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The use of ascorbic acid *in ovo* feeding for poultry embryos and its effect on egg hatchability, embryo mortality, chicks' weight, and gastrointestinal tract development

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Abstract – Ascorbic acid, also known as vitamin C, is essential for the development of eggs and chicks due to its various important roles in biological processes such as collagen synthesis, antioxidant activity, iron absorption, immune system support, neurological development and enzyme cofactor. The injection of nutrients in ovo is a valuable technique used in poultry production due to the possibility of early nutrient delivery to chick embryos and improvement of this development. Front this, this study aimed to evaluate the effects of ascorbic acid use to in ovo feeding for poultry embryos. A total of 350 Rhode Island Red fertile eggs with viable embryos were randomly distributed in seven treatments with 50 replicates (eggs) each. The treatments were control (untreated eggs), eggs subjected to a saline solution (0.50% NaCl) and solutions containing increased levels of ascorbic acid (0.25, 0.50, 0.75, 1.00, and 1.25%). Evaluation of hatchability, embryo mortality, chicks' weight, and gastrointestinal tract development were performed. The increased addition of ascorbic acid in solutions to in ovo feeding caused a linear decrease ($P \leq 0.05$) on hatchability, a linear increase ($P \leq 0.05$) on intermediary embryo mortality. It was also observed a linear decrease ($P \leq 0.05$) in late embryo mortality. In ovo injection of solutions with 0.50% of ascorbic acid provided heavier ($P \leq 0.05$) chicks at birth with better ($P \leq 0.05$) gizzard development. However, in ovo injection of ascorbic acid did not affect ($P > 0.05$) the general development of the gastrointestinal tract. In conclusion, in ovo injection of ascorbic acid affected hatching characteristics. Increasing levels of ascorbic acid resulted in a linear decrease on hatchability and a sudden increase in intermediary embryo mortality. In ovo injection of solutions with 0.50% of ascorbic acid provided heavier chicks at birth with better gizzard development. However, it did not affect the development of the gastrointestinal tract of chicks with 1 day-old.
Keywords: ascorbic acid, biotechnology, hatchability, in ovo, vitamin C.

1. Introduction

The in ovo nutrition during the pre-hatch stage is one of the most researched and applied biotechnologies by the poultry industry in the last few years (Uni et al., 2005). This is based on the concept of supplying exogenous nutrients to the embryo to increase the activity of the enzyme, stimulate tissues and cells development, and accelerate the maturation of the gastrointestinal tract (Uni et al., 1999; Uni et al., 2005; Campos et al., 2011).

The nutrients used for in ovo feeding may be involved in different physiological functions, such as energy sources (sucrose, dextrin, maltose, or glucose), activators of the immune system (vitamin E, copper or probiotics), stimulators of protein metabolism and anabolism (HMB or amino acids: methionine, lysine, threonine, arginine or leucine) and trophic agents in the intestinal mucosa (glutamine, zinc or butyric acid) (Ohta et al., 1999; Chen et al., 2010; Santos et al., 2010; Campos et al., 2011; Leitão et al., 2014; Saeed et al., 2019). However, positive responses from the use of these nutrients are not only dependent on the composition of the solution injected, but also on its volume and osmolarity (Campos et al., 2011; Uni et al., 2005).

Among the nutrients that play an essential role in the metabolism of birds, we may point out the vitamin C, a water-soluble vitamin that presents L-ascorbic acid activity and is present in two forms: ascorbic acid and dehydroascorbic acid (Rutz et al., 2014). Ascorbic acid acts as an enzymatic co-factor in several fundamental reactions in the animal organism, being crucial to collagen biosynthesis, synthesis and metabolism of neurotransmitters, maintenance of mucosal epithelium and vessel wall, formation of red blood cells, and the control of circulating corticosteroid levels (Teixeira and Abreu, 2011).

