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The influence of HVT FC 126 given by means of nebulization compared to parenteral vaccination on chickens immunocompetent cell transformation

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ABSTRACT

Marek's disease is a common lymphoproliferative disease of chickens, usually characterized by mononuclear cell infiltration of peripheral nerves and various other organs and tissues, including the iris and skin. A group of 70 newly-hatched chicks was vaccinated by means of nebulization and exposed for 60 seconds to the HVT FC 126 vaccine, while the other group of 70 chicks received the same vaccine by parenteral route (s.c.). The aim of this study was to compare the morphological patterns of the chicken's lymphocytes in their peripheral blood, before and after vaccination by means of either nebulization or parenteral injection. Image analysis was performed using the SFORM software (VAMSTEC, Zagreb, Croatia). A total of 50 blood smears from vaccinated chickens (20 by means of nebulization and 30 by parenteral injection), with an average of 100 cells per smear, were analyzed. The results showed that the peripheral blood lymphocytes of chickens vaccinated by means of nebulization, compared with parenteral vaccinated, had significantly higher values for the majority of the measured variables: area, outline, minimum and maximum radius, length, breadth and convex area, on all days after vaccination, except on day 4, indicating the significantly higher metabolic activity of those cells. In lymphocytes of chickens vaccinated parenterally, only the nucleo-cytoplasmic ratio was higher. The results show that morphological patterns of immunocompetent cell transformation could be used to evaluate immune responses to vaccination and vaccine efficacy. We conclude that nebulization as a mode of vaccination against Marek's disease stimulates the transformation of immunocompetent cells much earlier, thus shortening the time

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of immunosuppression and improving the immune response. This is of paramount importance for the practical application of Marek's disease vaccine.

Key words: chickens, Marek's disease, nebulization, parenteral vaccination, lymphocyte morphometry

Introduction

Marek's disease (MD) is lymphoproliferative contagious disease of chickens caused by a DNA virus of the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Mardivirus* (ISLAM et al., 2004). The three serotypes of the virus differ not only in genomic, but also biologic potential. Serotype 1 includes all oncogenic strains and their attenuated forms, serotype 2 is naturally non-oncogenic virus isolated from chickens, and serotype 3 is a non-oncogenic virus isolated from turkeys, known as the herpes virus of turkeys (HVT) (KAWAMURA et al., 1969; AFONSO et al., 2001; FILIPIČ et al., 2010). MD is therefore etiologically different from other lymphoid neoplasms of birds. It manifests as lymphocytic infiltrates in various organs, forming lymphocytic tumors (WITTER and SCHAT, 2003), and often as inflammatory and degenerative changes in neurons (neurolymphomatosis) (BAATEN et al., 2004).

After inhalation of the virus, macrophages transfer it to the lymphocytes, in which viral replication and cytolysis occur (NAIR, 2005). The virus can be detected earliest in the spleen three days after inhalation, and after four days it can be detected in the thymus and bursa of Fabricius, where it causes early cytolytic infection of B cells and a smaller number of T cells. This is followed by a latent phase affecting both B and T cells; late cytolytic activity mainly affects T cells and finally the transformation and development of $CD4^+$ T cell lymphoma occur (CALNEK, 2001; BAATEN et al., 2004; MILJKOVIĆ et al., 2008).

There is no treatment for Marek's disease. The disease is controlled by vaccination and by general hygiene measures. Vaccination is carried out in the hatchery during incubation by *in ovo* vaccination or immediately after hatching, mainly parenterally i.m. or s.c., by using several types of vaccines containing cell-associated virus, HVT, or simultaneously both viruses (GIMENO, 2008). MAZIJA et al. (1994) proved that freeze dried HVT vaccine may be applied by means of nebulization. Vaccination affects the blood cell count, particularly lymphocytes (GOTTSTEIN et al., 2015; FRIEDMAN et al., 1992). Morphometric parameters in all lymphoid organs - the diameter and volume of lymphoid follicles in the bursa of Fabricius, thymus volume and the absolute number of thymocytes, number and the diameter of lymphoid follicles and spleen volume are also affected (MILJKOVIĆ et al., 2008), as well as changes to the morphometric parameters of lymphocyte cytoplasm and nuclei in the peripheral blood (KARDUM et al., 2011; 2011a).

