Original Article

Efficacy of CpG-ODN Administration Routes on Humoral Responses against Newcastle disease in Broilers

Talebi [1] *, A., Arky-rezaei [2], S.

1. Department of Poultry Health and Diseases, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
2. Graduated, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

ABSTRACT

Un-methylated cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN) has been considered as a powerful vaccine adjuvant and recognition of CpG-ODN by chicken leukocytes promotes their ability to fight against infections. In our study, efficacy of different routes of CpG-ODN application as an adjuvant on immune responses (antibody titer together with leukogram) following vaccination against Newcastle disease (ND) has been evaluated in broiler chickens (Ross-308). The results indicated that routes of CpG-ODN administration influence immune responses and comparison effectiveness of CpG-OND delivery routes showed that group vaccinated by eye-drop application had the highest antibody titer than that of the group injected intramuscularly (im) and the difference was significant (p = 0.04) on day 35 of age. Antibody titer of the group treated with Clone 30 plus CpG-ODN via eye-drop route was higher than that of the group vaccinated with clone 30 alone on days 28 and 35 of age and the difference was significant (p = 0.04). Co-administration of both vaccine and CpG improved outcome of leukogram of the chickens on days 21 to 42 of age and among the treated groups, WBC of the group received both vaccine and CpG by eye-drop route significantly (p < 0.05) differed from that of the group vaccinated with clone 30 alone on days 28 and 35 but not on day 42 of age. Average final body weight of the control group did not significantly differ from those of the treated groups at end of the experiment. In conclusion, co-administration of ND vaccine plus CpG-ODN via eye-drop route improves immune responses.

Keywords: CpG, Antibody titer, ND, Broilers, Vaccine administration route

Efficacité des Voies d'Administration ODN-CpG sur les Réponses Humorales contre la Maladie de Newcastle chez les Poulets de Chair

Résumé: Les oligodésoxynucléotides non méthylés de cytosine-phosphate-guanosine (ODN-CpG) ont été considérés comme un puissant adjuvant vaccinal et la reconnaissance de l'ODN-CpG par les leucocytes du poulet favorise leur capacité à lutter contre les infections. Dans notre étude, l'efficacité des différentes voies d'application de l'ODN-CpG en tant qu'adjuvant des réponses immunitaires (évaluation du titre d'anticorps ainsi que du leucogramme) à la suite de la vaccination contre la maladie de Newcastle (ND) a été étudiée chez des poulets de chair (Ross-308). Les résultats montraient que les voies d'administration de l'ODN-CpG influent sur les réponses immunitaires. Le groupe vacciné par l'application de gouttes oculaires avait un titre d'anticorps plus élevé que celui traité pat injection intramusculaire (im) et la différence était significative (p = 0,04) au trente-cinquième jour. Le titre en anticorps du groupe traité par les gouttes oculaires comprenant le clone 30 combiné à l'ODN-CpG était supérieur aux jours 28 et 35 à celui du groupe vacciné uniquement avec le clone 30 (p = 0,04). La co-administration du vaccin et du CpG a amélioré les résultats du leucogramme des poulets du 21ème au 42ème jour. Parmi les groupes traités, le leucogramme du groupe ayant reçu à la fois le vaccin et le CpG par voie oculaire montrait des différences significatives (p <0,05) de celui du groupe vacciné uniquement avec le clone

30 aux 28ème et 35ème jours, mais au 42ème jour. Le poids corporel final moyen du groupe témoin ne différait pas significativement de celui des groupes traités à la fin de l'expérience. En conclusion, la co-administration du vaccin ND et de l'ODN-CpG améliore la réponse immunitaire après vaccination.

