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Non-ruminants

Glycosaminoglycans and vitamin C *in ovo* feeding affects bone characteristics of chicks

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ABSTRACT - Different levels of glycosaminoglycans (chondroitin sulfate and glucosamine sulfate) and vitamin C were injected *in ovo* to determine whether additive use influences the incubation parameters and bone characteristics of posthatch chicks. Hatchability was not affected by addition of glycosaminoglycans and vitamin C *in ovo*. However, chicks that received 4 µg additive showed 2.86% reduction in total mortality when compared with chicks from non-injected eggs. Moreover, tibia area and femur bone mineral density increased in chicks from eggs injected with 2.16 and 6.00 µg of additive, respectively. *In ovo* feeding with glycosaminoglycans and vitamin C can benefit bone development in embryos and reduce total mortality during the incubation period.

Key Words: ascorbic acid, bone mineral density, bone strength, chondroitin, glucosamine

Introduction

Upon reaching rapid body growth, efficient feed utilization, and maximum breast meat deposition, broilers begin to show disorders in their bone structure, with increased occurrence of angular and torsional deformities, tibial dyschondroplasia, rickets, osteochondrosis, and femoral necrosis (Almeida Paz et al., 2010). Bone deformities and fractures are severe health problems in modern, rapidly growing broilers, because genetic breeding programs aimed at superior performance have not included improvements in bone development (Almeida Paz et al., 2010).

In ovo feeding can be regarded as supplemental nutrition for the embryo and yolk sac (Santos et al., 2010), which may benefit embryo development and quality of posthatch chicks (Uni and Ferket, 2004).

Polysulfated glycosaminoglycans (GAG) such as glucosamine and chondroitin have anti-inflammatory action and are pharmacologically classified as slow-acting symptomatic drugs that reduce the symptoms of bone disorders such as osteoarthritis in humans and some animal species (Brandt et al., 2002). Additionally, they stimulate the synthesis of proteoglycans and collagen, increasing the proliferation of chondrocytes and bone matrix biosynthesis (Altman et al., 1989; Clark, 1991).

The use of GAG to minimize locomotor issues has been studied in several species, including human (Reginster et al., 2001; Simonaro et al., 2008), bovine (De Mattei et al., 2002), equine (Hanson et al., 1997; Fenton et al., 2000), and canine (Altman et al., 1989; Gonçalves et al., 2008; Melo et al., 2008; Eleotério et al., 2012). However, no research has been published regarding the use of GAG *in ovo* feeding or in the diet of broilers and laying hens.

Vitamin C, or ascorbic acid, participates in several biological processes. It helps in collagen synthesis and maintenance, is essential for the formation and maintenance of connective tissue, bone, and cartilage, and stimulates the immune system, increasing disease resistance (Berzina et al., 2013; Sgavioli et al., 2013; Chand et al., 2014).

Considering the existing research results about the benefits of vitamin C supplementation in broilers and chondroitin and glucosamine sulfates in other animal species, this study was conducted to evaluate the use of GAG and vitamin C *in ovo* feeding on bone system of developing posthatch chicks.

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Material and Methods

This study was approved by the local Committee of Ethics in Animal Use (case no. 011424/13) in Jaboticabal, SP, Brazil (21º14'05" South latitude and 48º17'09" West longitude, and average altitude of 615.01 m).

Four hundred and ninety (490) fertile eggs from 47-week-old Cobb® 500 broiler hens were distributed (67±1.16 g) into 14 trays (replicates) at seven eggs/treatment/tray (98 eggs/treatment). Eggs were incubated in incubators (CASP®, IHM Line e-V A 06, Amparo, São Paulo, Brazil) equipped with automatic temperature, humidity, and egg-turning control. The initial and final temperatures and humidity were 37.8 and 36.9 °C and 85.7 and 84.0%, respectively, with 504 h of incubation.

Eggs were distributed into five treatments, in a completely randomized experimental design, as follows: eggs non-injected (unperforated) and eggs injected with 0, 2, 4, or 6 µg additive/100 µL water on the fourth day of incubation.

According to Shim and Pesti (2011), in broiler embryos, the bone system starts to develop on the fourth day of incubation; for this reason, this date was chosen. Each 100 g of additive contained 30 g chondroitin sulfate, 30 g glucosamine, 5 g vitamin C (Synth, 99% purity, Diadema, Brazil), and 100 g excipient q.s. The chondroitin sulfate [$(C_{14}H_{21}NO_{14}S)n$, Infiniti Nutraceuticals, Inc.] had a purity of 91.35%, and the potassium glucosamine sulfate [$(C_6H_{14}NO_5)_2\ SO_4 \times 2KCl$, Zheijiang Freemen Inc.] had a sulfate content of 15.7%.

