Immunogenicity of Newcastle disease virus strain ZG1999HDS applied oculonasally or by means of nebulization to day-old chicks

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ABSTRACT Newcastle disease (**ND**) is one of the classic viral infections of poultry which resists all the efforts of eradication. Newcastle disease virus (**NDV**) strain ZG1999HDS was isolated during the outbreak in 1,999 at a broiler farm in Croatia. Previous trials in chickens confirmed it to be a lentogenic pathotype and immunogenic by stimulating humoral and cell mediated immunity. Further characterization by deduced amino acid sequence at the cleavage site of fusion protein confirmed its lentogenic nature, and in vitro tests its oncolytic capacity. Owing to its immunogenicity, strain ZG1999HDS is considered for vaccine development. In this study, 1-day-old chicks were vaccinated using strain ZG1999HDS oculonasally or by nebulization. Strain ZG1999HDS induced humoral immune response in both immunized groups The cell-mediated immune response occurred earlier in the group immunized by nebulization, as shown by a higher frequency rate of T and B lymphocytes, and significantly higher expression of IFN-α in respiratory organs and IFN-γ expression in the spleen. Viral genomic RNA was not detected in investigated organs. Thus, NDV strain ZG1999HDS is immunogenic when administered by means of nebulization or oculonasally without any adverse effects and is therefore suitable for further research and vaccine development. Further research is needed regarding its tropism.

Key words: Newcastle disease, cell-mediated immunity, flow cytometry, interferon-α, interferon- γ

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INTRODUCTION

Newcastle disease (**ND**) is a highly contagious viral infection of birds with significant economic impact on poultry industry. From the first occurence, ND remains a serious health issue with significant economic loss due to high mortality and costs of disease control. Specific control of ND includes vaccination with attenuated, lentogenic, or mesogenic strains (Beard and Hanson, 1984; Alexander, 1998; Catolli et al., 2010; Miller and Koch, 2013.). Compared with vaccination through drinking water, aerosol vaccination is more efficient and induces good protection in a shorter period, when antibodies appear in serum and mucosa 4 to 10 d after vaccination and reach peak levels after the third or fourth week (Alexander and Senne, 2008) and could insure 12 wk of protection. (Oberländer et al., 2020). Lentogenic strains, such as La Sota, are sufficiently immunogenic, and are widely used as a vaccine (Dimitrov et al., 2017.) Lentogenic strains may only be used in birds with low or no maternal antibodies, but not in areas where ND is endemic and caused by virulent strains, where more immunogenic mesogenic strains should be used (Al Garib et al., 2003). Vaccination against ND is performed as per guidelines in almost all European countries, including Croatia, with the aim of achieving a high level of specific immunity. In general, the duration of protective immunity induced with live vaccines is shorter if vaccinated flock is younger (Miller and Koch, 2013). Booster vaccinations are required in poultry flocks that remain in production for a longer time or the immunity should be passively transferred, as in layers and parent flocks (Dimitrov et al., 2017).

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The mechanism and organ systems involved in the production of specific antibodies also differ with respect to the manner of administration of the immunogen. Thus, when applied oculonasally or by drinking water, the vaccine virus will first come into contact with immunologic organs such as the Harderian gland and digestive system, which will respond to the applied antigen by B-lymphocyte migration into the area of viral entry (Alexander and Senne, 2008). But, if applied (by nebulization deeply into the respiratory system), despite the paucity of free macrophages (Härtle and Kaspers, 2014), respiratory system of birds (chickens), compared with mammals (rabbits) of the same body mass, proves to be 8 times more efficient, not only as a respiratory organ, but also as an immunogen-accepting organ (Brown et al., 1997).

Lentogenic strains of Newcastle disease virus (**NDV**) were not isolated in Croatia until 1999. Strain ZG1999HDS of NDV was isolated during enzootic outbreak in broiler chickens with flock mortality of 76.87% during 17 d from the onset of clinical signs. Until now, this strain been characterized for pathogenicity, mean death time (**MDT**), intracerebral pathogenicity index, and intravenous pathogenicity index (Mazija et al., 2011), the whole genome sequence (Nedeljković, 2011) as well as the deduced amino acid sequence at the cleavage site of the fusion glycoprotein (Runjić, 2006). Strain ZG1999HDS induces humoral and cell-mediated immunity, as well as the expression of interferon-gamma (**IFN-γ**) in the spleen 2 d after infection in commercial layers (Nedeljković, 2014). In addition, the strain shows significant differences in the lysis of the tumor cells when compared with the La Sota strain (Mazija et al., 2011). Owing to its immunogenicity, strain ZG1999HDS is considered a suitable candidate for vaccine development.

