UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CAMPUS DE JABOTICABAL

GLICOSAMINOGLICANOS E VITAMINA C NA INCUBAÇÃO E CRIAÇÃO DE FRANGOS DE CORTE

Elaine Talita Santos
Zootecnista

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Elaine Talita Santos
Orientadora: Profª. Drª. Silvana Martinez Baraldi Artoni
Co-orientadora: Profª. Drª. Sarah Sgavioli

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias - Unesp, Campus de Jaboticabal, como parte das exigências para obtenção do título de Doutor em Zootecnia.

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TÍTULO DA TESE: GLICOSAMINOGLICANOS E VITAMINA C NA INCUBAÇÃO E CRIAÇÃO DE FRANGOS DE CORTE

AUTORA: ELAINE TALITA SANTOS
ORIENTADORA: SILVANA MARTINEZ BARALDI ARTONI
COORIENTADOR: SARAH SGAVIOLI

Aprovada como parte das exigências para obtenção do Título de Doutora em ZOOTECNIA, pela Comissão Examinadora:

Profa. Dra. SILVANA MARTINEZ BARALDI ARTONI
Departamento de Morfologia e Fisiologia Animal / FCAV / UNESP - Jaboticabal

Prof. Dr. RICARDO DE ALBUQUERQUE
Faculdade de Medicina Veterinária e Zootecnia / USP - Pirassununga, SP

Profa. Dra. SELMA DE FÁTIMA GROSSI
Centro Universitário Moura Lacerda / Ribeirão Preto,SP

Profa. Dra. LIZANDRA AMOROSO
Departamento de Morfologia e Fisiologia Animal / FCAV / UNESP - Jaboticabal

Prof. Dr. OTTO MACK JUNQUEIRA
Departamento de Zootecnia / FCAV / UNESP - Jaboticabal

Jaboticabal, 06 de fevereiro de 2017

DADOS CURRICULARES DO AUTOR

Elaine Talita Santos - Nasceu no município de Campinas, Estado de São Paulo, no dia 20 de abril de 1986. Em agosto de 2006 iniciou o curso de Graduação em Zootecnia pela Universidade Estadual Paulista – UNESP, Campus de Dracena. Foi bolsista de Iniciação Científica pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), de julho de 2008 a junho de 2009. Também foi bolsista de Iniciação Científica pelo Programa Institucional de Iniciação Científica (PIBIC), de agosto de 2009 a julho de 2010. Participou do Grupo de Experimentação em Nutrição e Adubação de Plantas (GENAP), de março de 2007 a dezembro de 2010, realizando pesquisas e relatórios científicos, além da organização de eventos. Recebeu o título de Zootecnista em dezembro de 2010. Em março de 2011 iniciou o curso de Pós-Graduação em Zootecnia (Mestrado), pela Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP – Campus de Jaboticabal, São Paulo, durante o qual foi bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Recebeu o título de Mestre em Zootecnista em agosto de 2012. Em março de 2013 iníciou as atividades do curso de Doutorado na mesma instituição, onde foi bolsista pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) durante 39 meses. No período de outubro de 2015 a maio de 2016 fez estágio internacional na North Carolina State University (NCState), também fomentado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

“Cuidado meu amigo!!! O ego acadêmico é um grande perigo. Ele pode te aprisionar na bolha do Eu sabedoria máxima e te privar do saber humanizar. ”

(T.S.)

"A dificuldade é um severo instrutor."

(Edmund Burke)

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Protocolo nº 011424/13 do trabalho de pesquisa intitulado **"Injeção de glicosaminoglicanos e vitamina C na incubação sobre os parâmetros de desenvolvimento ósseo, desempenho zootécnico e rendimento de carcaça na criação de frangos de corte"**, sob a responsabilidade da Prof.ª Dr.ª Silvana Martinez Baraldi Artoni está de acordo com os Princípios Éticos na Experimentação Animal, adotado pelo Colégio Brasileiro de Experimentação (COBEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião extraordinária de 4 de junho de 2013.

Jaboticabal, 4 de junho de 2013.

Prof. Dr. Andrigo Barboza De Nardi
Coordenador - CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
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GLICOSAMINOGLICANOS E VITAMINA C NA INCUBAÇÃO E CRIAÇÃO DE FRANGOS DE CORTE

Resumo - O objetivo deste estudo foi verificar quais os efeitos do aditivo com glicosaminoglicanos (sulfato de condroitina e de glucosamina) e vitamina C *in ovo* e na dieta em frangos de corte. Para isso realizaram-se dois experimentos de incubação e um de criação, respectivamente. Foram incubados 490 ovos férteis de frangos de corte, distribuídos em delineamento inteiramente casualizado com cinco tratamentos: ovos não injetados e ovos injetados com 0, 2, 4 e 6 µg de aditivo/100 µL de água no quarto dia da incubação, com 14 repetições de sete ovos por tratamento. Os dados foram submetidos a análise de variância pelo procedimento Modelo Linear Geral (GLM) do SAS. Avaliou-se o efeito dos tratamentos em relação aos parâmetros de incubação, qualidade do pinto pós-eclosão e características ósseas. A nutrição *in ovo* com glicosaminoglicanos (GAGs) e vitamina C não interferiu nos parâmetros de incubação e promoveu melhora do desenvolvimento ósseo nos pintos pós eclosão. No segundo experimento de incubação foram incubados 2.160 ovos férteis de frangos de corte, distribuídos em um delineamento casualizado com dois tratamentos: ovos não injetados e ovos injetados 4 µg de aditivo/100 µL de água no quarto dia da incubação, com 15 repetições de 72 ovos por tratamento. Após a eclosão, 760 aves foram alojadas e distribuídas em esquema fatorial 2x2. Sendo dois tratamentos na incubação (ovos não injetados e ovos injetados com 4 µg de aditivo) e dois tratamentos durante a criação (dieta sem a inclusão de aditivo e dieta com 0,74 g de aditivo/100 kg de ração). O objetivo desse estudo foi determinar se a nutrição *in ovo* ou na dieta com glicosaminoglicanos (sulfato de condroitina e sulfato de glucosamina) e vitamina C influenciaria sobre a macroscopia óssea e da cartilagem, composição mineral, densitometria, resistência e histologia do fêmur e da tíbia, parâmetros sanguíneos, desempenho e rendimento de carcaça e de cortes de frangos de corte aos 42 dias de idade. Os efeitos de 4 µg de aditivo *in ovo* e 0,74 g de aditivo por kg de ração foram submetidos à análise de variância usando o procedimento do Modelo Linear Geral (GLM) do SAS®. Concluí-se que a nutrição *in ovo* ou durante a criação de frangos de corte, com sulfato de condroitina, sulfato de glucosamina e vitamina C, age no desenvolvimento ósseo e cartilaginoso das aves de forma positiva, quando

utilizados isoladamente, e podem contribuir na diminuição de problemas ósseos em frangos de corte.

Palavras-chave: ácido ascórbico**,** condroitina, densitometria óssea, glucosamina, ossos

GLYCOSAMINOGLYCANS AND VITAMIN C IN THE INCUBATION AND REARING OF BROILERS

Abstract - The purpose of this study was to verify the additive effects with glycosaminoglycans (chondroitin sulfate and glucosamine) and vitamin C *in ovo* feeding and diet of broilers. Two incubation experiments and one rearing experiment were carried out, respectively. A total of 490 Cobb® fertile broiler eggs were incubated and distributed in completely randomized experimental design, with five treatments: un-injected egg, in injection egg of 0, 2, 4 and 6 µg additive/100 µL water on day four of incubation. Data were subjected to analysis of variance by the General Linear Model procedure (GLM) of SAS®. Were evaluated the treatments effect in relation the incubation parameters, chick quality post hatching and bone characteristics. *In ovo* feeding with glycosaminoglycans (GAGs) and vitamin C did not interfere on incubation parameters and promoted improvement of bone development in chicks after hatching. Two thousand hundred sixty (2,160) Cobb® fertile broiler eggs were distributed into five teen trays (repetitions) with seventy-two eggs/treatment/tray. After hatching, 760 birds were housed and distributed in a 2x2 factorial design. Were two incubation treatments (un injected eggs and eggs injected with 4 µg of additive) and two rearing treatments (with 0.74 g of additive/100 kg of feed). The purpose of this study was to determine if *in ovo* feeding or feed nutrition with glycosaminoglycans (chondroitin sulfate and glucosamine sulfate) and vitamin C would influence on bone and cartilage macroscopic, mineral composition, densitometry, breaking strength and histology of the femur and tibia, blood parameters, performance and carcass yield of broilers at 42 days of age.The effects of 4 µg additive *in ovo* feeding and 0.74 g/kg additive in feed nutrition were submitted to analysis of variance using the General Linear Model procedure (GLM) of SAS®. We conclude that *in ovo* feeding or during broiler rearing, with chondroitin sulfate, glucosamine sulfate and vitamin C, act on the bone and cartilaginous system development of birds positively, when used isolated and may represent the solution to bone problems in broilers.

Keywords: ascorbic acid, bone, bone densitometry, chondroitin, glucosamine

CAPÍTULO I – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

Os frangos de corte são criados em sistemas intensivos de produção onde as aves são confinadas em alta densidade e seu período de vida até o abate é curto, sendo este de aproximadamente 40 dias (KNOWLES et al., 2008). Devido a essa vertiginosa velocidade do crescimento corporal e deposição de carne, os frangos passaram a apresentar distúrbios no sistema ósseo, aumentando a ocorrência de enfermidades como, deformidades nos digitos, deformidades angulares, discondroplasia tibial, osteoporose do fêmur proximal e necrose da cabeça femoral (OVIEDO-RONDÓN, 2008).

Esses problemas na estrutura óssea afetam não somente o desempenho zootécnico, mas também o bem-estar animal (ALMEIDA PAZ et al., 2009). Estima-se que as ocorrências de problemas de pernas em frangos de corte podem causar perdas economicas que variam entre 10 a 40% do lucro bruto, acarretando prejuízos principalmente por condenações e desclassificações na linha de abate (ALMEIDA PAZ et al., 2008).

