**Running title:** In Ovo Administration in Poultry Industry

**Influence of in ovo B-group vitamin administration on postnatal growth and immunity in replacement pullets of the Highsex Brown cross**

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**Novelty statement**

1) In Ovo injection should be used as an alternative method to improve hatchability and body weight of chicks.

2) B-vitamins injected into eggs in ovo had a positive influence on the reproduction organs formation.

3) In-Ovo injection of B vitamins in eggs airbag had significant effect on the immune system organ formation.

4) The activation of natural resistance of the body of replacement pullets through an increase in bactericidal, lysozyme and phagocytic activity has been established.

5) These findings support the use of in ovo injection to improve production in poultry farming.

**Abstract**

In ovo nutrient administration is a promising strategy for poultry improvement. Although B vitamins are critical for embryonic survival, in ovo administration of specific B vitamins during the late embryonic stage has not been comprehensively evaluated. In this study, the effects of B vitamins injected into eggs of the hybrid breed Highsex Brown in ovo on the 14th day of incubation in an air bag were evaluated. Fertilized eggs (n = 810) were divided into six groups: a control group (no injection) and groups treated with B1, B2, B6, B12, and Bc. The hatchability of eggs was 3.70% higher in the B12 group, 2.96% higher in the B6 group, and 2.22% higher in the B1 and B2 groups than in the control group. The weight of day-old chicks and live weight of birds at 17 weeks in the experimental groups significantly exceeded those in the control. The formation of reproductive organs in replacement pullets differed among groups. The B1, B2, and Bc groups exhibited the most efficient immune system organ formation. The mass of the bursa of Fabricius in these groups significantly exceeded that of the control group. Treatment with B6 and Bc had the most significant effect on the thymus weight. In all experimental groups, natural resistance was improved, as evidenced by increases in bactericidal, lysozyme, and phagocytic activity. In ovo injection with B-group vitamins increased the hatchability, growth indices, enzymatic function, reproductive organ development, blood profiles, immunity, and natural resistance of chicks.

**Keywords:** body weight; embryonic development; hatchability; in ovo injection; poultry farming

**Introduction**

Extensive research has focused on improving embryonic nutrition by in ovo injections to increase the rate of hatching, productivity, immune status, and health of chickens (Tufarelli *et al.*, 2021). Nutrient utilization begins on the first day of incubation, when both the white and yolk of the egg are food sources for the developing embryo. As a closed system, the proper supply of nutrients to the developing embryo is of paramount importance. Maternal nutrition is the only source of vitamins for eggs, and a nutritional deficiency leads to the death of embryos in the middle or end of the incubation period (Goel *et al.*, 2013; Ghane *et al.*, 2021; Dolgorukova *et al*., 2021).

An insufficiency in water-soluble B vitamins, which function as coenzymes, in the diet of chickens leads to high embryonic mortality. In addition, post-hatching food shortages, which often occur in chicks delayed by 24–48 hours, indicate the need to store energy and nutrients prior to hatching in order to maintain metabolic processes and regulate body temperature (Uni and Ferket, 2004). Several strategies have been proposed to increase efficiency in the early stages of development, such as incubator feeding immediately after hatching (Momeneh and Torki, 2018) and in ovo injection feeding (Joanna *et al.*, 2017), a novel method of providing nutrients for foetal growth until hatching (Chen *et al.*, 2009). This in ovo feeding technique is used to deliver nutrients directly to the developing embryo to improve postnatal growth, immune responses, and gastrointestinal development (Ohta *et al.*, 1999; Bhanja and Mandal, 2005; Konashi *et al.*, 2000; Dolgorukova *et al.*, 2019).

To address inconsistent results of previous studies and a general lack of comprehensive studies of multiple B vitamins in a single experiment, we studied the effects of thiamine (B1), riboflavin (B2), pyridoxine (B6), cobalamin (B12), and folic acid (Bc) provided in ovo on the embryonic development of chickens as well as the growth, development, and immune status of replacement pullets of the hybrid "Highsex Brown".