Nebulization (fogging) is the process of vaccine application deep into the respiratory system of poultry. Ultrasonic nebulizer uses ultrasound at a frequency of 2.1 MHz, which causes formation most (95%) particles ranging from 2 to 5 μm in diameter. This spray does not cause soaking of chickens and effectively reaches the lower respiratory system (Mazija and Štimac, 2003.; Mazija et al., 2009). The nebulization process was developed with the intention of administering the Marek's disease virus vaccine strain HVT FC126 (Mazija and Štimac, 2003; Gottstein et al., 2007, 2015) and thus greatly speed up the procedure otherwise done either intramuscularly or subcutaneously. Until now, live vaccine strains of NDV, Queensland V4 and Ulster 2C (Mazija et al., 2010), La Sota (Mazija et al., 2002, 2009), infectious bronchitis virus, strain H120 (Mazija et al., 2000a), the chicken pox virus (Mazija and Gottstein 2006), a pigeon pox virus (Gottstein et al., 2004), and Marek's disease–HVT FC126 (Mazija et al., 2000b; Gottstein et al., 2007; Gottstein et al., 2015) were applied by nebulization.

The aim of this study was to determine whether and to what extent ZG1999HDS strain of NDV promotes immune response when given oculonasally or by nebulization to newly hatched commercial layer chicks. Stimulative effect of strain ZG1999HDS to the cell-mediated immune response, resulting in the induction of interferon response and the proliferation of subpopulations of lymphocytes, was expected, which would justify the further use of the strain as a vaccine candidate.

MATERIALS AND METHODS

Animals and Trial Design

A total of 150 day-old male light hybrid chicks TETRA SL LL (Bábolna Tetra Ltd, Bábolna, Hungary) were placed in cages, where water and feed were given ad libitum. All required permits for performing the experiment were obtained (Veterinary and Food Safety Directorate, Ministry of Agriculture, Zagreb, Croatia, and the Ethics Committee of the Faculty of Veterinary Medicine, Zagreb, Croatia). The suspension of allantoic fluid containing strain ZG1999HDS of NDV was applied oculonasally to immunize chickens in O group with 0.02 mL of virus suspension in the right eye and nostril at a dose of 10^6EID$_{50}$/bird, whereas the chicks of group N were immunized by means of nebulization for 60 s, where the virus concentration was adjusted in such a way that each chicken was offered one full dose of vaccine (10^6EID$_{50}$) (Mazija et al., 2009). The chicks in the control group were not treated.

Before vaccination at day 1 and in week intervals till day 28, titer of antibodies against NDV was determined by hemagglutination inhibition (HI) test. During the experiment, 6, 12, 24 h, and 2, 3, 5, 7, and 14 d after vaccination, 6 birds were randomly chosen for blood sampling, and after sacrifice using carbon dioxide as per EU recommendations, samples of the spleen, bone marrow, lungs, esophagus, and small intestine were taken. Whole blood samples were used for hematology and flow cytometry, whereas tissue samples were used to determine the relative expression of interferon-alpha (**IFN-α**), IFN-γ, and the absolute quantification of the virus using qRT-PCR.

Determination of NDV-Specific HI Serum Antibody Titer

HI serological assay was used for detection of active development of specific antibodies for NDV, as well as their vertical transmission. The test was performed by standard beta-procedure (Allan and Gough, 1974; Alexander, 1998; Anonymus, 2012), in 96-well U-bottom microtiter plates, as previously described (Anonymus, 1992, 2012, 2019), and VG/GA strain of NDV was used as antigen (Avinew, Merial, France).

Determination of Total Leukocyte Count and Individual Subpopulations by Flow Cytometry

Chicken peripheral blood mononuclear cells (**PBMC**) were isolated from heparinized whole blood using a modified Ficoll density gradient (Dalgaard et al.,

2010). The 1 mL of whole blood was added to the test tube, mixed with PBS in a 1:1 ratio and 0.8 mL of 3% dextran was added to this solution and centrifuged at 50 × *g* at room temperature for 10 min (Jergović et al., 2017). With this process, we precipitated erythrocytes and platelets and collected the plasma supernatant with leukocytes. Upper layer, containing PBMC was carefully collected and layered onto Ficoll Histopaque-1077 (Sigma-Aldrich, St. Louis, MO in a 1: 1 ratio, then centrifuged at room temperature at 900 × *g* for 30 min to separate the PBMC in the layer between the supernatant and Ficoll. After centrifugation, a leukocyte ring was collected, transferred to new tubes, and washed twice in RPMI 1640 with L-glutamine, 10% fetal calf serum, penicillin/streptomycin, and centrifuged at 600 × *g* for 5 min. After the supernatant was discarded, the cells were suspended in 1 mL of cell medium, and counted under light microscope using Neubauer chamber. For immunophenotyping of PBMC and T cell subpopulations, 250,000 cells were stained with mouse monoclonal antibodies specific for chicken leukocyte antigens; CD45-APC (clone LT-40), macrophages/monocytes R-PE (clone KUL01), Bu-1 FITC (clone AV 20), CD3 SpectralRed (clone CT-3), CD8α-FITC (clone EP-72), CD4 R-PE (clone CT-4) (SouthernBiotech, Birmingham, AL). Unlabeled γδTCR antibody (clone TCR-1) was visualized with Zenon Alexa Fluor 647 (Thermo Fisher Scientific, Waltham, MA). Gating strategies are described and presented in Supplement 1 file. After exclusion of doublets, debris, and the small number of remaining erythrocytes and granulocytes by forward and side scatter, at least 20,000 cells were acquired in the lymphocyte region, on a LSRII flow cytometer (Becton Dickinson, Mountain View, CA). Multiparametric data analysis was performed using FlowJo software (Version 7.6.5, Tree Star, Inc., Ashland, OR).

