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Effect of *Zataria multiflora* essential oil on immune responses and faecal virus shedding period in broilers immunized with live Newcastle disease vaccines

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Summary

Zataria multiflora essential oil possesses immunomodulatory effects and the present study investigates its effect on immune responses to live Newcastle disease (ND) vaccines and faecal shedding period of vaccine strain virus in chickens. One hundred and eighty chickens from both sexes were divided into 6 equal groups: group 1: unvaccinated, group 2: vaccinated (B1 vaccine followed by La Sota 10 days later), group 3: vaccinated + levamisole (150 mg/kg/day, orally), groups 4, 5 and 6 were vaccinated and received *Z. multiflora* essential oil by SC injections at 0.1, 0.2 and 0.4 g/kg/day dosages, respectively. Levamisole and *Z. multiflora* essential oil were administered for 10 and 5 consecutive days post B1 vaccination. On days 0, 7, 14, 28 and 35 post booster vaccination (PBV), HI test was performed. Delayed type hypersensitivity (DTH) test was accomplished on day 14 PBV. Faecal virus shedding was determined during 15 days PBV with 3 day intervals by RT-PCR method. *Zataria multiflora* essential oil induced a dose-dependent increase in antibody titers in an extent higher than levamisole as well as a dose-dependent suppression of DTH reaction. Effect of *Z. multiflora* essential oil at the dosage of 0.1 g/kg on shortening of faecal virus shedding period was exactly the same as levamisole where higher doses demonstrated stronger decrease. In conclusion, *Z. multiflora* essential oil stimulates humoral immune responses and shortens faecal virus shedding period while suppressing cell-mediated immune responses in chickens vaccinated with live ND vaccines.

Key words: *Zataria multiflora*, Immune responses, Newcastle disease vaccines, Chickens

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Introduction

Newcastle disease virus (NDV) which belongs to the genus *Rubulavirus* of the family *Paramyxoviridea* occurs worldwide and has a considerable economic impact on the world poultry industry, due to loss of birds, diagnostic laboratory tests, preventive measures, *etc* in intensive farming units (Jørgensen *et al*., 1998) as well as domestic village chickens (Bouzari and Mousavi Morekani, 2006). The mildly virulent B1 and La Sota strains of NDV are currently the most widely used live-virus vaccines for prevention of Newcastle disease (ND) (Alexander, 1997). In spite of regular vaccinations, severe disease outbreaks occur in intensive farming units, which may be due to failure of vaccination (Binjawadagi *et al*., 2009). Recently, manipulation of immune responses by various agents, especially those with herbal origin, in order to improve efficacy of ND vaccination has been practiced with different outcomes. For instance, Zhai *et al*. (2011) demonstrated that ginseng stem and leaf saponins enhance antibody responses as well as cell-mediated immunity in chickens immunized with live ND vaccine, while Jafari *et al*. (2008) observed that inclusion of garlic powder in the diet of broilers has no appreciable beneficial effects on humoral immunity of broilers against ND vaccine.

Zataria multiflora, a suffruticose, perennial shrub with 40-80 cm height, is a member of *Labiatae* (*Lamiaceae*) family and

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is known by the common Persian name "Avishan-e-Shirazi ". This plant grows wild, on rocky and gravelly slopes, from southern to central parts of Iran and also in Pakistan and Afghanistan. *Zataria multiflora* is extensively used in the traditional medicine as antiseptic, analgesic, and carminative. It is also used as a condiment. The biological activities of the plant have been attributed to the essential oil, containing mainly phenolic compounds such as thymol and carvacrol (Hadian *et al*., 2011). Recent studies have demonstrated the immunomodulatory effects of essential oil of this plant *in vivo* (Shokri *et al*., 2006; Khosravi *et al*., 2007; Soltani *et al*., 2010) and *in vitro* (Amirghofran *et al*., 2011).

With regard to these facts, the present study was conducted to investigate the effect of *Z. multiflora* essential oil on immune responses to live ND vaccines in chickens and the duration of virus shedding in this species.

