EFFECT OF ASCORBIC ACID INJECTED IN PRE-INCUBATION EGG ON INCUBATION AND RESPIRATORY HEMATOLOGICAL PARAMETERS[1]

INJEÇÃO DE ÁCIDO ASCÓRBICO IN OVO PRÉ-INCUBAÇÃO SOBRE PARÂMETROS DE INCUBAÇÃO E HEMATOLÓGICOS RESPIRATÓRIOS

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SUMMARY

The present study evaluates the effect of ascorbic acid injection into fertile eggs on incubation and blood parameters. Eight hundred fertile eggs from broiler (Cobb®) breeders aged 36 weeks were weighed and homogeneously distributed in four incubators with automatic control of temperature, humidity and turning. The eggs were incubated at 37.5°C while relative humidity was kept at 60% until transfer (19 days incubation) and, then, increased to 70% on the last two days of incubation. The experimental design was completely randomized with five treatments, applied before incubation: eggs without holes and therefore not injected; eggs injected in the albumen with 100 µL of water plus 0, 2, 4 and 6% ascorbic acid. Statistical analysis was performed by SAS®. The data shows that the treatments did not compromise the development *in ovo* under thermoneutral conditions, except for the hatching, which was influenced by lack of skill in performing the injection technique. Furthermore, injection of 6% ascorbic acid increased hemoglobin content, improving the transport of gases in the blood of these chicks.

KEY-WORDS: Hatchability. Intra egg. Loss of mass. Vitamin C.

RESUMO

O presente estudo teve como objetivo verificar o efeito da injeção de ácido ascórbico em ovos férteis sobre parâmetros de incubação e sanguíneos. Oitocentos ovos férteis de matrizes de frango de corte (Cobb®) com 36 semanas de idade foram pesados e distribuídos homogeneamente em quatro incubadoras, com controle automático de temperatura, giro e umidade. Durante o período de incubação utilizou-se uma temperatura de 37,5ºC e 60% de umidade relativa até a transferência (19 dias de incubação) e 70% de umidade relativa nos dois últimos dias de incubação. O delineamento utilizado foi o inteiramente casualizado, com cinco tratamentos aplicados antes da incubação: ovos não perfurados e, portanto não injetados; ovos injetados com 100 µl de água no albúmem, com as seguintes concentrações: 0, 2, 4 e 6% de ácido ascórbico. As análises estatísticas foram realizadas pelo programa SAS ®. Os dados mostraram que os tratamentos utilizados não prejudicaram o desenvolvimento *in ovo* sob condições termoneutras, exceto a eclosão, a qual foi influenciada por falta de habilidade na execução da técnica de injeção. Além disso, a injeção de 6% de ácido ascórbico aumentou o teor de hemoglobina, com melhora no potencial de transporte de gases no sangue destes pintainhos.

PALAVRAS-CHAVE: Eclodibilidade. Intra ovo. Perda de massa. Vitamina C.

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INTRODUCTION

From the external piping, the chicks become exposed to adverse conditions, such as new antigens, heat stress and health challenges, against which their innate immune system acts on the frontline. *In ovo* injection of nutrients may be an alternative method for increasing hatchability, the quality of the birds at hatching and improve the innate immune system (OHTA et al., 2001). The use of ascorbic acid (vitamin C) as a nutritional additive/supplement during the fetal stage has shown dose-dependent positive effects of this vitamin on hatchability and body weight at hatching (GHONIM et al., 2009; MOHAMMED et al., 2011; NOWACZEWSKI et al., 2012). However, the literature reports on intra egg injection of vitamin C at later stages of embryo development. There is no data in the literature on the effects of injection of vitamin C *in ovo* pre-incubation on incubation and hematological parameters of chicks hatched from eggs incubated under thermoneutral conditions. Therefore, from the physiological point of view, it is very important to develop a management technique to maximize the production of better quality chicks. Normal chicks are those with the greatest potential to express their genetically determined performance and stronger innate immune defense system.

Thus, the present study examined the effects that the pre-incubation injection of ascorbic acid *in ovo* had on the quality of incubation (conductance, percentage and shell thickness, egg weight loss, hatchability and mortality rates), the quality of chicks (body weight and absolute and relative weight of the bursa of Fabricius and temperature of the body surface) and on respiratory and hematological parameters of chicks.

MATERIAL AND METHODS

The present study was approved by the Ethics Committee on Animal Use - CEUA of the Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, UNESP, Jaboticabal, under the experimental protocol no. 7377/10.

