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# METABOLIC PROFILE IN HENS SUBJECT OF LONG TERM HYPERIMMUNIZATION

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***Abstract***

*In the present paper, the metabolic profile was analysed in a group of adult Rhode Island hens extensively required for the production of IgY antibodies against bacterial entities. The results reveal changes in the leukocyte formula dominated by an increase in the percentage of heterophils and a decrease in the percentage of lymphocytes in the blood. An increase in the level of serum proteins with predominance of globulins is added to this. Increased uric acid levels were found also. Increased levels of GOT and GPT transaminases and γ-GT indicated an intensification of the protein metabolism, LDH and PA were not significantly modified. Regarding carbohydrate metabolism, it was found that serum glucose and lactate were maintained within physiological limits. The lipid profile was characterized by maintaining serum triglycerides and cholesterol within normal limits. Significant decrease in HDL and increase in LDL were interpreted as a decrease in the proteosynthetic capacity of the liver. The mineral profile was dominated by the increase of the level of sodium, iron, magnesium and calcium but by the decrease of the level of chlorine, potassium and phosphorus.*

***Key words****: immunoglobulin Y, hyperimunity, metabolic profile, hen.*

## INTRODUCTION

Immunoglobulins Y (IgY) are the major antibody in birds, and they are found in high concentrations in chicken egg yolk. As with the other immunoglobulins, IgY is a class of proteins which are formed by the immune system in reaction to certain foreign substances, and specifically recognize them. IgY is functionally similar to IgG, and also shares some similarity with IgE. That means, similarly to IgG and presumably IgE the main function of IgY is the defense against pathogens by binding them and thereby disabling them. Due to the functional similarities to IgG, IgY is sometimes referred to as "chicken IgG", or "bird IgG". Since chickens can lay eggs almost every day, and the yolk of an immunised hen's egg contains a high concentration of IgY, chickens are gradually becoming popular as a source of customised antibodies for research. Usually, mammals such as rabbits or goats are injected with the antigen of interest by the researchers to obtain the antibody (Davison et al., 2008). Xu et al. (2011) relived a significant antimicrobial activity of the IgY for refrigerated fish products, which allowed us to suggest its potential as a bio-preservative for seafood. To stimulate the production of antigen-specific IgY in a hen, the desired antigens are injected in the form of a vaccine containing an adjuvant. The latter are non-specific B-cell stimulating immune modulators which improve the immune response to an antigen (Goran et al., 2019; Shade et al., 2001). The synthesized antibodies are discharged into the egg yolk in the formation where it accumulates; eggs belonging to hyperimmuned hens with the same inoculums are harvested. The IgY contained in the respective eggs is woven by specific methods (Chiurciu et al., 2018; Chiurciu et al., 2020). Harvested eggs can be administered to animals in various forms; for example, IgY is extracted as a sterile aqueous solution which is administered orally or by aerosol, or the hyperimmune product can

be lyophilized. The most common site of injection is intramuscular. Although, the amount of IgY in the egg can be affected by various factors, such as age, breed of the chicken, antigen, adjuvant and injection route, IgY yields from 60 to 150 mg per egg have been reported. Interestingly, only 2-10% of the IgY in an immunized hen has been shown to be antigen-specific (Kovacs-Nolan and Mine, 2012). Beyond these achievements, the biological status of chickens subjected to such immunological solicitation is not sufficiently known. The aim of this paper is to determine the metabolic status of chickens subjected to the process of active hyperimmunization in order to produce IgY.

## MATERIALS AND METHODS

The research was conducted on 51-week-old female Rhode Island hens housed in a battery system in halls (5 capita/$m^2$) with monitored environmental parameters, free access to water and fed *ad libitum* on a standardized diet (main ingredients by g/kg: wheat 413, barley 315, oats 109, soybean meal 59, grass meal 26, fish meal 56, dicalcium phosphate 6, limestone 8, vitamin and mineral premix 7). The birds were inoculated with multiple PC2 antigen, respectively contained in inactivated strains: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* spp., *Escherichia coli, Enterococcus faecalis, Salmonella enteritidis, Salmonella typhimurium, Streptococcus mutans, Staphylococcus aureus, Streptococcus grup B, Proteus* spp., *Acinetobacter baumannii, Helicobacter pylori, Streptocccus pneumoniae, Clostridium difficile, Candida* spp. Administration protocol: 1 mL in 2 separate i.m. points (0.5 mL + 0.5 mL), in the pectoral muscles, 3 inoculations at intervals of 2 weeks each. Two weeks after inoculation, blood samples were taken by axillary vein puncture, samples were collected on anticoagulant (EDTA and heparin, depending on the tests to be performed). Morphological examinations of the blood were performed from the blood samples: number of erythrocytes, platelets, leukocyte formula, hemoglobin, hematocrit, derived erythrocyte parameters. Other blood samples were centrifuged to obtain the serum or plasma, depending on the situation. Plasma or serum was stored by freezing at −20°C until blood biochemistry tests were performed. Blood morphology analyzeses were performed using a cytometry flow analyser, Advia 2120i (Siemens, France). Blood biochemistry determination consisted of blood glucose, lactic acid, uric acid, lipid profile (triglycerides, cholesterol, HDL and LDL) and mineral profile, using an ELISA system. Separation of proteins was carried out by agarose gel electrophoresis (SPE P/N 655900, Beckman Coulter, Fullerton, USA), according to the manufacturer's instructions.