Morphometry is the quantitative description of geometrical structures in all dimensions (BAAK, 1985). Determination of the morphometric parameters of blood cells

using a computerized image analysis is applied in clinical laboratories, and provides the numerical objectification of the most subtle changes unreachable to visual inspection (OBERHOLZER et al., 1991). In human cytology and histology, morphometric parameters are used in comparison of normal cells in different physiological conditions, as well as for reliable differentiation between malignant and benign cells of different tumors. Digital analysis of the morphometric parameters of blood cells in addition may complement findings obtained by classical hematological methods, may be the basis for elaboration of reference values for each animal species, and may also provide a better quality standard for determination of aberrations in physiological and pathological conditions, as well as being a more precise starting premise in the diagnostics of hematological disturbances and diseases (POLJIČAK-MILAS et al., 2009).

The aim of this research was to determine the morphometric characteristics of lymphocytes in peripheral blood smears, before and after vaccination against Marek's disease in newly-hatched chickens parenterally vaccinated and chickens vaccinated by means of nebulization, using the HVT FC 126 strain. The goal was to determine whether there are differences in the morphometric parameters of peripheral blood lymphocytes between the two forms of vaccination mentioned, which might indicate possible differences in the speed of stimulation of immunocompetent cells, and thus a difference in the strength of the immune response in vaccinated chickens.

Materials and methods

Experimental chicks. The research was carried out on 140 chicks of the Lohmann light hybrid breed. During the 21 days of the experiment, the chicks were kept in 50 × 50 cm metal cages under controlled conditions, and water and feed were offered *ad libitum*. Newly-hatched chicks were vaccinated against Marek's disease using the commercial vaccine (Lyomarex® Merial, France) containing HVT FC 126. A group of 70 chicks was vaccinated by means of nebulization using the ultrasonic nebulizer Sonovac 095® (MAZIJA and ŠTIMAC, 1999; MAZIJA et al., 2009) whereby 70 doses were delivered per group of chickens for 60 seconds of exposure. In parallel with this form of vaccination, the other group of 70 chicks received the same vaccine by the parenteral route (s.c.) according to the manufacturer's instruction, whereby each chick received one dose of the vaccine.

Blood samples with heparin anticoagulant were collected by puncturing the jugular vein before vaccination (day 0), and on days 3, 4, 7, 10, 14 and 21 of the trial. Just after the blood was collected from each chick, blood smears were made and stained according to the Pappenheim method.

Blood smears and morphometric analysis of lymphocytes. The morphometric analysis of lymphocytes was carried out on standard-stained smears of chicken blood. A total of 50

blood smears of vaccinated chickens (20 by means of nebulization and 30 by parenteral injection), were analyzed. From each smear, an average of 100 to 105 lymphocytes, and a total of 11 440 objects (cells and nuclei) on all 50 smears, were analyzed. Computerized image analysis was carried out on a personal computer with an "SFORM" support system provided by VAMSTEC, Zagreb, Croatia. The system consists of a high-resolution camera (Pulinix) that digitalizes and transfers the image (x100 oil) from an Olympus BX 41 light microscope onto a personal computer. The margins of cytoplasm and nuclei were marked interactively, along with hand corrections by computer mouse (Figure 1). For lymphocyte cells and their nuclei the following parameters were determined: area and convex area in μm^2, outline, minimum and maximum radius, lymphocytic length and breadth, and the form factor and elongation factor of the cell in μm. From the obtained data for the nuclei area and the area of whole lymphocyte, the nucleo-cytoplasmic ratio (N/C) was calculated.

Statistical analysis. The statistical analysis of the results was carried out with the software STATISTICA 12 (StatSoft Inc. 2013). For each continuous variable, basic descriptive statistical indicators (arithmetic mean, median, minimum and maximum values) and measures of variability (coefficient of variance and standard deviation) were determined. Normality of data distribution was checked by the Kolmogorov-Smirnov test, which indicated the normal distribution of the data on all the examined variables, and according to that Student's *t*-test was used to analyze the significance of differences between the measured indicators in the groups. Tables and figures represent the mean value and standard deviation.

Results

The results of the basic morphometric characteristics of the lymphocytes and their nuclei are shown in Tables 1 and 2 and Figs 1-4.

