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Effect of *in ovo* Injection of Nicotonic Acid, Pantothenic Acid or Folic Acid on Immune System and Growth of Broiler Chickens

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Abstract

BACKGROUND: *In ovo* injection of nutrients as an early feeding method in birds directly supplies the nutrients to the developing embryo.

OBJECTIVES: This study was designed to evaluate the effects of in ovo injection of nicotonic acid, pantothenic acid and folic acid on the performance and immune system of broilers.

METHODS: 450 Ross 308 fertile eggs were divided into 5 groups and placed in a hatchery machine. Five experimental groups included *in ovo* injection of 0.121 mg of nicotonic acid, 0.052 mg of pantothenic acid, 0.007 mg of folic acid on the 14th day of incubation period, positive control or injection control (physiological serum injection) and negative control (non injecting control).

RESULTS: At the age of 18 days of the rearing period, injection of pantothenic acid and nicotinic acid increased the antibody titre against Newcastle Virus and folic acid and pantothenic acid reduced the SRBC titer. At 35 days of age, nicotinic acid and folic acid had lower SRBC titer than the negative control group. The highest lymphocyte to heterophilia ratio was observed in the pantothenic acid group and the lowest levels were seen in the folic acid group. *In ovo* injection of nicotinic acid and pantothenic acid caused weight loss in chicks during the first and second weeks of rearing period compared to positive and negative controls groups.

CONCLUSIONS: The results of this study indicated a positive effect of *in ovo* injection of pantothenic acid and nichotinic acid on some immune parameters of broiler chicks. In despite of the negative effect of *in ovo* injection of nicotinic acid and pantothenic acid on growth rate of chicks during the first and second week of age, there was a compensatory growth for the nicotinic acid group such that this treatment positively influenced the final weight of the broilers.

KEYWORDS: Broiler, folic acid, *In ovo* injection, nicotonic acid, pantothenic acid

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Introduction

The nutrients needed by the chicken embryo are met by hen, so the embryo only has access to the egg nutrient resevours after laying (Jha et al, 2019). In recent decades, the use of in ovo injection has been considered for early feeding in birds (Dal Pont et al., 2019). In this process, nutrients are fed to the embryo within the egg at various stages of the embryo's development.

Vitamins are nutrients, which are essential at very low levels. Pantothenic acid has an important role in coenzyme A, acyl carrier proteins synthesis (Lanska Douglas, 2012). Pantothenic acid is also involved in general metabolism and the previous reports have confirmed its positive effect on the health and performance of birds (Wang et al., 2016).

Folic acid has a critical role in one-carbon metabolism and is involved in DNA, RNA and protein methylation and DNA synthesis and maintenance (Leung et al., 2013). Folic acid also enhances the immunity (Delaney et al., 2013; Feng et al., 2011).

Niacin is involved in electron transport for intracellular respiration and there are reports on the positive effects of niacin on growth of poultry (Jiang et al., 2014). This study was designed to evaluate the effects of *in ovo* injection of nicotonic acid, pantothenic acid or folic acid on the live weight, carcass parameters and immune system of broilers.

Materials and Methods

In this study, 450 Ross 308 fertile eggs with 65 g average weight were obtained from breeder flock at 43 weeks of age. The eggs were individually weighed and divided into five experimental groups including 4 replicates. The experimental treatments were the non-injected group, the group injected with 1 ml physiological serum and three groups injected with 1 cc of physiological saline solution containing 0.121 mg of nicotinic acid, 0.052 mg of pantothenic acid or 0.007 mg of folic acid. The selected vitamin levels were based 75% of the recommended level throughout the starter phase of the rearing period (Aviagen, 2014). To prepare injectable solutions, nicotonic acid (Merck, Swizerland, 99.9% purity), Pantothenic acid (LeSen, China, 99% purity) and folic acid (DSM, Neiderland, 80% purity), in the amount of 0.211, 0.052 and 0.007 mg, were dissolved in 50 ml physiological serum, respectively.

In ovo injection of vitamins was carried out in the air cell of eggs on the 14th day of incubation and after separation of broken eggs. Before injection, the broad end of the eggs was disinfected using 96% ethyl alcohol. Disposable Insulin syringes with . 22 gauge needle were used for injection. In the wider part of the shell, eggs were pierced with a needle and the solutions were injected into the air cell. The temperature of the hatchery was 37.8 °C and the humidity was 65%. After completion of injection, the place was disinfected with alcohol, blocked using melted paraffin, and then transferred to the incubation trays.

