Impact of lycopene and astaxanthin on hematological and immunological parameters of laying hens

L V Shevchenko¹, V V Nedosekov¹, V A Davydovych¹, T N Rozhdestveskaya² and E I Drozdova²

¹ The National University of Life and Environmental Sciences of Ukraine (NULES), Kyiv, Ukraine
² Federal State Budget Scientific Institution “Federal Scientific Centre (VIEV), Moscow, Russia

E-mail: nedosekov1@rambler.ru

Abstract. The aim of this study was to determine the hematological profile and specific immunity of laying hens with the addition of oil extracts of lycopene or astaxanthin to the diet. The study used High Line W36 chickens that were vaccinated against Newcastle disease, infectious bronchitis, avian rhinotracheitis and egg drop syndrome. It was found that the addition of lycopene (20 mg/kg) and astaxanthin (10 mg/kg) for 30 days did not affect the hematological profile of laying hens. Increasing the content of lycopene to 40 and 60 mg/kg or astaxanthin to 20 or 30 mg/kg of feed for 30 days reduced the number of leukocytes and hemoglobin in the blood compared to the control, which received an equivalent amount of refined sunflower oil in the diet. Lycopene and astaxanthin supplements, regardless of dose and duration of administration, did not affect the titer of antibodies to Newcastle disease, infectious bronchitis, avian rhinotracheitis, and egg drop syndrome in serum of vaccinated laying hens. The obtained data can be used to justify the optimal dose and term of feeding of lycopene or astaxanthin supplements in the development of a model of carotenoid enrichment of chicken egg yolks.

1. Introduction
The use of carotenoids as antioxidants, anticancer and immunomodulatory agents is of great interest in human and animal nutrition. This is due to both the use of pure carotenoid supplements, including lycopene [1] and astaxanthin in human nutrition and through foods that include chicken eggs. Carotenoids, which do not have provitamin function in the body, have the ability to be deposited in the yolks of chicken eggs and give them an attractive color. Such carotenoids used to enrich egg yolks include lycopene and astaxanthin [2, 3]. Lycopene is becoming increasingly popular in poultry due to its ability to show antioxidant protection, to detect hepatoprotection, anticancer effect. Lycopene maintains oxidative balance in the body of birds by neutralizing free radicals and activating enzymes of the antioxidant defense system of the body [4]. Astaxanthin is widely used as a dye for aquaculture products and has a wide range of biological effects, including antioxidant, anti-inflammatory, stimulating immunity, prevention of cardiovascular disease and pigmentation in mammals [5]. This determines their value is not only to create a food product (chicken eggs) that meets the requirements of consumers not only for the color of the yolk, but also enriched with highly available biologically active substances valuable to the human body as nutraceuticals [6]. The ability of carotenoids to affect different...
types of immune reactions: nonspecific and specific immunity also involves their use as prophylactics. To date, the role of lycopene and astaxanthin in the functioning of the immune system of chickens has not been fully elucidated. Studies have focused on individual parts of nonspecific immunity [7, 8, 9] and some chronic and infectious of humans and animals diseases [10]. Highly productive crosses of laying hens are very sensitive to infectious viral diseases, which cause significant economic damage to poultry. Newcastle disease (NVD) is caused by a virus belonging to the genus Avulavirus and Paramyxoviridae. Clinical manifestations in chickens range from high morbidity and mortality to asymptomatic course. The severity of the infection depends on the virulence of the virus and age, immune status and susceptibility, and mortality can exceed 80% [11]. Infectious rhinotracheitis of birds (ART) is a highly contagious, respiratory disease in turkeys and chickens caused by avian metapneumovirus [12], accompanied by swelling of the head, reduced egg production (5 to 30%) and deterioration of egg quality (shell depigmentation). Infectious bronchitis is caused by the avian infectious bronchitis virus (IBV), which belongs to the Coronaviridae and characterized by respiratory symptoms, nephritis or decreased egg production and deterioration in the quality of eggs in chickens with a 30–40% mortality rate. Infectious bronchitis is endemic worldwide and leads to serious economic damage [13]. Egg drop syndrome (EDS) is caused by adenoviral infection in laying hens that characterized by the formation of soft shells and the production of eggs without shells in the apparently healthy birds and leads to a sudden decrease of 10 – 40% egg production or failure to reach the peak of egg production in poultry [14].