Ascorbic acid also has antioxidant action, an effective way to alleviate the adverse effects of heat stress on poultry production, in addition to increasing the degradation and decreasing the synthesis of glucocorticoids. In this way, the supply of ascorbic acid for birds in regions where there is a predominance of high environmental temperatures can be a tool to alleviate this environmental pressure (Teixeira and Abreu, 2011; Rutz et al., 2014). Considering the above, this study aimed to evaluate the effects of ascorbic acid use to in ovo feeding for poultry embryos.

2. Material and methods

All experimental procedures were performed in the Poultry Sector of the Federal University of Amazonas, Manaus, Amazonas, Brazil. The experimental protocols applied in this study were in accordance with the Brazilian guidelines for animal

welfare and were approved by the Animal Care and Use Committee of the College of Agrarian Science of the Federal University of Amazonas (protocol number 013/2019).

Standard Rhode Island Red fertile eggs (43.49±2.57 g, n = 400) were collected from the breeding of the Poultry Sector and immediately disinfected and stored in an incubator Model PETERSIME 168 (Coopemaq Incubation Equipment Co. Ltd., Urussanga, Brazil) within 24 h of lay under optimal incubation conditions (37.5 ºC and 65% RH). The incubator was previously disinfected with ultraviolet light, and the eggs in it were automatically turned every hour. Distilled water was manually added to a tank in the incubator twice daily. Eggs were randomly distributed into the incubator to avoid positional effects.

On d 17 of embryonic development, eggs were candled with a lamp, and those unfertilized or containing dead embryos were removed from the incubator. The remaining eggs with viable embryos (n = 350) were marked with a soft-lead pencil. These fertile eggs were distributed in a completely randomized experimental design, where the experimental groups, as shown in Table 1, were constituted of untreated eggs (control), a sterile saline solution (0.50% saline), and five solutions containing increased levels of ascorbic acid (0.5, 1.0, 1.5, 2.0, and 2.5%), with 50 eggs (replicates).

Treatments	Solutions	Osmolarity (mOsm/L)[2]
Control	Intact egg	-
Saline	0.5% NaCl	170.94
Solution 1	0.5% NaCl + 0.25 % AA	199.33
Solution 2	0.5% NaCl + 0.50 % AA	227.72
Solution 3	0.5% NaCl + 0.75 % AA	256.11
Solution 4	0.5% NaCl + 1.00 % AA	284.50
Solution 5	0.5% NaCl + 1.25 % AA	312.89

Table 1 – Experimental treatments containing controls groups and solutions with different levels of ascorbic acid (AA) to in *ovo* injection. The injected experimental solutions were calculated in 100 mL of solution base. Determined by the calculation of the molecular weight and the concentration of the substances in each solution.

The ascorbic acid used was manufactured by Midway International Labs Ltda© (Anápolis, Brazil). The feeding solution was prepared according to the method reported by Bhanja et al. (2004). The concentration of the solution to be injected in the eggs reported by Murphy (1994) was taken as a standard (Table 1). The vitamin mixture was dissolved in 200 ml of 0.50% sterile saline before injection. The solutions were adjusted to pH 7.0 adding NaOH and nullified for the microbial count through a sterile filter. Before the in ovo feeding procedure, the solutions were warmed up to 29 ºC in a water bath.

The ascorbic acid was dissolved in 200 ml 0.50% of sterile saline before in ovo feeding. At 17 days of embryonic development, the solutions were injected into the amnion cavity with a 21 G needle through a pinhole made at the blunt end of the egg above the airspace under laminar flow after disinfection with ethyl alcohol-laden swabs. The injection length was 20 mm. After injection, the pinholes in the eggs were sealed with molten paraffin and moved to a hatching machine Model PETERSIME 168 (Coopermaq Incubation Equipment Co. Ltd., Urussanga, Brazil) within 24 h of lay under optimal conditions (36.5 ºC and 75% RH). The hatching machine was disinfected with ultraviolet light and distilled water was added in a tank twice daily manually. The procedure followed the method described above within one hour, while eggs were kept outside the incubator for less than two hours.