At the end of the incubation period, the hatching chicks after counting and weighing were transferred to the poultry house. The hatchability of eggs in different groups was comparable, between 68 and 71 percent. The chicks were grown for 42-day experimental period under the same environmental conditions and diet formulated based on dietary requirements of the Ross 308 broiler (Aviagen, 2014). The mortality rate in all the experimental groups was less than 5%. Birds were vaccinated at 9 days old by injection of the

dual Newcastle and Avian influenza vaccine (Razi Institute, Karaj, Iran) and eye drop of dual bronchitis-B1 Newcastle virus vaccine, H120-B1 (bronchitis-B1 Newcastle virus) (Razi Institute, Karaj, Iran). ´At 15 days old Gumboro Nobilis D78 vaccine (MSD, Netherlands) was administrated in drinking water. At 18 days of age, Newcastle VITAPEST L vaccine (Ceva Santé Animale, France) and at 25 days of age Newcastle Avinew (*Boehringer Ingelheim*, Germany) were used in drinking water.

At the end of the experiment, a chick was selected from each replicate and blood samples were taken from wing vein. Blood samples were placed in tubes containing 50 µl EDTA to count the lymphocytes and heterocytes. The counting of white blood cells was done by observation and counting by eye after gisma staining using an optical microscope (Olympus UK Ltd., Essex, UK) (Grass and Siegel., 1983).

To determine the antibody titer of the bird against sheep red blood cells (SRBC), sheep blood was centrifuged for 10 min at a rate of 2500 rpm and each time the upper fluid was discarded and a same amount of chloride solution 0.9% was added. The washed red blood cells were diluted with sodium chloride solution so that a solution of 5% red blood cells was obtained. Injection of 0.1 ml SRBC at 18 and 35 days of rearing period (injection in the chest muscle) was carried out (Grasman, 2010) and blood samples were collected from left wing vein at 22 and 42 days of age (Grasman, 2010). The hemagglutination inhibition (HI) test using ELISA with a Newcastle virus (NDV) mono-specific antiserum was used to detect the titer against NDV (OIE, 2012). To measure the immunoglobulin G, Mercaptoethanol was used to create sediment. Immunoglobulin M was obtained by subtracting immunoglobulin G from total immunoglobulin (Cheema et al., 2003).

At the end of the rearing period, after recording live weight, 10 birds were slaughtered from each treatment and the weights of carcass, bursa of Fabricius, Thymus, Heart, Liver, Spleen and abdominal fat were recorded. Statistical analysis was performed using SAS 9.1 software and GLM procedure. The comparison of the meanings was done using Duncan's multiple range test and the 5% probability level was considered as a significant level.

Results

Tables 2 and 3 show the effect of *in ovo* injection of Nicotonic acid, Pantothenic acid, and Folic acid vitamins on antibody titers against SRBC and Newcastle Virus (NDV), IgG and IgM in serum at 18 and 35 days of age. At 18 days of age, in ovo injection of pantothenic acid and nicotinic acid resulted in an increase in the antibody titre against Newcastle Virus (NDV) compared to the control group and the folic acid injected group ($P<0.05$), but this parameter was not affected by experimental treatments at age 35 days. Antibody titers against sheep red blood cells were also influenced by experimental treatments. At the age of 18 days, injection of folic acid and pantothenic acid reduced the titer in comparison with the negative control group ($P<0.05$), and at 35 days of age, nicotinic acid and folic acid groups had a lower titer than the negative control group ($P<0.05$).

At 18 days, the level of IgG in serum of chicks in ovo injected with vitamins was lower than the negative control group ($P<0.05$), and a similar relationship was observed for IgM in chickens of pantothenic

acid group (P<0.05). At 35 days of age, there was no significant difference in serum IgG levels in different groups, but the IgM levels in the nicotinic acid and folic acid groups were lower than the negative control group (P<0.05).

Table 1. Ingredients and nutrient composition of basal diets fed (as-fed)

Ingredients	Starter (0-10 d)	Grower (11-24 d)	Finisher (25-42 d)
Corn	48.33	50.86	54.11
Soybean meal (44% CP)	42.52	39.34	35.31
Soybean Oil	4.67	5.71	6.64
Dicalcium phosphate	1.87	1.72	1.65
Calcium Carbonate	1.1	1	0.93
Mineral premix[2]	0.25	0.25	0.25
Vitamin premix[1]	0.25	0.25	0.25
DL-Methionine	0.37	0.31	0.3
L-Lysine	0.23	0.15	0.15
Salt	0.36	0.36	0.36
Salinomycin	0.05	0.05	0.05
Calculated analysis			
AMEn (Kcal/kg)	3000	3100	3200
CP	23	21.5	20
Calcium	0.98	0.9	0.85
Available P	0.49	0.45	0.43
Sodium	0.16	0.16	0.16
Lys	1.44	1.3	1.2
Met	0.71	0.6	0.61
Met+Cys	10.8	0.99	0.94

[1] Provided the following (per kg of diet): vitamin A 8,800 IU; vitamin D3 2,500 IU; vitamin E 11 IU; vitamin K3 2.2 mg; thiamine 1.5 mg; riboflavin 4 mg; pyridoxine 2.5 mg; pantothenic acid 8 mg; nicotinic acid 35 mg; folate 0.48 mg; cyanocobalamin, 0.01 mg; choline chloride 200 mg.