Materials and Methods

Animals and experimental design

One hundred and eighty chicks "Cobb 500" were purchased at 1-day-old; reared under strict bio security and fed *ad libitum*. Chicks were randomly allocated into 6 groups (n=30) and treated as follows:

Group 1: Unvaccinated
Group 2: Vaccinated
Group 3: Vaccinated + levamisole (Razak Pharmaceutical Laboratories, Tehran, Iran), 150 mg/kg/day by oral gavage (Munir *et al*., 2009)
Group 4: Vaccinated + *Z. multiflora* essential oil (Barij Essence Pharmaceutical Co., Kashan, Iran), 0.1 g/kg/day, S.C.
Group 5: Vaccinated + *Z. multiflora* essential oil, 0.2 g/kg/day, S.C.
Group 6: Vaccinated + *Z. multiflora* essential oil, 0.4 g/kg/day, S.C.

Maternal antibody titer against NDV was assayed on the entrance day of chickens by HI method (Grimes, 2002). When the maternal antibody titer reduced to 2^{-3}, all chicks (except group 1) were vaccinated with B1 vaccine (Razi Vaccine and Serum Research Institute, Tehran, Iran) via intraocular route. Ten days later a booster dose of La Sota vaccine (Razi Vaccine and Serum Research Institute, Tehran, Iran) was administered intra nasally.

Levamisole and *Z. multiflora* essential oil were administered for 10 and 5 consecutive days post B1 vaccination, respectively. *Z. multiflora* essential oil contained 29.5% thymol and 30.6% carvacrol as described by the manufacturer.

All methods used in the study were in compliance with the institutional ethical guidelines for use of animals in research.

HI test

On days 0, 7, 14, 28 and 35 post booster vaccination (PBV), HI test was performed on 5 birds of each group as described by Grimes (2002). Briefly, a two-fold serial dilution of serum samples was made in a 96-well V-shaped bottom micro titer plate containing 50 µl normal saline/well. 50 µl of 4 hemagglutination unit of NDV antigen was added to the first well and after mixing, 50 µl from the solution was transferred to the second well and so on. 50 µl of the solution from the last well was discarded. After incubation at room temperature for 30 min, 50 µl of 1% chicken erythrocyte suspension in normal saline was added and incubated for another 45 min. All samples were tested in duplicate. Positive and negative serum controls were included on each plate. The HI titer was determined as the highest dilution of serum causing complete inhibition of hemagglutination. Mean HI titers and standard deviations were calculated for each group.

Delayed type hypersensitivity test

Cell-mediated immune response was assayed by performing DTH test as described by Munir *et al*. (2009) with few modifications. 14 days PBV all birds were sensitized with 0.25 ml of 2, 4-dinitrochlorobenzene (DNCB) solution (10 mg/ml) by S.C. injection in the breast. After 14 days, these sensitized chickens were challenged with 0.25 ml of DNCB (1.5 mg/ml) injected in 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, 72, and 96 h post challenge by a Vernier

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caliper with the precision of 0.02 mm.

Evaluation of vaccine strain virus shedding period

During 15 days PBV faecal samples from 5 birds of each group were randomly collected with 3 day intervals and stored at -70°C. Virus detection was performed by reverse transcription polymerase chain reaction (RT-PCR) method.

A ten percent suspension of faecal samples in normal saline was prepared and after centrifugation at 7500 rpm for 10 min in 4°C, 200 µl of supernatant was used for RNA extraction which was performed by RNX™-plus (CinnaGen Co., Tehran, Iran) commercial kit according to manufacturer's instructions. Extracted RNA was reverse transcribed using AccuPower® RT PreMix (Bioneer Co., Daejeon, South Korea) kit. Twenty pmol/µl of P1F and P2R primer pair which yield specific amplification of a 362 bp fragment within the *F* gene were used with the following sequences: P1F: 5′ TTG ATG GCA GGC CTC TTG C 3′ and P2R: 5′ GGA GGA TGT TGG CAG CAT T 3′ as described by Oberdörfer and Werner (1998) for cDNA synthesis. Five µl of the cDNA was used for PCR amplification using AccuPower® PCR PreMix (Bioneer Co., Daejeon, South Korea). One µl of each of the above mentioned primers with 10 pmol/µl concentration was used for this purpose. The PCR thermocycling condition for the gene was as follows: an initial denaturation for 3 min at 95°C, 30 cycles with denaturation at 95°C for 45 s, primer annealing at 55°C for 30 s and primer extension at 72°C for 45 s and a final extension step at 72°C for 4 min. Five µl of PCR product was subjected to 2% 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 test 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 chickens of groups 1, 2, and 3 during the experimental period. However, a dose-dependent anaphylactic-like reaction was observed immediately after injection of *Z. multiflora* essential oil from day 3 post administration and so on in some chickens. Total death rates in chickens of groups 4, 5 and 6 were 10, 15 and 30%, respectively.

