Poultry Science Journal
ISSN: 2345-6604 (Print), 2345-6566 (Online) http://psj.gau.ac.ir
DOI: 10.22069/psj.2019.15929.1382

The Effects of *In ovo* Injection of Ascorbic Acid on Hatchability, Growth Performance, Intestinal Morphology, and Tibia Breaking Strength in 36h Post Hatch Fasted Broiler Chickens

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Poultry Science Journal 2019, 7(1): 43-49

Keywords
Ascorbic acid
In ovo Injection
Intestinal morphology
Tibia breaking strength

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Article history
Received: December 17, 2018
Revised: April 13, 2019
Accepted: April 15, 2019

Abstract

An experiment was conducted to evaluate the efficacy of intra-amnion administration of different doses of ascorbic acid (AA) on hatchability, growth performance, blood metabolites, jejunal morphology, and tibia breaking strength in 36h post hatch fasted broiler chickens. Two hundred eighty-eight Ross-308 fertile eggs at the 15th day of incubation were divided into four treatment groups, each containing four replicates of 18 eggs. The treatments included non-injected (control), injected into the amnion with 0.7 mL diluent (distilled water; sham treatment), and injected with 0.7 mL diluent containing 3 or 6 mg AA. The neonatal chicks were deprived of feed and water for 36 h. Hatched chicks were raised till 10 d of age. Hatch percentage was increased due to *in ovo* injection of 6 mg/egg AA. *In ovo* injection of different levels of AA had no significant effect on body weight gain, feed intake, and feed conversion ratio. *In ovo in*jection of AA, especially at the 6 mg level, significantly increased villus height, villus width, and villus height: crypt depth ratio at 3 d of age. Tibia resistance and breaking strength were improved by *in ovo* injection of 6 mg AA when compared to the control at 10 d of age. In conclusion, it seems *in ovo* injection of 6 mg AA/egg on 15th d of incubation could have a positive impact on hatchability, intestinal morphology, and bone characteristics in broiler chickens.

Introduction

In ovo feeding may help late-term embryos and newly hatchlings to overcome the limitation of egg nutrients (Foye *et al.*, 2006). Effects of *in ovo* injection of various biologically important compounds (e.g. amino acids, carbohydrates, vitamins, inulin, mannan oligosaccharide, daidzein, minerals and hormones) on hatchability and growth and development of chicks after hatching have been studied (Tako *et al.*, 2004; Keralapurath *et al.*, 2010; Cheled-Shoval *et al.*, 2011; Zhai *et al.*, 2011; Chamani *et al.*, 2012; Tako and Glahn, 2012; Eslami *et al.*, 2014; Amiri *et al.*, 2015; Hartono *et al.*, 2015; Yair *et al.*, 2015). Ascorbic acid (AA) does not exist in eggs and its production in the developing embryos may not be sufficient in particular toward the end of the incubation period when the embryo is most exposed to overheating (Nowaczewski *et al.*, 2012). So, AA synthesis in the neonatal chicks is apparently limited. Also, pathological and environmental stressors are known to alter AA synthesis in birds. AA plays an important role in the biosynthesis of corticosterone, a glucocorticoid hormone involved in gluconeogenesis to increase energy supply during stress (Frandson, 1986). Sophisticated results have been reported on the effect of *in ovo* injection of AA on hatchability (Ipek *et al.*, 2003; Nowaczewski *et al.*, 2012; Selim *et al.*, 2012; Hajati *et al.*, 2014) and growth performance of the hatchlings (Zakaria, 2001; Ghonim *et al.*, 2009; Selim *et al.*, 2012; Hajati *et al.*, 2014).

Delay in food and/or water access after hatch due to hatchery treatments such as vaccination, sexing, and transportation and also farm fasting may result in

Please cite this article as Soltani T, Salarmoini M, Afsharmanesh M & Tasharrofi S. 2019. The Effects of *In ovo* Injection of Ascorbic Acid on Hatchability, Growth Performance, Intestinal Morphology, and Tibia Breaking Strength in 36h Post Hatch Fasted Broiler Chickens. Poult. Sci. J. 7(1): 43-49.

additional stress in newly hatched birds (Karadas *et al.*, 2011). There are very few reports in the literature related to the effect of *in ovo* injection of AA on hatch percentage, intestinal morphology, bone breaking strength and meat quality warrant further research into this area. Thus, the objective of the present study was to investigate the effects of *in ovo* injection of AA at 15th d of incubation and 36 h post-hatch fasting on the above-mentioned traits.

