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ANTIHYPOXIC AND ENERGY STIMULATING EFFECTS OF COBALT GLYCINATE DURING EMBRYOGENESIS OF QUAILS (*Coturnix japonica*)

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A b s t r a c t

Hypoxic manifestations, including those associated with certain periods of bird embryogenesis, lead to slowdown in development, and in severe cases, to multifaceted morphological and functional disorders in embryos. Numerous studies have confirmed the effectiveness of biostimulants with pronounced antioxidant properties, which can neutralize negative effects of hypoxia and provide conditions for a faster transition to aerobic glycolysis. These biostimulants include cobalt glycinate, synthesized at Scriabin Moscow State Academy of Veterinary Medicine and Biotechnology. The choice of the biostimulant components was due to the properties of each component separately and their hypothetical complementary effect. In the present work, it was found for the first time that cobalt glycinate has an antihypoxic effect and stimulates energy metabolism in quail embryos and 1-day-old quails. The purpose of the work is to investigate the effect of cobalt glycinate on energy metabolism and to provide a background for correction of adverse effects of hypoxia that occur during embryogenesis in quails under incubation. The experiment was carried out on hatching eggs from Japanese quail (*Coturnix japonica*) of the same age (Shepilovskaya Poultry Farm, Moscow Province, 2020). The eggs were sorted in two batches (experimental and control, 220 eggs each). The experimental eggs were sprayed once with 0.05 % cobalt glycinate solution (an aerosol dispenser HURRICANE 2792, Curtis Dyna-Fog, USA). The control batch was not treated. The eggs were incubated in IUV-F-15-31 type incubators (Energomera, Russia; the temperature range from 38.1 to 36.8 °C, a 10-15 mm ventilation flaps' opening). Key categories of incubation waste, hatchability rate of eggs, hatching, live weight of 1-day-old juveniles, body temperature, and the quality as per Pasgar and Optistart scaled criteria were assessed. Blood of 1-day-old juveniles was sampled by decapitation. Blood antioxidant activity (AOA), the content of lipid peroxidation products were measured using a Beckman DU-7 spectrophotometer (Beckman Coulter, Inc., USA). Concentrations of total blood proteins, lipids, glucose were measured using an automatic biochemical analyzer DIRUI CS-600B (Dirui Industrial Co., Ltd., China). The content of lactate and pyroracemic acid was analyzed by tandem chromatography-mass spectrometry (an Agilent 6410 Triple chromatograph, Agilent Technologies Inc., USA). The ATP content was determined by bioluminescent method (a luminometer and reagents from Lumtek, Russia), pH by direct potentiometry (an E-Lyte 5 blood electrolyte analyzer, High Technology Inc., USA). In the test group, the number of the main incubation wastes (blood rings and died-in-shell birds) was 1.82 and 2.28 times less, respectively, than in the control group, while the hatching rate increased by 8.64 % ($p < 0,05$) and hatchability by 7.97 % ($p < 0.05$). Treatment with an optimal dosage of cobalt glycinate prior to incubation contributed to a decrease in free-radical reactions and lipid peroxidation. The greatest differences (20 %) occurred in the concentration of oxodiene conjugates ($p < 0.05$). The reduced LPO intensity may be due to the stimulating effect of cobalt glycinate on the antioxidant system, which resulted in an increase in AOA by 12.9 % ($p < 0.01$) compared to control. The blood concentration of ATP in quails of the test group was 1.4 times higher ($p < 0.01$) than in the control group. The ATF level, along with an increase in glucose by 8.73 % ($p < 0.01$), pyroracemic acid by 12.5 % ($p < 0.05$), pH by 0.67 % and a decrease in the lactate by 16 %, were indicative of a more efficient use of energy substrates by the birds. The likelihood of development of an uncompensated acidosis decreased in the birds of the test group. Along with this, the stimulation of energy metabolism caused a statistically significant ($p < 0.01$) increase in body temperature measured rectally and under the wing, by 0.4 and 0.3 °C, respectively (39.1 and 37.5 °C vs. 38.7 and 37.2 °C). An increase in the blood concentration of total proteins by 3.88 % ($p < 0.01$) and an increase in live weight by 8.34 % ($p < 0.05$) should be especially noted. Therefore, under industrial conditions, the pre-incubation treatment of Japanese quail eggs with 0.05 % solution of cobalt glycinate reduces the free radical level and, as a result, lipid peroxidation in 1-day-old quails. Additionally, cobalt glycinate stimulates energy metabolism, providing a faster transition of quails to aerobic glycolysis and reducing the likelihood of uncompensated acidosis. A higher concentration of ATP in 1-day-old individuals of the test group indicates both a better thermoregulatory function to ensure natural resistance and viability, and the absence of depleted energy metabolism during the previous periods of development, which determines the superiority in viability of embryos.