Each inoculated egg received the following constituents for the respective treatments: 2 µg of additive - 0.6 µg of chondroitin sulfate + 0.6 µg of glucosamine sulfate + 0.1 µg of vitamin C; 4 µg additive - 1.2 µg of chondroitin sulfate + 1.2 µg of glucosamine sulfate + 0.2 µg of vitamin C; and 6 µg additive - 1.8 µg of chondroitin sulfate + 1.8 µg of glucosamine sulfate + 0.4 µg of vitamin C.

The formulation of the additive injected *in ovo* was based on the veterinary medicine Condrodoton 500 µg (Vetnil®, Louveira, São Paulo, Brazil), recommended for dogs. The supplied quantity of chondroitin sulfate, glucosamine sulfate, and vitamin C in micrograms were thus converted according to the dog weight in pounds to the quantity in micrograms of those substances for broiler weight in pounds. Results of studies on broiler nutrition (Ferreira et al., 2015; Sgavioli et al., 2017) were also used to adjust the glucosamine and chondroitin doses in the additive injected.

For the additive injection, the eggs were held horizontally and, after cleaning with 70% ethanol, the shell was perforated near the thin end (the end opposite to the air cell) with a sterile needle [Injex, 13 × 0.38 (27.5 G1/2")] through which 100 µL aqueous additive solution were injected into the albumen, approximately 6 mm below the membrane. The solution was prepared with ultra-pure water autoclaved in the dark because of its photosensitivity (Sgavioli et al., 2013). After injection, the hole was sealed with a label identifying the treatment and replicate.

Hatchability (number of hatched chicks/number of incubated fertile eggs) and embryo mortality were determined according to embryo diagnosis phases (initial: 1-7 days; intermediate: 8-14 days; and late: 15-21 days of incubation, respectively).

The relative weight (%) of chicks post-hatch was obtained as the absolute weight of the chick (g) relative to the egg weight (g) (at day 0 of incubation).

Egg mass loss was calculated as the difference in egg weight before incubation and weight at the 18th day of incubation, expressed as a percentage of initial egg weight. Eggshell conductance was calculated as egg mass loss (g) divided by steam saturation pressure (23.86 mm/Hg at 25 ºC).

Eight birds per treatment whose average body weight was close to the average weight of the experimental unit were sacrificed at one day of age, in a total of 40 animals. Birds were stunned by individual exposure to CO_2 gas for 2 min in a flow-through system and then slaughtered by cervical dislocation.

Bone mineral density (BMD) (g/cm^2), area (cm^2), and length were determined in the left femur and tibia using dual-energy X-ray absorptiometry (Horizon Discovery Dxa Hologic®, Massachusetts, USA). Prior to sample scanning, the device was calibrated with a phantom, supplied by the manufacturer (Hologic®, Massachusetts, USA), as bones. For all analyses, a small-animal software (Hologic®, Massachusetts, USA) was used. Clean bones were placed with the cranial face in contact with an acrylic container with deionized water and scanned using a densitometer. The small-animal software was used to select the region for subsequent densitometric analysis. After the densitometric analysis, the bone length was assessed using a digital micrometer (MDC-Lite, 0.001 mm resolution, Mitutoyo, Suzano, SP, Brazil).

Left femur and tibia were used for the mechanical bone-strength tests (three-point bending). The tests were conducted using an EMIC® (DL 3000) universal testing machine. The load was applied at a rate of 5 mm/min with a force of 2000 N to determine the maximum permissible force (Fmax) of the bone and the deformation (bend) caused by Fmax. Bones were fixed on two supports (two points),

with span adjusted according to the size of the smallest bone. The force was then applied at the geometric mean point of the bones between the two supports (the middle third of the bone), and the equipment recorded the results. These variables express bone strength at the ends and in the middle third of the bone.

Right femur and tibia were used to determine bone calcium, phosphorus, and ash contents. The soft tissue was removed, and bones were boiled in deionized water for 5 min. After drying at room temperature, samples were immersed in petroleum ether for 48 h, dried in a forced-ventilation oven at 60 ºC for 48 h, and then ground in a ball mill. Bone mineral content was determined using wet analyses. Ash content was determined by burning the samples at 600 ºC. The methods were applied according to Silva and Queiroz (2002) and expressed as a percentage of defatted dry matter or mineral matter.