Isolation of Total RNA From Tissue Samples and IFN-α, IFN-γ and NDV Quantification

For the total RNA isolation from tissue samples, we performed the acid guanidine-thiocyanate-phenolchloroform extraction method (Chomczynski and Sacchi, 2006) using TRI Reagent (Sigma-Aldrich, Steinheim, Germany) as per the manufacturer's instructions. Ribonucleic acid pellet was resuspended in 50 µL of RNAse-free water and stored at −80°C until analysis. The relative expression of IFN-α and IFN-γ, and absolute quantity of virus genomic RNA (strain ZG1999HDS) was determined by qRT-PCR. The TaqMan RNA-to-CT 1-Step Kit (AppliedBiosystems, Foster City, CA) was used for analysis on a Rotor-Gene Q device (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Specific primers and probes used in the study are shown in Table 1.

The relative expression of IFN-α and IFN-γ of each sample in duplicate was determined relative to the expression of β-actin mRNA as a housekeeping gene, within 5 µL of sample RNA in a total of 15 µl of the reaction mixture, according to Pfaffl et al. (2002). Results are calibrated to the control group (C) at 6 h to be able to follow the expression change in the control group.

The amount of NDV RNA in tissue samples was determined using TaqMan RNA-to-CT 1-Step Kit (Applied-Biosystems) in parallel with standard curve for absolute quantification with five ten-fold serial dilutions of NDV RNA run in triplicates. Obtained results were analyzed using the associated Rotor-Gene Q Software program (Qiagen, Hilden, Germany).

Statistical Analyses

The results were analyzed by computer program STATISTICA 12 (StatSoft Inc., Tulsa, OK, 2016). The Kolmogorov-Smirnov test was used to test the normality of data distribution, and the one-way ANOVA or Kruskal-Wallis methods were used to analyze the differences between the experimental and control groups because of the normality of the data distribution.

RESULTS AND DISCUSSION

The aim of the study was to investigate the immunogenicity of the ZG1999HDS NDV strain applied by means of nebulization and oculonasally to one-day-old

Table 1. Specific primers and TaqMan probes.

Mark	Oligonucleotide sequence (5'→ 3')	Reference
qNDV-F (M+4100)	5′-AGTGATGTGCTCGGACCTTC-3′	Wise et al., (2004).
qNDV-R (M+4169)	5′-CCTGAGGAGAGGCATTTGCTA-3′	
qNDV-P (M-4220)	5′- HEX- TTCTCTAGCAGTGGGACAGCCTGC -BHQ 1 -3′	
qChIFN α F	5′- GGACATGGCTCCCACACTAC -3′	Jenkins et al., (2009).
qChIFN α R	5′-TCCAGGATGGTGTCGTTGAAG-3′	
qChIFN α P	5′-FAM-CAGCGCGTCTTGCTC-BHQ 1-3′	
qChIFN γ F	5′-GTGAAGAAGGTGAAAGATATCATGGA-3′,	Kaiser et al., (2000).
qChIFN γ R	5′-GCTTTGCGCTGGATTCTCA-3′	
qChIFN γ P	5′ FAM-TGGCCAAGCTCCCGATGAACGA-BHQ 1-3′	
Ch b-aktin–F	5′-ACCACAGCCGAGAGAGAAAT-3′	Filipović et al., (2013).
Ch b-aktin–R	5′-GACCTGACCATCAGGGAGTT-3′	
Ch b-aktin–P	5′-FAM-CGTCGCACTGGATTTCGAGCA-BHQ 1-3′	