Keywords: hypoxia, embryogenesis, quail, antioxidant, cobalt glycinate, hatchability rate

Hypoxic changes, including those physiologically determined and associated with the peculiarities of the processes occurring in different periods of bird embryogenesis, lead to a slowdown in development, and in severe cases, to multifaceted morphofunctional disorders in embryos [1-3]. According to M.T. Tagirova and O.V. Tereshchenko [4], after the allantois closure or hatching, the transition of embryos to the use of oxygen is delayed even under standard conditions of chicken egg incubation in poultry farming, which practically does not occur during natural hatching. This is inevitably accompanied by excessive accumulation of lactate and, as a result, aggravation of acidosis with a growing risk of its uncompensated form [5]. An increase in the concentration of lactic acid due to the imperfection of its utilization systems in the embryo causes irreversible negative effects in all cells and tissues of the embryo [4]. The described processes adversely affect the viability of the embryos and lead to a significant increase in incubation waste products such as blood rings and addled egg [6, 7].

An acute state of oxygen deficiency represses biological oxidation, critically reducing the synthesis of ATP that leads to an acute hypoenergetic state. With this, the intensity of substrate phosphorylation increases many times, which hinders production of the required amount of macroergic compounds in birds [4, 8]. The formation and functionality of the adaptation mechanisms necessary to successfully overcome stressful conditions is reduced [9]. An energy-deficient state also causes insufficient thermogenesis, and, therefore, insufficient immunobiological activity and resistance [10]. Under the influence of hypoxic stress during critical periods of embryonic development, the future functionality of organs and tissues in birds decrease, adaptive capabilities and productive qualities are not fully realized, causing significant production losses and a decrease in the profitability of not only an individual poultry enterprise, but also the poultry farming as a whole.

Numerous studies have confirmed the effectiveness of various biostimulants with pronounced antioxidant properties, which can neutralize the negative effect of hypoxia and ensure a faster transition to aerobic glycolysis [11-13]. The most effective were those that had not only antioxidant activity, but also the ability to maintain the functionality of the mitochondrial respiratory chain (MRC) [14, 15].

These biostimulants include cobalt glycinate synthesized at the Moscow State Academy of Veterinary Medicine and Biotechnology — Scriabin MVA [16]. The choice of biostimulant components was based on the properties of each component separately [17-19]. Thus, glycine is able to maintain the functionality of MRC, maintaining the energy synthesis [19, 20]. Along with this, it exhibits antioxidant properties due to glutathione in its structure. A significant content of aminoacetic acid is characteristic of keratins and collagen which are necessary for the formation of bones, cartilage and skin. It is also involved in the synthesis of purine bases which are part of DNA, RNA, coenzymes NAD^+, $NADP^+$, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), macroergic compounds [19, 20]. Glycine belongs to the glycogenic amino acids and supports carbohydrate metabolism.

It should be noted that both components of the biostimulant can influence tissue trophism, primarily due to the participation in the synthesis of heme (glycine as a participant in the first reaction and cobalt as an activator of a number of enzymes in this process) [19-22]. In addition, cobalt promotes the maturation of erythrocytes in the bone marrow by increasing the number of reticulocytes in the peripheral blood [18].