Humoral immune responses

Figure 1 demonstrates the results from the HI test. No NDV antibody titer was detected in chickens from group 1 during the sampling period. Peak of the antibody titer was observed 7 days PBV in all other groups except group 1. Antibody titers against NDV were significantly higher in groups 3, 4, 5 and 6 compared to the titer in group 2. This elevation was observed from day 7 PBV and throughout the study. Meanwhile, the titer in group 6 was increased significantly earlier at day 0 PBV (P=0.01). Levamisole administration resulted in antibody titers which were significantly higher than group 2, only on the 7th day post vaccination (P<0.001). Moreover, *Z. multiflora* essential oil treated chickens (groups 4, 5 and 6) had

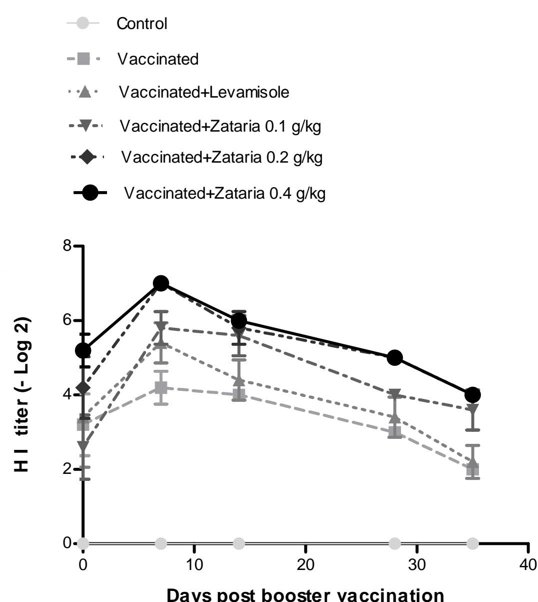

Fig. 1: HI antibody (-Log2±SD) titers at 0, 7, 14, 28 and 35 days following booster vaccination of chickens in different groups

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appreciably higher antibody titers than levamisole treated group on days 14, 28 and 35 (P<0.001 for all comparisons). On day 7, only groups 5 and 6 had significantly increased antibody titers as compared to group 3 (P<0.001). Although the antibody titers of chickens of groups 5 and 6 were higher than group 4 during the sampling period, the difference was significant only on the 14th and 35th days PBV (P<0.001).

Cell-mediated immune responses

The highest skin thickness was observed 24 h post challenge with DNCB in all groups. Although skin thickness of chickens in group 2 was higher than control group at 24, 48, 72 and 96 h post challenge, the difference was significant only at 48 h (P=0.03). Levamisole administration resulted in slight increase in skin thickness in comparison with group 2 at 48 and 72 h post challenge, this increase was not statistically significant. At 48 h post challenge skin thickness of birds treated with 0.4 g/kg *Z. multiflora* essential oil was significantly lower than birds in groups 2 and 3 (P=0.007 and P=0.001, respectively). At 72 h post challenge skin thickness of birds treated with 0.4 g/kg as well as 0.2 g/kg *Z. multiflora* essential oil were significantly lower than birds in group 2 (P=0.049 and P=0.031, respectively). At this time birds treated with all three *Z. multiflora* essential oil doses had significantly lower skin thickness than birds in group 3 (P=0.016, P=0.005 and P=0.003, respectively). At 96 h, although skin thicknesses of *Z. multiflora* essential oil treated chickens were still lower than group 2 and 3, the differences were not significant. Results from DTH test are depicted in Fig. 2.

Faecal virus shedding period

All samples from control group were void of virus during the whole sampling period. One hundred percent of samples from other groups were positive for virus on day 3 PBV. On day 6, although samples from vaccinated group were still 100% positive, virus was detected in 80% of samples from levamisole and 0.1 g/kg *Z. multiflora* essential oil treated chickens. At this time, all samples from 0.2 g/kg as well