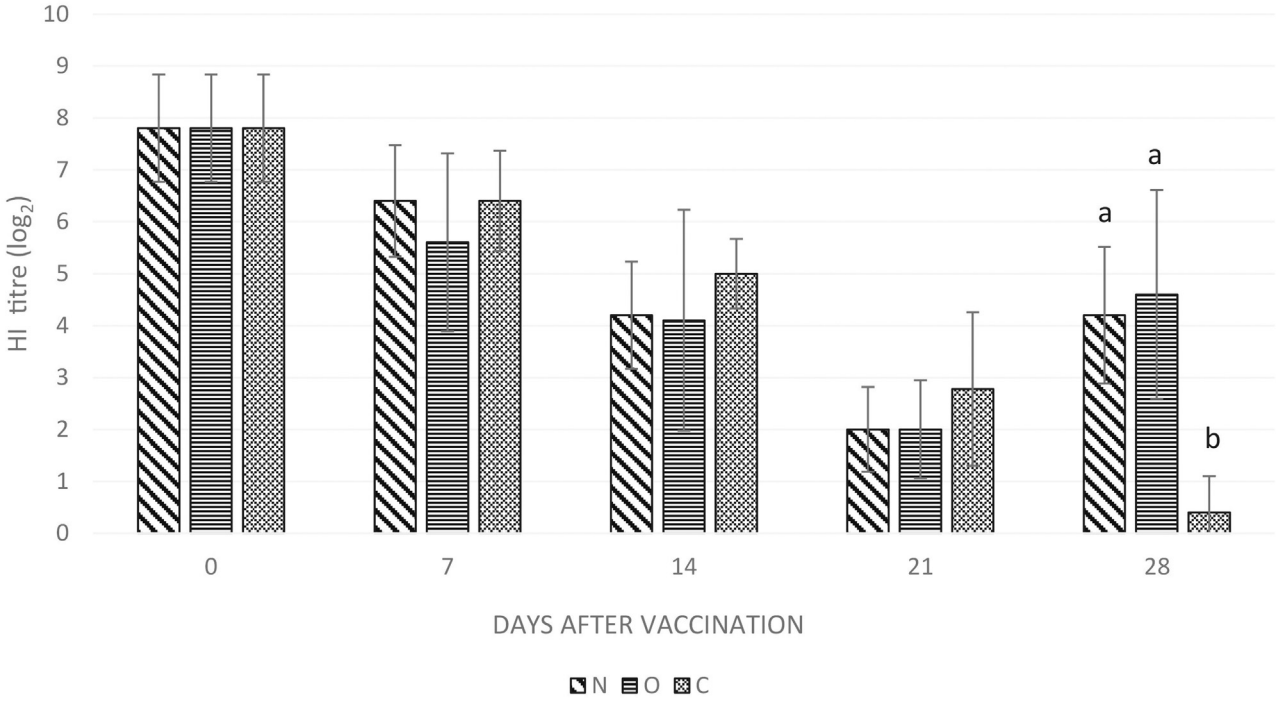

Figure 1. HI antibody titer values (log2) in chicken sera at weekly intervals after chicken immunization (mean ± SD). Significant differences between groups on day of sampling are indicated by different alphabet letters (a,b).

layer line chicks. The use of lentogenic strain La Sota by nebulization (Mazija et al., 2009, 2010) has shown to be simple for application in the hatchery to the newly hatched chicks, and to ensure protection of broiler chickens throughout production. It has also been shown that apathogenic enterotropic strains, such as Ulster 2C and Queensland V4, can be applied deep into the respiratory system without any vaccine reaction, and which