The important role of cobalt in the formation of nitrogenous bases, the formation of the primary structure of RNA and DNA, the synthesis of amino acids, and the intensity of carbohydrate and lipid metabolism has been reported [19, 20, 23]. It has also been proven that organic cobalt compounds are involved in reactions that suppress the synthesis of free radicals which are formed in excess during stress [24-29].

In general, the combination of antioxidant, membrane-protective, metabolism-stimulating properties of the biostimulator synthesized by us is of undoubted value, primarily for the intensive and full development of embryos under conditions of commercial incubation of poultry eggs. Glycine and cobalt have properties that cause a positive synergistic effect in leveling the negative consequences of hypoxia of various etiologies, and can become correctors of the conditions of energy-synthetic processes [15, 30].

In the present work, for the first time, it was found that cobalt glycinate has an antihypoxic and energy-stimulating effect on quail embryos and 1-day-old quails.

This work aims to reveal the effect of cobalt glycinate on energy metabolism to be further laid behind the procedure for correction of hypoxic processes that occur in quails during embryogenesis under commercial incubation.

Materials and methods. The experiment was carried out in 2020 at OOO Shepilovskaya Poultry Farm (Moscow Province, Serpukhov, Shepilovo village) on eggs of Japanese quail (*Coturnix japonica*) obtained from birds of the same age (63 days). The conditions for keeping the parent flock for all groups were the same, the birds were fed 2 times with barley-sorghum-soy type compound feed, balanced according to the existing standards of the RAAS. Experimental and control batches consisted of 220 eggs that were selected with regard to conditions and period of storage, the timing of laying and weight. The optimal biostimulant concentration has been identified in a series of previous experiments [16, 30, 31].

Before incubation, an experimental batch of eggs was once treated with a 0.05% solution of cobalt glycinate using a HURRICANE 2792 aerosol dispenser (Curtis Dyna-Fog, USA). The control batch was not treated (dry control). The eggs were placed in IUV-F-15-31 type incubators (Energomera, Russia; temperature range from 38.1 to 36.8 °C), adjusting the opening width of the ventilation dampers within 10-15 mm depending on the day of incubation.

The main categories of incubation waste, egg hatchability, hatched quail yield, body weight of 1-day-old chicks, and body temperature were assessed. In addition, 1-day-old birds were individually assessed according to the quality criteria of the Pasgar and Optistart scales.

Whole blood and serum were obtained from 1-day-old birds by decapitation. Antioxidant activity of blood plasma (AOA), products of lipid peroxidation, that is, alkadienes with isolated double bonds (IDB), diene conjugates (DC), triene conjugates (TC), oxodiene conjugates (ODC), Schiff bases (SB) were determined by colorimetric method. The optical density was measured on a Beckman DU-7 spectrophotometer (Beckman Coulter, Inc., USA), the concentration of total protein, lipids, glucose in blood serum on an automated biochemical analyzer DIRUI CS-600B (Dirui Industrial Co., Ltd., China) using commercial biochemical kits for veterinary medicine (ZAO DIAKON-DS, Russia). Concentrations of lactate and pyruvic acid (PVA) were determined by tandem chromatography-mass spectrometry using an Agilent 6410 Triple chromatograph (Agilent Technologies Inc., USA), the ATP concentration by bioluminescent method using a luminometer and a set of reagents Lumtek (Russia), pH was measured by direct potentiometry on a blood electrolyte analyzer E-Lyte 5 (High Technology Inc., USA) [32].

Statistical processing of the experimental data was carried out in Microsoft Office Excel 2007. The mean values (*M*) and standard errors of the means (±SEM) were calculated. The statistical significance of differences was assessed by Student's *t*-test.

Results. Pre-incubation treatment of eggs with cobalt glycinate at an optimal concentration contributed to a decrease in the intensity of free-radical reactions and, as a result, in lipid peroxidation in quails from the experimental group. This creates the prerequisites for maintaining the integrity of cell structures, including mitochondria, which is necessary for biological oxidation that provides the embryos with the main pool of macroergic compounds during periods of embryogenesis not associated with hypoxia [27].