Therefore, the aim of our study was to investigate the impact of lycopene and astaxanthin in different doses on the hematological profile and the level of immune protection of chickens vaccinated against Newcastle disease, infectious rhinitis, infectious bronchitis and oviposition syndrome.

2. Materials and methods

The experiment was conducted at the Laboratory of Veterinary Medicine Faculty NULES on 14-week-old High Line W36 laying hens (45 heads). All the chicks were divided into 3 analog groups (15 birds each). At 23 weeks (after adaptation and reaching the peak of egg production), studies were started that included three periods over 90 days (table 1).

| Group               | Diet                                          |
|---------------------|------------------------------------------------|
|                     | 1-30 days                                      |
| Control             | BD¹ + 0.33 g/kg of refined sunflower oil       |
| Lycopene diet       | BD² + 0.66 g/kg of refined sunflower oil       |
| Astaxanthin diet    | BD³ + 1.0 g/kg of refined sunflower oil        |
|                     | 31-60 days                                    |
| Control             | BD¹ + 20 mg/kg of lycopene (LP20)              |
| Lycopene diet       | BD² + 40 mg/kg of lycopene (LP40)              |
| Astaxanthin diet    | BD³ + 60 mg/kg of lycopene (LP60)              |
|                     | 61-90 days                                    |
| Control             | BD¹ + 10 mg/kg of astaxanthin (AST10)          |
| Lycopene diet       | BD² + 20 mg/kg of astaxanthin (AST20)          |
| Astaxanthin diet    | BD³ + 30 mg/kg of astaxanthin (AST30)          |

Note: DB – basic diet, the same superscripts ¹, ², ³ show the same content of refined sunflower oil in the diet.

As a source of lycopene were used 6% oil extract of lycopene extracted from tomatoes (LycoRed, Israel), as well as astaxanthin – 10% oil extract of astaxanthin obtained from the biomass of the alga Haematococcus pluvialis (ALGAE Technologies, Israel). Laying hens were vaccinated according to the program (table 2).

In this experiment, we considered antibody titers to infectious rhinotracheitis (ART), infectious bronchitis (IBV), Newcastle disease (NDV) and egg drop syndrome (EDS). The conditions for feeding and keeping laying hens were provided as described in previously published materials [15, 16].

| Age of chickens, days | Name of disease                      | Name of vaccine                  | Method of vaccination |
|-----------------------|-------------------------------------|----------------------------------|-----------------------|
| 1                     | Infectious bronchitis, Newcastle disease | Nobilis®Ma5+Clone30 (live lyophilized vaccine) | spray                 |
10 Infectious bronchitis Nobilis®Ma5+Clone30 (live lyophilized vaccine) Watering

24 Infectious bronchitis, Newcastle disease Nobilis®Ma5+Clone30 (live lyophilized vaccine) Watering

50 Infectious bronchitis, Newcastle disease Nobilis®Ma5+Clone30 (live lyophilized vaccine) Watering

75 Infectious bronchitis Nobilis®Ma5+Clone30 (live lyophilized vaccine) Watering

85 Newcastle disease AviPro ND LASOTA (live lyophilized vaccine) Watering

95 Infectious rhinotracheitis, infectious bronchitis, Newcastle disease, egg drop syndrome Nobilis®RT+IBmulti+ND+EDS (inactivated lyophilized vaccine) intramuscularly

On the 31st, 61st, and 91st days of the experiment, blood was drawn from the axillary vein from 5 hens of each group in two tubes in the morning before feeding. Blood with the anticoagulant EDTA was taken in one test tube to determine the formed elements, hematocrit and hemoglobin in the blood. In the second tube, blood was collected without anticoagulant to obtain serum. Serum for studies was obtained by sparing centrifugation at 2000 rpm for 10 min and stored at -20 °C.

All experiments were performed in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Scientific Experiments or Other Scientific Purposes of 1986, as well as the Law of Ukraine "On Protection of Animals from Cruelty" of 21.02.2006 № 3447-IV in the edition of 04.08.2017.