Eight hundred fertile eggs from broiler (Cobb®) breeders aged 36 weeks were weighed and used in a completely randomized design with 5 treatments as follows: control, no-injection; and four treatments consisting of an intra-egg injection of 100 µL water plus 0, 2, 4 and 6% of ascorbic acid. The eggs from each treatment were stored in four incubators, with 40 eggs each, while egg average weight per repetition and treatment was 67 ± 2g. Incubators (Premium Ecológica IP200) equipped with automatic temperature control and egg turning every 2h, were maintained at relative humidity of 60% until transfer (19 days incubation) and 70% in the last two days of incubation.

The water and ascorbic acid solution was injected before the start of incubation. The egg shell area was cleaned with 100% ethanol, and then punctured with a sterile needle (Injex, 13 x 0.38 (27.5 G1/2")). The solution with ascorbic acid (Synth, 99% purity) was injected into the albumen, approximately 6 mm below the skin. The eggs were placed horizontally, and the solution was injected on the egg thinner pole (opposite to the air cell). After injection, the hole was closed with treatment and repetition identification label. Ascorbic acid was diluted in Milli-Q water, autoclaved and kept in the dark due to its photo sensitivity.

The variables analyzed were: hatching and hatchability rates, embryo mortality, eggshell percentage and thickness, conductance, weight loss, body surface temperature, relative body weight, absolute and relative weight of the bursa of Fabricius, hematocrit (HCT), hemoglobin (HGB), total red blood cells (RBC) and mean corpuscular volume of erythrocytes (MCV).

Hatching/hatchability rate

We evaluated the hatching (number of chicks born/number of hatched eggs) and hatchability (number of chicks born/number of fertile eggs incubated).

Percentage and shell thickness, weight loss, body surface temperature and conductance

To determine eggshell percentage and thickness at the end of the incubation, 10 eggs per treatment were analyzed, after removing both inner and outer membranes and cuticle as described by Rahn et al. (1981) while keeping shell fragments in boiling aqueous solution of NaOH 0.5%. Then, the shells were washed in distilled water and kept at room temperature for 72 hours for drying and subsequent analysis. Shell weight is expressed as % of the pre-incubation egg weight. The shell thickness was obtained from the measurement of the equatorial region fragments of shells, using a digital micrometer (Mitutoyo - 0.001 mm resolution).

The percent mass loss is given by the difference between the weight of the egg before incubation and after 18 incubation days. The conductance of the shell was calculated by weight loss (g) until the transfer/saturation vapor pressure (23.86 mm/Hg at 25°C).

Temperatures of the wing, head, shank and back of male chicks were measured using an infrared thermometer to determine the mean body surface temperature (T), given by the formula: = mean surface temperature $(0.12 \times T_{wing}) + (0.03 \times T_{head}) + (0.15 \times T_{shank}) + (0.70 \times T_{back})$, as described by Richard (1971).

Quality of chicks

The weight and bursal index (BI), bursa weight/body weight ratio, were obtained from eight hatched female chicks per treatment after killing them by cervical dislocation followed by decapitation. The relative weight (%) of newly hatched chicks was given by the absolute body weight (g), obtained after drying the fluff, relative to egg weight (g).

Blood parameters

Eight female chicks per treatment were used to determine blood parameters (HCT, HGB, RBC, MCV). Blood was collected from the jugular vein and stored in eppendorf vials containing 15µL anticoagulant/1 mL blood (Glistab, cat. 29, Labtest Diagnostica) on ice for use in the analysis on a blood cell counter (Celm, Mod 550), with two readings per chick.

Statistical Analysis

Data were subjected to analysis of variance by the General Linear Model (GLM) of SAS® (SAS Institute, 2002). The significantly different means (5% probability) were compared by orthogonal and polynomial contrasts: Contrast 1 - comparison between control and means of treatments with 0, 2, 4 and 6% ascorbic acid; Contrasts 2, 3 and 4 - three regression models were used: linear, quadratic and cubic models in order to check polynomial effects regarding ascorbic acid levels. The hatchability and hatching frequencies were compared using the chi-square at 5% probability.

RESULTS AND DISCUSSION

Hatching and hatchability rates were influenced significantly ($p<0.05$) by the treatments (Table 1). It was noted that injected eggs, regardless of ascorbic acid, had lower hatching/hatchability rates.