A estrutura óssea é composta por um tecido heterogêneo complexo, constituído por células em vários estágios de diferenciação, com quatro funções principais: suportar a musculatura, auxiliar na movimentação, promover o crescimento da ave e servir como reserva mineral (MACARI et al., 2002). O osso constitui-se de 22% de matriz óssea orgânica, 9% de água e 69% de materiais inorgânicos, sendo a matriz orgânica composta predominante por colágeno (90%), que participa no processo de mineralização óssea (ARAÚJO et al., 2006).

O período de incubação pode influenciar a saúde óssea dos frangos de corte (OVIEDO-RONDÓN et al., 2006 a, b). Durante esse período, diversos tipos de suplementos nutricionais como vitaminas, minerais e probióticos podem ser utilizados na nutrição *in ovo* (TAKO et al., 2004; UNI & FERKET, 2004). A suplementação *in ovo* é uma estratégia utilizada durante a incubação para favorecer o desenvolvimento dos sistemas das aves, prevenir o aparecimento de doenças desenvolvidas na fase adulta e aumentar as reservas nutricionais dos pintos pós eclosão (UNI & FERKET, 2004).

Os glicosaminoglicanos (GAGs) polissulfatados, como a glucosamina e a condroitina, possuem ação anti-inflamatória e condroprotetoras, que podem auxiliar na prevenção e no tratamento de alterações ósseas. Essas substâncias reduzem a perda de proteoglicanos e de colágeno, ao inibirem enzimas degradativas da cartilagem (hialuronidase, catepsina, elastase, colagenase e metaloproteinases neutras). Também estimulam a síntese de proteoglicanos e colágeno, sendo capazes de aumentar a proliferação dos condrócitos e a biossíntese da matriz óssea (ALTMAN et al., 1989; CLARK, 1991).

O uso de GAGs tem efeito protetor claramente evidenciado em humanos (LIPPIELLO, 2000; BORGES, 2003). Estudos *in vivo* em coelhos indicam que a glucosamina e o sulfato de condroitina proporcionam preservação da matriz óssea e retardo na progressão de alterações da cartilagem de coelhos com doença articular degenerativa (LIPPIELLO, 2000). Em humanos o tratamento com associação de sulfato de glucosamina e sulfato de condroitina foi eficaz contra osteoartrose e sintomas a ela relacionados (BRANDÃO, 2009).

Paralelo aos GAGs, a vitamina C, é um nutriente essencial para a formação do colágeno e sua deficiência resulta na síntese de molécula de colágeno instável, caracterizada por matriz extracelular anormal e interrupção da ossificação endocondral. Lesson & Summers (2001), relataram que a suplementação de dietas com vitamina C melhora a condição dos ossos das pernas em aves estressadas. Além disto, a vitamina C está envolvida na formação e manutenção do tecido conjuntivo, ossos e cartilagens e possui ação imune estimulante (SPINOSA et al., 2002).

Contudo, não existem trabalhos na literatura que visam prevenir alterações ósseas nas pernas de frangos de corte com a injeção de GAGs (sulfato de glucosamina e sulfato de condroitina) e vitamina C *in ovo* em frangos de corte. Sendo assim, é pertinente a investigação dessas substâncias *in ovo* e na dieta, uma vez que sua utilização pode favorecer a melhora da estrutura óssea, evitando possíveis problemas locomotores e, consequentemente beneficiando a produtividade e o bem-estar de frangos de corte.

2. REFERENCIAL TEÓRICO

2.1 Tecido ósseo

O tecido ósseo pode ser definido como tecido conjuntivo especializado, dinâmico, vascular, formado por matriz orgânica mineralizada e por população heterogênea de células (WATKINS & SEIFERT, 2000). Esse tecido possui funções mecânicas, biológicas e químicas (RHO et al., 1999).

Os processos de modelagem e remodelação é responsável pela formação dos ossos e ocorre por meio da atividade sincronizada de osteócitos, osteoblastos e osteoclastos (LANYON, 1973; UHTHOFF & JAWORSKI,1978; YAIR et al., 2012).

O desenvolvimento do osso é classificado como endocondral, no caso do modelo de cartilagem servir de precursor do tecido ósseo que será formado, ou intramembranoso, se o osso for formado sem a intervenção de um precursor cartilaginoso. Nos ossos chatos predomina o desenvolvimento por ossificação intramembranosa e nos ossos curtos e longos o desenvolvimento é por processo endocondral (MACARI, 1994).

O osso constitui-se de 22% de matriz orgânica, 8% a 9% de água e de aproximadamente 70% de materiais inorgânicos. A matriz orgânica (osteoide) tem como componente predominante o colágeno (90%), que participa no processo de mineralização óssea. Os outros 10% correspondem à substância amorfa (BANKS, 1991; PIZAURO Jr, 2002).

Segundo Suchý et al. (2009), aproximadamente 70 a 80% da massa óssea em frangos de corte é determinada geneticamente, enquanto que os outros 20 a 30% resultantes são atribuídos a fatores externos, sendo a nutrição, fator importante (EASTELL & LAMBERT, 2002), capaz de influenciar diretamente mineralização óssea (HUYGHEBAERT,1997).

Nos últimos dias da incubação o embrião tem reservas limitadas de cálcio e fósforo para a mineralização óssea (YAIR & UNI, 2011). Logo após a eclosão o desenvolvimento ósseo passa por um período crítico, dado que a mineralização do esqueleto é pobre em minerais, o consumo de ração é baixo e o trato gastrointestinal é imaturo (ANGEL, 2007).

2.2 Problemas ósseos em frangos de corte

A seleção genética para o rápido crescimento e o aumento de carne na região do peito em frangos de corte causaram graves transtornos ao sistema esquelético das aves (GARNER et al., 2002). Algumas doenças do sistema locomotor ósseo se tornaram comuns, como por exemplo, discondroplasia tibial, osteomielite ou necrose da cabeça femoral, deformidades angulares, síndrome do osso negro, entre outras (OVIEDO-RONDÓN, 2008; BALDO et al., 2013).

A discondroplasia tibial, comumente chamada de “problema de perna” caracteriza-se por massa cartilaginosa anormal, de cor branca opaca, não vascularizada e com pouca mineralização na cartilagem de crescimento da epífise proximal da tíbia, podendo se estender para a epífise distal dos fêmures e úmeros (FARQUHARSON & JEFFERIES, 2000). Histologicamente ocorre aumento e acúmulo na quantidade de condrócitos pré-hipertróficos e ausência de células hipertróficas (PINNES et al., 1998; PRAUL et al., 2000).

A oesteomielite, também conhecida como necrose da cabeça femoral (MCNAMEE & SMYTH, 2000) é outra claudicação que ocorre no sistema ósseo de frangos de corte, provocando condronecrose bacteriana por meio da contaminação com o *Staphylococcus aureus* (DINEV, 2009). Essa doença causa lesões na epífise proximal do fêmur favorecendo a degeneração articular ou da placa de crescimento da cartilagem e do osso. Com isso, ocorre a separação entre a placa de crescimento e o osso metafisário, além de necrose avascular com a separação e degeneração da massa anormal de cartilagem pré-hipertrófica, com fratura espontânea da cabeça femoral, metáfise ou colo do fêmur (JULIAN, 1985; THORP et al., 1992).

A deformidade angular óssea pode ser observada nos primeiros dias de vida, aproximadamente com seis a oito dias de idade ou na terceira e quarta semana da fase de crescimento. Devido à deformidade anatômica e à dor, o frango reduz o deslocamento até o bebedouro e comedouro, tendo o desempenho e o bem-estar afetados (JULIAN, 1998). Essa deformidade angular óssea está relacionada com o rápido crescimento, o que acarreta em comprometimento do alinhamento e remodelação adequada dos ossos do tibiotarso resultando em curvaturas ou torção das pernas (MCNAMEE & SMYTH, 2000).

A síndrome do osso negro ou “black bone” é causada por um problema na ossificação intramembranosa (BALDO et al., 2013) o que prejudica a qualidade da carne e o bem-estar das aves durante a criação (WHITEHEAD, 2010). Ela é definida como condição em que a superfície do tecido ósseo e muscular adjacente ficam vermelho escuro, marrom ou preto, após o cozimento (SMITH & NORTHCUTT, 2004).

Os consumidores rejeitam carnes com ossos escurecidos, o que pode afetar a comercialização da carne de frango. Algumas redes de “fast-food” evitam a compra de frango com osso, visando a diminuição do problema (LYON & LYON, 2002; BALDO et al., 2013).

2.3 Nutrição *in ovo*

De acordo com Yair et al. (2012) nos últimos dias da incubação as propriedades mecânicas e geométricas do tibiotarso e do fêmur permanecem inalteradas ou podem se deteriorarem nas linhagens de frango de corte atuais.

Em 2003 Uni & Ferket introduziram o conceito de administração de alto volume (0,4 a 1,2 mL) de nutrientes para o líquido amniótico de ovos de frangos de corte e perus durante a incubação, visando "alimentar" o embrião, que consome o líquido amniótico antes da eclosão. Esse conceito vem sendo estudado por outros pesquisadores que comprovaram efeitos positivos sobre o embrião. (FOYE et al., 2007; CHEN et al., 2009).

O ovo contém quantidade finita de nutrientes para que ocorra o desenvolvimento, crescimento embrionário e eclosão. Muitas vezes esses nutrientes podem estar praticamente esgotados durante a eclosão (FOYE et al., 2006). Assim, a nutrição *in ovo* serve como ferramenta para superar limitações precoces do crescimento e desenvolvimento pós eclosão dos frangos de corte (FOYE et al., 2006).