Hematocrit was determined in the blood by centrifugation of whole blood in capillaries using a CM-3 MICROmed centrifuge (Ukraine) and determination of the ratio of blood plasma to formed elements in %. All blood cells were counted in swabs by manual method. The sum of erythrocytes (RBC) was expressed in 10\(^{12}\)/l. To determine the number of leukocytes, whole blood samples were stained using Leikodif 200 reagent kits (LDF 200) (Erba Lachema, Czech Republic). The sum of leukocytes (WBC) was expressed in 10\(^{9}\)/l. Differential leukocyte counting was performed in Pappenheim-stained whole blood smears [17]. To do this, counted 100 cells and determined their percentage to the total number of leukocytes.

Hemoglobin in the blood (Hb) was determined using reagents from Pointe Scientific Inc. (USA) and semi-automatic analyzer Pointe 180 (Poland).

Titters of specific antibodies to infectious rhinotracheitis (ART), infectious bronchitis (IBV), Newcastle disease (NDV) and egg drop syndrome (EDS) were determined using ELISA tests (BioChek®, Reeuwijk, the Netherlands) according to the manufacturer's instructions.

Statistical processing of the obtained results was performed using the ANOVA program, the data in the tables are presented in the form of x ± SD (mean ± standard deviation). The difference between the values in the groups was determined using the Tukey test. The difference was considered probable at p<0.05 (taking into account the Bonferroni correction).

3. Results

Enrichment of the diet of laying hens for 30 days with lycopene at a dose of 20 mg/kg or astaxanthin at a dose of 10 mg/kg did not affect hematological parameters: hematocrit, hemoglobin content, as well as the number of erythrocytes and leukocytes in the blood compared to control (table 3).

Table 3. The effect of lycopene at a dose of 20 mg/kg and astaxanthin at a dose of 10 mg/kg of feed on the hematological parameters of laying hens, x ± SD, n = 5.

| Indicator                              | Control         | LP20            | ASTI10          |
|----------------------------------------|-----------------|-----------------|-----------------|
| Hematocrit, %                          | 29.38±2.51      | 28.62±0.73      | 26.96±1.23      |
| Hemoglobin, g/l                        | 96.42±8.87      | 97.54±8.09      | 104.38±8.39     |
| Erythrocytes, 10\(^{12}\)/l            | 2.57±0.12       | 2.50±0.12       | 2.82±0.12       |
The effect of lycopene at a dose of 40 mg/kg and astaxanthin at a dose of 20 mg/kg of feed on the hematological parameters of laying hens, x ± SD, n = 5.

| Indicator         | Control       | LP40          | AST20         |
|-------------------|---------------|---------------|---------------|
| Leukocytes, 10^9/l| 23.87±0.80    | 22.08±0.60    | 22.70±1.07    |
| Eosinophils, %    | 5.00±0.61     | 5.00±0.50     | 5.00±1.22     |
| Heterophiles, %   | 41.20±2.27    | 42.20±2.01    | 44.80±2.70    |
| Lymphocytes, %    | 48.20±2.48    | 49.20±3.44    | 43.40±3.46    |
| Monocytes, %      | 4.20±1.14     | 6.00±1.37     | 4.80±1.64     |
| Basophils, %      | 1.40±1.04     | 1.60±0.57     | 2.00±0.79     |

Note: see table 1

The ratio of eosinophils, heterophiles, lymphocytes, monocytes and basophils of LP20 and AST10 in the blood chickens was at the control level and did not exceed the normative parameters.

**Table 4.** The effect of lycopene at a dose of 40 mg/kg and astaxanthin at a dose of 20 mg/kg of feed on the hematological parameters of laying hens, x ± SD, n = 5.

| Indicator         | Control       | LP40          | AST20         |
|-------------------|---------------|---------------|---------------|
| Hematocrit, %     | 25.14±0.58    | 22.28±0.77    | 24.00±1.20    |
| Hemoglobin, g/l   | 106.18±3.25   | 97.28±5.65    | 104.16±6.67   |
| Erythrocytes, 10^12/l | 2.72±0.12 | 2.45±0.14     | 2.70±0.14     |
| Leukocytes, 10^9/l | 25.22±1.49a   | 19.40±0.37b   | 21.01±0.62ab  |
| Eosinophils, %    | 2.40±0.57     | 5.40±1.82     | 1.40±0.57     |
| Heterophiles, %   | 46.20±1.98    | 40.00±2.57    | 41.20±4.16    |
| Lymphocytes, %    | 42.20±2.84    | 44.40±2.77    | 48.00±4.57    |
| Monocytes, %      | 6.00±2.06     | 6.20±0.65     | 7.60±0.67     |
| Basophils, %      | 3.20±1.08     | 4.00±0.61     | 1.80±0.82     |