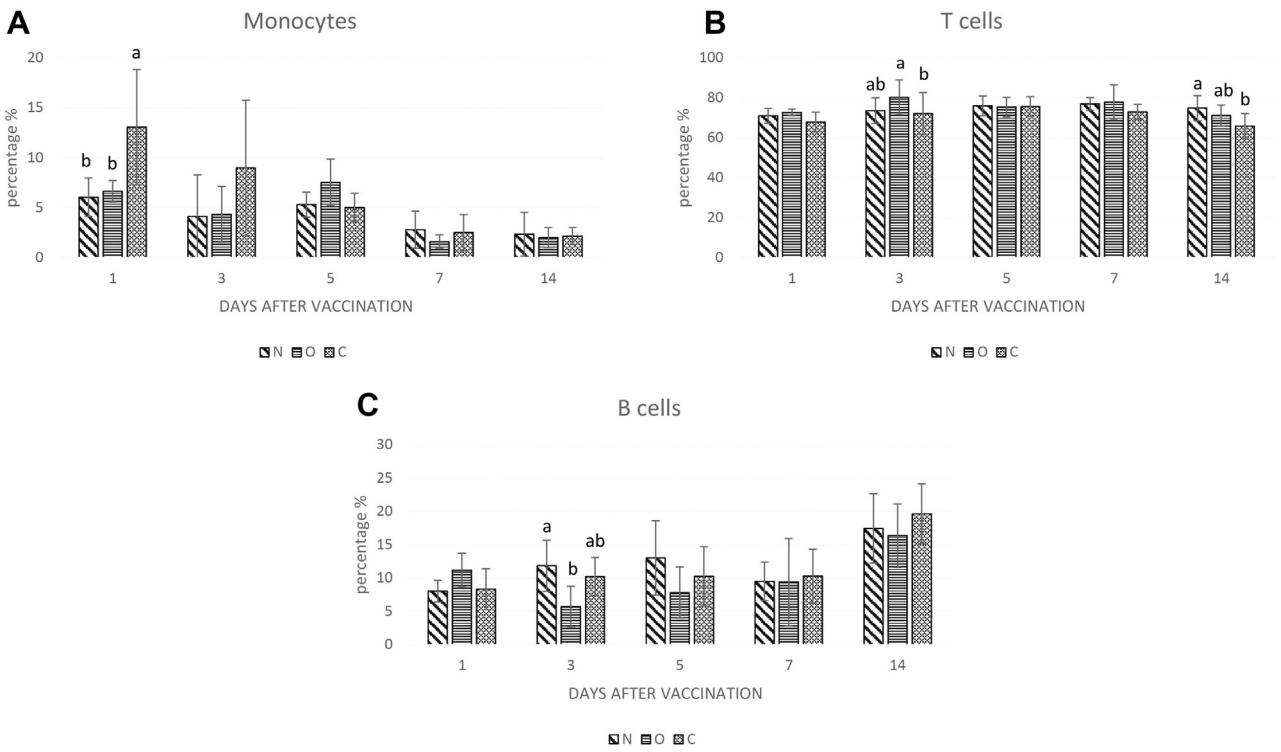

Figure 2. Leukocyte panel—relative proportion (%) of monocytes (A), T (B) and B (C) cells in CD45+ parent population (mean ± SD) in chicken blood during the experiment. Significant differences between groups on day of sampling are indicated by different alphabet letters (a,b).