Immediately post-hatch, the chicks were weighed before sacrifice for sample collection, and hatch weight (HW) was recorded. It was also evaluated the hatchability (birth chicks per fertile eggs injected), intermediary mortality (dead embryos between 16 and 18 days of incubation), late mortality (dead embryos between 19 and 21 days of incubation without pecked the eggshell), pipped eggs (dead embryos between 19 and 21 days of incubation that pecked the eggshell), and the proportion of chick weight per its respective egg weight according to the method described by Damasceno et al. (2017).

From hatchability results, five viable chicks with 1 day-old from control, saline solution, solution 1 and solution 2 were randomly selected, slaughtered by cervical dislocation and the yolk sac, heart, liver, pro-ventricle, and gizzard were dissected, drained out of blood, and weighed. The same procedure was used to evaluate the length of the gastrointestinal tract and its regions (oropharynx plus esophagus, duodenal loop, jejunum plus ileum, cecum, and colon plus rectum) according to the method described by Damasceno et al. (2017).

Data were subjected to one-way ANOVA in Statistical Analysis System (SAS Inc., Cary, NC). Polynomial regression was used to determine the injection effect. The regression procedure of curve estimation was applied in the determination of the regression model. A linear or quadratic model was decided, derived from the scatter diagram plot. Hatchability was determined as the proportion of hatched egg numbers to total fertile egg numbers (Bhanja et al., 2004). Significant differences were found when the probability value (p-value) was less than 0.05.

3. Results and Discussion

It was observed that the ascorbic acid, even included in low concentration, caused relatively medium osmolarity values (Table 1). The literature reported that solutions to in ovo feeding should be maintained between 400 and 600 mOsm (Pedroso et al., 2006; Retes et al., 2017). Normally, carbohydrates present high values of molecular weight (up to 400 g/Mol), while amino acids present medium values (ranging from 100 to 200 g/mol). This is important information as the molecular weight of a nutrient is the main factor influencing the osmolarity of the solutions used in ovo. We observed that ascorbic acid presented an intermediate molecular

weight (proximate 176,12 g/mol) between the values found for amino acids and carbohydrates, the main nutrients used in studies on in ovo feeding (Retes et al., 2017; Alves et al., 2020).

The results (Table 2) from the in ovo injection of ascorbic acid presented a significant effect (P≤0.05), where increased levels of ascorbic acid caused a linear egg hatch decrease (y = -9.7143x + 58.857 R^2 = 0.84). At the same time, there was observed a significant effect (P≤0.05) from the use of ascorbic acid on intermediary mortality, where increased levels of ascorbic acid caused a linear increase in the results (y = -9.7143x + 58.857 R^2 = 0.84). The control (intact egg) and saline solution presented better results

Treatment	Hatchability (%)	Intermediary mortality (%)	Late mortality (%)	Pipped eggs (%)
Control	76.00	12.00	8.00	4.00
Saline	72.00	18.00	8.00	2.00
0.5% AA	22.00	72.00	4.00	2.00
1.0% AA	4.00	90.00	2.00	4.00
1.5% AA	10.00	84.00	4.00	2.00
2.0% AA	6.00	90.00	4.00	0.00
2.5% AA	0.00	100.00	0.00	0.00
P-value	0.01	0.01	0.02	0.14
Effect	NL	PL	NL	ns
CV (%)	18.52	7.51	19.08	17.72

Table 2 – Effects of *in ovo* feeding of ascorbic acid at day 17 of embryonic development on hatchability and embryo mortality of Rhode Island Red chicks in Manaus, Brazil. All data represent the mean of 50 replicates per treatment. Control is the non-injected treatment; saline is 0.50% sterile saline solution; AA. = ascorbic acid concentration. All *in ovo* feeding test ascorbic acid was dissolved in saline solution. Q = Quadratic. PL = Positive Linear. CV = Coefficient of variation.

(P≤0.05) of hatchability and intermediary embryo mortality.