Note: see table 1, different superscript letters a, b indicate values that probably differed in one row of the table (p<0.05) according to the results of comparison using Tukey test with Bonferroni correction.

Increasing the dose of lycopene in the diet of chickens LP40 and astaxanthin in the diet of chickens AST20 did not affect the hematocrit, erythrocyte count and hemoglobin content in the blood compared with the control (table 4). Additions of lycopene LP40 reduced (p<0.05) the number of leukocytes in the blood of chickens, while astaxanthin AST20 did not affect this indicator compared to the control. Both supplements in the diet of chickens LP40 and AST20 did not change the ratio of subpopulations of leukocytes: eosinophils, heterophiles, lymphocytes, monocytes and basophils. A further increase in the dose of astaxanthin in the diet of laying hens AST30 reduced the hematocrit (p<0.05) compared with the control, but in the group with the addition of lycopene LP60 this was not observed.

Supplements LP60 and AST30 caused a decrease in the number of leukocytes in the blood in chickens (p <0.05) compared with the control. Astaxanthin showed a stronger effect on this indicator than lycopene (p<0.05) (table 5).

**Table 5.** The effect of lycopene at a dose of 60 mg/kg and astaxanthin at a dose of 30 mg/kg of feed on the hematological parameters of laying hens, x ± SD, n = 5.

| Indicator         | Control       | LP60          | AST30         |
|-------------------|---------------|---------------|---------------|
| Hematocrit, %     | 32.36±2.30a   | 27.16±1.87ab  | 23.84±1.57b   |
| Hemoglobin, g/l   | 118.14±0.91a  | 108.42±4.58   | 111.66±5.06   |
| Erythrocytes, 10^12/l | 2.94±0.12a | 2.42±0.12b    | 2.35±0.13b    |
| Leukocytes, 10^9/l | 26.02±0.79a   | 20.86±0.44b   | 17.94±0.78b   |
| Eosinophils, %    | 4.80±0.89     | 4.00±1.06     | 5.40±1.52     |
| Heterophiles, %   | 45.00±1.73    | 41.80±3.11    | 45.60±3.75    |
| Lymphocytes, %    | 41.80±1.14    | 41.60±3.55    | 39.80±2.75    |
| Monocytes, %      | 5.00±1.77     | 7.80±0.42     | 7.40±1.15     |
| Basophils, %      | 3.40±0.84     | 4.80±0.89     | 1.80±0.89     |

Note: see tables 1, 4
Increasing the dose of lycopene and astaxanthin in the diet of laying hens LP60 and AST30 reduced the number of erythrocytes (p<0.05), but did not change the content of hemoglobin in the blood compared to control. The effect of additives in groups LP60 and AST30 on the ratio of subpopulations of leukocytes in the blood of chickens: eosinophils, heterophiles, lymphocytes, monocytes and basophils has not been established. Immature and pathological forms of erythrocytes and leukocytes were not detected in the blood of chickens.

Additions of lycopene at doses of 20, 30 and 60 mg/kg, as well as astaxanthin at doses of 10, 20 and 30 mg/g of feed throughout the experiment (90 days) did not affect the titers of antibodies to Newcastle disease (NDV), infectious bronchitis (IBV), infectious rhinitis and tracheitis (ART) and egg drop syndrome (EDS) in the serum of vaccinated chickens (tables 6 – 8).