**Material and Methods**

The study was conducted in accordance with the European Convention for the Protection of Animals used for Scientific Purposes (2003) and the ethical standards Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes. Blood sampling and poultry handling were practiced in accordance with the ethical guidelines of the L.K. Ernst Federal Science Center for Animal Husbandry, guidelines established at the farm, and local poultry care laws and policies. The farm owner provided consent for the use of eggs and chicks in this study.

The effects of B vitamins injected in eggs by the in ovo method on hatchability, the rearing of replacement young stock of the hybrid breed Highsex Brown, and productivity were evaluated under the breeding conditions of the II order of the Svetly Joint Venture of the JSC Agrofirma Vostok Volgograd region (Table 1). The age of hens and roosters of the parent flock was 42 weeks.

Before injecting the drug, eggs are treated with a disinfectant. Then, using a small drill, a hole was made on the blunt end of the egg, through which the drug was injected. The hole was then filled with warm wax. The manual injection of eggs during the incubation period is a labour-intensive process and is used only for research; in industrial conditions, machines are used for the vaccination of embryos (Khodorovich, 2021).

In our studies, injection was performed on the 14th day of incubation. Fertilized eggs (n = 810) were divided into six groups: control (no injection), group I (vitamin B1), group II (vitamin B2), group III (vitamin B6), group IV (vitamin B12), and group V (vitamin Bc). The eggs were disinfected with alcohol (30°C) and scanned under an ovoscope. A hole of no more than 1 mm in diameter was made. A syringe (needle 0.3 mm in diameter) was used to infect a vitamin solution through the shell membrane into the air bag. The pinhole site was sealed with sterile paraffin immediately after injection. The injected eggs were returned to the incubator (Dolgorukova and Mikheeva, 2020).

Before injection, weighed portions of vitamins were dissolved in 0.5 ml of sterile water.

The live weight of day-old chicks was determined weekly using electronic scales (Polaris PKS 0323 DL, Polaris Co., Ltd, China, and CAS SW–10W, CAS Co., Ltd., Korea). The reproductive and immune system organs of the replacement pullets were examined after slaughter, adhering to the European Convention for the Protection of Animals used for Scientific Purposes (2003) and the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes.

All organ samples were dried by wiping to remove excess moisture. The weights of the ovaries and oviducts were determined using an electronic scale (EHA-501). The length of the oviducts was measured with a ruler. Immune system organs of the replacement pullets (bursa of Fabricius, spleen, and thymus) were weighed on an electronic scale (EHA-501). The bactericidal activity in serum (Deryabin and Polyakov, 2006), lysozyme activity (Fogelson *et al.*, 1954), and phagocytic activity of leukocytes (Shirshev *et al.*, 2014) were evaluated following previously described methods.

The data were processed using Microsoft Office. The Student's *t*-test was conducted at three probability levels. P < 0.05 was considered statistically significant.

**Results and Discussion**

As summarized in Table 2, the injection of vitamin B12 (experimental group IV) during embryogenesis had the greatest effect on the development of chickens. Hatching increased by five heads compared to the control and the hatchability increased to 91.85%, compared with 88.15% in the control, consistent with the results of Teymouri *et al.* (2020). In experimental group III, in which vitamin B6 was administered in ovo, chicken hatching increased by four heads and the hatchability was 91.11%, which was 2.96% higher than that in the control, consistent with the results of Bhanja *et al.* (2007). In experimental group I (vitamin B1) and experimental group II (vitamin B2), three more chickens were obtained than the number in the control and hatchability increased in both groups by 2.22%. Similar data were obtained by Bakyaraj *et al.* (2012); however, Goel *et al.* (2013) found no increase in egg hatchability. In experimental group V (folic acid Bc), the egg incubation results were equivalent to those in the control, with a hatchability of 88.15%. A previous study (Ismail *et al.*, 2019) also reported no effect of in ovo folic acid injections on egg hatchability. In experimental groups I, II, III, and IV, an increase in the hatchability of eggs was achieved by reducing the number of dead embryos at the end of incubation and hatching.