[2] Provided the following (per kg of diet): Mn (MnSO4, H2O) 75 mg; Zn (ZnO) 64 mg; Fe (FeSO4, H2O) 75 mg; Cu (CuSO4, 5H2O) 6 mg; I (KI) 0.86 mg; and Se (Na2SeO3) 0.2 mg.

Table 2. Effect of *in ovo* injection of Nicotonic acid, Pantothenic acid, and Folic acid on serum antibody titers and at 18 days of age (log2)

	(Newcastle disease Virus (NDV	IgM[1]	IgG[1]	SRBC
Negative Control	4.04[b]	1.64[a]	2.60[a]	4.24[a]
Positive Control	5.27[a]	1.26[ab]	1.55[b]	2.82[b]
Nicotinic acid	5.16[a]	1.66[a]	1.66[b]	3.33[ab]
Pantotenic acid	4.92[a]	1.07[b]	1.46[b]	2.53[b]
Folic acid	4.00[b]	1.28[ab]	1.57[b]	2.85[b]
SEM	0.27	0.13	0.28	0.34
P Value	0.01	0.05	0.03	0.04

[a-b]Means in the same column without a common superscript differ significantly (P <0.05).
[1] The titers are for SRBC injected birds.

Table 3. Effect of *in ovo* injection of Nicotonic acid, Pantothenic acid, and Folic acid on serum's antibody titers and at 35 days of age (log2)

	Newcastle disease Virus (NDV)	IgM[1]	IgG[1]	SRBC
Negative Control	5.50	1.83[a]	1.66	3.50[a]
Positive Control	treatment was removed from Due to the deterioration of most positive blood samples, this the comparisons			
Nicotinic acid	4.50	1.10[c]	1.50	2.60[b]
Pantotenic acid	4.94	1.58[ab]	1.70	3.29[ab]
Folic acid	5.05	1.31[bc]	1.47	2.79[b]
SEM	0.32	0.14	0.22	0.28
P Value	0.33	0.018	0.79	0.05

[a-c]Means in the same column without a common superscript differ significantly (P <0.05)
[1] The titers are for SRBC injected birds.

Table 4 shows the effect of in ovo injection of vitamins on white blood cells. The highest heterphils to lymphocytes (H/L) ratio was observed in the pantothenic acid group and the lowest levels were seen in the folic acid group (P<0.05). The highest levels of lymphocyte and heterophile were recorded in folic acid and nicotinic acid groups, respectively (P<0.05). The total number of white blood cells counted in the folic acid group was higher than the negative control group (P<0.05).

The only carcass and organs parameter affected by experimental treatments was the relative weight of the liver that increased in the nicotinic acid group compared with the control groups (P <0.05) (Table 5). *In ovo* injection of nicotinic acid and pantothenic acid

caused weight loss in chicks during the first and second weeks of rearing period compared to the positive and negative control groups (P<0.05) (Fig. 1). However, the body weight at the 6th week of rearing period in the nicotinic acid injected group was significantly higher than other groups (P<0.05) except for the negative control group (Fig. 1).

Table 4. Effect of *in ovo* injection of Nicotonic acid, Pantothenic acid, and Folic acid on white blood cells

	H/L	Monocytes	Lymphocytes	Heterophiles	White blood cells
Negative Control	0.83^{b}	2.82	54.04^{c}	43.13^{b}	28179^{b}
Positive Control	0.74^{bc}	2.71	56.12^{bc}	41.20^{b}	29153^{ab}
Nicotinic acid	0.63^{bc}	2.66	60.16^{ab}	37.16^{bc}	30079^{ab}
Pantotenic acid	1.11^{a}	2.75	47.85^{d}	49.40^{a}	28995^{ab}
Folic acid	0.53^{c}	2.70	63.64^{a}	33.64^{c}	32286^{a}
SEM	0.07	0.29	1.94	2.00	1109
P Value	0.0001	0.99	0.0001	0.0001	0.05

$^{a-d}$Means in the same column without a common superscript differ significantly (P <0.05)