Material and Methods

Two hundred eighty-eight Ross-308 fertile eggs were obtained from a commercial broiler breeder flock (Mahan Company, Kerman, Iran) at 49 weeks of age. The eggs were selected according to quality and weight (57 ± 2 gr) as specified by Zhai *et al*. (2011). In this experiment four treatment groups, each containing four replicates of 18 eggs was used. The treatments included non-injected (control), injected with 0.7 mL diluent (distilled water; sham), and injected with 0.7 mL diluent containing 3 or 6 mg AA. The AA (L- ascorbic acid, 99%) was purchased from Sigma-Aldrich Company.

Immediately after hatching and drying, 12 chicks (6 male and 6 female) from each replicate were transferred to the floor pens (1×1.2 meters). Chicks are commonly fasted for the first 36 to 72 h post-hatch because of the logistics of commercial production (Decuypere *et al.*, 2001). Therefore, for simulation of the commercial conditions, the neonatal chicks were deprived of feed and water for 36 h. Afterward, all birds had free access to feed and water. The ingredients and composition of the starter basal diet (1 to 10 days of age) are shown in Table 1. Diets were fed in mash form and formulated to meet the nutrient requirements of Ross-308 broiler chickens. The initial 32 (±2) ºC room temperature was gradually reduced to 24 (±2) ºC by the third week and remained constant until the end of the experiment. The room's relative humidity was set at 50±5%. During the study, the birds received a lighting regimen of 23L:1D.

Table 1. Ingredient and nutrient composition of the starter diet

Ingredients (%)	Starter (1-10 d)
Corn	53.42
Soybean meal	37.80
soybean oil	4.00
Calcium carbonate	1.30
Dicalcium phosphate	1.89
DL- Methionine	0.39
L- Lysine HCl	0.33
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
Salt	0.37
Chemical composition	
ME (Kcal/kg)	2985
Crude protein (%)	21.70
Lysine (%)	1.40
Methionine (%)	0.71
Methionine+ Cystine (%)	1.06
Calcium (%)	1.04
Available Phosphorus (%)	0.50
Sodium (%)	0.16

[1]Vitamin premix provided the following per kilogram of diet: vitamin A, 9000 IU; vitamin E, 36 IU; cholecalciferol, 2000 IU; vitamin K3, 2 mg; thiamine, 1.8 mg; riboflavin, 6.6 mg; pantothenic acid, 10 mg; niacin, 30 mg; choline chloride, 250 mg; biotin, 0.1 mg; folic acid, 1 mg; pyridoxine 3.0 mg; vitamin B12, 0.015 mg; BHT, 1 mg.
[2]Trace mineral premix provided the following in milligrams per kilogram of diet: iron, 50 mg; zinc, 85 mg; manganese, 100 mg; iodine, 1 mg; copper, 10 mg; selenium, 0.2 mg.

***In ovo* injection procedure**

At 15th d of incubation, the injection site (broad end of the egg) was disinfected with alcohol and then 0.7 mL of each solution was injected into the amnion, using a 23-gauge needle with the depth of 25 mm. The holes were then sealed using commercial glue. All eggs, including those belonging to the non-injected control group remained outside the setter for 20 minutes during the injection procedure. An incubator with automatic temperature and relative humidity control was used. All eggs were kept at 37.5ºC and 60% relative humidity until they were transferred to the hatcher (19th d of incubation), after which 70% relative humidity was applied and egg rotation was stopped. On the 19th d of incubation, the eggs were transferred to the hatcher.

Hatchability and growth performance

The hatching percentage and birth weight were recorded. Hatchability is the percentage of fertile

eggs that hatch. Feed intake (FI) and body weight gain (BWG) of broilers were recorded at 10 days of age and feed conversion ratio **(**FCR) was calculated.