By comparing the morphometric characteristics of lymphocytes in the group of chickens vaccinated by means of nebulization with the parenterally vaccinated group statistically significant differences were found in the lymphocyte area throughout the entire period of research. For the other investigated single parameters, the outline, minimum and maximum radius, length and breadth of lymphocytes statistically significant differed on the 3^{th}, 7^{th}, 10^{th}, 14^{th} and 21^{th} days after vaccination. Regarding shape factors, statistically significant differences were found for the convex area on days 3, 7, 10, 14 and 21, for FF on days 3, 4, 7 and 10, and for EF on days 4 and 7 of the trial (Table 1 and 2). In the tables it is obvious that during the entire trial the area of lymphocytes in the blood of the nebulized chickens was significantly higher than that in the parenterally vaccinated chickens. However, it should be noted that values of the area of lymphocytes in the blood of both groups of chickens on days 3 and 4 after vaccination were lower than the values

of the area prior to immunization (0 day) (P = 0.0038; P = 0.0000, respectively), on the 7th day in nebulized chickens the area values exceeded the values of day 0 (P = 0.008), and an increasing trend in the values of the area continued until the end of the experiment (P = 0.0000; P = 0.0000, P = 0.0000, respectively). In the parenterally vaccinated chickens the area of lymphocytes reached the values from prior to immunization on the 14th day after vaccination, and on the 21st day lower values of the area were measured once again compared to those of day 0 (P = 0.000) (Fig. 2). Values for the outline, minimum and maximum radius, length and breadth of lymphocytes were also significantly higher in nebulized chickens, compared to the parenterally vaccinated on the 3rd, 7th, 10th, 14th and 21st days of the experiment, however 4 days after vaccination no significant differences were found. Regarding changes in the values of morphometric parameters according to the values of day 0, a similar pattern was noted for the area in the nebulized chickens. On the 3rd and 4th days after vaccination lower values for the outline, minimum and maximum radius, length and breadth were noted than those measured on day 0, on the 7th day values reached the values of day 0, and further an increasing trend was recorded up to the end of the experiment. In contrast, in parenterally vaccinated chickens, the values of outline, minimum and maximum radius, length and breadth seldom reached values measured prior to vaccination during the entire experiment (Table 1 and 2). During the experiment the regularity of cells (convex area) also changed. Cells proved to be more regular in parenterally vaccinated chicks, while the values for the nuclei followed the cell values (Tables 1 and 2). In nebulized chickens, the values of the lymphocyte convex area began to decline on the 3th day (P = 0.0022) and 4th day (P = 0.000) in comparison to values prior vaccination (0 day). On day 7 the values of the convex area exceeded the values on day 0 (P = 0.0026) and continued to rise until the end of the experiment (P = 0.0001 on day 10, P = 0.0000 on days 14 and 21). In contrast, in parenterally vaccinated chickens the convex area values declined and remained lower than the values on day 0 until day 10 after vaccination (P = 0.000 on days 3, 4, 7, and 10). On the 14th day after vaccination the values reached Day 0 values, but on the 21st day they again dropped below the values measured prior to vaccination (Fig. 3). Comparing all the morphometric parameters of the lymphocytes' nuclei, on days 3 and 4 no significant differences were found between the two modes of vaccination, except FF on the 3rd day. However, on days 7, 10, 14 and 21 significantly higher values of the area, outline, minimum and maximum radius, length and breadth, FF and EF of lymphocytes in the blood of nebulized chickens were measured, except EF on days 7, 14 and 21, and FF on days 10 and 14 (Table 1 and 2). Regarding the morphometric parameters of the lymphocytes' nuclei, a similar pattern of initially decreasing values of all measured parameters up to day 4 in comparison to day 0, and increasing values up to the 21st day in the blood of nebulized chickens was observed. In parenterally vaccinated chickens the values of these parameters were lower up to the 10th day of the trial, after which an increase to the 14th day followed, and then again a

Table 1. Morphometric characteristics of lymphocytes in chicken's peripheral blood, before (Day 0) and after vaccination against Marek's disease (3rd, 4th and 7th days) by means of nebulization (N) and parenteral vaccination (P)