**Table 6.** The antibody titer in the serum of laying hens to NDV, IBV, ART and EDS at feeding lycopene at a dose of 20 mg/kg and astaxanthin at a dose of 10 mg/kg of feed, log2, x ± SD, n = 5.

| Indicator | Control   | LP20     | AST10     |
|-----------|-----------|----------|-----------|
| NDV       | 14.22±0.12| 14.30±0.04| 14.23±0.07|
| IBV       | 14.01±0.21| 13.69±0.30| 14.08±0.17|
| EDS       | 13.43±0.10| 13.51±0.03| 13.35±0.07|
| ART       | 14.28±0.01| 14.27±0.02| 14.25±0.01|

Note: see table 1

**Table 7.** The antibody titer in the serum of laying hens to NDV, IBV, ART and EDS at feeding lycopene at a dose of 40 mg/kg and astaxanthin at a dose of 10 mg/kg of feed, log2, x ± SD, n = 5.

| Indicator | Control   | LP40     | AST20     |
|-----------|-----------|----------|-----------|
| NDV       | 13.96±0.13| 14.27±0.05| 14.17±0.10|
| IBV       | 13.35±0.40| 13.24±0.25| 13.10±0.29|
| EDS       | 13.43±0.03| 13.49±0.03| 13.44±0.05|
| ART       | 14.13±0.10| 14.19±0.10| 14.24±0.01|

Note: see table 1

**Table 8.** The antibody titer in the serum of laying hens to NDV, IBV, ART and EDS at feeding lycopene at a dose of 60 mg/kg and astaxanthin at a dose of 30 mg/kg of feed, log2, x ± SD, n = 5.

| Indicator | Control   | LP60     | AST30     |
|-----------|-----------|----------|-----------|
| NDV       | 14.29±0.19| 14.41±0.20| 13.97±0.15|
| IBV       | 12.78±0.40| 13.50±0.30| 12.99±0.46|
| EDS       | 14.14±0.03| 13.89±0.23| 13.67±0.21|
| ART       | 14.84±0.07| 14.89±0.11| 14.65±0.15|

Note: see table 1

**4. Discussion**

An important hallmark for assessing the health and immune status of the body are hematological parameters, which may reflect preclinical changes in internal homeostasis in animals [18]. The number of erythrocytes, leukocytes and the ratio of their subpopulations in chickens allow to assess the effectiveness of dietary supplements of lycopene and astaxanthin in the diet and predict their effect on productivity and quality and biological value of products. In our studies, enrichment of the diet of laying hens with lycopene at a dose of 20 mg/kg or astaxanthin at a dose of 10 mg / kg feed did not affect the hematological profile compared to control. This is consistent with the results of studies by Olson et al., [3], which found that lycopene even at doses of 420 and 840 mg/kg diet did not affect immune responses (inflammatory, skin hypotension to basophils, response to antibodies 1 and 2 degrees). This is also evidenced by data obtained using laboratory animals: mice and rats in the determination of acute, subchronic and chronic toxicity of lycopene [19].
With increasing doses of lycopene and astaxanthin in the diet of laying hens, the level of expression of changes in the hematological profile in our experiment increased. Lycopene at a dose of 40 mg/kg and astaxanthin at a dose of 20 mg/kg caused a decrease in the number of leukocytes in the blood of chickens. A further increase in the dose of lycopene to 60 mg/kg and astaxanthin to 30 mg/kg of feed in our experiment caused a probable decrease in hemoglobin and white blood cell count. This probably indicates a decrease in the proliferative capacity of the organs of immunopoiesis in healthy chickens under the influence of carotenoid supplements. Confirmation of our results is also the data of [20], which indicate the immunosuppressive effect of lycopene in the diet of broiler chickens at a dose of 100 mg/kg 5% of the drug, which is associated with inhibition of lymphocyte proliferation through mechanisms dependent on early cell activation [21]. Our conclusions are consistent with previous studies to study the effects of β-carotene on nonspecific immunity of broiler chickens [22]. At the same time, we did not notice significant changes in the ratio of subpopulations of leukocytes in the blood of chickens, which is consistent with the data obtained by Petri end Lundebye [23] in rats. The decrease in the number of leukocytes in the blood of laying hens due to lycopene and astaxanthin in our experiment probably did not affect the functional capacity of the humoral link of specific immunity, in the synthesis of antibodies in response to vaccination against Newcastle disease, infectious rhinotracheitis and infectious bronchitis. All of the above diseases are caused by viruses, which involves prevention in the form of vaccinations with live or inactivated vaccines. The effectiveness of the formation of specific antibodies to the pathogens of these infections in chickens is determined by a number of factors, including the presence in the diet of biologically active compounds that can reduce the negative effects of post-vaccination stress and stimulate the formation of stable immunity. Information on the possible role of astaxanthin in the immune response of chickens is scarce, but there are data obtained using beagle dogs, in which dietary astaxanthin increased the concentration of IgG and IgM and the population of B cells, which allowed the authors to conclude that cell-mediated and humoral immune response in dogs during vaccination, reduction of cell DNA damage and inflammation [24]. The possible role of lycopene in maintaining immunological stress in laying hens in our experiment is probably also associated with a reduction in oxidative damage to lymphocyte DNA [25]. Throughout our experiment (90 days), dietary supplements of lycopene or astaxanthin did not affect antibody titers in the serum of healthy vaccinated laying hens against Newcastle disease, infectious bronchitis, infectious rhinotracheitis, and egg drop syndrome compared with controls. Our results are consistent with data [26], which did not show a significant improvement in the titer of antibodies to cholera in ducks under the influence of β-carotene and astaxanthin. This is evidenced by data obtained by [27], which proved that carotenoids, such as β-carotene, do not have an indirect effect through the maternal body on the immune status of partridges, indicating the absence of functional changes in the organs of immunopoiesis responsible for humoral immunity in chickens under the influence of lycopene and astaxanthin. This assumption is consistent with the results obtained by studying the effect of β-carotene, lutein and canthaxanthin on the immune status of chickens vaccinated against Newcastle disease. In this study, it was shown that carotenoids do not affect the antibody titer in vaccinated chickens against Newcastle disease [20]. It was also confirmed that dietary supplements of other carotenoids did not affect the titer of antibodies to Live Bovine Respiratory Syncytial Virus in the serum of cattle [28].