Blood metabolites

Blood samples were taken randomly from two male chicks per pen at 3 and 10 d after hatch. The serum was separated and then centrifuged at 900 × g for 10 min. Serum glucose and cholesterol levels were measured using Pars-Azmoon kits and an autoanalyzer (Roche Cobas-Mira analyzer).

Intestinal morphology

Two male chicks per pen were sacrificed by cervical dislocation at 3 and 10 d post hatch, then 2 cm tissue sample from the middle of jejunum were cut off, washed in physiological saline solution, and fixed in 10% buffered formalin (100 mL of 40% formaldehyde, 4 g phosphate, 6.5 g dibasic sodium phosphate and 900 mL of distilled water) for 24 h. Tissues were dehydrated by transferring through a series of alcohols with increasing concentrations, placed into xylol and embedded in paraffin. A microtome was used to make 5 cuts that were 5 µm. The cuts were stained with hematoxylin-eosin. Measurements for villi length and width were taken from the tip of the villus to the valley between individual villi, and measurements for crypt depth were taken from the valley between individual villi to the basolateral membrane. Ten measurements per slide was made for each parameter and averaged into one value (Thompson and Applegate, 2006). In the morphometric study, images were captured using a light microscope (Leica) and a system that analyzed computerized images (Leica Queen 550).

Bone characteristics

After slaughter at d 10, right and left tibias were taken from 2 birds per pen; soft tissues were removed and stored at −20°C. Right tibia of each bird was collected to determine bone resistance and breaking strength using an Instron Materials Tester (model 5500, Instron Corp, Canton, MA). Left tibia of each bird was collected to determine the percentage of tibia ash on a fat-free dry weight basis, according to AOAC (2005; method 932.16).

Statistical analysis

Data were statistically analyzed using the GLM procedure of SAS software (SAS Institute, 2005). The statistical model was completely randomized design. Differences among treatments were determined by Duncan's multiple range tests. The level of significance was reported at $P < 0.05$.

Results

The hatch percentage of the fertile eggs was increased ($P < 0.01$) by *in ovo* injection of 6 mg AA/egg (Table 2). Although, *in ovo* injection of either level of AA caused higher ($P > 0.05$) hatching weight, but the effect was not statistically significant (Table 2). BWG, FI, and FCR of chickens are presented in Table 2. According to the results, *in ovo* injection of AA had no significant effect ($P > 0.05$) on these traits during 1 to 10 d of age.

The amount of glucose and cholesterol in blood serum at 3 and 10 d post hatch are shown in Table 3. *In ovo* injection of 6 mg AA resulted in significantly higher ($P < 0.01$) glucose (just at 10 d post-hatch) and lower ($P < 0.01$) cholesterol (at either age) levels when compared to the control and sham groups.

Table 2. Effects of *in ovo* injection of ascorbic acid on hatchability, hatchlings weight, and also body weight gain, feed intake, and feed conversion ratio of the broiler chicks during 1-10 d of age

	control	sham	3 mg ascorbic acid	6 mg ascorbic acid	*SEM*	*P*-value
Hatchability (%)	70.83^{b}	69.44^{b}	69.44^{b}	83.33^{a}	1.74	0.0002
Hatchlings weight (g)	46.56	46.25	47.60	47.91	0.60	0.103
BWG (g/bird/day)	12.23	11.84	11.17	12.07	0.25	0.063
FI (g/bird/day)	20.99	20.16	19.97	20.28	0.35	0.233
FCR	1.71	1.70	1.78	1.68	0.03	0.088

SEM= standard error of the mean.

Means with no common superscript within each row are significantly ($P < 0.05$) different.

Table 3. Effects of *in ovo* injection of ascorbic acid on blood metabolites of chicks at 3 and 10 d post-hatch (mg/dL)

Age		control	sham	3 mg ascorbic acid	6 mg ascorbic acid	*SEM*	*P*-value
	3 d post-hatch						
Glucose		205.50^{ab}	184.25^{b}	189.25^{b}	219.25^{a}	6.72	0.010
Cholesterol		508.50^{ab}	579.50^{a}	411.25^{bc}	373.25^{c}	27.44	0.0004
	10 d post-hatch						
Glucose		275.50^{b}	318.25^{b}	283.75^{b}	390.50^{a}	13.06	0.0002
Cholesterol		183.25^{a}	174.00^{a}	144.50^{b}	155.00^{b}	4.15	0.0001

Means with no common superscript within each row are significantly ($p < 0.05$) different.