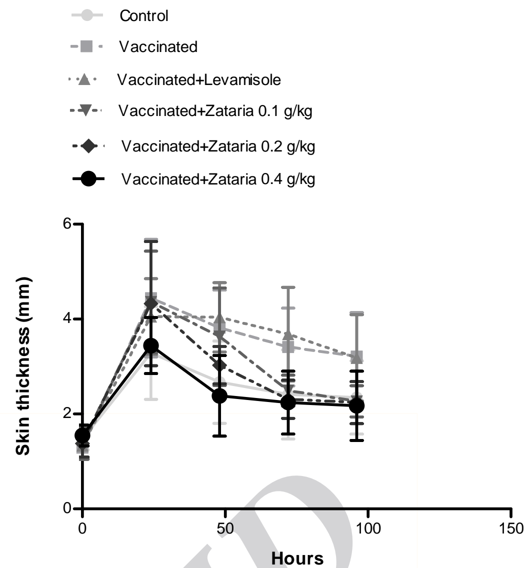

Fig. 2: Skin thickness (Mean±SD) of chickens in different groups, measured at 0, 24, 48, 72 and 96 h post challenge with 2, 4-dinitrochlorobenzene

as 0.4 g/kg groups became negative, which continued till the end of sampling period. On the 9th day the percentage of virus positive samples in groups 2, 3 and 4 was 20%. On day 12 only group 2 was still positive for virus (20%) and all other samples were completely void of virus.

Discussion

The present study aimed to evaluate possible effects of *Z. multiflora* essential oil on immune responses of chickens vaccinated with live ND vaccines. To this end, HI antibody titer and delayed type hypersensitivity reaction were measured as a manifestation of humoral and cell-mediated immunity responses, respectively. Moreover, faecal shedding period of vaccine strain was determined as a model of field virus shedding feature.

Immunostimulatory effect of *Z. multiflora* essential oil on humoral responses has been shown by Khosravi *et al*. (2007) in rabbits inoculated with *Candida albicans* antigens. This is consistent with the outcome of the present study where *Z. multiflora* essential oil administration resulted in a dose-dependent increase in antibody titers against ND vaccine virus in an extent even higher than levamisole. In the study performed by Soltani *et al*. (2010), *Z.*

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multiflora essential oil significantly increased blood lymphocyte count in common carps. Although the mechanism responsible for elevation in antibody titers due to administration of *Z. multiflora* essential oil could not be deduced from the present study, increase in peripheral lymphocyte number may be involved. Obviously, this speculation needs further investigation in future studies. Effect of *Z. multiflora* essential oil at the dose of 0.1 g/kg on shortening of faecal virus shedding period was exactly the same as levamisole whereas stronger doses of 0.2 or 0.4 g/kg resulted in further decrease of the duration. This obvious reduction in virus shedding period could be described by higher antibody titers of *Z. multiflora* essential oil treated birds. Saderi and Abbasi (2011) demonstrated that aqueous extract of *Z. multiflora* has appreciable antiviral effect against adenoviruses. Therefore, direct antiviral effects by this herb could also be involved in reducing NDV shedding period. Moreover, enhancement of innate immunity by *Z. multiflora* essential oil which has been proved in mice (Shokri *et al*., 2006) may play a role in this regard, although these speculations need to be investigated in future studies.

DNCB has been shown to induce a DTH reaction in chickens (Awadhiya *et al*., 1982). This reaction is a convenient model to investigate the basic mechanisms of the cellular immune response and its regulation in the chicken (Huynh and Chubb, 1987).

The effects of *Z. multiflora* essential oil on cell-mediated immunity responses have been controversial. In the present study, *Z. multiflora* essential oil induced a dose-dependent suppression of DTH reaction. Khosravi *et al*. (2007) observed that the essential oil of this herb stimulates cellular immunity responses against *Candida albicans* antigens and Con-canavalin A mitogen in rabbits, while Amirghofran *et al*. (2011) showed the inhibitory effects of hexan extract of *Z. multiflora* on phytohaemagglutinin mitogen-induced proliferation of peripheral blood lymphocytes. These discrepancies may be due to different experimental systems and/or herbal preparation used. It should be mentioned that leukocyte migration assays have demonstrated that increased levels of cell-mediated immunity do not always correlate with hemagglutination-inhibition of NDV using serum from vaccinated chickens (Agrawal and Reynolds, 1991; Rehmani and Spradbrow, 1995). This may explain the obvious rise in antibody titer despite cell-mediated immunity suppression in the present study.

In conclusion, *Z. multiflora* essential oil stimulates humoral immune responses and shortens faecal virus shedding period while suppressing cell-mediated immune responses in chickens vaccinated with live ND vaccines.

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