	1/3	0/3	0/3	2/3	2/3	2/3
15	0/3	1/3	0/3	0/3	0/3	1/3	1/3	0/3

Data presented as number of samples positive for virus/number of total samples. Co: Control; Ch: Challenged with H9N2 influenza virus; Ch+L1: Challenged with H9N2 influenza virus and treated with 15 mg kg^{-1} levamisole for three consecutive days just before virus inoculation and Ch+L2: Challenged with H9N2 influenza virus and treated with 15 mg kg^{-1} levamisole for three consecutive days just before virus inoculation which was repeated 10 days PI.

Highest antibody titer was observed 9 days post challenge in all other groups. HI titers of birds in groups 2, 3 and 4 were significantly higher than control group from day 9 and so on with $p<0.02$ for all comparisons. Levamisole administration in both regimens had no appreciable effect on antibody titers during the sampling period as compared to birds in group 2 ($p>0.05$).

Cell-mediated immune responses: The highest skin thickness of all groups was observed 24 h post challenge with DNCB. Skin thickness of birds in group 2 was significantly higher than control group at 48 and 72 h post challenge ($p = 0.012$ and $p = 0.009$ respectively); Levamisole administration in repeated regimen (group 4) resulted in skin thickness significantly higher than group 2 at 48 and 72 h ($p = 0.011$ and $p = 0.031$ respectively). The difference in skin thickness of two levamisole treated groups was significant at 48 h with $p = 0.043$. Results are depicted in Fig. 2.

Virus shedding period: All samples from control group were void of virus during the whole sampling period, while both tracheal and fecal samples from group 2 became positive for virus from day 3 post inoculation and so on. 60% of total fecal samples from group 2 were positive for virus while in birds treated with levamisole the percentage reduced to 26.6 and 20% in groups 3 and 4 (34.4 and 40% lower than group 2 respectively). For tracheal samples the percentage was 73.3% in group 2, 40% in group 3 and 26.6% in group 4 (33.3% and 46.7% lower than group 2 respectively). Results of PCR test on fecal and tracheal samples are summarized in Table 1.

DISCUSSION

Levamisole is a synthetic anthelmintic agent with immunomodulatory properties. Its use as an immunostimulant in avian species dates back to 1979 when Soppi *et al.* (1979) demonstrated that levamisole is able to enhance both humoral and cellular immune responses in normal chickens. The effect was probably mediated by the activation of the T cell function and included only antibody responses to thymus dependent antigen. To our knowledge, although effects of levamisole on immune responses against influenza have been evaluated in humans (Obrosova-Serova *et al*., 1984; Pike *et al*., 1977) and chickens (Mayahi *et al*., 2007), quails were not addressed before. This motivated us to evaluate the effect of levamisole on CMI, antibody responses and virus shedding period of H9N2 AI viruses in this species with regard to the recent progressive interest in quail industry and the potential of this species as an intermediate host for both avian and human influenza viruses (Wan and Perez, 2006) accompanied by incomplete protection of inactivated vaccines (Ebrahimi *et al*., 2011).

Infection with AI viruses elicits a humoral antibody response at both systemic and mucosal levels. Antibodies against the surface proteins (HA and NA) are neutralizing and protective (Suarez and Schultz-Cherry, 2000). In our study inoculation of H9N2 virus resulted in an obvious antibody response as compared to non infected birds, which clearly

demonstrate that inoculation of virus was successful and birds were infected.

In a study performed by Obrosova-Serova *et al*. (1984) levamisole activated antibody production in young subjects in response to administration of a live influenza A (H3N2) vaccine and enhanced the protective effect of vaccination. The senile subjects vaccinated with inactivated influenza A vaccine (H3N2 and H1N1) had a good immune response and the use of levamisole was not reflected in antibody rises. Mayahi *et al*. (2007) showed that daily administration of 2 mg levamisole from two days before vaccination of chickens with killed H9N2 influenza vaccine, or 4 mg levamisole at the time of vaccination against influenza disease can increase influenza antibody titer in this species. This is inconsistent with our results where levamisole administration in both regimens had no appreciable effect on antibody titers during the sampling period.

To describe the discrepancy, it should be mentioned that the intensity of antibody response varies with species where quails produce lower antibody responses than chickens (Davison *et al*., 1996; Suarez and Schultz-Cherry, 2000), this may partially describe lack of an enhanced antibody response to levamisole in our study, although the precise effect of levamisole on antibody producing cells in quails needs to be further investigated.

The host immunity, especially cell mediated immunity, is important in the pathogenesis of AI viruses. In the present study, levamisole administration at repeated regimen significantly enhanced DTH responses in quails; moreover, virus shedding was influenced. Fewer fecal samples of birds treated with levamisole were positive for virus and these samples (especially from birds treated repeatedly) became void of virus earlier than birds in group 2. Tracheal samples of birds treated with levamisole remained virus free until day 9 PI and birds treated with levamisole repeatedly, became virus negative earlier that group 2.

Levamisole activates macrophages and enhances production of the key cytokine IFN-γ which leads to stimulation of maturation of cells which are involved in CMI (Symoens and Rosenthal, 1977; Szeto *et al*., 2000). Pike *et al*. (1977) suggested that levamisole may be useful in enhancing depressed cellular immune function in patients with acute influenza. More over; Consistent with our results, in a study performed by Kwon *et al*. (2008), treating H9N2 infected chickens with cyclosporin A, as a suppressor of CMI such as CD8+ T-cells and expression of IFN-γ mRNA, was correlated with high viral load in the oropharynx and cloaca of these birds which suggests that T-cell-mediated responses is important in influenza viral clearance. More definitive tests for evaluation of CMI are needed to clarify how levamisole affects these responses and shedding of H9N2 AI virus in quails.

CONCLUSION

In conclusion, levamisole administration in a repeated regimen enhances CMI responses against H9N2 AI viruses and reduces virus shedding period in experimentally infected quails. This may pave the road for further investigations on potential positive effects of this agent on prevention and management of H9N2 AI infections in quail industry.

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