Table 5. Effect of in ovo injection of Nicotonic acid, Pantothenic acid, and Folic acid on the relative weights of organs of broilers

	Abdominal fat	Carcass	Spleen	Liver	*bourse of Fabricius*	Heart	Thymus
Negative Control	1.75	65.37	0.13	1.86^{b}	0.17	0.46	0.47
Positive Control	1.84	60.82	0.14	1.91^{b}	0.25	0.44	0.52
Nicotinic acid	2.07	61.76	0.10	2.24^{a}	0.16	0.50	0.61
Pantotenic acid	1.95	64.61	0.12	2.14^{ab}	0.17	0.46	0.53
Folic acid	1.86	64.71	0.12	2.14^{ab}	0.17	0.48	0.56
SEM	0.001	0.80	0.57	0.31	0.63	0.25	0.12
P Value	0.14	3.20	0.20	0.01	0.06	0.20	0.40

$^{a-b}$Means in the same column without a common superscript differ significantly (P <0.05)

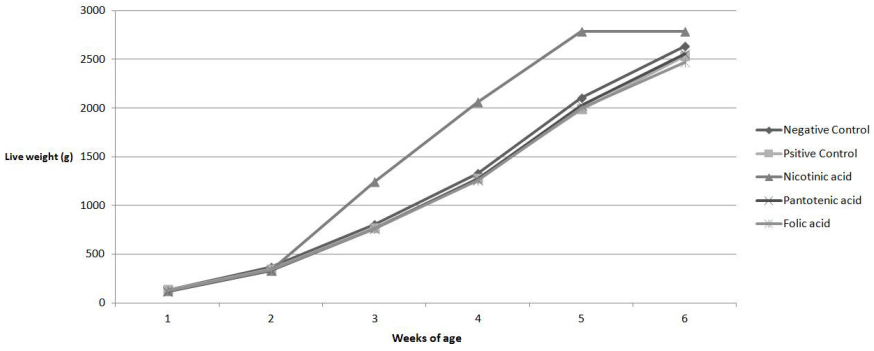

Figure 1. Effect of *in ovo* injection of water-soluble vitamins on the live weight of broilers chickens.

Discuusion

The Heterophil-to-lymphocyte ratio (H/L) is known as an index to stress (Stefaniak et al., 2019). Stressors, such as fasting or water deprivation and environmental extremes increase the H/L ratio (McFarlane et al., 1989). Then the lower H/L ratio in birds from eggs *in ovo* injected with folic acid could be a positive indicator of bird's health. There are not many reports on the effects of *in ovo* injection of B-complex vitamins on the immune system of poultry. One of the few reports is available from Li et al. (2016) who showed that *in ovo* injection of folate injection improved immunity by increasing plasma lysozyme activity and plasma IgG and IgM concentrations, and changes in the expression of immune-linked genes. These researchers reported the best effects with*in ovo* injection of 150 ng folic acid. In the present study, the reduction of blood immunoglobulin levels was observed by vitamins injection, which seems inconsistent with the previous report and further studies need to clearify the mechanism of the observerd effects.

In this study, the relative weight of the liver that increased in the nicotinic acid group was compared with the control groups. Liver is the main source of lipid synthesis in birds and changes in the liver weight could be due to increased fat content. Folic acid is a cofactor of coenzyme A that plays a vital role in lipid metabolism and fatty acid synthesis (Feng et al., 2011). The liver, as the main source of metabolism in the body, plays an important role in the production of IGF-2 in the bloodstream (Liu et al., 2016). There are also reports that there is a positive relationship between liver weight and IGF-2 gene expression in ducks (Jianmin et al., 2014).

The finding of this study showed a negative effect of in ovo injection of nicotinic acid and pantothenic acid on weight gain in chicks during the first and second weeks of rearing period compared to the positive and negative control groups. However, the body weight at the 6th week of rearing period in the nicotinic acid injected group was increased. In a study by Wang et al. (2016), consumption of about 26 mg of pantothenic acid improved the weight gain of geese. Bootwalla and Harms (1991) indicated that the level of 4.8 mg of pantothenic acid in the diet was the best level to increase the weight of broiler chicks. Qi et al. (1998) reported that dietary supplementation with pantothenic acid increased the metabolism of calcium and phosphorus, which results in higher body weight gain. The researchers suggested 10 mg / kg pantothenic acid for best performance. Positive effects of *in ovo* injection of folic acid on the weight gain of chicks have also been reported.