These results differ from those observed by Nowaczewski *et al.* (2012) who reported no significant difference between the control and the experimental groups on hatchability. However, the results found in this study corroborated those observed by Damasceno *et al.* (2017) and Rufino *et al.* (2019a,b) who also reported greater embryo mortality, especially intermediary, from the *in ovo* injection of solutions with high concentrations of nutrients, regardless of molecular weight of the nutrient studied. Uni *et al.* (2005), Pedroso *et al.* (2006) and Alves *et al.* (2020) reported that a high inclusion of nutrients with high volume of free compounds in these solutions tends to cause a sudden increase in osmolarity. This can make it difficult for poultry embryos to accept the solutions and, consequently, causing high mortality soon after the in ovo injection procedure (Retes *et al.*, 2017; Peebles, 2018; Alves *et al.*, 2020, Das *et al.*, 2021), as we observe in the results of this study.

The late embryo mortality also presented a significant effect (P≤0.05), where increased levels of ascorbic acid caused a linear decrease in the results (y = -1.7143x + 12.286 R^2 = 0.79). This result indicates a clean concentration of embryo mortality in the intermediary period, helping to link with the information reported by the literature and reported above.

From this, it is important to mention that the use of NaCl is important to stabilize the pH and balance the osmolarity of the solution. This can facilitate the transport of free nutrients to the embryo's organism, allowing the *in ovo* feeding to cause good effects on embryo development (Retes *et al.*, 2017; Peebles, 2018; Alves *et al.*, 2020, Das *et al.*, 2021). However, there are contexts where not even the NaCl use is sufficient to turn the concentration of the nutrient in the solution acceptable to the embryo. Generally, these contexts are related to osmotic balance disequilibrium caused by high nutrient concentration in the solution, toxic compounds present in the nutrient, and the volume of the injected solution (Al-Murrani, 1982; Ohta *et al.*, 1999; Salmanzadeh *et al.*, 2016; Retes *et al.*, 2017; Peebles, 2018; El-Kholy *et al.*, 2019; Kop-Bozbay and Ocak, 2019). A disequilibrium in one of these tend to cause a significant reduction in hatchability and, consequently, an increase in embryo mortality.

On the other hand, the use of ascorbic acid *in ovo* caused a significant effect (P≤0.05) on the chick's weight, where the *in ovo* injection of solutions with 0.50% of ascorbic acid provided heavier chicks at birth (Table 3).

Based on the literature (Retes et al., 2017; Peebles, 2018; Alves et al., 2020, Das et al., 2021), when the nutrient is included in an ideal range, the in ovo feeding tends to increase the concentration of available nutrients and improve the embryo development of chicks, reflecting in an increase on its body weight (Foye et al., 2007). Even the ascorbic acid not being a nutrient with high-energy content, this can play an essential role in the metabolism (Teixeira and Abreu, 2011; Rutz et al., 2014). In addition, it may help the poultry embryo to accelerate its development and hatch presenting greater physiological maturity.

However, in the results of heart and main organs of gastrointestinal tract weight, the use of ascorbic acid in ovo feeding caused a significant effect (P≤0.05) on the gizzard weight. Higher levels of ascorbic acid caused a linear increase on these results (y = 0.249x + 1.41 R^2 = 0.94) (Table 4).

Treatment[2]	Hatching weight (g)	Proportion of chick weight per its respective egg weight
Control	32.43	0.69
Saline	34.62	0.72
0.5% AA	34.11	0.76
1.0% AA	37.34	0.72
1.5% AA	33.53	0.72
2.0% AA	31.24	0.71
2.5% AA	-	-
p-value	0.01	0.12
Effect[3]	Q	ns
CV (%)[4]	6.28	5.44

Table 3 – Effects of *in ovo* feeding of ascorbic acid at day 17 of embryonic development on hatching weight of Rhode Island Red chicks at 1 day old in Manaus, Brazil. All data represent the mean of 50 replicates per treatment. Control is the non-injected treatment; saline is 0.50% sterile saline solution; AA = ascorbic acid concentration. All in ovo feeding test ascorbic acid was dissolved in saline solution. ns – non-significant. CV = Coefficient of variation.