Alguns benefícios da nutrição *in ovo* para o embrião pós eclosão foram relatados por diferentes autores, como: aumento da capacidade digestiva (TAKO et al., 2004, 2005; FOYE, 2006; SMIRNOV et al., 2006; BOHORQUEZ et al., 2007), aumento da taxa de crescimento e da eficiência alimentar (KORNASIO et al., 2011), redução da incidência de distúrbios esqueléticos e melhora na mineralização óssea (De OLIVEIRA 2007; YAIR et al., 2013) e maior desenvolvimento e produção de carne de peito (MOORE, 2005; KORNASIO et al., 2011).

Diversos nutrientes podem ser incluídos na nutrição *in ovo*, entre eles, ácidos graxos, aminoácidos, carboidratos, minerais, vitaminas, entre outros. As limitações quanto as escolhas dos nutrientes devem estar relacionadas com o volume, osmolaridade e viscosidade da solução a ser injetada *in ovo* (UNI et al., 2005). A nutrição *in ovo* com minerais, vitaminas e carboidratos favorecem o desenvolvimento esquelético com benefícios claros sobre o desenvolvimento do sistema ósseo das aves (YAIR et al., 2011).

2.4 Glicosaminoglicanos

Os glicosaminoglicanos (GAGs) são polímeros lineares compostos por unidades dissacarídicas repetitivas onde uma das unidades é invariavelmente uma hexo amina (D-glucosamina ou D-galactosamina) e a outra um ácido hexurônico (glucurônico ou idurônico) ou uma galactose em sequência não ramificada, apresentando substituições de grupamentos sulfatos em várias posições da cadeia polissacarídica (CARNEY & MUIR, 1988).

Sulfato de glucosamina e sulfato de condroitina são exemplos de GAGs sulfatados (GANDHI & MANCERA, 2008), capazes de tratar lesões ósseas (VAUGHAN-SCOTT & TAYLOR, 1997). Os GAGs sulfatados também têm sido utilizados profilaticamente para prevenir perdas de componentes da cartilagem óssea (MCLLWRAITH, 2016). Em humanos a deficiência de GAGs está relacionada com doenças articulares ósseas graves (SIMONARO et al., 2008). Quando à formação de GAGs no organismo de cães e gatos ocorrem de maneira ineficiente, pode acarretar em mucopolissacaridose (MPS). Estudos com diferentes modelos animais revelaram que a MPS causa anormalidade morfogenética primária na placa de crescimento ósseo, tecidos ósseos e estrutura do osso cortical (CLARKE, 2011).

O sulfato de glucosamina, um sal da glucosamina sintetizado artificialmente, ajuda o fluido sinovial a ser resistente e elástico, proporcionando o reestabelecimento do sistema locomotor em casos de lesões (JEROSCH, 2011). A deficiência de glucosamina pode reduzir o índice de produção destas importantes macromoléculas causando o enfraquecimento de tecidos. Durante o trauma a quantidade de glucosamina normalmente sintetizada pelo corpo é insuficiente para o completo reparo (SWEETMAN, 2002). Portanto, o sulfato de glucosamina auxilia o fluido sinovial a ficar

resistente e elástico, proporcionando assim uma tentativa do reestabelecimento do sistema locomotor. A glucosamina é incorporada nas proteínas plasmáticas durante o metabolismo, resultando em 26% de biodisponibilidade, após sua ingestão oral (BARCLAY et al., 1998).

O sulfato de condroitina tem sido investigado em estudos bioquímicos, pois atua na fisiologia da cartilagem articular (PIPITONE, 1991). A condroitina possui propriedades condropotetoras como: ação inibitória de enzimas de degradação da cartilagem (DIAZ et al., 1996) e propriedades condroestimuladoras, que promovem aumento da síntese de proteoglicanos pelos condrócitos (BEALE et al., 1990). O sulfato de condroitina tem modo de ação retardado em casos de tratamento contra osteoartrite (AO) em humanos. Isso significa que os primeiros efeitos dessa substância em relação a dor e mobilidade na AO, ocorre depois de duas a três semanas de terapia (D. UEBELHART, 2008)

Todos os GAGs, exceto o ácido hialurônico são encontrados na forma de proteoglicanos (TOFFOLLETO et al. 2005). Segundo Toffoleto (2005) os condrócitos sintetizam os vários tipos de proteoglicanos presentes nas cartilagens, que formam junto com a matriz colagênica um complexo supramolecular, que dão a este tecido a função de uma mola biológica, capaz de resistir a forças de alta compressão. Portanto, o tratamento com GAGs polissulfados respondem bem a lesões nas articulações de animais (FRISBIE et al., 2016).

Apesar dos problemas locomotores em aves merecerem a atenção de inúmeros pesquisadores, são escassos trabalhos na literatura para prevenir esta disfunção com o uso de glicosaminoglicanos em frangos de corte, sendo estes comuns para outras espécies de animais, como bovinos (de MATTEI et al., 2002) equinos (FENTON et al., 2000a; HANSON et al., 1997) e cães (GONÇALVES et al., 2008; MELO et al., 2008; ROMANO et al., 2006; BIASI et al., 2005; FERNANDES et al., 1998).

2.5 Vitamina C

A vitamina C ou ácido ascórbico é classificada como uma vitamina não essencial para aves, pois as mesmas sintetizam em quantidade suficiente para atender a demanda do organismo (RUTZ et al., 2014). A síntese de vitamina C em frangos de corte ocorre predominantemente nos rins, utilizando como substratos a glicose, manose e frutose (KHAN et al., 2012). Porém, a suplementação com vitamina C na dieta pode trazer benefícios aos frangos de corte, como por exemplo, melhora do perfil colágeno e da fertilidade em machos, redução do nível de cortisol e aumento da tolerância ao estresse térmico (KHAN et al., 2012; RUTZ et al., 2014; MAHMOUD et al., 2014). Esta vitamina é sensível à oxidação (TOLBERT et al., 1975), calor, luz e exposição à agentes pró-oxidantes que reduzem sua atividade (LINSTER & SCHAFTINGEN, 2007).

Estudos *in vitro* com osteoblastos de animais e humanos demostraram que a vitamina C é um componente da formação biológica de células e da massa óssea, e age como precursora da mineralização da matriz óssea estimulando a diferenciação osteoblástica (MORTON et al., 2001). Em dietas suplementadas com vitamina C, Farquharson et al. (1993) relataram redução na incidência de discondroplasia tibial em frangos de corte.

O colágeno contém alguns aminoácidos como a hidroxiprolina e hidroxilisina, formados por um processo dependente de vitamina C, que implica na transferência enzimática de grupos hidroxila para a prolina e a lisina nas cadeias procolágeno. A ausência da Vitamina C, cofator neste processo, prejudica a formação do colágeno (LIBBY & AIKAWA, 2002).

A vitamina C também atua na conversão da vitamina D para a forma metabólica colecalciferol, essencial para a regulação do cálcio e para o processo de calcificação óssea (BAINS, 1985).

A injeção *in ovo* de vitamina C pode ser alternativa à suplementação dietética desta vitamina para pintos. Ipek et al. (2004) em estudo com injeção de vitamina C *in ovo* no 13° dia da incubação, observaram efeito positivo para a eclosão com a injeção de 3 e 5 mg por ovo, diluídos em 0,5 ml de solução.

NOWACZEWSKI et al. (2012), em estudo com a injeção de 3 e 6 mg de vitamina C, dissolvido em 0,1 mL de solução, realizados nos dias 13, 15 e 17 de

incubação, na câmara de ar, observaram aumento da eclodibilidade para o grupo que recebeu 6 mg de vitamina C, no 15º dia da incubação, em relação ao grupo controle (sem perfuração).

Wilson & Jaworski (1992) afirmaram que a concentração plasmática de vitamina C diminuiu no 15º dia da incubação em ovos de galinha White Leghorn, juntamente com a elevação da corticosterona no sangue, que é geralmente associada ao estresse do embrião e com a redução da biossíntese de vitamina C em aves (KUTLU & FORBES, 1994). Portanto, a vitamina C pode ser considerada como agente anti-estresse (PARDUE & THAXTON, 1985), pois reduz a corticosterona, principalmente em embriões incubados em altas temperaturas.

Zakaria & Al-Enezi (1996) em estudo com injeção de vitamina C realizadas aos 11, 15, 17 e 19 dias da incubação, com concentrações de 0,5, 1, 3, e 12 mg por ovo, diluídos em 0,1 mL de solução, observaram melhor eclodibilidade para os ovos injetados com 3 mg da vitamina, enquanto que a dose de 12 mg acarretou em piora desta característica. Além disto, os autores observaram melhores resultados para os ovos injetados com a vitamina nos 11 e 15 dias da incubação; enquanto este efeito foi minimizado nos ovos injetados no dia 19 da incubação.

SELIM et al. (2012) em estudo com a injeção de soluções (0,1 mL de óleo de milho; 0,1 mL de óleo de milho + 10 mg de vitamina E; 0,1 mL de solução salina e 0,1 mL de solução salina + 3 mg de vitamina C) aos 12 dias da incubação de patos, observaram que a injeção *in ovo* da solução, com ou sem vitamina C diminui a eclodibilidade para (68%) em comparação com o controlo não injetado (74%).

GHONIM et al. (2009) ao compararem a injeção de 3 mg de vitamina C, com a imersão dos ovos no ácido em diluição de 20 g/L e a pulverização (30 g/L) em ovos de patos aos 14 dias da incubação, relataram melhora para a eclodibilidade dos ovos pulverizados e imergidos em vitamina C, quando comparado com os ovos injetados.

MOHAMMED et al. (2011) ao avaliarem a imersão de ovos de matrizes em soluções (controle, sem imersão; imersão em soluções contendo 0, 5 e 10 g de vitamina C/L de água destilada durante dois minutos) e exposição a 24ºC durante seis horas no 16º dia da incubação, observaram melhora na eclodibidade dos ovos imergidos em 5g de vitamina C.

SHAFEY et al. (2002) imergiram ovos antes do início da incubação em diferentes soluções (controle, sem imersão; imersão em água; imersão em soluções contendo 10, 20 e 30 g de vitamina C), e observaram que a imersão dos ovos de matrizes com 29 semanas de idade, em solução contendo 10 g de vitamina C aumentou a condutância e a eclodibilidade destes ovos, em relação ao grupo controle. Os autores observaram também que este tratamento aumentou a perda de massa dos ovos.