Mots-clés: CpG, titre en anticorps, ND, poulets de chair, voie d'administration du vaccin

INTRODUCTION

Vaccination is the most practical method of prevention of avian infections in poultry industry world-wide and vaccines require inclusion of effective adjuvants to promote broad protective immune responses (Gupta et al., 2014; Mount et al., 2013). Previous studies have demonstrated that CpG-ODNs are known to stimulate immune responses (Gomis et al., 2003) and have an immunoprotective effect against avian pathogens (Hartley et al., 2012; Gunawardana et al., 2015). Three classes of CpG-ODN with distinct immuno-stimulatory activities have been identified (Völlmer et al., 2004) and effectiveness of different CpG-ODN (Fu et al., 2013) motifs, as adjuvant, have also been evaluated (St Paul et al., 2014). Co-administration of CpG-ODN with commercial live vaccines (Zhang et al., 2008) as well as with inactivated vaccines (Fu et al., 2013) enhances humoral immune responses including immunoglobulin M (IgM), immunoglobulin G (IgG) and immunoglobulin A (IgA) production (Ameiss et al., 2006; Zhang et al., 2008). Regarding to mechanism of action of CpG, it has been reprted that CpG-ODN involved in induction of pro-inflammatory immune response in chicken monocytes (Haiqi et al., 2011), triggering Th1 response (Azmi et al., 2014) and potenting of humoral as well as cellular responses, through Toll-like receptor 9 mediated signaling pathway (Scheiermann and Klinman, 2014; U-Taynapun et al., 2016; Yu et al., 2017). In 21st century, there is still a need to incorporate more suitable adjuvants (Gupta et al., 2014) or multiadjuvants (Mount et al., 2013) in the vaccines to promote immune responses in order to provide optimum protection against important avian diseases including ND which is the fourth most problematic disease in poultry industry (Dimitrov et al., 2017). Various routes of application of CpG-ODN may affect its efficacy (Chrząstek et al., 2014) because delivering of CpG-ODN to the initiation sites of immune responses (Yu et al., 2017) is essential for its application as a vaccine adjuvant. Therefore finding of a suitable vaccination route for co-administering of CpG-ODNs with various poultry vaccines is in much interest in poultry industry and this study was conducted to evaluate the efficacy of different methods of application of class B CpG-ODN on antibody titer and leukogram induced by vaccination against ND in broilers.

MATERIAL AND METHODS

Chickens. All procedures involving chickens were carried out with the approval of the Faculty of Veterinary Medicine of Urmia University as a DVM thesis. One hundered and fifty one-day-old broiler chicks (Ross-308 strain) were leg labeled, kept in boxes, and fed ad libitum with a diet based on Ross-308 husbandry catalogue. The chicks were divided into 6 treatmens (3 replicates per treatment) including group A (no vaccine and no CpG-ODN) as a control (V⁻ & CpG⁻), group B (no vaccine but with CpG-ODN by im route) as a control for efficacy of CpG-ODN alone (V⁻ & CpG^{im}), group C (only vaccine by eye-drop route) as a control for efficacy of vaccine alone (V$^{eye-drop}$ & CpG⁻), group D (vaccine by eye-drop route plus CpG-ODN by im route) (V$^{eye-drop}$ & CpG^{im}), group E (vaccine by

im route plus CpG-ODN by im route) for evaluation of im route (V^{im} & CpG^{im}), and group F (vaccine plus CpG-ODN by eye-drop) for evaluation of eye-drop route (V$^{eye-drop}$ & CpG$^{eye-drop}$).

Vaccines and vaccination routes. Based on maternally derived antibody (MDA) (El-Tayeb et al., 2013; Miller & Koch, 2013; Jacobs et al., 2014) of chicks, live ND clone 30 vaccine (Nobilis ND clone 30, Intervet) was used for vaccination of chikens of group C, D and F by eye-drop route, and of chickens of group E by im administration on day 9 of age and for re-vaccination on day 21 of age. Chickens of group A and B were not vaccinated.

CpG ODN. The synthetic class B CpG-ODN was purchased from Cinna Gen Inc (Cinna Gen, Tehran, Iran) according to referred sequence (5′-TCGTCGTTG TCGTTTTGTCGTT-3′) as previously reported (Gomis et al, 2003; Chrząstek et al., 2014). The CpG-ODN was stored at –20 ℃ until used and diluted in PBS at 20g/l before administered 50μg/chicken as an adjuvant for chickens groups B (control for CpG), D, E, and F on day 9 and 21 of age.