Quantitative results were obtained by software for quantitative gel analysis. Total protein was determined based on the biuret reaction by proteins following the Lowry method. For the preparation of the leukocyte formula, smears were performed which were stained by the MGG method. Glutamateoxalatetransaminase (GOT), glutamatepyruvatetransaminase (GPT), γ-glutamil transferase (γ-GT), lactic dehidrogenase (LDH), andalkalinephosphatase (AP) activities were determined using the method described by Manta et al. (1976). The determination of the number of blood platelets was performed by fixing and staining the blood by the Natt Herick method followed by dilution with a Potain pipette.The data obtained were centralized using the Excel 2010 program and the statistical processing was performed using the GraphPad program for Windows, version 8.0.2, GraphPad Software, Inc. The differences between the control group and the experimental groups were considered significant for values of $P \leq 0.05$.

## RESULTS AND DISCUSSIONS

The results of the analysis of erythrocyte morphological changes are presented in Table 1. The analysis of the data presented in Table 1 shows improvements in the series of erythrocytes materialized in the significant increase in the number of erythrocytes ($P = 0.003$) compared to the untreated control hens. Thus, there is a 22% increase in the number of erythrocytes, followed by an increase in haematocrit (Ht) of a similar percentage (20.3%), as well as an increase in the amount of hemoglobin: 12.2% higher than the control. It is found that the hemoglobin content could not

follow the ascending course of erythrocyte count and Ht. The presentation of these results of erythrocyte series changes cannot be explained in itself, without further analysis of the main metabolic aspects. On the other hand, the lack of data in the literature on this subject also makes it difficult to interpret these modifications which were found by us. These data obviously reveal an intensification of the erythropoiesis processes, generated either by a high energy and oxygen consumption, as a result of some metabolic intensification or by other reasons. Almost all other research focuses on the clinical effects of InY and less on the biological status of chickens used in this hyperimmunization process. Regarding the leukocyte formula, the data presented in Table 1 reveal first of all an increase in the number of leukocytes, of 52.1% vs. control.

This increase in the number of leukocytes is due in main to heterophils pseudoeosinophils, whose percen-tage in the leukocyte formula increased by 11.1% vs. control, an increase statistically significant ($P<0.05$). On the other hand, the percentage of lymphocytes, eosinophils and monocytes was significantly and accordingly reduced in the group of hyperimmunized chickens compared to the control group.

Table 1. Comparative presentation of the blood morphology in hyperimmune hens vs. control

| Group | Erythron | | | | | |
|---|---|---|---|---|---|---|
| | Number ($\times10^6/\mu L$) | Ht (%) | Hb (g/dL) | VEM (fL) | HEM (pg) | CHEM (%) |
| Control hens | 3.86±1.02 | 35.5±4.22 | 9.43±3.31 | 9.1±2.21 | 2.44 ± 0.3 | 26.5±6.21 |
| Hyperimmune hens | 4.72 ± 0.9 | 42.6 ± 6.6 | 10.5 ± 0.5 | 9.02 ± 1.9 | 2.22 ± 0.8 | 24.6 ± 2.2 |
| P value | 0.003 | 0.03 | 0.002 | 0.05 | 0.10 | 0.03 |
| | Leukocytes | | | | | |
| | Number ($\times10^3/\mu L$) | Pseudoeosinophils (%) | Eosinophils (%) | Lymphocytes (%) | Monocytes (%) | Basophils (%) |
| Control hens | 21.3±4.32 | 27.0±4.19 | 3.0±0.56 | 62.0±16.52 | 8.0±1.88 | 0.3 ± 0.0 |
| Hyperimmune hens | 32.5 ± 7.4 | 30.3 ± 7.7 | 4.0 ± 2.3 | 59 ± 22.5 | 6.6 ± 3.2 | 0.0 ± 0.0 |
| P value | 0.000 | 0.007 | 0.04 | 0.002 | 0.022 | 0.143 |
| | Thrombocytes ($\times10^3/\mu L$) | | | | | |
| Control hens | 90.9 ± 8.0 | | | | | |
| Hyperimmune hens | 89.0 ± 21.0 | | | | | |
| P value | 0.133 | | | | | |