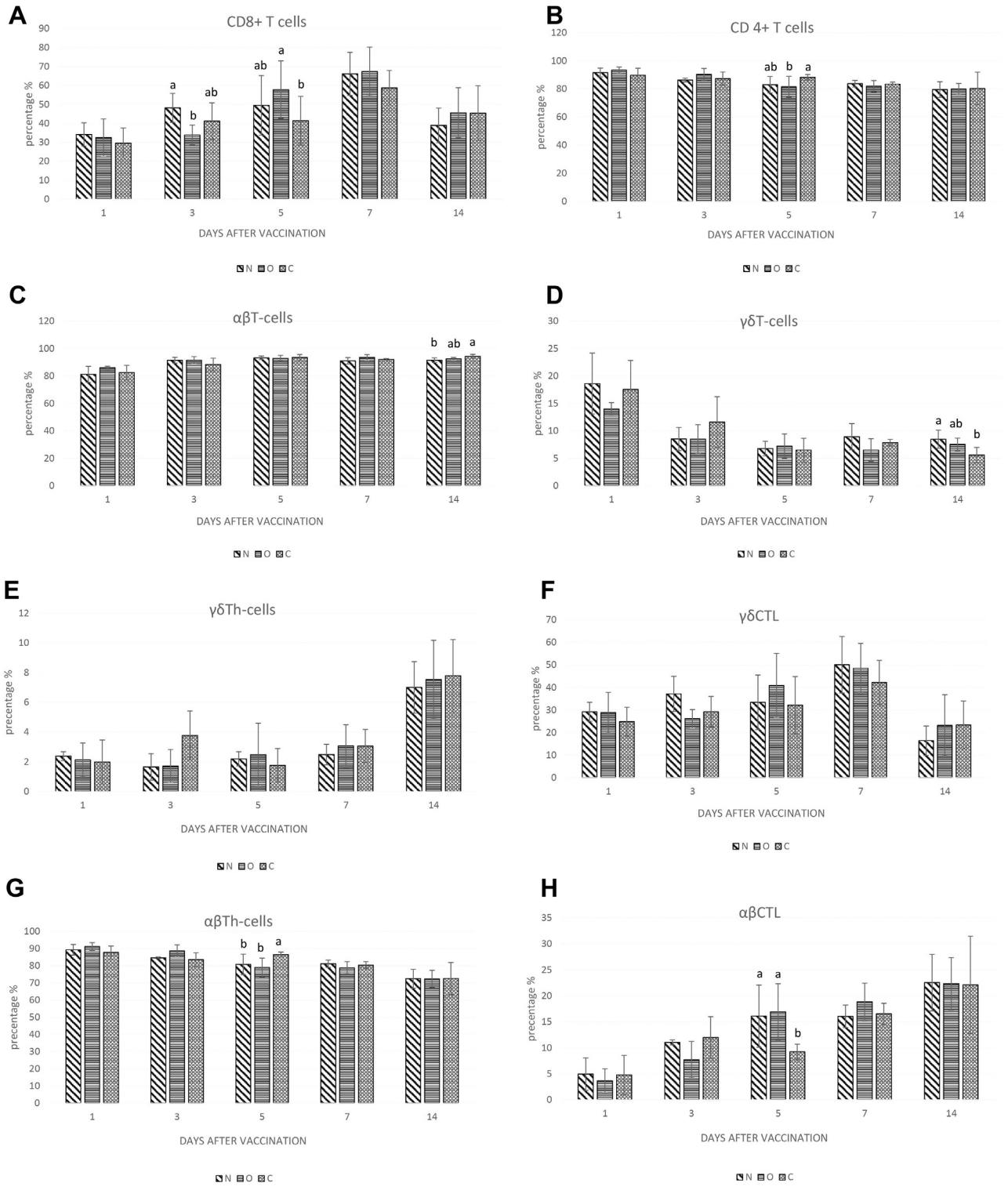

Figure 3. T-cell panel—relative proportion (mean ± SD) of live CD45+ T-cell subpopulations in chicken blood during the experiment. (A) CD8+ T-cells, (B) CD4+ T-cells, (C) αβT-cells, (D) γδT-cells, (E) γδTh-cells, (F) γδCTL, (G) αβTh-cells, and (H) αβCTL). Significant differences between groups on day of sampling are indicated by different alphabet letters (a,b).

also promoted long-term protection (Mazija et al., 2010). The chickens in this study had high maternal HI antibody titers (over 7.5), which accounted for the high passive protection of chickens at the early stage of life (Figure 1). The maternal antibody titer decreased in all groups, more intensively in vaccinated than in control, but increased only in the vaccinated chickens between day 21 and 28. Such a long period for seroconversion can be expected because of extremely high titer of maternal antibodies which could hinder immune response to vaccination (Bertran et al., 2018).

Previous studies of vaccine administration by means of nebulization have shown that seroconversion can be expected as early as 14 d in commercial chickens with

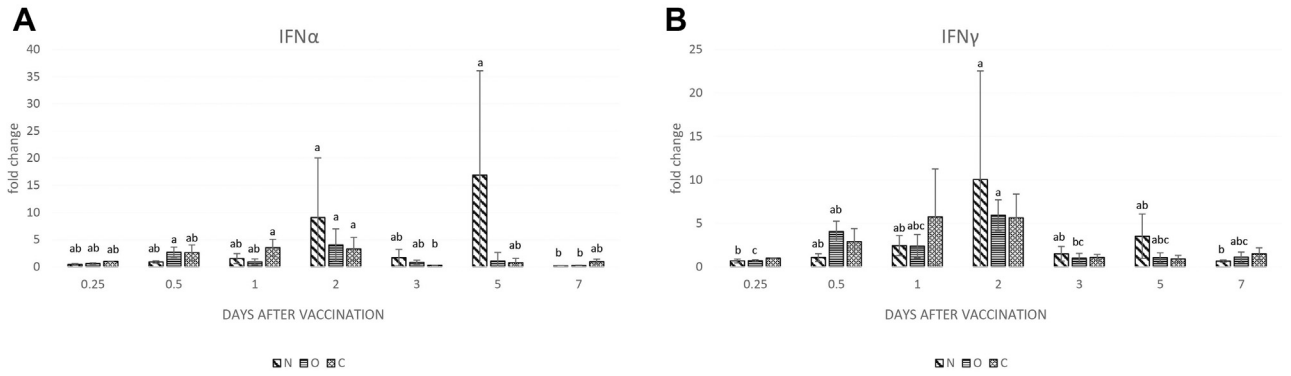

Figure 4. Relative change in mRNA expression of IFN-α and IFN-γ in the spleen (mean ± SD) normalized relative to control (calibrator) group 6h after vaccination with expression level 1. Significant differences between days of sampling within groups are indicated by different alphabet letters (a,b,c). Abbreviations: IFN-α, interferon-alpha; IFN-γ, interferon-gamma.

inherited HI titers ranging from 3.5 to 5 and in SPF chickens as early as 7 d (Mazija et al., 2009; Ervaćinović, 2010). In the mentioned study, the titer peak is after 21 d with La Sota strains, while with apathogenic strains with intestinal tropism result in measurable seroconversion in 28 d, with the titer peak 35 d after vaccination (Mazija et al., 2010). Owing to similarity of ZG1999HDS strain with the F strain, which by its properties is classified as lentogenic strain of lower pathogenicity than La Sota strain, such response is to be expected. This is supported by the results of studies on the F strain, which show that the MDT is even 184 h (Dey et al., 2014), and the MDT for ZG1999HDS is over 150 h. In our study, there were no postvaccinal reactions in the vaccinated groups, what was expected because of the low pathogenicity of tested strain, compared with La Sota strain which can cause reaction at this age despite high maternal antibody titers (Al-Garib et al., 2003).