The effects of incubation treatments (eggs non-injected and injected with 0, 2, 4, or 6 µg additive) on all studied parameters were analyzed statistically using the model described below:

$$Yij = \mu + Ti + eij,$$

in which Y = studied parameter; µ = mean value of the parameter; Ti = treatment (0, 2, 4, or 6 µg additive); and eij = residual error.

Data were subjected to analysis of variance by the General Linear Model procedure (GLM) of SAS® (Statistical Analysis System, version 9.2) and analyzed for the presence of outliers (Box-and-Whisker plot), normal distribution of studentized errors (Cramer-Von-Mises test), and homogeneity of variances (Brown-Forsythe) (Littell et al., 2006).

Means were compared by 5% probability polynomial orthogonal contrasts, as follows: contrast 1 – comparison between the non-injected egg treatment versus the average of treatments with 0, 2, 4, and 6 µg of additives injected; contrasts 2 and 3 – comparisons using linear and quadratic regression models (0, 2, 4, and 6 µg of additive) (Robbins et al., 1979) to check the effects of additive application.

For embryonic mortality, data were analyzed for frequency by Fisher's exact test at the 5% probability level.

Results

Hatchability, egg mass loss, conductance, chick relative weight (Table 1), and embryonic mortality (Table 2) did not differ based on the analyzed contrasts (P>0.05).

In the embryo diagnosis, there was an effect for intermediate (P = 0.0083), late (P = 0.0083), or total (P = 0.0167) mortality, with the lowest mortality found in the treatments injected with 0, 2, and 4 µg additive, respectively (Table 2).

Tibial area and femur bone mineral density (BMD) responded quadratically (P = 0.0315 and P = 0.0333, respectively) (Table 3). According to equations $Area_{tibia} = 0.0038\ additive^2 - 0.0195\ additive + 0.511$ ($R^2 = 0.98$) and $BMD_{femur} = -0.0003\ additive^2 + 0.0013\ additive + 0.0196$ ($R^2 = 0.60$), additive injections of 6 and 2.16 µg increase the tibial area and femur BMD, respectively.

There was a contrast-1 effect (non-injected eggs vs. injected eggs) for the femur area (P = 0.0175). Smaller femur areas were observed in injected eggs (0.37 cm^2) when compared with non-injected eggs (0.40 cm^2) (Table 3).

Table 1 - Glycosaminoglycans (chondroitin sulfate + glucosamine sulfate) and vitamin C *in ovo* feeding on hatchability, egg mass loss, eggshell conductance, and relative chick weight

Treatment	Hatchability (%)	Egg mass loss[2] (%)	Eggshell conductance	Relative chick weight[3] (%)
Non-injected eggs	84.29	11.08	0.464	73.73
0 µg of additive	84.76	10.91	0.457	73.81
2 µg of additive[1]	83.81	10.25	0.429	72.69
4 µg of additive	87.14	10.33	0.432	72.74
6 µg of additive	83.81	10.42	0.437	72.79
SEM	1.76	0.17	0.01	0.34
CV (%)	18.42	18.73	18.74	2.84
		Contrast probability		
Non-injected vs. injected eggs	0.8953	0.1691	0.1688	0.4544
Linear effect[4]	0.9792	0.3988	0.3968	0.4539
Quadratic effect[4]	0.7687	0.3031	0.3011	0.5063

SEM - standard error of the mean; CV - coefficient of variation.
[1] Additive composition: each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine, and 5 mg of vitamin C (excipient q.s. – 100 g). Probability: P>0.05.
[2] Egg mass loss: difference in egg weight before incubation and at the 18th day of incubation, expressed as a percentage of initial egg weight.
[3] Relative weight: absolute weight (g) of the chicks relative to egg weight (g) (at day 0 of incubation).
[4] Treatments used for the regression calculation: 0, 2, 4, and 6 µg of additive.

Table 2 - Glycosaminoglycans (chondroitin sulfate + glucosamine sulfate) and vitamin C *in ovo* feeding on embryonic mortality frequency (initial, intermediate, and late)

Treatment	Initial (0-7 days)	Intermediate (8-14 days)	Late (15-21 days)	Total (0-21 days)
		%		
Non-injected eggs	0.00	2.62	13.10	15.72
0 µg of additive	3.05	1.52	10.67	15.24
2 µg of additive[1]	3.47	3.47	9.25	16.19
4 µg of additive	0.00	3.22	9.65	12.86
6 µg of additive	0.00	6.07	10.12	16.19
Probability	0.0500	0.0083[2]	0.0083[2]	0.0167[2]

[1] Additive composition: each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine, and 5 mg of vitamin C (excipient q.s. – 100 g).
[2] Significant at P<0.05 (Fisher's exact test).