De acordo com Vick & Brake (1986) os ovos mergulhados por dois minutos em solução contendo 3 g de vitamina C tiveram a maior condutância, no entanto, apresentaram queda na eclodibilidade (SHAFEY et al., 2002).

3. OBJETIVOS

3.1 Geral

Verificar se a suplementação *in ovo* com sulfato de glucosamina, sulfato de condroitina e vitamina C influencia os parâmetros densitométricos e morfométricos do sistema ósseo de frangos de corte.

3.2 Específicos

i.) Se a suplementação *in ovo* com GAGs e vitamina C durante a incubação influencia os parâmetros de incubação e desenvolvimento ósseo de pintos pós eclosão.

ii.) Se a suplementação com GAGs e vitamina C *in ovo* e na dieta influenciam os parâmetros zootécnicos, rendimento de carcaça e de cortes comerciais, desenvolvimento ósseo e parâmetros sanguíneos de frangos de corte.

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CAPÍTULO II – GLICOSAMINOGLICANOS E VITAMINA C *IN OVO* AFETAM CARACTERÍSTICAS ÓSSEAS DE PINTOS DE CORTE PÓS ECLOSÃO

RESUMO - Anomalias esqueléticas, deformidades ósseas e fraturas causam perdas significativas na produção de frangos de corte durante a criação e o processamento. Glicosaminoglicanos (sulfato de condroitina e sulfato de glucosamina) e vitamina C podem ajudar o desenvolvimento ósseo em frangos de corte. Os parâmetros de incubação não foram afetados (P>0,05) pela adição de glicosaminoglicanos e vitamina C *in ovo*. No entanto, os pintos de corte que receberam este aditivo apresentaram aumento significativo (P<0,05) na área óssea da tíbia, densidade mineral óssea do fêmur e resistência óssea do fêmur (força máxima e deformação). Em conclusão, a nutrição *in ovo* com glicosaminoglicanos (GAGs) e vitamina C promove o desenvolvimento ósseo de embriões de pinto de corte. A ausência de literatura com GAGs na nutrição *in ovo* mostra que esses resultados são inovadores. Mais estudos são necessários para determinar o nível ótimo de glicosaminoglicanos e vitamina C, para melhor compreender os efeitos do aditivo no desenvolvimento ósseo.

Palavras-chave: ácido ascórbico**,** cinzas, condroitina, densidade mineral óssea, glucosamina, resistência óssea

CHAPTER II – GLYCOSAMINOGLYCANS AND VITAMIN C *IN OVO* FEEDING AFFECTS BONE CHARACTERISTICS OF CHICKS POST-HATCHING

Artigo redigido de acordo com as normas da revista British Poultry Science

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

Running head: glycosaminoglycans and vitamin c in bone development of chicks

Abstract: 1. Skeletal abnormalities, bone deformities and fractures cause significant losses in broiler production during rearing and processing. Glycosaminoglycans (chondroitin sulfate and glucosamine sulfate) and vitamin C can help the bone development in broilers.

2. Hatchability was not affected (P>0.05) by the addition of glycosaminoglycans and vitamin C. However, broilers receiving this additive showed a significant increase (P<0.05) on tibia area, femur bone mineral density and femur bone strength (maximum force and deformation).

3. In conclusion, *in ovo* feeding with glycosamynoglicans and vitamin C promotes bone development of embryos. The absence of literature with GAGs *in ovo* feeding shows these results becomes innovative.

4. Further studies are required to determine the optimal level of glycosaminoglycans and vitamin C to better understand the effects of additive on bone development.

Key words: ascorbic acid, ash, bone mineral density, bone strength, chondroitin, glucosamine

INTRODUCTION

With the fast body growth, efficiency of food utilization and maximum breast meat deposition, broiler them begin to show disorders in the bone structure, increasing the occurrence of angular deformity and torsional, tibial dyschondroplasia, rickets, osteochondrosis and femoral necrosis (Almeida Paz *et al.*, 2010).

During incubation period the embryo has mineral resources for the skeleton development, but these resources can be also used to the development and embryonic growth (Angel, 2007).

In ovo feeding can be regarded as a supplemental nutrition for the embryo, besides the yolk sac (Dos Santos *et al.*, 2010), which may favor the development of the embryo and the quality of chicks post-hatch (Uni and Ferket, 2004).

Glucosamine sulfate is derived from natural monosaccharide glucosamine sulfate (Reginster *et al.*, 2001; Wang *et al.*, 2007). Glucosamine is a constituent of glycosaminoglycan found in cartilage and synovial fluid (Hamerman, 1989), with different pharmacological action on cartilage and joint tissues (Reginster *et al.*, 2001). When administered exogenously, glucosamine exerts specific pharmacological effects on osteoarthritic cartilage and chondrocytes (Hamerman, 1989; Rovati *et al.*, 2012; Kucharz *et al.*, 2016).

Chondroitin sulfate it's compound of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid (Nakano et al., 2010). The chondroitin is also found in high concentrations in cartilage tissues (Nakano *et al.*, 2012).

The glycosaminoglycans (GAGs) polysulfated as glucosamine and chondroitin have anti-inflammatory action and are pharmacologically classified as symptomatic drugs slow-acting, that reduce the symptoms of bone disorders such as osteoarthritis in humans and some animal

species (Brandt *et al.*, 2002). They reduce the loss of proteoglycans and collagen, to inhibit cartilage degrading enzymes (hyaluronidase, cathepsin, elastase, collagenase and neutral metalloproteinases). Also stimulates the synthesis of proteoglycans and collagen, increasing the proliferation of chondrocytes and bone matrix biosynthesis (Altman *et al.*, 1989; Clark, 1991).

GAGs may be used in the prevention and treatment of bone human disorders (Reginster *et al.*, 2001; Simonaro *et al.*, 2008), horses (Fenton *et al.*, 2000) and dogs (Gonçalves *et al.*, 2008; Melo *et al.*, 2008)

Ascorbic acid participates in several biological processes; it assists collagen synthesis and maintenance; it is essential for formation and maintenance of connective tissue, bone and cartilage; it, stimulates the immune system and enhances disease resistance (Berzina *et al.*, 2013; Sgavioli *et al.*, 2013; Chand *et al.*, 2014). Finally, ascorbic acid influences the development of the leg bones in stressed birds (Lesson and Summers, 2001).

However, no research has been published regarding the application of GAGs to prevent broiler locomotor issues. The purpose of this research was study the use of GAGs and vitamin C *in ovo* feeding, considering this additive might avoid possible locomotor problems in commercial broilers production.

MATERIALS AND METHODS

This study was conducted in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and the experimental procedures were approved by the local Committee for Ethical Animal Use (CEUA - protocol n. 011424/13) of the College of Agricultural and Veterinary Sciences, Sao Paulo State University, Jaboticabal, SP, Brazil.

Birds, housing and experimental design

Four hundred and ninety (490) Cobb® fertile broiler eggs from 47-week-old breeders were distributed according to average egg weight (67 ± 1.16g) into 14 trays (repetitions) with seven eggs/treatment/tray (48 eggs/treatment). The eggs were incubated (CASP® model IHM Line e-V A 06, Amparo, Sao 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; 85.7 and 84.0%, respectively, with 504 hours of incubation duration.

The eggs were distributed in to a completely randomized experimental design, with five treatments: un-injected egg, in injection egg with 0, 2, 4 and 6 μg additive/100 μL water on fourth day of incubation. Each 100 g of additive contained 30 g of chondroitin sulfate, 30 g of glucosamine and 5 g of vitamin C (Synth, 99% purity, Diadema, Brazil) and vehicle q.s 100 g. The chondroitin sulfate [(C14H21NO14S)n, Infinit Mutraceutic Inc.] had a purity of 91.35% and the potassium glucosamine sulfate [(C6H14NO5)2 SO4 x 2KCl, Zheijiang Freemen Inc.] had sulfate content of 15.7%.

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 x 0.38 (27.5 G1/2")]. Then, 100 μL of aqueous additive solution was injected in the albumen, about six mm below the membrane. The solution was prepared with ultra-pure water autoclaved in the dark due to its photosensitivity. After injection, the hole was sealed with a label identifying the treatment and repetition.

Hatchability, embryo mortality and body weight

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

The relative weight (%) of chicks post hatching was obtained from the absolute chicks weight (g) in relation to the egg weight (g).

Egg mass loss and eggshell conductance

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

Eight birds/treatment were killed on day of age using a total of 40 animals. The birds were killed by electro sensitization followed by bleeding.

Mineral density, area and length bone

Bone mineral density (BMD) in (g/cm^2), area (cm^2) and lenght were determined in the left femur and tibia. Bone mineral density, area and lenght were measured using dual-energy X-ray absorptiometry (DXA), calibrated by the manufacturer (Hologic®), with a small animal software of Hologic®. Clean bones were placed in 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 were assessed using a digital micrometer (model MDC-Lite, 0.001-mm resolution, Mitutoyo, Suzano, SP, Brazil)

Bone breaking strength

The left femur and tibia were used for the mechanical bone strength tests (three-point bending). The tests were conducted using an EMIC® (model DL 3000) universal testing machine (UMT). The load was applied at a rate of five mm/min with a force of 2000 N to determine the maximum permissible force (Fmax) of the bone and amount of deformation (bend) caused by the Fmax. The bones were fixed on two supports (two points), with the span adjusted according to the size of the smallest bone. Force was then applied at the bone's geometric mean point between the two supports (the middle third of the bone) and the equipment recorded the results. These variables express bone strength at the ends of the bone and the middle third.