SEM= standard error of the mean.

In ovo injection of AA, especially at the 6 mg level, significantly increased villus height ($P < 0.01$), villus width ($P < 0.05$), and villus height: crypt depth ratio ($P < 0.01$) at 3 d of age (Table 4). The same results were observed at 10 d of age, except villus width, which did not differ ($P > 0.05$) between the treatments.

Table 4. Effects of *in ovo* injection of ascorbic acid on jejunal morphology at 3 and 10 d post-hatch (μm)

Treatments	villus width		villus height		crypt depth		villus height : crypt depth	
	3 d	10 d	3 d	10 d	3 d	10 d	3 d	10 d
control	47.5^{b}	116.0	352.5^{c}	991.5^{c}	43.5^{ab}	107.5^{ab}	8.1^{b}	9.22^{b}
sham	49.5^{ab}	118.0	351.0^{c}	988.4^{c}	41.0^{b}	105.0^{b}	8.6^{ab}	9.41^{b}
3 mg ascorbic acid	53.0^{a}	122.5	417.0^{b}	1022.5^{b}	44.0^{ab}	104.5^{b}	9.4^{a}	9.78^{a}
6 mg ascorbic acid	54.0^{a}	118.5	463.0^{a}	1080.5^{a}	48.5^{a}	109.4^{a}	9.5^{a}	9.87^{a}
SEM	1.30	2.60	6.89	2.21	1.64	1.50	0.27	0.98
P-value	0.014	0.39	0.0001	0.0001	0.046	0.019	0.0078	0.001

Means with no common superscript within each column are significantly ($P < 0.05$) different.
SEM= standard error of the mean.

Effects of *in ovo* feeding of AA on tibia ash, resistance and breaking strength at 10 d post-hatch are shown in Table 5. These traits were improved by *in ovo* injection of 6 mg AA ($P < 0.05$) compared to the control at 10 d of age. No differences were detected for bone ash.

Table 5. Effects of *in ovo* injection of ascorbic acid on tibia characteristics at 10 d post- hatch

Treatments	Resistance (Newton)	breaking strength (Newton)	Ash (%)
control	44.75^{b}	34.00^{bc}	35.96
sham	52.50^{ab}	42.25^{ab}	34.32
3 mg ascorbic acid	35.50^{b}	30.50^{c}	31.77
6 mg ascorbic acid	64.25^{a}	51.00^{a}	34.35
SEM	4.06	3.02	1.70
P-value	0.0021	0.001	0.413

Means with no common superscript within each column are significantly ($P < 0.05$) different.
SEM= standard error of the mean.

Discussion

The results of the present study suggested that *in ovo* injection of 6 mg AA could decrease hatching stress and improve hatchability percentage. The results are in agreement with some previous studies (Zakaria and Al-Anezi, 1996; Ipek *et al.*, 2003; Nowaczewski *et al.*, 2012; Hajati *et al.*, 2014). Also, Ghonim *et al.* (2009) indicated that embryonic mortality and hatchability percentages were significantly improved by AA dipping and spraying methods in ducklings. In contrast to our finding, Bhanja *et al*. (2007) and Selim *et al*. (2012) reported that *in ovo* injection of AA had no effect on hatchability. Improved hatchability could be attributed to the fact that AA is an antioxidant that could reduce synthesis and secretion of corticosteroids and consequently alleviate stress in the last phase of incubation. Newly laid eggs do not have AA, but it will appear by endogenous biosynthesis of 4-day-old embryos which is not an adequate amount of AA in artificial incubation (Nowaczewski *et al.*, 2012).