This result may be related to the body weight results, because the stimuli to develop the digestive organs from *in ovo* injection of nutrients tends to accelerate the functional maturity of several areas of the gastrointestinal tract (Uni *et al.*, 1999; Uni *et al.*, 2012; Chen *et al.*, 2017; Xu *et al.*, 2019). *In ovo* injected nutrients may act as a tool to overcome the growth constraints imposed by limited digestive capacity in modern embryos, enhancing its intestinal function and maturation prior to hatching, resulting in larger and heavier chicks at birth (Tako *et al.*, 2004; Foye *et al.*, 2006; Leitão *et al.*, 2010; Leitão *et al.*, 2014).

Treatment	Yolk sac (g)	Heart (g)	Liver (g)	Pro-ventricle (g)	Gizzard (g)
Control	4.98	0.39	0.99	0.40	1.72
Saline	4.37	0.44	1.00	0.44	1.80
0.5% AA	4.30	0.34	0.94	0.39	2.19
1.0% AA	4.59	0.39	0.97	0.42	2.42
p-value	0.13	0.19	0.08	0.19	0.01
Effect	ns	ns	ns	ns	PL
CV (%)	13.67	10.06	13.91	16.46	14.72

Table 4 – Effects of *in ovo* feeding of ascorbic acid at day 17 of embryonic development on organ weigh of Rhode Island Red chicks in Manaus, Brazil. All data represent the mean of 50 replicates per treatment. Control is the non-injected treatment; saline solution is 0.50% sterile saline solution; AA = ascorbic acid concentration. All *in ovo* feeding test ascorbic acid was dissolved in saline solution. Q = Quadratic. NL = Negative Linear. ns = non-significant. CV = Coefficient of variation.

However, when we analyzed the results of biometry of the gastrointestinal tract areas (Table 5), we did not observe a significant effect (P>0.05) of in ovo injection of ascorbic acid. This result may indicate that the increase in gizzard from in ovo injection of ascorbic acid was a punctual effect that positively influenced the chick weight, but did not influence the general development of the gastrointestinal tract. In addition, due to the extremely low birth rate in the treatments using ascorbic acid levels above 0.50%, we analyzed chicks subjected to solutions containing up to 0.50% of ascorbic acid, which may have limited the analysis. However, if we consider that levels of ascorbic acid above 0.50% caused great embryo mortality, it is very logical to imagine that these levels could not cause a positive effect on gastrointestinal tract development.

Treatment	Total length (cm)	Oropharynx + oesophagus (%)	Duodenal loop (%)	Jejunum + ileum (%)	Cecum (%)	Colon + rectum (%)
Control	39.50	7.37	5.50	19.50	5.54	3.33
Saline	38.00	7.40	4.90	18.50	5.50	3.30
0.5% AA	39.80	7.20	5.20	19.30	5.60	3.10
1.0% AA	38.00	7.50	4.80	18.50	5.55	3.20
p-value	0.15	0.17	0.13	0.14	0.17	0.14
Effect	ns	ns	ns	ns	ns	ns
CV (%)	10.56	14.86	11.25	17.63	13.30	15.50

Table 5 – Effects of *in ovo* feeding of ascorbic acid at day 17 of embryonic development on gastrointestinal tract length of Rhode Island Red chicks in Manaus, Brazil. All data represent the mean of 50 replicates per treatment. Control is the non-injected treatment; saline solution is 0.50% sterile saline solution; AA = ascorbic acid concentration. All *in ovo* feeding test ascorbic acid was dissolved in saline solution. Q = Quadratic. PL = Positive Linear. NL = Negative Linear. ns = non-significant. CV = Coefficient of variation.

4. Conclusion

In conclusion, in ovo injection of ascorbic acid affected hatching characteristics. Increasing levels of ascorbic acid resulted in a linear decrease in hatchability and a sudden increase in intermediary embryo mortality. In ovo injection of solutions of 0.50% of ascorbic acid provided heavier chicks at birth with better gizzard development. However, in ovo injection of ascorbic acid did not affect the general development of the gastrointestinal tract of chicks with 1 day-old.

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