Object	Parameter	0. day	N 3	P 3	P<0.05	N 4	P 4	P<0.05	N 7	P 7	P<0.05
Cell	Area (μm²)	38.74 ± 11.10	36.69 ± 10.18	33.97 ± 12.07	0.0010	34.98 ± 9.99	33.54 ± 9.44	0.0394	41.88 ± 15.03	30.57 ± 9.33	0.0000
	Outline (μm)	24.13 ± 3.64	23.18 ± 3.33	22.52 ± 4.44	0.0264	22.82 ± 3.40	22.70 ± 3.59	NS	24.45 ± 4.29	21.32 ± 3.33	NS
	Max. radius (μm)	3.98 ± 0.60	3.87 ± 0.61	3.72 ± 0.72	0.0033	3.76 ± 0.54	3.70 ± 0.56	NS	3.97 ± 0.68	3.49 ± 0.52	0.0000
	Min. radius (μm)	2.90 ± 0.48	2.84 ± 0.42	2.72 ± 0.52	0.0006	2.76 ± 0.46	2.73 ± 0.42	NS	3.10 ± 0.60	2.61 ± 0.44	0.0000
	Length (μm)	7.48 ± 1.14	7.26 ± 1.14	6.95 ± 1.30	0.0005	7.07 ± 1.03	6.95 ± 1.04	NS	7.53 ± 1.33	6.55 ± 0.98	0.0000
	Breadth (μm)	6.69 ± 1.00	6.53 ± 0.90	6.25 ± 1.16	0.0002	6.35 ± 0.93	6.30 ± 0.88	NS	6.98 ± 1.24	6.00 ± 0.89	0.0000
	Convex area (μm²)	40.09 ± 11.37	37.85 ± 10.48	35.21 ± 12.52	0.0022	36.13 ± 10.19	34.85 ± 9.83	NS	42.93 ± 15.27	31.61 ± 9.57	0.0000
	Form factor	0.82 ± 0.08	0.84 ± 0.06	0.82 ± 0.08	0.0001	0.83 ± 0.07	0.81 ± 0.08	0.0018	0.85 ± 0.05	0.83 ± 0.07	0.0000
	Elongation factor	1.12 ± 0.09	1.11 ± 0.10	1.11 ± 0.08	NS	1.11 ± 0.08	1.10 ± 0.08	0.0041	1.08 ± 0.05	1.09 ± 0.07	0.0041
Nucleus	Area (μm²)	29.46 ± 7.67	27.79 ± 6.58	27.08 ± 8.82	NS	27.33 ± 7.92	26.73 ± 7.00	NS	31.32 ± 9.45	23.96 ± 6.74	0.0000
	Outline (μm)	21.39 ± 3.13	20.33 ± 2.68	20.39 ± 4.03	NS	20.15 ± 2.99	20.48 ± 3.22	NS	21.61 ± 3.98	18.99 ± 2.82	0.0000
	Max. radius (μm)	3.56 ± 0.52	3.43 ± 0.47	3.37 ± 0.44	NS	3.34 ± 0.45	3.33 ± 0.48	NS	3.57 ± 0.62	3.14 ± 0.44	0.0000
	Mini. radius (μm)	2.29 ± 0.48	2.31 ± 0.36	2.31 ± 0.63	NS	2.36 ± 0.44	2.36 ± 0.36	NS	2.45 ± 0.42	2.16 ± 0.38	0.0000
	Length (μm)	6.65 ± 0.95	6.40 ± 0.86	6.29 ± 1.13	NS	6.28 ± 0.87	6.24 ± 0.88	NS	6.72 ± 1.17	5.87 ± 0.84	0.0000
	Breadth (μm)	5.74 ± 0.82	5.59 ± 0.67	5.52 ± 0.96	NS	5.59 ± 0.84	5.60 ± 0.73	NS	5.93 ± 0.86	5.24 ± 0.71	0.0000
	Convex area (μm²)	30.87 ± 8.04	28.84 ± 6.87	28.26 ± 9.30	NS	28.30 ± 8.06	27.94 ± 7.37	NS	32.56 ± 10.09	24.93 ± 7.00	0.0000
	Form factor	0.80 ± 0.08	0.84 ± 0.07	0.81 ± 0.09	0.0000	0.83 ± 0.08	0.80 ± 0.09	NS	0.83 ± 0.08	0.82 ± 0.08	0.0000
	Elongation factor	1.16 ± 0.11	1.14 ± 0.10	1.14 ± 0.10	NS	1.12 ± 0.09	1.11 ± 0.09	NS	1.13 ± 0.11	1.12 ± 0.09	NS
Nucleo-cytoplasmic ratio (N/C)		0.77 ± 0.11	0.77 ± 0.10	0.80 ± 0.07	0.0000	0.79 ± 0.13	0.80 ± 0.08	NS	0.76 ± 0.10	0.79 ± 0.07	0.0002

Values are expressed as mean value ± standard deviation. Total number of analyzed lymphocytes (n_1) = 5720, total number of analyzed objects (cell and nuclei) (n_2) = 11440.