Bone mineral composition

The right femur and tibia were used to determine bone calcium, phosphorus, and ash content. The soft tissue was removed and the bones boiled in deionized water for five minutes. After drying at environmental temperature, the samples were immersed in petroleum ether for 48 hours, dried in a forced-ventilation oven at 60º C for 48 hours, and then ground in a ball mill. Bone mineral content was analyzed at the Feed Analysis Laboratory of the College of Agriculture and Veterinary Sciences of the São Paulo State University (UNESP - Jaboticabal), Jaboticabal, SP Brazil, using wet analyses. Ash content was determined by burning the samples at 600º C. The methods were applied according to Silva and Queiroz (2002), and expressed as percentage of defatted dry matter or mineral matter.

Weight and length liver

Relative weight of each liver (Bel S1002, 1000g x 0.01g, Piracicaba, SP, Brazil) was calculated as percentage relative to each chicks body weight. The length of left and right lobe liver were assessed using a digital micrometer (model MDC-Lite, 0.001-mm resolution, Mitutoyo, Suzano, SP, Brazil).

Statistical analysis

The effects of incubation treatments (0, 2, 4 and 6 µg additive) on all studied parameters were statistically analyzed using the model described in equation (1).

$$Yij= \mu + Ti + eij. \text{ Eq. 1,}$$

where Y = the studied parameters, µ = the mean value of the parameter, Ti= treatments (0, 2, 4 and 6 µg additive), and eij = the residual error.

Data were subjected to analysis of variance by the General Linear Model procedure (GLM) of SAS® (SAS Institute, 2002). Data were 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).

The comparison of means was performed by 5% probability polynomial orthogonal contrasts: contrast 1: comparison between the treatment un injected versus the control average of treatments with 0, 2, 4, and 6 µg additive; contrasts 2, 3, and 4: comparisons using regression models: linear, quadratic, and cubic (Robbins *et al*., 1979), with the purpose of verifying the effects of additive application. For embryonic mortality data were analysis for frequency by Chi-square test at 5% probability

RESULTS

The hatchability, egg mass loss, conductance, chicks relative weight (Table 1) and embryonic mortality (Table 2), were not different by the contrasts analyzed (P> 0.05).

Insert Table 1

However, injected eggs with 4 µg of additive had higher hatchability (P> 0.05), when compared to other treatments (87.14%) and lower intermediate and late mortality (3.51 and 12.86%, respectively) when compared to the other treatments. (P> 0.05).

Insert Table 2

For tibia area and femur bone mineral density (BMD) there was quadratic effect (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 +0.0012 additive + 0.0196 (R^2=0.60), additive injection with 6 and 2 µg increases the tibia area and femur BMD, respectively.

There was contrast 1 effect (un-injected eggs versus injected eggs) to the femur area (P = 0.0175), a lowest chicks femur area with injected eggs (0.37 cm^2) when compared with chicks femur area of un-injected eggs (0.40 cm^2) (Table 3).

There was contrast 1 effect (un-injected eggs versus injected eggs) for femoral bone mineral density (P = 0.0296), with highest chicks femur bone mineral density of injected eggs (0.020 g/cm^2), when compared to chicks femur BMD of un-injected eggs (0.017 g/cm ^{2}) (Table 3).

Insert Table 3

The femur maximum force and deformation there was cubic effect (P = 0.0234 and P = 0.0175, respectively) (Table 4). According to equations: Maximum force$_{femur}$= 0.1819 additive^3 -1.7613 additive^2 +4.31 additive +7.6 (R^2=0.98) and Deformation$_{femur}$= 0.0144 additive^3 -0.1363 additive^2 +0.31 additive + 0.6 (R^2=0.97), additive injection 2 μg increases the femur maximum force and the deformation.

Insert Table 4

The femur and tibia concentration of phosphorus (P) there was a linear effect (P = 0.0343 and P = 0.0353, respectively) for all injected 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 increase, there is a reduction in P concentration of the chicks femur and tibia.

Insert Table 5

There was no treatments effect (P > 0.05) for the liver parameters analyzed in birds (Table 6).

Insert Table 6

DISCUSSION

In ovo feeding of chondroitin sulfate, glucosamine sulfate and vitamin C were evaluated in the ability to promote changes in bone and cartilages development post-hatch. Promoting better embryo development of cartilage and skeletal system during on incubation can be solution to the broilers problems during growth phase.

The greater area of the femur bone mineral density (BMD) was observed with injection of 6 and 2 µg of the additive (Table 3), respectively, demonstrate the efficiency of glycosaminoglycans (GAGs) and vitamin C in bone development of broiler chickens post-hatching. This is an interesting data *in ovo* feeding evidenced by first time in the literature, and those findings suggest that embryos chickens can be need high concentrations of glucosamine sulfate, chondroitin sulfate and vitamin C during the incubation.

Biomechanical parameters are direct indicators of bone quality and include by bone mineral density (Barreiro *et al.*, 2011) and bone maximum flexibility strength (Reis *et al.*, 2011). The maximum force to bone break is correlated with the bone resistance to break, therefore, greater maximum force refers to a bone that is more resistant and less likely to break. While the deformation found at maximum force, it relates with the bone ability to undergo physical deformation, without its breaking. Therefore, greater value of the maximum force and flexibility of this bone, occurs to avoid rupture. The femur better maximum force and deformation performed on egg chickens injected with 2 µg additive (Table 4). These results highlight the importance of *in ovo* feeding with GAGs and vitamin C in bone strengthening of post-hatching chickens.

Bone deformities and fractures are severe health problems in modern, rapidly growing broilers, because the genetic improvement on performance has not included genetic improvement on bone development as well (Almeida Paz *et al.*, 2010). The use of GAGs to minimize locomotor issues has been studied in several species. For instance, 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; Eleotério *et al.*, 2012). However, no research has been published regarding the use of GAGs *in ovo* feeding or feed nutrition of broilers and laying hens.

The GAGs stimulate the proteoglycans and collagen synthesis and are capable of increasing the chondrocyte proliferation and the biosynthesis of the matrix (Clark, 1991). These effects support the hypothesis that the changes in the joint cartilage can be alleviated, and the use of the GAGs might complement the treatment (Kantor *et al*., 2014). Being the most used GAGs, the glucosamine sulfate and chondroitin sulfate.

Ascorbic acid participates in several biological processes as, collagen synthesis and maintenance, formation and maintenance of connective tissue, bone and cartilage, stimulate the immune system and enhances disease resistance (Berzina *et al*., 2013; Sgavioli *et al*., 2013; Chand *et al*., 2014).

There are several studies on the effects on the *in ovo* feeding with injection of ascorbic acid (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) when injecting 6 µg of ascorbic acid *in ovo* before incubation, were not observed beneficial effects on bone development (femur and tibia densitometry, mineral density and bone strength, respectively) of birds at 42 days of age.

A lower percentage of bone phosphorus can influence in bone mineralization and also affecting the broiler bone mechanical quality. According to Vargas *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 the bones. However, the lowest tibia and femur phosphorus mineral composition did not affect the density and bone strength parameters ($P>0.05$) (Table 5). Thorp and Waddington (1997) observed that broiler bones with high amount of calcium had fewer fractures during processing. Crespo *et al.* (2002) also found a lower femur fractures incidence in adults birds and turkeys with high percentage of calcium.

The absence of treatments effects on embryo mortality (Table 2), especially initial mortality and hatchability and incubation parameters (Table 1), indicate that *in ovo* feeding with GAGs and vitamin C at fourth day of incubation, did not result in compromised embryo development, showed positive results for hatchability of eggs injected with 4 µg of the additive (Table 1).

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

Effect of percentage vitamin C injected on hatchability was recorded by Ipek et al. (2004) and Nowaczewski *et al*. (2012), which observed improvement in the hatchability when injecting different concentrations of ascorbic acid *in ovo* at 13th days; 13,15 and 17th days of incubation, respectively.

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

Jochemsen and Jeurissen (2002) they reported that the *in ovo* injection age is performed can affect where the product is injected. According to Ohta and Kidd (2001), *in ovo* injection of products must be performed in extra embryo cavity or yolk sac at 7th day of incubation to avoid hatchability reduction and benefit the embryo development.

In addition, the liver morphometry of the birds was not impaired by treatments (Table 6), these results are interesting, can indicate that the additive injection did not affect the development of the embryos hepatic system. According to Barclay *et al*. (1998), approximately 87% of glucosamine an oral dose was absorbed from the gastrointestinal tracts of dogs and the oral

bioavailability in humans following first-pass metabolism by liver was 26%. Radiolabeled glucosamine initially appears in the plasma compartment and subsequently distributes into liver, kidney and cartilage (Barclay *et al*., 1998).

In conclusion, *in ovo* feeding with GAGs and vitamin C promotes bone development of embryos. The absence of literature with GAGs *in ovo* feeding shows these results becomes innovative and supports the hypothesis that *in ovo* feeding in the initial incubation phase indicates a possible solution for bone development problems of broilers. Our research *in ovo* feeding with GAGs and vitamin C has established a new science of neonatal nutrition. However, many studies must be done *in ovo* feeding to use this additive in commercial scale.

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Table 1. *Additive injection of GAGs (chondroitin sulfate+glucosamine sulfate) and vitamin C in ovo on hatchability, egg mass loss, eggshell conductance and relative chicks weight.*

Treatments	Hatchability	Egg mass loss (%)	Eggshell conductance	Relative chicks weight (%)
Un-inject eggs	84.29	11.08	0.464	73.73
0 µg adittive[1]	84.76	10.91	0.457	73.81
2 µg additive	83.81	10.25	0.429	72.69
4 µg additive	87.14	10.33	0.432	72.74
6 µg additive	83.81	10.42	0.437	72.79
SEM	15.50	1.97	0.08	1.90
CV (%)	18.42	18.73	18.74	2.84
		Probability		
Un-inject eggs vs inject eggs	0.8953	0.1691	0.1688	0.4544
Linear effect	0.9792	0.3988	0.3968	0.4539
Quadratic effect	0.7687	0.3031	0.3011	0.5063
Cubic effect	0.5452	0.6584	0.6597	0.7557

[1]Additive composition: Each 100 g of additive contained 30 g of chondroitin sulfate, 30 g of glucosamine, 5 g of vitamin C and vehicle q.s 100 g. Probability: P>0.05.