Antioxidant activity of blood plasma (AOA), products of lipid peroxidation, that is, alkadienes with isolated double bonds (IDB), diene conjugates (DC), triene conjugates (TC), oxodiene conjugates (ODC), Schiff bases (SB)

1. Lipid peroxidation and activity of antioxidant defense system in 1-day-old Japanese quails (*Coturnix japonica*) hatched after pre-incubation egg treatment with 0.05% cobalt glycinate solution (*M*±SEM, *n* = 5; OOO Shepilovskaya Poultry Farm, Moscow Province, Serpukhov, Shepilovo village, 2020)

Parameter	Group	
	control (without treatment)	experiment
AOA, %	49.60±1.43	56,00±1,00**
IDB, OD/ml	7.00±0.32	6,20±0,37
DC, OD/ml	2.30±0.03	2,16±0,04
TC, OD/ml	0.93±0.04	0,74±0,03
ODC, OD/ml	0.90±0.04	0,75±0,01*
SB, OD/ml	0.50±0.03	0,36±0,05*

Note. AOA — antioxidant activity of blood plasma, IDB — alkadienes with isolated double bonds, DC — diene conjugates, TC — triene conjugates, ODC — oxodiene conjugates, SB — Schiff bases; OD — optical density.
* and ** Differences vs. control are statistically significant at $p < 0.05$ and $p < 0.01$.

In 1-day-old quails from the experimental group, there was a decrease in lipid peroxidation vs. control (Table 1). The largest differences (20%) occurred in the concentration of ODC ($p < 0.05$). This confirms the assumption that it is possible to preserve the integrity of membrane phospholipids, including due to the prevention of the formation of hydroperoxides [33, 34]. A relatively high ODC value in the control quails indicated a more intense free radical processes [35] and modification of the membrane bilayer with an increase in the ionic permeability, which leads to lower ATP synthesis and disruption of cell functionality [33, 36-39].

In test birds, the concentration of Schiff bases decreased 1.38-fold vs. control ($p < 0.05$) that can be considered as a positive phenomenon. So, according to Yu.A. Vladimirov [33], this indicator characterizes the ability of endogenous aldehydes (fragments of the acidic components of phospholipids) to bind to the amino groups of proteins, which leads to the formation of intermolecular crosslinks that negatively affect the functionality of organelles as a whole [40]. In addition, a decrease in production of Schiff bases means that allergic reactions and autoimmune pathologies become less likely [41].

It should be noted that the reduced LPO intensity may have been associated with the stimulating effect of cobalt glycinate on the antioxidant system, which resulted in an increase in AOA by 12.9% ($p < 0.01$) compared to control. I.S. Lugovaya [42] proved that regression of excessive lipid peroxidation preserve the integrity of cell structures and the activity of enzymes, including those necessary for the formation of energy production which is generally consistent with our data.

2. Blood biochemical parameters of 1-day-old Japanese quails (*Coturnix japonica*) hatched after pre-incubation egg treatment with 0.05% cobalt glycinate solution (M±SEM, n = 5; OOO Shepilovskaya Poultry Farm, Moscow Province, Serpukhov, Shepilovo village, 2020)

Parameter	Group	
	control (without treatment)	experiment
ATP, μmol/l	2,89±0,21	4,01±0,14**
Glucose, mmol/l	9,62±0,16	10,54±0,15**
Pyruvic acid, mmol/l	0,24±0,01	0,27±0,01*
Lactate, mmol/l	1,09±0,04	0,94±0,05
pH	7,42±0,03	7,47±0,02
Total protein, г/л	27,20±0,14	28,30±0,21**
Total lipids, mmol/l	2,56±0,08	2,69±0,09

N o t e. ATP — adenosine triphosphoric acid.
* and ** Differences vs. control are statistically significant at p < 0.05 and p < 0.01.