Table 2. (extension). Morphometric characteristics of lymphocytes in chicken's peripheral blood after vaccination against Marek's disease (10th, 14th and 21st days) by means of nebulization (N) and parenteral vaccination (P)

Object	Parameter	N 10	P 10	P<0.05	N 14	P 14	P<0.05	N 21	P 21	P<0.05
Cell	Area (μm²)	42.00 ± 12.18	35.93 ± 12.61	0.0000	43.10 ± 10.58	38.89 ± 12.79	0.0000	50.73 ± 14.15	34.12 ± 11.01	0.0000
	Outline (μm)	24.82 ± 3.80	23.08 ± 4.09	0.0000	25.16 ± 3.45	23.66 ± 4.19	0.0000	26.82 ± 4.06	21.96 ± 3.57	0.0000
	Maximum radius (μm)	4.03 ± 0.60	3.75 ± 0.62	0.0000	4.08 ± 0.56	3.85 ± 0.66	0.0000	4.40 ± 0.67	3.63 ± 0.61	0.0000
	Minimum radius (μm)	3.12 ± 0.50	2.83 ± 0.54	0.0000	3.18 ± 0.43	3.00 ± 0.52	0.0000	3.45 ± 0.54	2.77 ± 0.44	0.0000
	Length (μm)	7.63 ± 1.13	7.06 ± 1.23	0.0000	7.75 ± 1.05	7.29 ± 1.24	0.0000	8.36 ± 1.27	6.86 ± 1.14	0.0000
	Breadth(μm)	7.02 ± 1.03	6.46 ± 1.10	0.0000	7.11 ± 0.91	6.73 ± 1.13	0.0000	7.70 ± 1.14	6.28 ± 0.97	0.0000
	Convex area (μm²)	43.26 ± 12.46	37.12 ± 12.85	0.0000	44.31 ± 10.92	39.94 ± 13.19	0.0000	51.88 ± 14.88	34.97 ± 11.32	0.0000
	Form factor	0.84 ± 0.06	0.82 ± 0.07	0.0014	0.84 ± 0.05	0.85 ± 0.05	NS	0.87 ± 0.04	0.87 ± 0.04	NS
	Elongation factor	1.08 ± 0.07	1.09 ± 0.06	NS	1.09 ± 0.08	1.08 ± 0.06	NS	1.08 ± 0.08	1.09 ± 0.07	NS
Nucleus	Area (μm²)	31.62 ± 7.68	28.25 ± 10.35	0.0000	35.91 ± 9.10	31.26 ± 10.46	0.0000	38.63 ± 11.08	27.64 ± 8.80	0.0000
	Outline (μm)	21.88 ± 3.11	20.66 ± 3.91	0.0000	23.06 ± 3.27	21.55 ± 3.92	0.0000	23.51 ± 3.59	19.87 ± 3.20	0.0000
	Maximum radius (μm)	3.62 ± 0.49	3.39 ± 0.59	0.0000	3.75 ± 0.50	3.50 ± 0.63	0.0000	3.91 ± 0.60	3.30 ± 0.56	0.0000
	Minimum radius (μm)	2.44 ± 0.48	2.35 ± 0.52	0.0090	2.79 ± 0.46	2.58 ± 0.49	0.0000	2.86 ± 0.50	2.44 ± 0.39	0.0000
	Length (μm)	6.82 ± 0.92	6.36 ± 1.14	0.0000	7.11 ± 0.96	6.60 ± 1.16	0.0000	7.42 ± 1.14	6.22 ± 1.06	0.0000
	Breadth (μm)	5.98 ± 0.77	5.66 ± 1.01	0.0000	6.45 ± 0.86	6.01 ± 1.02	0.0000	6.62 ± 0.99	5.61 ± 0.87	0.0000
	Convex area (μm²)	32.96 ± 8.13	29.45 ± 10.71	0.0000	37.07 ± 9.38	32.37 ± 10.95	0.0000	39.74 ± 11.44	28.42 ± 9.11	0.0000
	Form factor	0.82 ± 0.07	0.81 ± 0.07	NS	0.84 ± 0.08	0.83 ± 0.06	NS	0.86 ± 0.06	0.86 ± 0.05	0.0426
	Elongation factor	1.14 ± 0.10	1.12 ± 0.09	0.0127	1.10 ± 0.07	1.09 ± 0.07	NS	1.12 ± 0.10	1.11 ± 0.09	NS
Nucleo-cytoplasmic ratio (N/C)		0.77 ± 0.10	0.79 ± 0.09	0.0120	0.83 ± 0.08	0.800.08	0.0000	0.77 ± 0.13	0.81 ± 0.08	0.0000

Values are expressed as a mean value ± standard deviation. Total number of analyzed lymphocytes (n_1) = 5720, total number of analyzed objects (cell and nuclei) (n_2) = 11440.

decrease to values lower than those measured prior to vaccination (Fig. 4). During the trial, except on the 14th day, only the nucleo-cytoplasmic ratio was higher in the lymphocytes of parenterally vaccinated chickens compared to the nebulized ones.