Table 2. *Additive injection of GAGs (chondroitin sulfate+glucosamine sulfate) and vitamin C in ovo on embryonic mortality frequency (initial, intermediate and late).*

Treatments	Initial 0-7 days	Intermediate 8-14 days	Late 15-21 days
		%	
Un-inject eggs	1.12	5.61	15.72
0 µg adittive[1]	0.95	6.67	15.24
2 µg additive	2.86	7.62	16.19
4 µg additive	1.17	3.51	12.86
6 µg additive	2.86	4.76	16.19
Probability	0.2650	0.2410	0.2200

[1]Additive composition: Each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine and 5 mg of vitamin C and vehicle q.s 100 g. Probability: P>0.05.

Table 3. *Additive injection of GAGs (chondroitin sulfate+glucosamine sulfate) and vitamin C in ovo on femur and tibia length, bone area and bone mineral density (BMD) of chicks post-hatch.*

Treatments	Femur			Tibia		
	Length (cm)	Area (cm^2)	BMD (g/cm^2)	Length (cm)	Area (cm^2)	BMD (g/cm^2)
Un-inject eggs	2.04	0.40	0.017	2.99	0.49	0.023
0 µg adittive[1]	2.03	0.37	0.020	2.97	0.51	0.021
2 µg additive	2.09	0.37	0.020	2.97	0.49	0.023
4 µg additive	2.10	0.36	0.022	3.05	0.49	0.022
6 µg additive	2.11	0.37	0.018	3.08	0.53	0.021
SEM	0.08	0.02	0.002	0.10	0.04	0.004
CV (%)	4.31	5.84	11.59	3.47	7.62	20.72
			Probability			
Un-inject eggs vs inject eggs	0.3374	0.0175*	0.0296*	0.6248	0.5865	0.6328
Linear effect	0.1662	0.9607	0.6325	0.0570	0.2940	0.8109
Quadratic effect	0.5069	0.5261	0.0333*	0.7487	0.0315*	0.5339
Cubic effect	0.7804	0.5329	0.0622	0.4569	0.7235	0.9047

[1]Additive composition: Each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine and 5 mg of vitamin C and vehicle q.s 100 g. *Significant $P<0.05$.

Table 4. *Additive injection of GAGs (chondroitin sulfate+glucosamine sulfate) and vitamin C in ovo on femur and tibia maximum force and deformation of chicks post hatch.*

Treatments	Femur		Tibia	
	Maximum force	Deformation	Maximum force	Deformation
	(N)	(mm)	(N)	(mm)
Un-inject eggs	8.47	0.62	6.45	0.88
0 µg adittive[1]	7.60	0.60	5.79	1.15
2 µg additive	10.63	0.79	5.75	1.13
4 µg additive	8.30	0.58	5.59	1.13
6 µg additive	9.34	0.66	6.06	1.47
SEM	1.78	0.13	1.26	0.34
CV (%)	21.13	21.93	21.90	29.73
	Probability			
Un-inject eggs vs inject eggs	0.6002	0.6386	0.3178	0.0641
Linear effect	0.4275	0.9008	0.8066	0.1753
Quadratic effect	0.2257	0.4068	0.6567	0.2322
Cubic effect	0.0234*	0.0175*	0.7590	0.6149

[1]Additive composition: Each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine and 5 mg of vitamin C and vehicle q.s 100 g. *Significant P<0.05.

Table 5. *Additive injection of GAGs (chondroitin sulfate+glucosamine sulfate) and vitamin C in ovo on femur and tibia ashes, calcium and phosphorus contents of chicks post hatch.*

Treatments	Femur			Tibia		
	Ash	Ca	P	Ash	Ca	P
	(% DM[2])	(% Ash)		(% DM[2])	(% Ash)	
Un-inject eggs	25.12	38.13	18.97	25.57	34,61	17.77
0 µg adittive[1]	24.58	38.59	19.30	25.02	35,15	18.21
2 µg additive	24.80	39.59	19.22	25.76	35,74	18.03
4 µg additive	26.02	38.45	18.96	25.80	36,47	18.04
6 µg additive	24.64	37.83	18.68	24.57	34,21	17.46
SEM	1.02	1.28	0.19	1.03	0.68	0.21
CV (%)	9.77	8.02	2.62	9.97	4.95	2.91
	Probability					
Un-inject eggs vs inject eggs	0.9281	0.7550	0.7841	0.8208	0.3748	0.5168
Linear effect	0.7684	0.5684	0.0353*	0.7884	0.5329	0.0343*
Quadratic effect	0.4418	0.5375	0.6300	0.3605	0.0638	0.3752
Cubic effect	0.4332	0.6444	0.8596	0,9024	0.3379	0.4239

[1]Additive composition: Each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine and 5 mg of vitamin C and vehicle q.s 100 g. *Significant $P<0.05$.[2] Dry matter.

Table 6. *Additive injection of GAGs (chondroitin sulfate+glucosamine sulfate) and vitamin C in ovo on relative liver weight and length of right and left lobe of chicks post hatch.*

Treatments	Relative liver weight	Right lobe length	Left lobe length
	(%)	(cm)	(cm)
Un-inject eggs	1.96	1.57	2.03
0 µg adittive[1]	2.09	1.74	2.14
2 µg additive	2.09	1.68	2.10
4 µg additive	2.18	1.86	2.06
6 µg additive	1.91	1.66	2.14
SEM	0.21	0.16	0.18
CV (%)	12.64	10.59	9.61
		Probability	
Un-inject eggs vs inject eggs	0.3875	0.0570	0.4222
Linear effect	0.3869	0.8689	0.9217
Quadratic effect	0.2726	0.3933	0.5120
Cubic effect	0.3910	0.0990	0.7684

[1]Additive composition: Each 100 mg of additive contained 30 mg of chondroitin sulfate, 30 mg of glucosamine and 5 mg of vitamin C and vehicle q.s 100 g.

CAPÍTULO III – NUTRIÇÃO *IN OVO* E DURANTE A CRIAÇÃO COM GLICOSAMINOGLICANOS E VITAMINA C SOBRE PARÂMETROS ÓSSEOS DE FRANGOS DE CORTE

RESUMO - O objetivo deste estudo foi determinar se a nutrição *in ovo* e durante a criação com glicosaminoglicanos (sulfato de condroitina e sulfato de glucosamina) e vitamina C influenciariam a macroscopia óssea e da cartilagem, a composição mineral (BMC), a densidade mineral (BMD), a resistência óssea, a avaliação histológica (ossos e cartilagem), os parâmetros sanguíneos, o desempenho e o rendimento de carcaça e de cortes comerciais em frangos de corte. Para isso, ovos férteis de matrizes de corte (Cobb®) foram submetidos a dois tratamentos na incubação: ovos não injetados e ovos injetados com 4 µg de aditivo/100 mL de água, no quarto dia da incubação. Após a eclosão, os pintos dos dois tratamentos da incubação foram submetidos a tratamentos durante a fase de crescimento (dieta sem e com 0,74 g de aditivo/kg de ração). Utilizou-se o delineamento inteiramente casualizado em arranjo fatorial 2x2, sendo dois tratamentos durante a fase de incubação e dois tratamentos durante a fase de crescimento. Os dados foram submetidos à análise de variância utilizando o procedimento do Modelo Linear Geral (GLM) do SAS® (SAS Institute, 2002). Na nutrição *in ovo* com 4 µg de aditivo o maior peso da cartilagem da epífise proximal do fêmur foi observado para as aves alimentadas com 0,74 g de aditivo/kg de ração (P=0,0098). A maior espessura da cartilagem da epífise proximal da tíbia foi observada nas aves que receberam nutrição *in ovo* com 4 µg de aditivo e dieta com 0,74 g de aditivo/kg de ração (P=0,0453). Os maiores valores de cinzas, fósforo, cálcio, BMD e BMC foram para o fêmur e a tíbia das aves que receberam 4 µg de aditivo *in ovo* e não receberam aditivo durante a criação ou para as aves do tratamento de ovos não injetado e dieta com 0,74 g de aditivo/kg de ração durante a criação. A nutrição *in ovo* com 4 µg de aditivo reduziu (P=0,0008) o número de condrócitos da cartilagem da epífise proximal da tíbia e aumentou (P=<0001) o número de osteócitos da diáfise da tíbia de frangos aos 43 dias de idade. Concluímos que a nutrição *in ovo* ou durante a criação de frangos de corte, com sulfato de condroitina, sulfato de

glucosamina e vitamina C, age no desenvolvimento dos ossos e sistema cartilaginoso das aves de forma positiva, quando utilizados isoladamente, e podem representar a solução para problemas ósseos em frangos de corte.

Palavras-chave: ácido ascórbico, cartilagem, condroitina, densidade mineral óssea, glucosamina

CHAPTER III - *IN OVO* FEEDING AND DURING REARING WITH GLYCOSAMINOGLYCANS AND VITAMIN C ON BONE PARAMETERS OF BROILERS

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ABSTRACT

The purpose of this study was to determine if *in ovo* feeding and rearing feed with glycosaminoglycans (chondroitin sulfate and glucosamine sulfate) and vitamin C would influence on bone and cartilage macroscopic, mineral composition (BMC), mineral density (BMD) and area, bone breaking strength, histological evaluation (bone and cartilage), blood parameters, performance and carcass yield of broilers. Fertile eggs from breeders (Cobb®) were submitted to incubation treatments as follow: un injected egg and injection egg of 4 μg additive/100 μL water on day four of incubation. After hatching, chicks from the two incubation treatments were submitted to treatments during the growth phase (diet without and with 0.74 g of additive/kg of feed). A completely randomized design in 2x2 factorial arrangement was applied, with two treatments during the incubation phase and two treatments during the growth phase. The data were submitted to analysis of variance using the General Linear Model procedure (GLM) of SAS® (SAS Institute, 2002). *In ovo* feeding with 4 μg of additive resulted in highest cartilage weight of the femur proximal epiphysis for birds feeding with 0.74 g of additive/kg of feed (P=0.0098). The tibia cartilage thickness of the proximal epiphysis, the highest thickness was observed for birds that received feed with 4 μg *in ovo* feeding and in feed with 0.74 g/kg of additive (P=0.0453). The highest values of ashes, phosphorus, calcium, BMD and BMC were highest for the femur and tibia, were with 4 μg of additive *in ovo* feeding and not feeding with additive during the rearing or for birds of treatment of un injected eggs and diet with 0.74 g of additive/kg of feed during rearing. *In ovo* feeding with 4 μg of the additive reduced (P=0.0008) the chondrocytes number of tibia cartilage of the proximal epiphysis (P=<.0001) and increased osteocytes number of tibia diaphysis of broiler at 43 days of age. We conclude that *in ovo* feeding or during broiler rearing, with chondroitin sulfate, glucosamine

sulfate and vitamin C, act on the bone and cartilaginous system development of birds positively, when used isolated and may represent the solution to bone problems in broilers.