Our immunophenotyping results of chicken blood cells in this experiment could indicate a possible activation of a cellular immune response in the experimental groups. Thus, on d 3, the proportion of B lymphocytes in the N group with respect to the O group increased significantly (Figure 2C), whereas in the O group, the proportion of T lymphocytes on the same day increased

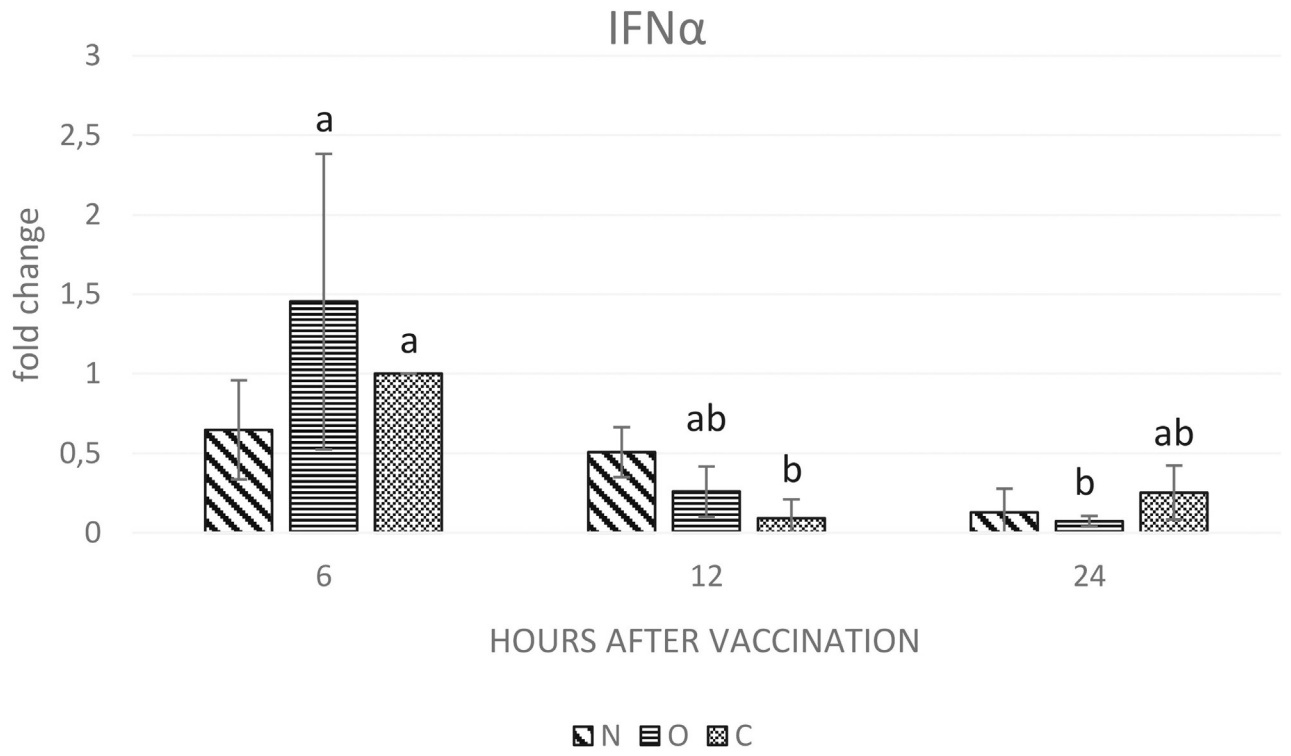

Figure 5. Relative change in mRNA expression of IFN-α in the lungs (mean ± SD) normalized relative to control (calibrator) group 6 h after vaccination with expression level 1. Significant differences between days of sampling within groups are indicated by different alphabet letters (a,b). Abbreviation: IFN-α, interferon-alpha.

significantly relative to the control group C (Figure 2B). Activation of B cells certainly indicates activation of a cellular immune response (Tizard, 2017), which is obviously more effective after nebulization, then by oculonasal administration of the same strain. On the other hand, activation of T lymphocytes in the N group is slightly milder than in the O group but has a prolonged effect. Immunophenotyping results show that on day 3, a significant increase in B lymphocytes was observed after administration by nebulization compared with oculonasal administration, suggesting a more active stimulation of the immune response after administration of used strain by that application. The proportion of monocytes, as the major circulating antigen-presenting cells, drops during experiment.