The weights of day-old chickens in groups II (B2), III (B6), IV (B12), and V (folic acid) were slightly higher than the control values and the weight in group I (B1) was significantly higher than that in the control by 0.47 g (P < 0.05).

For further rearing, the day-old chicks of the final hybrid from the Highsex Brown cross were divided by sex. From each group, after separation, 50 heads of replacement pullets were selected and the growth and development of each treatment group were monitored until the onset of physiological maturity. Although the live weight of day-old chicks exceeded that of the control only in the first experimental group, in the process of growing replacement pullets, higher weights were observed in all experimental groups than in the control group. By the onset of physiological maturity (17 weeks), the body weights of replacement pullets were 79.7 g higher in experimental group I (5.32%; Р < 0.01), 47.4 g higher in group II (3.17%; Р < 0.05), 65.3 g higher in group III (4.36%; Р < 0.01), 72.2 g higher in group IV (4.82%; Р < 0.01), and 68.0 g higher in group V (4.54%; P < 0.01) than in the control group.

When growing replacement pullets, the formation of reproductive organs is a key determinant of further productivity. The state of the reproductive organs of replacement pullets at the age of 17 weeks is shown in Figure 1. The length of the oviduct of pullets in experimental group I (B1) was higher than that of the control by 2.27 cm (6.28%; P < 0.01), the length in group III (B6) was 2.07 cm longer (5.77%; P < 0 01), the length in group IV (B12) was 2.39 cm longer (6.66%; P < 0.01), and the length in group V (folic acid) was 2.24 cm longer (6.24%; P < 0.01). In pullets of experimental group II (B2), the length of the oviduct exceeded that of the control by 1.42 cm (3.96%; P < 0.05). The masses of the oviduct of replacement pullets in groups I, II, III, IV, and V were higher than those of the control by 12.04% (P < 0.01), 7.56% (P < 0.05), 8.57% (P < 0.05), 10.07% (P < 0.01), and 9.88% (P < 0.01), respectively.

The mass of the ovary in replacement pullets differed among experimental groups during embryonic development. Treatment with vitamin B1 (group I) and vitamin B12 (group IV) had the greatest effects on the mass of ovaries, with masses at physiological maturation of 22.69 and 22.63 g, which were higher than that in the control by 19.29% (P < 0.01) and 18.97% (P < 0.01), respectively. The ovarian weights were also 16.61% (P < 0.01) and 17.61% (P < 0.05) higher in groups III (B6) and V (folic acid) than in the control group and 14.14% higher in group II (B2) than in the control group (P < 0.05).

The intensive development of the reproductive organs of replacement pullets in the experimental groups synchronized the timing of puberty, allowing them to reach 50% of productivity by the age of 20 weeks.

The masses of the central (thymus and bursa of Fabricius) and peripheral (spleen) organs of the immune system of birds (17 weeks old) in each group are presented in Table 3.

Results of our studies have also shown that the treatment of embryos in ovo with vitamins B1 (thiamine), B2 (riboflavin), and Bc (folic acid) effectively promote the formation of immune system organs. The masses of the bursa of Fabricius in experimental groups I, II, and V were significantly higher than that in the control by 34.97% (P < 0.01), 32.34% (P < 0.01), and 28.99% (P < 0.01) and the mass spleen was higher by 22.18% (P < 0.01), 19.72% (P < 0.01), and 15.85% (P < 0.05). In groups III (V6) and IV (V12), increases in the masses of these organs were observed; however, the differences were not significant. The thymus mass was 20.57% (P < 0.05) and 16 .71% (P < 0.01) higher after vitamin B6 (pyridoxine, group III) and Bc (folic acid, group V) than in the control group. In experimental groups I (B1), II (B2), and IV (B12), the differences in thymus mass compared with that in the control group were 9.51%, 3.34%, and 13.11%.