Liu et al. (2016) reported that *in ovo* injection of 150 µg folic acid increased the weight of the chicks compared to the control group. In that study, IGF-2 expression was increased. The correlation between plasma IGF-2 levels and chick embryo weight has already been reported (Lu et al., 2007). Folic acid is a potent antioxidant agent (Letelier et al., 2010) and plays a major role in the immune system (Duthie et al., 2010) and this can help the bird to cope with oxidative stress. In the study of Jiang et al. (2014), the body weight of broiler chickens fed 60 mg/kg nicotinic acid was improved, an observation in line with the higher final body weight of chickens in the nicotinic acid *in ovo* injected group in the present study. In the present study, the *in ovo* injection of folic acid had the highest effect on the weight of the chicks.

Although the broiler breeder diets contain enough vitamin supplements, it does not seem that the level of vitamins in the diet is a limiting factor for the development of the fetus. However,*in ovo* injection of nutrients provides nutrients and vitamins directly to the growing fetus, which can affect the efficiency of nutrient uptake for developing fetus (Jha et al., 2019). The results of this study indicated a positive effect of *in ovo* injection of pantothenic acid and nicotinic acid on some immune parameters of broiler chicks, and in particular, *in ovo* injection of nicotinic acid positively influenced the final weight of the broilers. Actually the final body weight is a more important economical trait than any effect on body weight loss in earlier steps of growth and could suggest a compensatory growth.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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اثر تزریق درون تخم مرغی اسید نیکوتینیک، اسید پانتوتنیک یا اسید فولیک بر سیستم ایمنی و رشد جوجه‌های گوشتی

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چکیده

زمینه مطالعه: تزریق درون تخم مرغی مواد مغذی به عنوان یک روش تغذیه زودهنگام در پرندگان، مواد مغذی را به طور مستقیم در اختیار جنین در حال تکامل قرار می‌دهد.

هدف: مطالعه حاضر به منظور بررسی اثرات تزریق درون تخم مرغی اسیدنیکوتینیک، اسید پانتوتنیک و اسید فولیک بر بازدهی و سیستم ایمنی جوجه‌های گوشتی طراحی گردید.

روش کار: ۴۵۰ قطعه تخم مرغ نطفه‌دار به ۵ گروه تقسیم شده و در ماشین جوجه کشی قرار داده شدند. پنج گروه آزمایشی عبارت بودند از تزریق درون تخم مرغی ۰/۱۲۱ میلی‌گرم اسید نیکوتینیک، ۰/۰۵۲ میلی‌گرم اسید پانتوتنیک، ۰/۰۰۷ میلی‌گرم اسید فولیک در روز چهاردهم جوجه‌کشی، گروه کنترل مثبت یا کنترل تزریق (تزریق سرم فیزیولوژیک) و گروه کنترل منفی (کنترل بدون تزریق).

نتایج: در سن ۱۸ روزگی از دوره پرورش، تزریق اسید پانتوتنیک و اسید نیکوتینیک تیتر آنتی بادی علیه ویروس نیوکاسل را افزایش داد و اسید فولیک و اسید پانتوتنیک تیتر SRBC را کاهش دادند. در سن ۳۵ روزگی، اسید نیکوتینیک و اسید فولیک تیتر SRBC پایین تری در مقایسه با گروه کنترل منفی داشتند. بالاترین نسبت لنفوسیت به هتروفیل در گروه اسید پانتوتنیک مشاهده شد و پایین‌ترین سطوح در گروه اسید فولیک مشاهده گردید. تزریق درون تخم مرغی اسید نیکوتینیک و اسید پانتوتنیک باعث کاهش وزن در جوجه‌ها طی اولین و دومین هفته پرورش در مقایسه با گروههای کنترل مثبت و کنترل منفی شد.

نتیجه گیری نهایی: نتایج این مطالعه نشان دهنده اثرات مثبت تزریق درون تخم مرغی اسید پانتوتنیک و اسید نیکوتینیک بر پارامترهای ایمنی جوجه‌های گوشتی بود و به ویژه، تزریق درون تخم مرغی اسید نیکوتینیک اثر مثبتی بر وزن نهایی جوجه‌های گوشتی داشت. علیرغم اثر منفی تزریق درون تخم مرغی اسید نیکوتینیک و اسید پانتوتنیک بر نرخ رشد جوجه‌ها طی هفته‌های اول و دوم زندگی، یک رشد جبرانی برای گروه اسید نیکوتینیک مشاهده گردید به طوریکه در این تیمار وزن نهایی بدن جوجه‌های گوشتی به طور مثبتی تحت تاثیر قرار گرفت.

واژه‌های کلیدی:

تزریق درون تخم مرغی، اسید نیکوتینیک، اسید پانتوتنیک، اسید فولیک، جوجه‌های گوشتی

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