Blood sampling. On day 0, three chicks of each group were scarified as previously described (Alcorn, 2008) and their blood was considered as one-day-old samples. On day 7, blood samples were taken from jugular veins (Alcorn, 2008) of the chicks using 1ml syringes and were labeled by number of birds and date. On day 14 and weekly interval until 42 days of age, blood samples were taken and processed as previously discribed (Alcorn, 2008; Collet, 2013). For haematological examination, blood samples were mixed with ethylenediaminetetraacetic acid (EDTA) and used immediately (Collett, 2013).

Performance. Weight gain and feed-intake as well as feed conversion ratio (FCR) were measured in order to evaluate effects of vaccine, CpG and vaccination routes on performance of the chickens.

Haemagglutination inhibition (HI) test. HI test was used to evaluate antibody titers of the serum samples taken on different age and also to determine range of antibody titer among the chickens of each group as recommended (Miller and Koch, 2013).

Leukogram. Whole blood samples were collected into tubes containing EDTA as anticoagulant at a 1:10 dilution. Blood smears were stained with the classic Wright's stain. Absolute count of white blood cells (WBC) was determined by routine methods as previously described (Cample, 1992; Walberg, 2001).

Statistical analysis. The data for all variables were subjected to one-way analysis of variance (ANOVA) as a completely randomized design using the SPSS software (Version 21; SPSS Inc., Chicago, USA). The means for treatments showing significant differences in the ANOVA were compared using Post-Hoc Tukey and paired-samples t-tests. Differences were considered significant at $p < 0.05$.

RESULTS

Performance. The effects of vaccination methods together with vaccinal agents including CpG-ODN on performance of chickens vaccinated against ND is shown in Figure 1. Average weights of unvaccinated groups (A and B) were higher than those of the vaccinated groups but the differences were not significant ($p > 0.05$) at the end of experiment.

Antibody response. The result of this study on efficacy of CpG-DNA on immune response of chickens to ND vaccination is presented in Figure 2. Maternally derived antibody of the chicks against ND gradually declined in all the groups and it's levels in groups A (V$^-$ & CpG$^-$) and B (V$^-$ & CpG^{im}) was undectable on day 21 of age. As shown in Figure 2, antibody titer of the group vaccinated with Clone 30 plus CpG-ODN via eye-drop route was higher than that of the group vaccinated with clone 30 alone on days 28 and 35 of age and the difference was significant ($p = 0.04$). Concerning routes of CpG-OND delivery, the group with eye-drop application also had the highest antibody titer than that of the group injected intramuscularly and the difference was significant ($p = 0.041$) on day 35 of age.

Leukogram. Effects of CpG-ODN on leukogram of chickens vaccinated against ND are presented in Figure 3 and Table 1. WBC counts of all the groups were increased gradualy during the experimental period, but those of the treated groups were higher than those of unvaccinated groups on day 21 until end of the experiment. As shown in Figure 3, differences were significant ($p < 0.01$) on 21, 28 and 35 of age but on day 42 of age, only those of the groups D, E and F significantly ($p < 0.05$) differed from that of the group

A. Among the treated groups, WBC of the group F (V[eye-drop] & CpG[eye-drop]) significantly ($p < 0.05$) differed from those of the groups C and E. As shown in Table 1, significant ($p < 0.05$) difference were also observed when heterophils and lymphocytes percentage of the groups were compared at the end of the experiment.

DISCUSSION

Comparison average weight of the groups (Figur 1) showed some differences among them but lack of
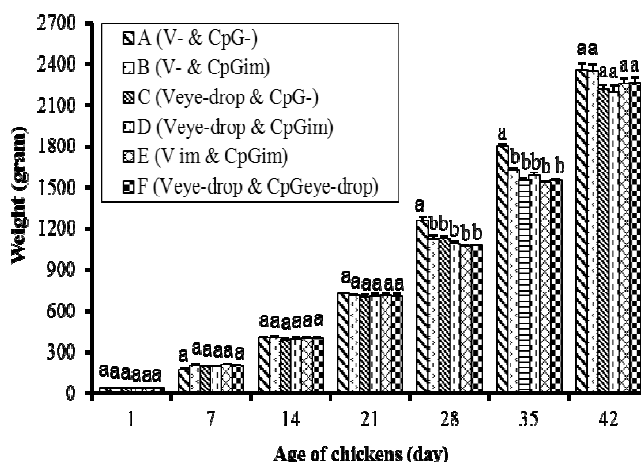