There was a contrast-1 effect (non-injected eggs vs. injected eggs) for femoral BMD (P = 0.0296), whose highest values were found in injected (0.020 g/cm^2) compared with non-injected eggs (0.017 g/cm^2) (Table 3).

The maximum strength and deformation of the femur did not differ based on the analyzed contrasts (P>0.05) (Table 4).

Femur and tibia concentration of phosphorus (P) responded linearly (P = 0.0343 and P = 0.0353, respectively) for all injection levels (Table 5). According to the equations $P_{tibia} = -0.112$ additive + 18.271 ($R^2 = 0.78$) and $P_{femur} = -0.106$ additive + 19.358 ($R^2 = 0.95$), when the additive injection concentration increases, there is a reduction in the phosphorus concentration of the femur and tibia of chicks.

Discussion

In ovo feeding with chondroitin sulfate, glucosamine sulfate, and vitamin C was evaluated in terms of ability to promote changes in bone and cartilage development post-hatch. Despite the increase in femur and tibia area and femur BMD observed with *in ovo* feeding (GAG + vitamin C), the phosphorus concentration in the femur and tibia declined as the injected concentrations of *in ovo* feeding were elevated.

The maximum derivative of equations for tibia area and femur BMD revealed that *in ovo* feeding with 2.16 and 6 µg of additive maximizes the response to the cited characteristics, respectively.

Glycosaminoglycans and vitamin C can influence the formation of long bones, as these substances stimulate the synthesis of the proteoglycans and collagen and are capable of increasing the proliferation of the chondrocytes and biosynthesis of the matrix (Clark, 1991; Sgavioli et al., 2017). They are essential in the endochondral ossification process, responsible for the longitudinal growth of long bones, due to epiphyseal disk calcification (Anderson et al., 2005).

Table 4 - Glycosaminoglycans (chondroitin sulfate + glucosamine sulfate) and vitamin C *in ovo* feeding on femur and tibia maximum force and deformation of posthatch chicks

Treatment	Femur		Tibia	
	Maximum force (N)	Deformation (mm)	Maximum force (N)	Deformation (mm)
Non-injected eggs	8.47	0.62	6.45	0.88
0 µg of additive	7.60	0.60	5.79	1.15
2 µg of additive[1]	10.63	0.79	5.75	1.13
4 µg of additive	8.30	0.58	5.59	1.13
6 µg of additive	9.34	0.66	6.06	1.47
SEM	0.39	0.03	0.24	0.07
CV (%)	21.13	21.93	21.90	29.73
	Contrast probability			
Non-injected vs. injected eggs	0.6002	0.6386	0.3178	0.0641
Linear effect[2]	0.4275	0.9008	0.8066	0.1753
Quadratic effect[2]	0.2257	0.4068	0.6567	0.2322

SEM - standard error of the mean; CV - coefficient of variation.
[1] Additive composition: each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine, and 5 mg of vitamin C (excipient q.s. – 100 g).
[2] Treatments used for the regression calculation: 0, 2, 4, and 6 µg of additive.

Table 3 - Glycosaminoglycans (chondroitin sulfate + glucosamine sulfate) and vitamin C *in ovo* feeding on femur and tibia length, bone area, and bone mineral density (BMD) of posthatch chicks

Treatment	Femur			Tibia		
	Length (cm)	Area (cm^2)	BMD (g/cm^2)	Length (cm)	Area (cm^2)	BMD (g/cm^2)
Non-injected eggs	2.04	0.40	0.017	2.99	0.49	0.023
0 µg of additive	2.03	0.37	0.020	2.97	0.51	0.021
2 µg of additive[1]	2.09	0.37	0.020	2.97	0.49	0.023
4 µg of additive	2.10	0.36	0.022	3.05	0.49	0.022
6 µg of additive	2.11	0.37	0.018	3.08	0.53	0.021
SEM	0.02	0.01	0.001	0.02	0.01	0.001
CV (%)	4.31	5.84	11.59	3.47	7.62	20.72
	Contrast probability					
Non-injected vs. injected eggs	0.3374	0.0175[3]	0.0296[3]	0.6248	0.5865	0.6328
Linear effect[2]	0.1662	0.9607	0.6325	0.0570	0.2940	0.8109
Quadratic effect[2]	0.5069	0.5261	0.0333[3]	0.7487	0.0315[3]	0.5339

SEM - standard error of the mean; CV - coefficient of variation.
[1] Additive composition: each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine, and 5 mg of vitamin C (excipient q.s. – 100 g).
[2] Treatments used for the regression calculation: 0, 2, 4, and 6 µg of additive.
[3] Significant at P<0.05.