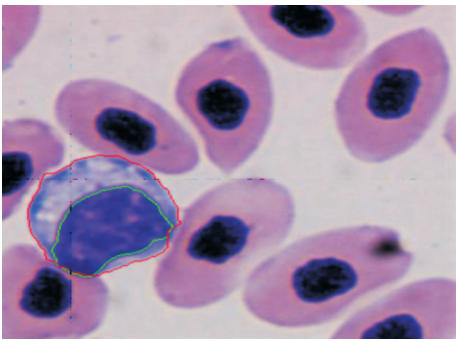

Fig. 1. Image analysis of lymphocyte cells (red) and nuclei (green) in a chicken peripheral blood smear carried out on a personal computer with an "SFORM" supporting system

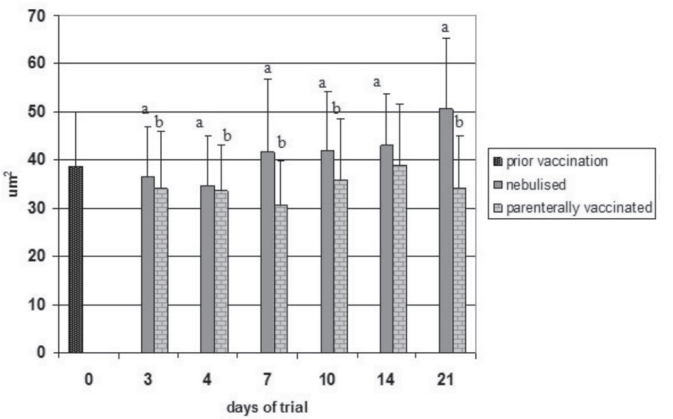

Fig. 2. Variations in lymphocyte area in nebulized and parenterally vaccinated groups of chickens during the trial, compared to lymphocyte area prior vaccination. Values are expressed as mean value ± SD. a - significantly different values compared to values prior vaccination in nebulized chickens. b - significantly different values compared to values prior vaccination in parenterally vaccinated chickens.

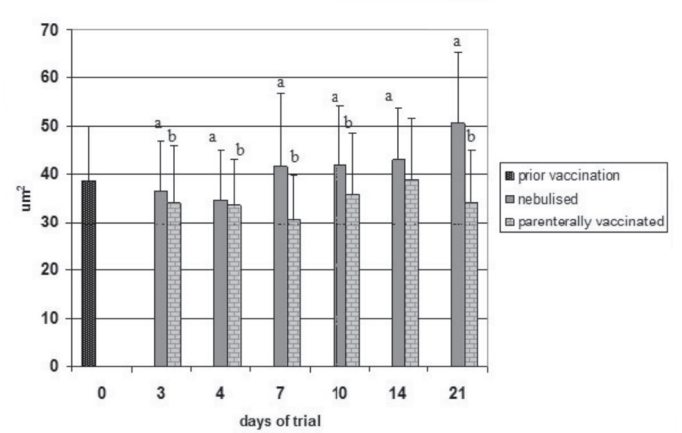

Fig. 3. Variations in the convex area of lymphocytes in nebulized and parenterally vaccinated groups of chickens during the trial, compared to the lymphocyte convex area prior to vaccination. Values are expressed as mean value ± SD. a - significantly different values compared to values prior to vaccination in nebulized chickens. b - significantly different values compared to values prior to vaccination in parenterally vaccinated chickens.

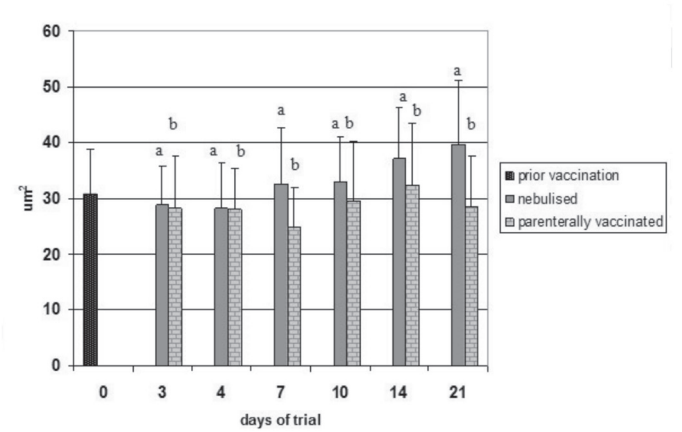

Fig. 4. Variations in the convex area of lymphocyte nuclei in nebulized and parenterally vaccinated groups of chickens, during the trial, compared to the lymphocyte nuclei convex area prior to vaccination. Values are expressed as mean value ± SD. a - significantly different values compared to values prior to vaccination in nebulized chickens. b - significantly different values compared to values prior to vaccination in parenterally vaccinated chickens.