Uni & Ferket (2003) stated that injecting high concentrations of the solutions may interfere with the osmotic balance and affect embryo development while reporting 800 mOsm as the limit. In the present study, the solution injected *in ovo* had lower osmolarity (113 mOsm), which indicates that the lower hatching rate was not due to the injected ascorbic acid changing the osmotic balance of the eggs excessively. The results of this study differ from those obtained by Pires et al. (2011), who observed an increase in hatching rate with injection of 1% ascorbic acid *in ovo* pre-incubation. Percentage-dependent effect of ascorbic acid on the hatching rate has also been reported by Zakaria & Al Anezi (1996), Elibol et al. (2001), Ipek et al. (2004) and Nowaczewski et al. (2012), who observed improvement in hatchability of eggs with injection of 3 and 6 mg of ascorbic acid at later stages of embryo development. This fact shows that the effect of vitamin A on the development *in ovo* varies with solution concentration and the stage of embryo development in which the injection is performed.

Jochemsen & Jeurissen (2002) reported that the age at which the inoculation procedure is performed can affect the site where the product is applied. Ohta et al. (1999) observed a hatching reduction after amino acid inoculation *in ovo* was performed before incubation. According to Ohta & Kidd (2001), product injection *in ovo* should be made either in the extra embryonic cavity or in the yolk sac to prevent hatching reduction; however, the authors injected at seven days of incubation and not in the pre-incubation as in the present study.

The data from this study show that the inoculation method was not suitable and that embryos were possibly perforated since they were injected in the middle of the egg. More training is required to perform this procedure regarding site selection and injecting the solution because any movement of the egg can move or pierce the yolk sac, or even the embryo.

Table 2 shows that eggshell percentage and thickness were not affected by treatments ($p>0.05$) at the end of the incubation. Gas exchange between egg internal and external environments depends on total pore number and size, shell thickness as well as incubator temperature, humidity, air ventilation and the speed, angle and frequency of egg rotation, factors that interfere with heat loss and conductance (MORITA et al., 2010). Therefore, shell thickness influences shell gas exchange (ANCEL & GIRARD, 1992) and water loss. The data indicate that regardless the ascorbic acid injection, the same proportion of shell was used for embryonic and fetal development during incubation. The calcium necessary for embryonic and fetal development comes from the eggshell (TUAN, 1983; GRIZZLE et al. 1992); therefore, the thinner the shell at the end of incubation, the greater the amount of calcium used for the *in ovo* development.

Table 01 - Effect of ascorbic acid injection on the frequency of hatchability and hatching.

Treatments	Hatchability (%)	Hatching (%)
Control	90.20	88.46
Ascorbic Acid, 0%	67.35	65.35
Ascorbic acid, 2%	67.00	65.69
Ascorbic acid, 4%	66.34	64.42
Ascorbic acid, 6%	72.28	70.19
Probability	0.0004*	0.0005*

* Significant at 5% probability by chi-square test.

Table 02 - Effect of ascorbic acid injection on the percentage of eggshell, weight loss, conductance, shell thickness, body weight and surface temperature of chicks at hatching.

Treatments	**Shell Percentage (%)**	**Weight loss (%)**	**Conductance**	**Shell thickness (mm)**	**Relative body weight (%)**	**Body surface temperature (° C)**
Control	7.74	8.12	0.21	0,460	74.80	32.28
Ascorbic Acid, 0%	7.88	8.20	0.21	0,480	74.95	32.20
Ascorbic acid, 2%	8.19	8.08	0.20	0,494	73.63	32.69
Ascorbic acid, 4%	7.89	7.88	0.20	0,482	76.00	32.47
Ascorbic acid, 6%	7.78	7.64	0.19	0,481	75.31	31.80
Probability	0.4745	0.8294	0.8263	0.1417	0.1669	0.1180
Coefficient of variation	7.04	16.03	17.01	6.01	7.53	1.73

Table 03 - Effect of injection of ascorbic acid on body weight, absolute and relative weight of the bursa of Fabricius of female chicks at hatching.

Treatments	Body Weight (%)	Absolute weight (g)	Relative weight (%)
Control	74.80	0.056	0.124
Ascorbic Acid, 0%	74.95	0.051	0.110
Ascorbic acid, 2%	73.63	0.049	0.109
Ascorbic acid, 4%	76.00	0.048	0.105
Ascorbic acid, 6%	75.31	0.048	0.107
Probability	0.1669	0.6842	0.7621
Coefficient of variation (%)	7.53	23.48	24.08

Table 04 - Effect of ascorbic acid injection on hematocrit (HCT), hemoglobin (HGB), total number of red blood cells (RBC), erythrocyte mean corpuscular volume (MCV) of female chicks at hatching

Treatments	HCT (%)	HGB (g/dL)	RBC (10^6/mm^3)	VCM (µm^3)
Control	31.35	11.22	2.34	137.57
Ascorbic Acid, 0%	32.70	9.50	2.47	135.18
Ascorbic acid, 2%	27.35	9.22	1.95	140.70
Ascorbic acid, 4%	15.25	5.70	1.16	131.38
Ascorbic acid, 6%	30.90	13.22	2.33	129.50
Probability	0.0548	0.0430 *	0.0837	0.8107
Coefficient of variation (%)	37.65	40.85	40.40	9.85
Linear effect	-	0.0307 *	-	-

* Significant at 5% probability. Hemoglobin = 0.3817 x + 8.2633 (R^2 = 0.1029).