Figure 1. Effects of administration routes of CpG-ODN and ND vaccination on weight of the chickens. Group A (control), group B (CpG-ODN alone), group C (ND clone 30 alone), group D (vaccine by eye-drop route and CpG by im route), group E (im route), group F (eye-drop route). Different letters on each column indicate significant (p < 0.05) difference.
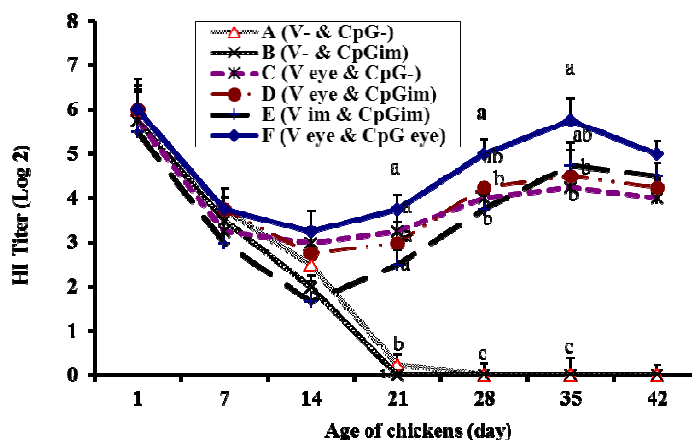

Figure 2. Newcastle disease HI titers (Log 2) of chickens of the groups. Group A (control), group B (CpG-ODN alone), group C (ND clone 30 alone), group D (vaccine by eye-drop route and CpG by im route), group E (im route), group F (eye-drop route). Different letters on each sampling date express significant (p < 0.05) difference.

significance differences at the end of the experiment may indicates that negative effects of vaccination routes on final average body weight of broiler chickens were negligible. Lack of detectable level of antibody in serum of chickens group A (environmental control group) and group B (control for CpG-ODN) from age of 21st day to end of the experiment indicates that there was no enviromental contamination during the experimental period. Comparison of the unvaccinated groups with the vaccinated groups showed that there was a difference on day 21 to the end of experiment and the significant ($p < 0.05$) difference was resulted from vaccination as indication for desirable vaccination with no environmental contamination in the unvaccinated groups. Regarding to comparison of antibody titers of vaccinated groups, some differences among them were observed on day 21 to the end of experimental period and the highest level of antibody response was in the rangeas previously reported (Miller and Koch, 2013). Comparison of the results on day 21 showed that: 1) the significant difference ($p = 0.001$) between vaccinated and unvaccinated groups was a clear indication for lack of environmental contamination on 3rd week of experiment; 2) there was no significant difference between group A (V⁻ & CpG⁻) and group B (V⁻& CpG^{im}), indicating that CpG-ODN alone may induce innate responses (Mena et al., 2003) but did not induce specific response when measured by HI to ND antigen; 3) the difference among treated groups were not significant ($p > 0.05$), indicating that routes of vaccination and administration of CpG did not affect on immune responses in a short period as expected. Comparison of the results on day 28 showed that: 1) the difference between group C (V$^{eye-drop}$ & CpG⁻) and group D (V$^{eye-drop}$ & CpG^{im}) was not significant ($P > 0.05$), indicating that administration of CpG-ODN with im route had no significant effect at least in short period; 2) the significant ($p = 0.047$) difference between group C (V$^{eye-drop}$ & CpG⁻) and group F (V$^{eye-drop}$ & CpG$^{eye-drop}$) also demonstrated the effects of CpG-ODN by eye-drop route in vaccinated