Keywords: ascorbic acid, bone mineral density, cartilage, chondroitin, glucosamine

INTRODUCTION

The genetic selection for fast growth and more meat deposition in the breast of broilers may result in economic losses during rearing. Among other factors, these losses involve the occurrence of locomotor problems, related with bone development deficiency in birds (Nääs *et al*., 2012). The bone deformations and fractures incidence are serious problems in the poultry production and cause decrease in performance due to the difficulty of access to water and feed (Almeida Paz *et al*., 2008). The occurrence of bone problems during birds rearing is responsible for economic losses worldwide (Almeida Paz *et al*., 2010).

In ovo feeding is a strategy used to improve the development of specific embryo systems or increase the nutritional reserves of chicks post-hatching (Langley-Evans, 2015, Roto *et al*., 2016). This management implicates in solutions injection, where the content may vary according to purpose, but is based on the use of vitamins, minerals, amino acids, probiotics or a pool of nutrients (Bednarczyk *et al*., 2011; Yair & Uni, 2011; Roto *et al*., 2016). According to Oviedo-Rondón *et al*. (2013) the egg nutritional composition can benefit the embryonic development, with positive effect during the birds.

The glucosamine sulfate assists the synovial fluid stay more resistant and elastic, providing restoration of the locomotor system in case of injury (Toffoletto *et al*., 2005), they also have chondroprotective properties, which inhibit the cartilage enzymes degradation (Diaz

et al., 1996) and chondrostimulatory properties, which further increased of proteoglycans synthesis by chondrocytes (Beale *et al*., 1990).

The chondroitin sulfate has been investigated in biochemical studies because it is involved on the articular cartilage physiology (Pipitone, 1991). In experiments with cultures of collagen matrix cells, chondroitin sulfate was positive on cell adhesion and cell proliferation (Wollenweber *et al*., 2006).

The glucosamine sulfate and chondroitin sulfate are examples of glycosaminoglycans (GAGs) (Gandhi & Mancera, 2008). The polysulfated glycosaminoglycans are able to treat bone lesions (Kantor *et al*., 2004). They have anti-inflammatory action, reduce the proteoglycan and collagen losses, increase chondrocyte proliferation and bone matrix biosynthesis (Altman *et al*., 1989; Clark, 1991).

The vitamin C is involved in several biological processes, including collagen formation and maintenance, is essential for the development of connective tissue, bone and cartilage and stimulates the immune system by improving disease resistance (Chand *et al*., 2014). *In vitro* studies with animal and human osteoblasts, the vitamin C demonstrated to be a component of biological cell formation and the bone mass and acted as precursor to bone matrix mineralization stimulating osteoblastic differentiation (Morton *et al*., 2001). Diets supplemented with vitamin C reduced the incidence of bone problems, as tibial dyschondroplasia in broilers (Farquharson *et al*., 1993).

Although there are data on *in ovo* feeding (Sgavioli *et al*., 2016) and nutrition during rearing (Lohakare *et al*., 2005; Ogunwole, 2015) with vitamin C, the goal to promote the birds bone development and prevent bone problems, there is no published data on effect of *in ovo* feeding with GAGs, well as its combination with vitamin C and the provision of both during

broiler rearing, with the purpose of support the bird's cartilage development and thus to prevent broilers leg problems.

In the present study, we examined the effects of GAGs and vitamin C (*in ovo* feeding and feed nutrition) on bone and cartilage macroscopic, mineral composition, mineral density and area, bone breaking strength, histological evaluation (bone and cartilage), blood parameters, performance and carcass yield of broilers. This study is very important, because it can provide data on the potential use of GAGs and vitamin C and prevent possible locomotor problems in commercial broilers production due to the improvement in structure of the locomotor system (bones and joints).

MATERIALS AND METHODS

This study was conducted in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and the experimental procedures were approved by the local Committee for Ethical Animal Use (CEUA - protocol n. 011424/13) of the College of Agricultural and Veterinary Sciences, Sao Paulo State University, Jaboticabal, SP, Brazil.

Birds, housing and experimental design

Two thousand hundred sixty (2,160) Cobb® fertile eggs from 50-week-old breeders were distributed according to average egg weight (67 ± 1g) into five teen trays (repetitions) with seventy-two eggs/treatment/tray. The eggs were incubated (CASP® model IHM Line e-V A 06, Amparo, SP, Brazil) equipped with automatic temperature, humidity, and egg-turning control. The eggs were turned every 2 hours, with initial and final temperatures and humidity were 37.8 and 36.9 °C; 85.7 and 84.0%, respectively, with 504 hours of incubation duration.

A completely randomized design in 2x2 factorial arrangement was applied, with two treatments during the incubation phase (un injected egg and injection egg of 4 μg additive/100 μL water on day four of incubation) and two treatments during the growth phase (diet without and with additive).

For each 100 g of additive injected contained: 30 g of chondroitin sulfate, 30 g of glucosamine and 5 g of vitamin C (Synth, 99% purity, Diadema, Brazil) and vehicle q.s. 100 g. The chondroitin sulfate [(C14H21NO14S)n, Infinit Mutraceutic Inc.] had a purity of 91.35% and the potassium glucosamine sulfate [$(C6H14NO_5)^2$ SO_4 x 2KCl, Zheijiang Freemen Inc.] had sulfate content of 15.7%.

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 x 0.38 (27.5 G1/2")]. Then, 100 μL of aqueous additive solution was injected in the albumen, about 6 mm below the membrane. The solution was prepared with ultra-pure water autoclaved in the dark due to its photosensitivity. After injection, the hole was sealed with a label identifying the treatment and repetition.

After hatching, the male with one-day old chicks were housed in 76 pens with 19 birds each, resulting in 10 replicates per treatment, totalizing 760 birds. The chicks were reared until 42 days of age. The following vaccination program was completed during the experimental period: IBD (mild strain) on 7^{th} day of age using eye drops; Newcastle disease, and IBD (hot strain) through the drinking water, using powdered milk as carrier (2 g L^{-1}) on day 14^{th}.

The house controlled of temperatures were done with ventilation, exhaust fans and pad cooling system. The temperature and relative humidity were daily recorded using two digital thermo-hygrometers (Instrutemp, ITHT 2250, SP, Brazil) placed at the bird's height in two equidistant places. The average and absolute maximum temperatures were 30°C and 34.1°C,

respectively, while the average and absolute minimum temperatures recorded were 18.6°C and 13.4°C, respectively. The average and absolute maximum relative humidity were 73.36% and 36.18%, and the average and absolute minimum relative humidity was 27.82% and 15.00%, respectively.

The broilers were reared on wood shavings using the amount of 1.2 kg of dry substrate per pen. The adopted light regimen was 24L: 0D (light: dark) regimen during the trial.

Feed and water were provided *ad libitum*. The corn and soybean-meal based diets (Table 1) were formulated according to rearing phase (starter: 1 to 21 days old; grower-finisher: 22 to 42 days old), following the nutritional requirements established by Rostagno *et al*. (2011). For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C, respectively.

For the following analyzes, 6 birds per treatment were sacrificed with 43 days of age, totaling 24 birds. After the stunned with CO_2 the birds were killed by exsanguination by cutting the jugular vein and collected the left femur and tibia bone, respectively.

Bone macroscopy

For bone macroscopy analysis, the right femur and tibia were weighed in a precision analytical balance (Toledo®, model Adventurer AR1530, São Bernado do Campo, SP, Brazil) and the proximal, diaphysis and distal epiphysis of tibia and femur thicknesses of mesured with micrometer (Mitutoyo, model MDC-Lite, Suzano, SP, Brazil).

Were used the bone saw machine (Becker®, model SBP-17, Brusque, SC, Brazil) for sawed the bone in the longitudinal direction. The trabecular bone tissue was gently removed and weighed in a precision analytical balance (Toledo®, model Adventurer AR1530, São Bernado do Campo, SP, Brazil). After removal of the trabecular bone tissue, the bones were

again weighed (Toledo®, model Adventurer AR1530, São Bernado do Campo, SP, Brazil) to obtain the compact bone weight. The proximal and distal epiphyses thickness of compact bone was measured with digital micrometer (Mitutoyo, model MDC-Lite, Suzano, SP, Brazil).

Joint cartilage macroscopic

The right femur and tibia cartilages of the proximal and distal epiphysis were evaluating after bone macroscopic analysis. The weight was determined using precision balance (Toledo®, Adventurer AR1530, Sao Bernado do Campo, SP, Brazil).

Was used the digital micrometer (Mitutoyo, MDC-Lite, Suzano, SP, Brazil) for measured the length, width and thickness of the cartilage proximal and distal epiphyses of tibia and femur. The cartilages were positioned with the posterior portion downwards and the measured thickness were performed on the middle portion of left and right sides.