Table 2. Serum biochemical profile in hyperimmune hens (expressed as g/dL of plasma)

| Group | Total protein (g/dL) | Uric acid (mg/dL) | Glucose (mg/dL) | Lactate (mg/L) | Triglycerides (mg/dL) | Cholesterol (mg/dL) | HDL (mg/dL) | LDL (mg/dL) |
|---|---|---|---|---|---|---|---|---|
| Control | 5.44 ± 0.55 | 4.4 ± 0.4 | 188.8 ± 21.3 | 190.0±45 | 434.0 ± 32.0 | 154 ± 32 | 55.0±6.6 | 165±21 |
| Hyperimmunized | 6.16 ± 0.90 | 14.4 ± 2.1 | 163.6 ± 32.5 | 420 ± 101 | 843.5 ± 83.2 | 286 ± 56 | 32.1±5.0 | 213±43 |
| P value | 0.021 | 0.000 | 0.003 | 0.000 | 0.000 | 0.002 | 0.005 | 0.032 |

Serum protein profile is presented in Figure 1. According to the data presented in Figure 1, six protein fractions were identified by electrophoresis: albumins, $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ and $\gamma$-globulins. The concentration of albumin (3.82 g/dL) found by us in adult chickens from the control group (Table 2) is in agreement with that reported by Peltonen and Sankari (2011) in adult chickens. Alpha and beta globulins are acute-phase proteins, and gamma globulins are immune globulins. There is a complex change in protein fractions in hyperimmunized chickens compared to the control. Firstly, a doubling of the IgG protein (IgY) fraction occurred. There is also an increase in fractions $\beta1$ and $\beta2$ whose interpretation will be difficult to achieve. Finally, the albumin, $\alpha_1$ and $\alpha_2$ fractions remained relatively unchanged in the experimental groupsvs control.

Changes in lipid metabolism maybe reflected in their mobilization resulting in increased circulating total triglycerides, cholesterol and LDH.

The metabolic enzyme profile was also represented by the determination of the activity of GOT, GPT, $\gamma$-GT, LDH and PA enzymes (Figures 2 and 3). Transaminase activity (normal range 33-43 IU/L in hen) doubled in the serum of hyperimmunized vs. control

chickens, increasing from 86.6, 48.4 and 12.6 IU to 163.3, 165.4 and 54 IU, respectively. On the other hand, the activity of LDH decreased significantly ($P<0.05$) while PA were no significance altered: around 300 UI ($P<0.05$). A decreased activity of LDH, associated with an intensified activity of LDH and transaminases, leads to a decrease in anaerobic glucose metabolism in favour of protein consumption. This is also confirmed by a significant decrease in blood plasma glucose ($P = 0.003$). A physiological intensification of the serum transaminases activity reveals the intensification of the amino acid metabolism, of the protein metabolism, respectively. The intensification of protein catabolisme is also reflected in the increase in plasma uric acid concentration, as a product of protein catabolisme in birds.

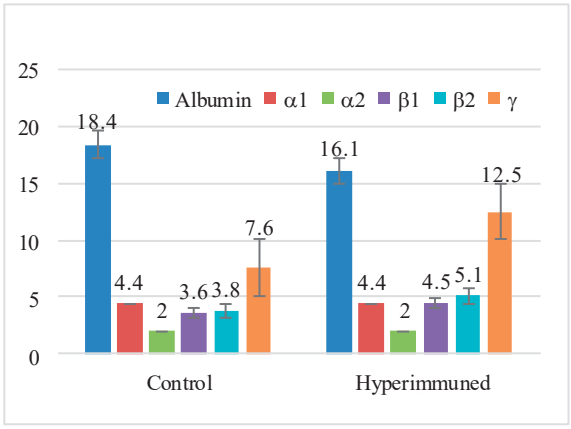


Figure 1. Serum protein profile in hyperimmunized hens (values in g × $L^{-1}$ of serum)