The injection of embryos with various B vitamins contributed to the activation of natural defence factors (Fig. 2).

The bactericidal activity in the blood serum of replacement pullets in all experimental groups differed significantly from that of the control, with the greatest difference observed in groups III (B6) and V (folic acid) (i.e. 5.93 and 6.15%; both P < 0.01). Lysozyme activity levels in experimental groups I, II, III, and V were higher than that in the control group by 16.92% (P < 0.05), 15.85% (P < 0.05), 21.02% (P < 0.01), and 21.36% (P < 0.01); in experimental group the IV, the difference was negligible. The phagocytic activity of leukocytes differed substantially in response to treatment in ovo with B vitamins. In groups III (B6), IV (B12), and V (folic acid), the phagocytic activity of leukocytes relative was 10.20% (P < 0.001), 7.74% (P < 0.01), and 9.93% (P < 0.001) higher than that in the control group. In experimental groups I (B1) and II (B2), this indicator also exceeded levels in the control by 4.91% (P < 0.05) and 4.32% (P < 0.05), consistent with the results of Goel *et al.* (2013) and Ghane *et al.* (2021).

**Conclusion**

The results of this study revealed that B group vitamins administered in ovo affect the hatchability, growth, enzymatic function, reproductive organ formation, immune system activation, blood status, and natural resistance of the organism, thereby affecting egg productivity and the economics of the production of food eggs (Fig. 3).

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**Author Contributions**

Study conception and design: IFG, ZBK, MIS, VNN;

Methodology and Data curation: IFG, ZBK, MIS;

Measurements and Acquisition: AVR, EAS;

Analysis of data: AVR, AAM, DAM, EYuA;

Interpretation of data and Drafting of manuscript: IFG, ZBK, EYuA.

**Conflict of Interest**

All authors declare no conflict of interest

**Data Availability**

Data presented in this study will be available on a fair request to the corresponding author

**Ethics Approval**

The study was conducted in accordance with the European Convention for the Protection of Animals used for Scientific Purposes (2003) and the ethical standards Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes. Blood sampling and poultry handling were practiced in accordance with the ethical guidelines of the L.K. Ernst Federal Science Center for Animal Husbandry, guidelines established at the farm, and local poultry care laws and policies. The farm owner provided consent for the use of eggs and chicks in this study.

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**Table 1:** Experimental design

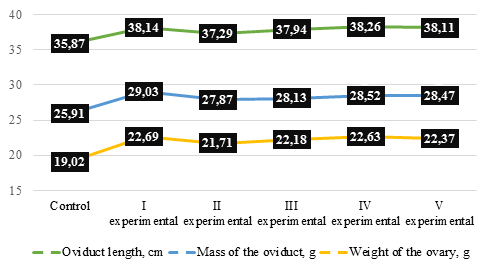
|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Vitamins in ovo, µg/egg | Control | Experimental groups | | | | |
| I | II | III | IV | V |
| Thiamine (B1) | - | 100 | - | - | - | - |
| Riboflavin (B2) | - | - | 100 | - | - | - |
| Pyridoxine (B6) | - | - | - | 50 | - | - |
| Cobalamin (B12) | - | - | - | - | 50 | - |
| Folic acid (Bc) | - | - | - | - | - | 150 |