chickens as shown in Figure 2; 3) the significant ($p = 0.01$) difference between group D (V$^{eye-drop}$ & CpG^{im}) and group F (V$^{eye-drop}$ & CpG$^{eye-drop}$) observed during this study may refelect the effectiveness of administration route of CpG-ODN. Comparison of the results on day 35 of age showed that: 1) significant ($p < 0.05$) difference between unvaccinated groups (A and B) and vaccinated groups (C, D, E and F) confirmed that environmental status of the experimental was also not contaminated on 5th week; 2) significant differences between group F with groups C and D ($p = 0.01$, $p = 0.04$, respectively), indicating the effects of CpG and it's route of application the effects. Comparison of the results on day 42 of age showed that antibody titers of the vaccinated groups were declined and differences among the treated groups (C, D, E, and F) were not significant ($p > 0.05$), while continued lack of detectable antibody titer in unvaccinated chicken confirmed the safty of environment during the experimental period. Overall as shown in Figure 2, the results clearly demonstrate that higher and rapid stimulation effects of eye-drop route adminstration of both vaccine and CpG-ODN on antibody production level of vaccination against ND. The immunoenhancing effect of CpG-ODN on ND titer observed in our study is in agreement with those previously reported (Linghua et al., 2007). The results of this study showed that ocular method had the highest effect on immunoglobulin G (IgG=IgY) responses to clone 30 vaccine, while it has been reported that oral route specifically enhances both the immunoglobulin M (IgM) and immunoglobulin A (IgA) responses (Huang et al., 2007) and intranasal deleevery of CpG-ODN stimulated both T-helper type1 (IgG2) and type 2 (IgA) responses (Zhang et al., 2007; Zhang et al., 2008) as well as intramuscular co-injection of CpG-ODN with ND vaccine induces a stronger immune responses (serum IgG titer and lymphocytes proliferation) in chickens (Linghua et al., 2007). As shown in Figure 2, antibody titer of the group received clone 30 plus CpG-ODN in eye-drop route was higher than that of the

group treated with clone 30 alone may emphesize on adjuvant effects of CpG-ODN on serum antigen-specific antibody titer. Our observationson enhanced antibody titer following dual vaccination, is in agreement with the results as previously reported (Barri, 2004). Some differences between our findings and previous reports (Barri, 2004) could be related to

chickens (Chrząstek et al., 2014). Overall, there are more encouraging reports on stimulatory effects of CpG-ODN on innate immune responses and enhancement on antigen-specific humoral responses together with promoting resistance of birds to avian pathogenic agents, indicating very important immunological consequences for vaccine development
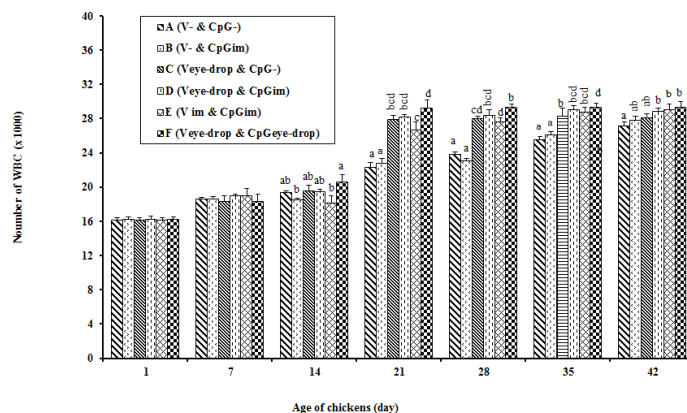

Figure 3. White blood cells (WBC) count of chickens in the groups. Group A (control), group B (CpG-ODN alone), group C (ND clone 30 alone), group D (vaccine by eye-drop route and CpG by im route), group E (im route), group F (eye-drop route). Different letters on each column express significant (*p* < 0.05) difference.