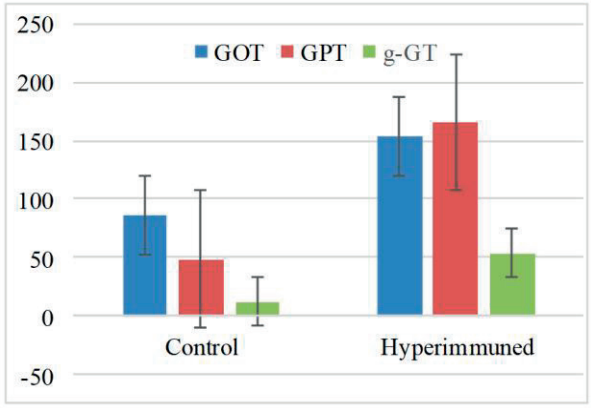


Figure 2. Blood plasma levels of GOT, GPT and γ-GT in hyperimmuned hens vs. control expressed as IU

The interest in using IgY in various therapies is growing. IgY is used for control of coccidiosis as a vaccine: results of studies provided the first successful demonstration of inducing protective passive immunity against avian coccidiosis by passively transferring antibodies (oral feeding) obtained from egg yolk of the hyperimmunized hens. Larger trials in the field conditions confirmed the efficacy of this passive immunity strategy and this technology is being applied commercially in Mexico (USDA, 2006; Horie et al., 2004). One research study found that some people with osteoarthritis feel less joint pain or swelling after taking a specific "immune egg" powder formulation for 2 months (Horie et al., 2004). Developing research suggests that a preparation of purified antibodies from hyperimmune eggs harvested from hens immunized against strains of rotavirus can modestly decrease rotaviral diarrhoea in children (Mine and Kovacs-Nolan, 2002). But an increased muscular strength and enhanced muscle repair with hyperimmune egg protein supplementation was reported (Scheelt et al., 2006).

On the other hand, the production and use of IgY provoke interest for the study of the metabolic profile of birds involved in IgY production in particular and of metabolism in birds in general. Unfortunately, the number of works dedicated to the study of bird metabolism used in the production of IgY antibodies is insufficient or almost non-existent. Kaab et al. (2019) measured the level of three acute phase proteins in red mite-infested chickens: the acute phase proteins: alpha-1 acid glycoprotein, serum amyloid-A, and ceruloplasmin were measured. Others identified proteins were associated with the egg formation.

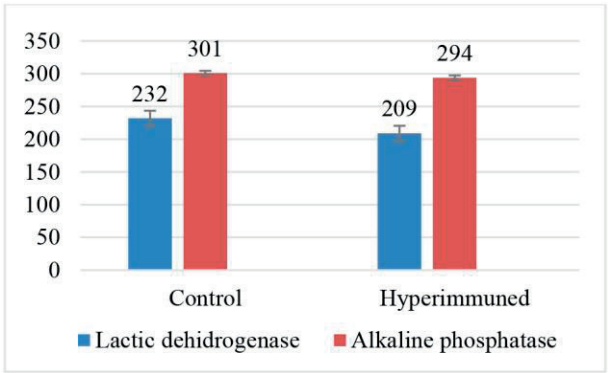


Figure 3. Blood plasma levels of LDH and AP in hyperimmuned hens vs. control expressed as IU

Peltonen and Sankari (2011) also performed a serum protein profile in chickens; the study provides a comparative picture of the protein

profile of the blood plasma looked at the effect of age and gender. The increase in the level of γ-globulin fraction in our study reveals the real activation of the avian immune system in overload conditions. The physiological significance of the increase of two other globulins, β1 and β2 remains to be explained.

The plasma mineral profile of the hyperimmune hens was dominated by the significant increase of the level of sodium (12%), iron (8.9%), magnesium (14.2%) and calcium (4.3%) but by the decrease of the level of chlorine (6.6%), potassium (6.4%) and phosphorus (10.0%) in our study. Our control found values are in agreement with Dobrzański et al. (2011). The variation of mineral concentration in blood of laying poultry is an important issue due to intense skeleton development (young birds) or egg formation (adult birds). Some of the elements are significant in laying hens feeding, due to large requirements of birds on calcium and phosphorus, which may be supplied in a specified ratio and chemical form (Dobrzański et al., 2011). Studies on the effect of hyperimmunization on the avian mineral profile are few. Most likely, other studies are needed to elucidate the effects of such biological stress on metabolism in general and on mineral metabolism in particular.

## CONCLUSIONS

Hens subjected of intensive hyperimmunization treatments are characterized by increased erythrocytes and neutrophils. Among the serum proteins, Y globulins increase significantly. Blood biochemical profile is dominated by decreased blood sugar but increased triglycerides and uric acid while LDH and PA are not significantly modified. However, these changes do not alter the basic physiological status of these birds.

## ACKNOWLEDGEMENTS

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