In ovo injection of AA had no significant effect on BWG, FI, and FCR during the starter phase. The BWG results are in agreement with the findings of Ghonim *et al*. (2009). But, Zakaria (2001), Selim *et al*. (2012), and Hajati *et al*. (2014) reported that *in ovo* injection AA resulted in greater BW. The results for FCR are in agreement with those of Ghonim *et al.* (2009) and Hajati *et al.* (2014), but Selim *et al.* (2012) reported better FCR due to *in ovo* injection of AA. The results on FI are in agreement with the findings of Ghonim *et al.* (2009) and are not in agreement with those reported by Hajati *et al*. (2014), and Selim *et al*. (2012), who reported higher FI due to *in ovo* injection of AA. Improvement in growth performance due to *in ovo* supplementation of AA should be explained by its antioxidant role and its interference with the synthesis of anti-stress hormones of the adrenal glands. Biosynthesis of AA is limited in very young birds and several stressors (for instance: hatch process, transport, and fasting) may alter the biosynthesis or use of AA or both (Pardue and Thaxton, 1986). Anyway, the results on broiler growth performance after hatch are very contradictory.

According to the results, *in ovo* injection of AA resulted in significantly higher glucose and lower cholesterol levels in serum. Our findings are in agreement with Hajati *et al*. (2015), who reported that dietary supplementation of vitamin C leads to higher serum glucose. Also, some other studies reported that cholesterol level in serum (Dorr and Nockels, 1971; EL-gendi *et al.*, 1999) and egg yolk (El-Gendi *et al.*, 1999) could be decreased by the dietary addition of

AA. *In ovo* injection of AA in our study probably reduced synthesis and secretion of corticosteroids (Frandson, 1986) and consequently decreased muscle wasting because of corticosteroids catabolic effects on protein metabolism (McKay and Cidlowski, 2003) and lessened the need for yolk as a protein source which was followed by depression of serum cholesterol derived from yolk use.

In ovo injection of AA significantly improved jejunal morphometric parameters at 3 d of age. no report is available on the effect of *in ovo* injection of AA on intestinal morphology, but similar results reported for *in ovo* injection of different nutrients such as: dextrose (Amiri *et al.,* 2015); 25-hydroxycholecalciferol (Chou *et al.,* 2009), and carbohydrates (Mousavi *et al.,* 2009). It is well known that fasting decreases villus maturation and epithelial layer improvement (Geyra *et al.,* 2001) but, *in ovo* feeding of different nutrients (including carbohydrates, proteins, minerals, and vitamins) improves secretion of intestinal mucus and speeds up maturation of villus epithelial (Bohorquez *et al.,* 2011).

Tibia resistance and breaking strength were improved by *in ovo* injection of AA. There appear to be no reports on the effect of *in ovo* injection of AA on tibia ash, resistance and breaking strength. Similarly, Weiser *et al.* (1990) reported that dietary AA supplementation positively affected bone properties in broilers. Newman and Leeson (1997) revealed that dietary AA can affect bone breaking strength in laying hens. In contrast, Keshavarz (1996) showed that dietary AA addition did not affect the mineralization of poultry bones. The main role of AA in bone metabolism is in the formation of osteoid (Bhattacharya, 2010) and it is necessary for bone development as a cofactor for the conversion of vitamin D_3 to its active form of 1, 25 $(OH)_2$ D_3 (McDowell, 2000; Kutlu, 2001) and also required for the hydroxylation of proline which is necessary for the synthesis of procollagen (Leeson and Summers, 2001).

In conclusion, *in ovo* injection (into the amnion) of 6 mg AA/egg on 15th d of incubation, improved hatchability, jejunal morphology, and tibia resistance and breaking strength and had no adverse effect on growth performance of the birds. This may indicate an insufficient endogenous synthesis of AA by chick embryo. The discrepancy of our findings in some parts with the findings of other researchers could be due to the differences in methodology of *in ovo* injection (for example, age of embryo, injection site inside the egg, and injection volume). Therefore, further investigations are needed to highlight the effect of *in ovo* injection of AA on embryo growth and post-hatch growth performance in broiler chicks.

Acknowledgments
The authors are grateful to the office of the vice president in research at Shahid Bahonar University of Kerman, Iran, for providing the facilities and financial support of the experiment.

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