Although there are opposite results regarding the role of carotenoids in the functioning of the immune system of birds. For example, cocks consuming β-carotene and canthaxanthin had higher antibody titers in response to Newcastle disease (NDV) vaccination than control birds [29], and increased carotenoid levels in the diet of chickens caused an increase in the mass of the fabric bursa and enhanced the immune response when infected with E. tenella [28]. Feeding a diet rich in carotenoids increased the immune response in domestic chickens [31] and wild birds [32]. The links between immune function and carotenoids in animal diets have also been experimentally proven in a number of studies [33, 34, 35, 36]. It should be noted that the effects found by the authors of the studies did not always coincide with each other. This difference in the results of studies by different scientists on the effects of carotenoids on immune function may be due to the role they play in the body, which is in different states: infectious disease, stress, intoxication, carcinogenesis, and a healthy body. Therefore, free radicals and singlet
oxygen, which are formed in the cells of the body, can perform both physiological and pathological functions [37]. Probably, lycopene and astaxanthin can have different effects on the immune status of animals: in a body that has an excess of free radicals and is in a state of illness or stress, their antioxidant effect is regarded as positive. In the case of using supplements for a healthy body (for example, in our experiment), we did not notice the effect of stimulating the immune response. However, the intensity of immunity to infectious diseases of laying hens by the titer of antibodies to Newcastle disease, infectious rhinotracheitis, infectious bronchitis and the egg drop syndrome in the serum is considered to be quite high.

5. Conclusions
The use of lycopene at a dose of 20 mg/kg and astaxanthin at a dose of 10 mg/kg of feed does not affect the number of leukocytes and their subpopulations, erythrocytes, hematocrit and hemoglobin content in the blood of laying hens. Increasing the content of lycopene to 40 and 60 mg/kg or astaxanthin to 20 or 30 mg/kg of feed for 30 days reduces the number of leukocytes and hemoglobin in the blood of laying hens. Additions of lycopene at doses of 20, 40 and 60 mg/kg and astaxanthin at doses of 10, 20 and 30 mg/kg of feed for 30 days each did not affect the titer of antibodies to Newcastle disease, infectious bronchitis, avian rhinotracheitis and egg drop syndrome in serum of vaccinated laying hens. The obtained research results can be promising in the development of a model of enrichment of lycopene or astaxanthin in chicken eggs.

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