Table 5 - Glycosaminoglycans (chondroitin sulfate + glucosamine sulfate) and vitamin C *in ovo* feeding on femur and tibia ashes, calcium, and phosphorus contents of posthatch chicks

Treatment	Femur			Tibia		
	Ash (% DM)	Ca (% Ash)	P (% Ash)	Ash (% DM)	Ca (% Ash)	P (% Ash)
Non-injected eggs	25.12	38.13	18.97	25.57	34.61	17.77
0 µg of additive	24.58	38.59	19.30	25.02	35.15	18.21
2 µg of additive[1]	24.80	39.59	19.22	25.76	35.74	18.03
4 µg of additive	26.02	38.45	18.96	25.80	36.47	18.04
6 µg of additive	24.64	37.83	18.68	24.57	34.21	17.46
SEM	0.44	0.55	0.10	0.45	0.34	0.10
CV (%)	9.77	8.02	2.62	9.97	4.95	2.91
	Contrast probability					
Non-injected vs. injected eggs	0.9281	0.7550	0.7841	0.8208	0.3748	0.5168
Linear effect[2]	0.7684	0.5684	0.0353[3]	0.7884	0.5329	0.0343[3]
Quadratic effect[2]	0.4418	0.5375	0.6300	0.3605	0.0638	0.3752

DM - dry matter; SEM - standard error of the mean; CV - coefficient of variation.
[1] Additive composition: each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine, and 5 mg of vitamin C (excipient q.s. – 100 g).
[2] Treatments used for the regression calculation: 0, 2, 4, and 6 µg of additive.
[3] Significant at P<0.05.

There are several studies on the effects of *in ovo* feeding with injection of vitamin C (Ghonim et al., 2009; Mohammed et al., 2011; Nowaczewski et al., 2012) in broilers. However, scientific papers that correlate vitamin C *in ovo* feeding and bone development in broilers are scarce. Sgavioli et al. (2015) injected 6 µg ascorbic acid *in ovo* before incubation and did not observe beneficial effects on bone development (femur and tibia densitometry, mineral density, and bone strength) of birds at 42 days of age.

Therefore, our hypothesis is that *in ovo* feeding with GAG may be more effective in promoting bone development of posthatch chicks than vitamin C. However, to confirm this hypothesis, further research should be conducted to evaluate the isolated effects of each additive on the bone development of birds.

Higher phosphorus concentration in the femur and tibia were observed when the eggs were injected with water only; therefore, inclusion of GAG and vitamin C linearly reduces the concentration of phosphorus in the analyzed bones. A lower percentage of bone phosphorus can influence bone mineralization and affect bone mechanical quality, in broilers. According to Vargas Junior et al. (2004), calcium and phosphorus are the most essential minerals for bone formation, since 98% of the calcium and 80% of the phosphorus in the body are found in bones. However, the lower tibia and femur phosphorus mineral composition did not affect the density or bone-strength parameters.

According to Uni and Ferket (2003), high concentrations of solutions can interfere with osmotic equilibrium and affect embryo development. These authors described that the maximum osmolarity of the solution to be injected *in ovo* is 800 mOsm. The solution applied *in ovo* in the present study has a lower osmolarity than this limit (86 mOsm).

The lack of treatment effects on initial mortality indicates that *in ovo* feeding with GAG and vitamin C at the fourth day of incubation did not result in compromised embryo development. Moreover, positive results for total mortality were obtained with *in ovo* feeding with 4 µg of additive (2.86% mortality reduction when compared with the unperforated treatment). This is unprecedented information, because it shows for the first time in the literature that *in ovo* feeding with GAG and vitamin C can reduce embryo mortality.

However, Sgavioli et al. (2015) reported a decrease in egg hatchability after injecting increasing levels of vitamin C before incubation. This shows that the effect of *in ovo* feeding on hatchability varies with the concentration of the solution, stage of embryonic development, and egg region where the solution was injected.

Jochemsen and Jeurissen (2002) asserted that the *in ovo* injection site can be determined according to egg incubation day. According to Ohta et al. (2001), *in ovo* injection of products must be performed in the extra embryo cavity or yolk sac at the 7th day of incubation to avoid decreased hatchability and to benefit embryo development.

Conclusions

In ovo feeding with glycosaminoglycans and vitamin C can change the bone development of chicks and reduce their mortality. Our research testing *in ovo* feeding with glycosaminoglycans and vitamin C has established a new science of neonatal nutrition.

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