Discussion

Marek's disease, as a highly contagious disease caused by a virus, infects chicken by respiratory route. Due to the easy spread of the disease among newly-hatched chicks it represents a major problem in modern poultry production all over the world.

With the discovery of a vaccine in the middle of the last century (CHURCHILL et al., 1969) the era of combatting this disease began. Single or combinations of attenuated strains 1 (*Gallid herpesvirus 2*), naturally apathogenic strains 2 (*Gallid herpesvirus 3*) and apathogenic HVT (*Herpesvirus Turkey)* have been used as the standard vaccines for decades in the fight against the virulent virus of Marek's disease (ISLAM et al., 2004; BUBLOT and SHARMA, 2004; MIŠKOVIĆ et al., 2013).

Blood cell count is significantly influenced by vaccination, with early lymphopenia as a possible reflection of initial immunosuppression, followed by the stimulus of a specific immune response (GOTTSTEIN et al., 2015), transient depletion of B cells of different degrees after application of a bivalent (HVT + SB-1) or MDV serotype 1 Rispens vaccine (FRIEDMAN et al., 1992). Other authors also pointed to initial immunosuppressive features and the stimulation of immune response development. CALNEK et al. (1998) pointed out MDV-induced immunosuppression which includes both humoral and cellular immunity associated with lymphopenia due to cytolysis of B and T cells, together with the reduction of cell count in the thymus and in the follicles of the bursa cortex. MILJKOVIĆ et al. (2008) showed changes to morphometric parameters in all lymphoid organs: in the bursa of Fabricius a significant reduction in the diameter and volume of lymphoid follicles was noticed (the volume of the follicles' medulla and the number of cells in the cortex of the follicles), reduction of thymus volume and the absolute number of thymocytes in the thymus. In contrast, in the spleen, an increase in the volume, number and diameter of lymphoid follicles occurred.

After exposure to antigens and stimulation of the immune system, scattered lymphocytes may become larger (a prelude to blast transformation or plasmacytoid differentiation) and have dark blue granular cytoplasm (SCHALM, 2010). This leads to changes (increase and/or abnormalities of cells and nuclei, changes of their mutual relations, etc.) that can be quantified by morphometry.

Our previous study of the morphometric parameters of lymphoid cells in chicken's peripheral blood smears following vaccination against Marek's disease by means of nebulization (KARDUM et al., 2011) showed that after initial immunosuppression, characterized by reduced values of the morphometric characteristics of lymphoid cells in peripheral blood, this subsequently leads to the development of an immune response that is visible as transformation and morphological changes, in the irregularities of shapes and in a significant increase in lymphoid cells. In this study, we confirmed the results of the previous study. On the 3rd and 4th day after vaccination by means of

nebulization reduced values of morphometric parameters were measured, but from the 7th day onwards the values exceeded the initial values and continued to increase until the end of the experiment. In contrast to this mode of vaccination, values of lymphocytes in parenterally vaccinated chickens reached values measured on day 0 on the 14th day, but on the 21st day lower values were measured than those on day 0. An immune response requires rapid and extensive cell growth; therefore the metabolic and biosynthetic demand of lymphocytes becomes dramatically increased after vaccination (MACIVER et al., 2008). Furthermore, in *in vitro* stimulated lymphocytes by the mitogens, a number of complex biological processes could be activated, resulting in the increase in the rate of synthesis of specific cell proteins (HALL et al., 1984). The results of this research showed that peripheral blood lymphocytes of chickens vaccinated by means of nebulization compared to those parenterally vaccinated had significantly higher values in the majority of measured variables: area, outline, minimum and maximum radius, length, breadth and convex area, on the 3rd, 7th, 10th, 14th and 21st days after vaccination, indicating the higher metabolic activity of those cells. In the lymphocytes of parenterally vaccinated chickens, only the nucleo-cytoplasmic ratio was higher, except on the 14th day, because of the greater decrease in the area of whole cells of parenterally vaccinated chickens compared to nebulized ones, which was most expressed on the 21st day of the experiment. These results demonstrate that nebulization, as a mode of vaccination against Marek's disease, stimulates the transformation of immunocompetent cells much earlier, thus shortening the time of immunosuppression and improving the immune response. This is of a paramount importance for the control of Marek's disease by vaccination.