The percentage of weight reduction until transfer time is a parameter used commercially to determine the degree of embryo development (SANTOS et al., 2009) while conductance, gas exchange ability between the egg and the environment, is related with water (CAMPOS & SANTOS, 2003) and metabolic heat (HAMIDU et al., 2007) losses. The treatments did not affect significantly ($p>0.05$) mass loss and conductance and, therefore, the *in ovo* solution injection did not influence neither embryo development nor gas exchange during incubation.

The treatments did not affect significantly ($p>0.05$) body surface temperature, thus indicating that there is no dose-dependent effect of ascorbic acid on the control of body temperature.

The effects of stressors or modulators on bird lymphoid organs can be assessed by weight and organ weight/body weight index (WYATT et al., 1986; ROSALES et al. 1989; REVIDATTI et al., 2002). In this study, the ascorbic acid injection *in ovo* did not affect body weight and bursal index ($p>0.05$) (Table 4), these data corroborate Selim et al. (2012), who found no change of bursa weight when duck eggs were injected with ascorbic acid. The weight of lymphoid organs reflects the body ability to produce lymphoid cells during an immune response (RIBEIRO et al., 2008) and, together with the relative weight of the bursa of Fabricius, shows the defense potential of the bird. The values found for the egg and body weight ratio are within the 73-80% range, considered normal for chicks according to Henry & Burke (1997). Alloui et al. (2005) classified the bursal index of broilers as excellent ($> 0.20\%$), good ($0.18\% < x \leq 0.20\%$), medium ($0.15\% < x \leq 0.18\%$) and bad ($\leq 0.15\%$). The results of this study show that bursal index at the hatching did not reach 0.15%, considered a bad index

for broilers, according to the classification of Alloui et al. (2005). This low bursal rate shows that, regardless the ascorbic acid injection, the newly hatched chicks have low protection ability resulting from acquired immunity, which may be related to the fact that lymphoid organ development occurs after hatching (PARAMITHIOTIS & RATCLIFFE , 1994; GLICK, 2000).

Blood parameters

HGB values were significantly affected ($p<0.05$) by the injections (Table 4). The results showed a linear effect on the levels of ascorbic acid solution injected *in ovo* prior to incubation. The second half of egg incubation is characterized by intense fetal growth, which involves increased metabolism (TULLETT, 1990; FRENCH, 1997; MEIJERHOF, 1999; TAZAWA & WHITTOW, 2000) and, therefore, increased demand for gas exchange (to obtain O_2 and eliminate CO_2). According to Moura & Pedroso (2003) ascorbic acid is related to an increase of RBC, HGB and HCT values, so this study also investigates whether ascorbic acid injection *in ovo* influenced such hematological respiratory variables. Considering that hemoglobin is related to the transport of gases, these data suggest that increasing the concentration of the inoculated ascorbic acid solution increased respiratory rate, and consequently, the hematopoietic process and respiratory potential, without changing shell conductance.

The increase of the HGB value might be associated with dehydration (CAMPBELL, 1994). However, treatment did not affect either the relative weight of chicks at hatching or the presence of seemingly dehydrated chicks, thus ruling out this possibility. Although the hatchability of eggs injected with ascorbic acid was lower than the eggs of the control treatment, one cannot fail to consider that higher HGB grants greater gas exchange potential to the chicks.

The other hematologic characteristics - HCT, RBC, MCV - were not affected significantly ($p>0.05$) by the treatments. These data corroborate those of Ghonim et al. (2009), who reported no effect of the injection of ascorbic acid on the erythrocyte of broiler chicks; however, the authors only looked at the effects of this vitamin in chicks resulting from eggs injected on the 14^{th} incubation day.

CONCLUSION

The data show that injecting or not (control) ascorbic acid solutions *in ovo* did not hinder the embryo development under thermoneutral conditions, except hatchability and hatching, which were influenced by the lack of skill during the inoculation technique. The injection of 6% of ascorbic acid increased the hemoglobin content, with potential improvement in the transport of gases in the blood of these chicks.

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