The blood ATP concentration in the experimental quails was 1.4 times higher ($p < 0.01$) vs. control. The high ATP level, increased glucose (by 8.73%, $p < 0.01$), PVA (by 12.5%, $p < 0.05$), pH (by 0.67%) and a decreased lactate (by 16%) indicate a more efficient use of energy substrates in birds derived from eggs treated with cobalt glycinate due to a faster transition to aerobic glycolysis which is more beneficial in terms of energy supply (Table 2). In quails from the experimental group, the likelihood of developing an uncompensated form of acidosis decreased. Stimulation of energy metabolism also led to a statistically significant ($p < 0.01$) increase in temperature measured rectally and under the wing, by 0.4 and 0.3 °C, respectively (39.1 and 37.5 °C vs. 38.7 and 37.2 °C), which indicates better physiological state and natural resistance of young birds [43-45].

Note, there was an increase in the blood concentration of total protein by 3.88% ($p < 0.01$) with an increase in body weight by 8.34% ($p < 0.05$) (8.88±0.21 g in control, 9.62±0.19 g in the experimental birds, n = 5). That is, protein monomers were mainly used not for energy but for growth and development, which is necessary for economically important qualities of an individual in further ontogenesis.

Total protein, total lipids, and glucose in the control birds were close to the lower limits of the reference values [46], which indicates an overexpenditure of macroergic compounds in embryogenesis. Obviously, this was necessary to increase the efficiency of adaptation mechanisms and, at the same time, testified to the exhaustion of the body due to the impact of stressors caused by the conditions of commercial incubation.

3. Incubation biocontrol parameters (%) **1-day-old Japanese quails (*Coturnix japonica*) hatched after pre-incubation egg treatment with 0.05% cobalt glycinate solution** (M±SEM, n = 220; OOO Shepilovskaya Poultry Farm, Moscow Province, Serpukhov, Shepilovo village, 2020)

Group	Incubation waste					Egg hatchability		Hatched quails	
	1	2	3	4	5	total	to control	total	to control
Control	6,82±1,70	3,64±1,26	8,18±1,85	3,64±1,26	2,27±1,00	80,98±2,65		75,45±2,90	
Experimental	5,45±1,53	1,82±0,90	5,91±1,59	1,36±0,78	1,36±0,78	88,94±2,11*	+7,97	84,09±2,47*	+8,64

N o t e. 1 — unfertilized eggs (including falsely unfertilized), 2 — blood rings, 3 — dead embryos, 4 — addled eggs, 5 — weak embryos.
* Differences vs. control are statistically significant at p < 0.05.

According to Yu.S. Yermolova [47], the antioxidant balance and, as a result, optimized energy exchange improve embryonic viability and functional integrity of organs and tissues, which is consistent with our data (Table 3). Thus, in the experimental group, we found a significant decrease in all categories of incubation waste, especially those associated with hypoxia. In particular, the proportion of blood rings and addled eggs [15] was less than control by 1.82 and 2.28 times, respectively, with a significant increase in the number of hatched quails (by 8.64%, $p < 0.05$) and egg hatchability (by 7.97%, $p < 0.05$). Besides, there is an increase in the quality of 1-day-old young birds, the scores on the Pasgar and Optistart scales are higher by 1.0 ($p < 0.05$) and 1.3 ($p < 0.01$), respectively. The data obtained are obviously largely due to the integrity of all cell membrane structures and, therefore, their functionality, which is necessary for tissue respiration of a growing embryo [48].

Thus, in Japanese quails, the treatment of eggs with a 0.05% solution of cobalt glycinate prior to commercial incubation optimizes metabolic processes, including due to the preservation of enzyme activity by reducing lipid peroxidation. This ensures a faster transition of quails to aerobic glycolysis and a decrease in the likelihood of uncompensated acidosis incidence. As a result, individuals from the experimental group were superior to the control birds in terms of embryonic viability. Along with this, cobalt glycinate at the optimal concentration has an energy-stimulating effect. A higher concentration of ATP in 1-day-old chicks of the experimental group indicates no depletion in energy metabolism during previous development. This provides better thermoregulation which characterizes natural resistance and biological responsiveness.

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