Research on the mechanisms of immune protection of HVT *via* the respiratory route (MAZIJA et al., 1994.; GOTTSTEIN et al., 2015), resulted in the introduction of vaccination by means of nebulization using an ultrasonic nebulizer (MAZIJA and ŠTIMAC, 1999), which proved to be superior over subcutaneous application (MIŠKOVIĆ at al., 2013). Previous research also showed that virus delivery via the respiratory system (ABDUL-CAREEM et al., 2009) could induce better immunity against Marek's disease, but the induction of local immunity in the respiratory system and interruption of viral transmission, which is not well defined, should be elucidated (HAQ et al., 2013). Also, delivery of vaccine in combination with different adjuvants can induce significantly better immunoprotection, but still not sterile immunity (PARVIZI et al., 2012; HAQ et al., 2013). The work of GOTTSTEIN et al. (2015) led to the assumption that nebulization as a mode of vaccine delivery can enhance the immune response to the Marek's disease virus, imitating the natural way of infection via the respiratory system. As a method of mass administration it can be an excellent form of application of recombinant HVT vaccines against major poultry diseases, as well as a simple method of implementation of the DIVA program (YONGQING et al., 2011). Results of comparative analysis of the morphometric parameters of lymphocytes, in parenterally vaccinated chickens and chickens vaccinated against Marek's disease by means of

nebulization, and differences established in the morphological (morphometric) patterns of the transformation of immunocompetent cells, are in accordance with this assumption.

Furthermore, these results show that the morphometric analysis of immunocompetent cells may have a role in the practical use of a vaccine against Marek's disease, in the evaluation of immune responses to vaccination, vaccine efficacy and evaluation of possible revaccination, respectively, promoting effective methods of vaccination.

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SAŽETAK

Marekova je bolest limfoproliferativna zarazna bolest kokoši, obično karakakterizirana mononuklearnim staničnim infiltacijama u perifernim živcima i ostalim organima i tkivima, uključujući šarenicu i kožu. Skupina od 70 netom izleglih pilića cijepljena je protiv Marekove bolesti cjepivom od soja FC 126 herpesvirusa purana postupkom nebulizacije izlaganjem aerosolu tijekom 60 sekundi, dok je druga skupina od 70 pilića primila isto cjepivo parenteralnim načinom (supkutano). Cilj ovog istraživanja bio je usporediti morfometrijske značajke limfocita u perifernoj krvi pilića prije i nakon cijepljenja kako postupkom nebulizacije tako i parenteralnim načinom primjene. Kompjutorska analiza slike učinjena je na osobnom računalu korištenjem programa „SFORM" (VAMSTEC, Zagreb, Hrvatska). Ukupno je pretraženo 50 obojenih razmaza periferne krvi cijepljenih pilića (20 postupkom nebulizacije i 30 parenteralno), te je analizirano prosječno 100 limfocita po uzorku. Rezultati su pokazali da su limfociti u perifernoj krvi pilića cijepljenih postupkom nebulizacije u odnosu na parenteralno cijepljene bili značajno veći u većini istraživanih pokazatelja: površini, opsegu, minimalnom i maksimalnom polumjeru, dužini i širini, te ispupčenosti odnosno konveksnosti površine, u svim danima

pokusa osim četvrtoga, što ukazuje na pojačanu metaboličku aktivnost tih stanica. U limfocitima parenteralno cijepljenih pilića samo je omjer jezgre i citoplazme bio veći. Postignuti rezultati pokazuju morfološke značajke transformacije imunokompetentnih stanica, koje se mogu rabiti u procjeni imunosnog odziva na cjepivo, učinka cijepljenja i mogućeg docjepljivanja. Zaključili smo da nebulizacija kao metoda cijepljenja protiv Marekove bolesti stimulira transformaciju imunokompetentnih stanica mnogo ranije, skraćujući time vrijeme imunosupresije i pojačavajući imunosni odziv. To je od osobite važnosti za praktičnu primjenu cjepiva protiv Marekove bolesti.

Ključne riječi: pilići, Marekova bolest, nebulizacija, parenteralno cijepljenje, morfometrija limfocita