**Table 2:** Egg incubation results

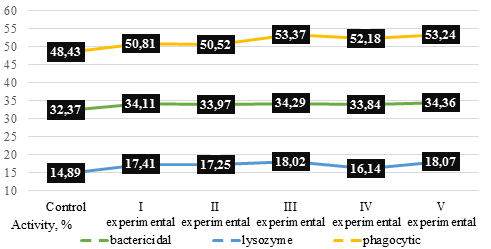
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| --- | --- | --- | --- | --- | --- | --- |
| Values | Control | Experimental groups | | | | |
| I | II | III | IV | V |
| Eggs with live embryos: | | | | | | |
| pieces | 135 | 135 | 135 | 135 | 135 | 135 |
| Waste classification in the incubation process: | | | | | | |
| "blood ring" – death of embryos 1–7 days | | | | | | |
| pieces | 3 | 2 | 3 | 2 | 2 | 3 |
| % | 2.22 | 1.49 | 1.49 | 1.49 | 1.49 | 2.22 |
| dead-in-shell – death of embryos 8–18 days | | | | | | |
| pieces | 7 | 6 | 6 | 5 | 5 | 7 |
| % | 5.19 | 4.44 | 4.44 | 3.70 | 3.70 | 5.19 |
| addled egg – death on hatching, 19–21 days | | | | | | |
| pieces | 6 | 5 | 5 | 5 | 4 | 6 |
| % | 4.44 | 3.70 | 3.70 | 3.70 | 2.96 | 4.44 |
| Young stock bred | | | | | | |
| goals | 119 | 122 | 122 | 123 | 124 | 119 |
| Hatchability of eggs | | | | | | |
| % | 88.15 | 90.37 | 90.37 | 91.11 | 91.85 | 88.15 |
| Weight of chickens | | | | | | |
| g | 36.73±0.11 | 37.21±0.13\* | 36.77±0.10 | 36.79±0.12 | 36.75±0.09 | 36.81±0.12 |
| Mean ± standard deviation.  \*P ˂ 0.05 compared with data for the control group. | | | | | | |

**Table 3:** Metric indicators of the immune system organs (n = 10)

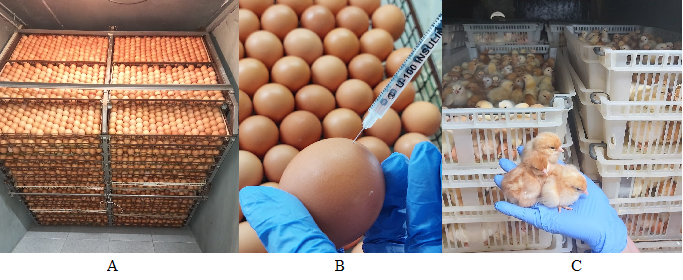
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| --- | --- | --- | --- | --- | --- | --- | --- |
| Values | Live weight of pullets, g | Weight | | | | | |
| Bursa Fabricius | | Spleen | | Thymus | |
| g | % | g | % | g | % |
| Control | 1495.9±16.14 | 2.69±0.18 | 0.18 | 2.84±0.17 | 0.19 | 3.89±0.19 | 0.26 |
| I | 1578.1±17.39\*\* | 3.63±0.21\*\* | 0.23 | 3.47±0.19\* | 0.22 | 4.26±0.21 | 0.27 |
| II | 1546.5±13.27 | 3.56±0.19\*\* | 0.23 | 3.40±0.46\* | 0.22 | 4.02±0.15 | 0.26 |
| III | 1563.7±15.32\*\* | 2.97±0.15 | 0.19 | 3.28±0.15 | 0.21 | 4.69±0.22\* | 0.30 |
| IV | 1569.9±17.99\*\* | 2.98±0.17 | 0.19 | 3.14±0.16 | 0.20 | 4.40±0.17 | 0.28 |
| V | 1565.8±11.69\* | 3.47±0.16\*\* | 0.22 | 3.29±0.13\* | 0.21 | 4.54±0.20\* | 0.29 |
| Mean ± standard deviation.  \*\*P ˂ 0.01, \*P ˂ 0.05 compared with data for the control group. | | | | | | | |



**Fig. 1:** State of the reproductive organs



**Fig. 2:** State of natural resistance against infections in the blood



**Fig. 3:** Overview of the experiment: A – incubation; B – in ovo injection; C – day-old chicks