Table 1. Some leukogram parameters of the groups at end of the experiment

Groups	Lympho(%)	Hetero(%)	H:L ratio	Mono(%)	Esino(%)	Baso(%)
A (V⁻ & CpG⁻)	54.6ᵃ	33.6ᵃ	0.61ᵃ	3.6	5.6	2.6ᵃ
B (V⁻ & CpG⁺)	34.8ᵇ	53.8ᵇ	1.52ᵇ	3.2	4.4	4.2ᵃ
C (Vᵉʸᵉ⁻ᵈʳᵒᵖ & CpG⁻)	59.2ᵃ	31.2ᵃ	0.52ᵃ	3.2	3.8	2.6ᵃ
D (Vᵉʸᵉ⁻ᵈʳᵒᵖ & CpGⁱᵐ)	33.8ᵇ	55.8ᵇ	1.66ᵇ	3.4	3.6	3.2ᵃ
E (Vⁱᵐ & CpGⁱᵐ)	31.4ᵇ	57.0ᵇ	1.85ᵇ	4.6	4.4	2.2ᵃᵇ
F (Vᵉʸᵉ⁻ᵈʳᵒᵖ & CpGᵉʸᵉ⁻ᵈʳᵒᵖ)	25.2ᵇ	60.4ᵇ	2.42ᵇ	3.0	6	5.2ᵃᶜ
Significance:		*P < 0.05*		Not significant		*P < 0.05*

Lympho (lymphocyte), hetero (heterocyte), Mono (monocyte), Esino (esinophil), Baso (basophil). Different letter on each column expressed significant (*p< 0.05*) difference.

motif of CpG-ODN, dose of injection (Xie et al., 2003), the inclusion of spacers between multiple copies of the motif and vaccination dates. Moreover, CpG-ODNs increase the immunogenicity of co-administered vaccines by various mechanisms including promoting the function of antigen-presenting cells (Bode et al., 2011; Scheiermann and Klinman, 2014) and it's immuno-stimulatory activity appeared to be in dose-dependent manner as administration of 50 to 75 µg of CpG-ODN can accelerate the production of antigen specific antibodies of all three isotypes in commercial

even though with 1000-fold reduction in antigen amounts in some cases (Gomis et al., 2003; Huang et al., 2007).

Leukogram. As shown in figure 3, comparison of the results on day 14 of age showed a significant (*p* < 0.05) difference between the groups F and E within 5 days PI and may indicate that co-administration of ND vaccine plus CpG via im route did not strongly affect the WBC of the chickens at least on short time. Comparison of the results on day 21 of age (12 days PI) showed that: 1) a significant (*p* = 0.01) difference between the

control groups (A and B) and the treated groups, indicating that vaccination alone or plus with CpG-ODN affect WBC counts of the chickens; 2) lack of a significant ($p > 0.05$) difference among the groups C and D together with presence of a significant ($p = 0.04$) difference between the groups F and E, may indicate the effectiveness of eye-drop routes. Comparison of the results on days 28 and 35 of age showed that: 1) a significant ($p = 0.01$) difference between the control groups (A and B) and the treated groups is still exist as it was observed on day 21 of age, indicating the safety of the experiment; 2) WBC of the group F ($V^{eye-drop}$ & $CpG^{eye-drop}$) significantly differed from that of the group C ($V^{eye-drop}$ & CpG^-) on days 28 and 35 of age ($p = 0.01$ and $p = 0.03$, respectively), indicating that higer WBC of the group F observed during this study was due to CpG-ODN co-administration with ND vaccine; 3) a significant ($p = 0.01$) difference between WBC of the groups F and E only on day 28 of age together with lack of a significant difference between the groups D and E may also confirm the effectiveness of CpG-ODN co-administration via eye-drop route. Regarding to comparison the results on day 42 of age, significance differences between the group A (control group) with the groups D, E and F ($p = 0.03$, $p = 0.02$ and $p = 0.1$, respectively) together with lack of significant differences among the groups A, B and C also confimed that co-administration of CpG-ODN increased WBC counts of the vaccinated chickens. As shown in Table 1, a significant ($p < 0.05$) difference among heterophils and lymphocytes percentage of the groups A and C with those of CpG-ODN treated groups (B, D, E, F) may indicate that co-administration of CpG-ODN plus ND vaccine activated leukocytes of the chickens.

In conclusion, co-administration of ND vaccine plus CpG (perticulary via eye-drop route) induced higher humoral response as well as influence leukocytes activity without affecting performance (body weight) of broiler chickens and may contribute toward development of more effective vaccines.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

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