But if we look at the level of T-cell subpopulations, we can see that proportion of CD8+ T lymphocytes on the third day of the trial in N group is significantly higher, indicating faster activation of the cellular immune response compared with oculonasally administered virus (Figure 3A). Such rapid reactivity is in favor of faster stimulation of the complete immune response, with emphasis on cellular immunity, if vaccine is delivered by nebulization, with consequently high enough achieved titer of specific antibodies as well.

Analyses of CD8 + T-cell subpopulations at the level of αβ (Figure 3H) and γδ cytotoxic T-cells (Figure 3F) showed their proportion higher in both vaccinated groups on day 5 of the experiment than in the control group, and significantly for αβ Tc cells. This indicates that the cellular immune response is stimulated regardless of the route of administration of the vaccine, but it is certainly faster by the nebulization process. Evidence of a significant increase in αβ cytotoxic T lymphocytes and a significant decrease in αβ helper T (Figure 3G) lymphocytes in the experimental groups on day 5 of the trial may indicate that the immune response was defined and the agent was brought under its control. This sequence of the immune response is precisely characteristic of stimulating the Th1 type of immune response, characterized by an increase in the number of B and helper T-cells, followed by cytotoxic T-cells as carriers of the cellular immune response (Kaiser, 2010).

Cytokine expression plays an important role in the evaluation of the immune response, especially cellular immune response. The study analyzed the expression of cytokines IFNα and IFNγ in different organs, with cytokine expression in the spleen, with the highest value in evaluating the cellular immune response to a given antigen (Dalgaard et al., 2010; Sharma and Rautenschlein, 2013). The expression of IFNγ best provides insight into the cellular immune response (Kapczynski and Kogut, 2008; Rue et al., 2011). In the present study, the results show that the ZG1999HDS strain applied by the nebulization significantly stimulated the expression of both cytokines in the spleen on day 2, and in particular on day 5 of the experiment compared with 6 h after vaccination (Figure 4.). Oculonasal vaccination stimulated both cytokines slightly 12 h and 2 d after vaccination compared with 6 h after vaccination, whereas the activity in the control group rose slightly 1 and 2 d after vaccination in both IFN following same pattern.

This result follows findings in CD8+ cells, suggesting a faster and more intense immune response to the NDV strain administered by the nebulization process and confirming a better effect of stimulating the immune response to ZG1999HDS strain than the oculonasal one. The expression of IFN-γ in the spleen is higher in the group vaccinated with the nebulization procedure, and its profile clearly follows the movement curves of cytotoxic T lymphocytes, confirming a stronger reactivity during the immune response after administration by this method.

In addition, the expression of IFNα in the lungs 12 h after administration by nebulization increased compared with the control group, indicating promotion of local protection very early and interference that can protect from wild-type virus infection (Figure 5).

On the other hand, the expression of the analyzed cytokines in other peripheral organs, such as the intestine and esophagus (data not shown), indicates their better expression after administration of strain ZG1999HDS oculonasally, which is to be expected because this is more effective way to administer virus to those organs.

The tropism of the virus itself could not be determined in this study because the genome of used virus strain we did not detect in the organs. For the spleen and bone marrow, as organs not directly related to the mucous membranes to which the virus was delivered, these results are expected. Previous studies have shown that highly lentogenic strains can rarely be found in the spleen (Rauw et al., 2009; Kapczynski et al., 2013), and especially not in the case of high maternal antibody titers, as was the case in our research. Because the most caudal part of the lungs at the entrance of the abdominal air sacs were sampled and searched, a negative finding of viral RNA in the lungs may indicate that virus multiplication; however, predominantly occurs at the point of entry of the virus, that is, in the anterior airways, such as the trachea and primary bronchi. That this part of the lung tissue was inadequate for analyses is also shown by the very poor detection of cytokine mRNAs, which was completely absent for IFNγ. For the esophagus, the negative finding can be explained either by the absence of reactive centers in it or by inappropriate sampling of them. The distal part of the esophagus was sampled in which, in accordance with the literature, there are reactive centers in the folds of the mucosa in which the pathogen is retained and where the immune response begins (Olah et al., 2003). On the other hand, the end portion of the small intestine with cecum tonsils was also sampled, which was also negative for the strain applied. It is possible owing to the extremely poor pathogenicity of the strain and the high level of maternal antibodies, especially IgA, which can be found in gastrointestinal tract after ingestion of egg white (Hamal et al., 2006). Strain was inactivated in the cranial intestinal parts and thus prevented in spreading

into the caudal regions. However, despite the negative findings, viral entry was proven by formation of specific antibodies to NDV as confirmed by the HI titer. In association with this finding, positive immune response, and better expression of cytokines in the respiratory system, compared with the digestive, could be an indicator of its tropism.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2021.01.024.

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