Bone mineral composition

The right femur and tibia were used to determine bone calcium, phosphorus, and ash content. The soft tissue was removed and the bones boiled in deionized water for five minutes. After drying at environmental temperature, the samples were immersed in petroleum ether for 48 hours, dried in a forced-ventilation oven at 60°C for 48 hours, and then ground in a ball mill. Bone mineral content was analyzed at the Feed Analysis Laboratory of the College of Agriculture and Veterinary Sciences of the Sao Paulo State University (UNESP - Jaboticabal), Jaboticabal, SP Brazil, using wet analyses. Ash content was determined by burning the samples at 600°C. The methods were applied according to Silva & Queiroz (2002), and expressed as a percentage of defatted dry matter or mineral matter.

Mineral density, mineral content, area bone

Bone mineral density (BMD) (g/cm^2), bone mineral content (BMC) (g), area (cm^2) were determined in the left femur and tibia, in the whole bone, diaphysis, proximal and distal epiphysis, using dual-energy X-ray absorptiometry (DXA), calibrated by the manufacturer (DPX-Alpha, Lunar® - Hologic, Waltham, MA, USA), and a small animal software (DPX-Alpha, Lunar®).

Bone breaking strength

The left femur and tibia were used for the mechanical bone strength tests (three-point bending). The tests were conducted using an EMIC® (DL 10.000, cell Trd 21, software, Tesc 3.13, São José dos Pinhais, PR, Brazil) universal testing machine (UMT). The pre-load force applied was 10 N, and the time of adaptation was five second. The speed of using the power was five mm min^{-1} using the cell force of 50 kg to determine the maximum force (N), the maximum deformation at full force (mm) and the rigidity (N m^{-1}). The equipment was calibrated to allow the diaphysis length of 6 cm for all bone samples, as this value was the maximum duration of support found amongst the smallest select sample. The bones were fixed on a two-points support with the span adjusted according to the size of the smallest bone. Force was then applied at the bone's geometric mean point between the two supports (the middle third of the bone), and the equipment recorded the results. These variables express the bone strength at the ends of the bone and at the middle third.

Histological evaluation of tibia: bone and cartilage

For the bone histological evaluation, it was collected one cm from bone diaphysis and proximal epiphysis cartilage, both of the right tibia. Were meansured the osteocytes bone number and chondrocytes cartilage number.

The bone and cartilage samples were fixed in a solution of formaldehyde (10%) during 24 h and decalcified in a solution of formic acid (5%) during 14 days at room temperature, washed in distilled water and processed according to usual methods for light microscopy. The samples were dehydrated in a series of increasing ethanol concentrations (70%, 80%, 90%, 95% and 100%). Afterward, they were diaphanized in ethanol-xylene solution (1:1) followed by xylene (100%, 3x), and embedded in a mixture of xylene-paraffin (1:1) followed by paraffin, spending approximately 45 minutes in each solution. Histologic semi-serial cuts with 6 mm thick were coated with hematoxylin-eosin.

Thirty-six slides were made, with nine replications/treatment. On each slide was placed four semi-serial cuts. Were obtained four images per cut, totaling 144 images per treatment. The pictures were obtained using a system of registering and analyzing the image (Leica QWin; Leica Imaging Systems, Wetzlar, Germany) with objective *lens ×40.* For the cells count in the images an area of 3.11 square micrometers was stipulated.

Blood parameters

At 42 days of age, six birds per treatment were stunned with CO_2 and killed by exsanguination by cutting the jugular vein for blood collect. The blood variables analysis was: hematocrit (HCT) (% PCV), hemoglobin (HGB) 3 (g/dL), pH, pressure of carbon dioxide (pCO2) (mm Hg), pressure of oxygen (pO2) (mm Hg), base excess (BEecf) (mM), total of carbon dioxide (TCO2) (mM), hemoglobin oxygen saturation (SO2) (%), sodium (Na) (mM), potassium (K) (mM), ionized Calcium (iCa) (mM), bicarbonate ($HCO3^{-)}$ (mM) and glucose

(mg/dL). Blood amounts were collected from the jugular vein using a syringe containing sodic heparin (15 µl heparin/1 mL blood; Glistab, cat. 29th, Labtest Diagnostica) and immediately analyzed using a portable clinical analyzer (I-STAT Co., Abbott Laboratories, USA, Cg8+cartridge). The pH, pCO2 and pO2 were adjusted to the average surface temperature according to Richards *et al*. (1971).

Wing, head, leg, and back surface temperatures were recorded using an infrared thermometer (Instrutemp, ITTI 550, São Paulo, SP, Brazil) to obtain the average body surface temperature (T). Temperature was calculated using the equation (1), according to Richards *et al.* (1971).

$$T=(0.12 \times T_{wing}) + (0.03 \times T_{head}) + (0.15 \times T_{leg}) + (0.70 \times T_{back}) \quad \text{Eq. (1).}$$

Performance and carcass yield

The weight gain, feed intake and feed conversion ratio were evaluated for all phases (1-21, 22-42, 1-42 days of age, respectively). The viability and productive efficiency index were evaluated for the total rearing period (1-42 days of age).

At 43 days of age, 16 birds per treatment were select according to the average weight of each replicate (with variation of 10% above and 10% below the average) and submitted to eight hours' pre-slaughter fast. The broilers were weighed individually again before slaughtered, then stunned with CO_2 and killed by exsanguination by cutting the jugular vein. Carcasses were scalded, plucked and eviscerate. Were obtained the yield from the carcass (without feet, head and neck), breast, thigh and drumstick, wing and back, respectively.

For the following analyzes, eight birds per treatment were sacrificed with 43 days of age, totaling 32 birds. The left and right tibia of each bird were removed and marked.

Statistical analysis

The effects of incubation treatments (without and 4 μg additive *in ovo* feeding (IOF)) and of rearing treatments (without and 0.74 g/kg additive in feed nutrition (FN)) and their interactions (IOF x FN) on the variables studied were analyzed according to the experimental model: $Y_{ijk} = \mu + (IOF)_i + FN)_J + (IOF \times FN)_{ij} + e_{ijk}$. The data were checked for the presence of outliers and meeting the assumptions of normality of observational mistakes and homogeneity of variances. After not finding issues that compromise these assumptions, data were submitted to analysis of variance using the General Linear Model procedure (GLM) of SAS® (SAS Institute, 2002). When means differed significantly by the F test, the analysis orthogonal were performed to test the linear, quadratic and cubic effects *in ovo* feeding and feed nutrition on the studied parameters.

RESULTS

Bone macroscopy

There was no effect (P>0.05) of the treatments for the macroscopic characteristics evaluated in femur and tibia: bone weight, proximal epiphysis thickness, diaphysis and distal epiphysis of the bones, trabecular and compact bone tissue weight and proximal and distal epiphysis thickness of the compact bone.

Joint cartilage macroscopic

In ovo feeding with 4 μg of additive resulted in highest cartilage weight of the femur proximal epiphysis for birds feeding with 0.74 g of additive/kg of feed (P=0.0098). However, when did not occurred the *in ovo* feeding the greatest cartilage weight of the proximal femoral

epiphysis occurred for the birds that did not receive the additive in feeding (P=0.0273) (Table 6).

There was interaction among the factors for cartilage weight of the proximal femoral epiphysis (P=0.0213), cartilage thickness of the tibia proximal epiphysis and tibia distal epiphysis (P = 0.0216 and P=0.0402, respectively).

For the tibia cartilage of the distal epiphysis, greatest thickness occurred for the treatment without inclusion of the additive *in ovo* and diet (P=0.0095). However, the tibia cartilage thickness of the proximal epiphysis, the highest thickness was observed for birds that received feed with 4 μg of additive *in ovo* feeding and in the additive feed with 0.74 g/kg (P=0.0453) (Table 6).

Bone mineral composition

There was factor interaction (P<0.05) for bone mineral composition of femur and tibia, with highest values of ashes, phosphorus and calcium for the femur and tibia, respectively, with 4 μg of additive *in ovo* feeding and not feeding with additive during the rearing. In addition, these results, the highest concentration of phosphorus and calcium (P=0.0230 and P=0.0344, respectively) was observed for bird's treatment: eggs not injected during incubation and supply of 0.74 g of additive/kg of feed during rearing (Table 7).

Mineral density, mineral content, bone area

In ovo feeding reduced the proximal epiphysis area (Table 3) and diaphysis area (Table 4) of the femur (P=0.0202 and P<0.0001, respectively). Also for area, the additive inclusion

during rearing increased the femur area and the femur distal epiphysis area (P=0.0088 and P=0.0345, respectively) (Tables 2 and 5).

In relation to bone mineral density (BMD) and bone mineral composition (BMC), there was interaction between the factors for the proximal epiphysis, diaphysis and distal epiphysis of femur (P<0.05). In general, BMD and BMC were highest for femur that received injected *in ovo* with 4 μg of additive and without additive inclusion in diet and for birds of treatment of un injected eggs and diet with 0.74 g of additive/kg of feed during rearing (Tables 2, 3, 4 and 5).

Bone breaking strength

There was effect of additive in diet (P=0.0376) during rearing on femur stiffness with the better stiffness to birds bones that did not receive additive in diet, when compared to birds that received 0.74 g of the additive/kg of feed. The femur and tibia deformation at maximum strength and displacement were not been affected by the treatments (P>0.05) (Table 8).

Histological evaluation of tibia: bone and cartilage

In ovo feeding with 4 μg of the additive reduced (P=0.0008) the chondrocytes number of tibia cartilage of the proximal epiphysis (P=<.0001) and increased osteocytes number of tibia diaphysis of broiler at 43 days of age. The inclusion of 0.74 g of additive/kg of feed in broiler diet during rearing, increased (P=0.0074) the osteocytes number of the tibia diaphysis, when compared to broilers that did not receive additive in diet (Table 8).

Blood parameters

In ovo feeding with 4 μg of additive increased the blood levels of partial pressure of carbon dioxide (P=0.0211), sodium concentration (P=0.0143) and ionized calcium (P=0.0114),

when compared to birds of un injected egg (Table 9). The other blood parameters were not affected (P>0.05) by the treatments.

Performance and carcass yield

The inclusion of 0.74 g/kg of additive in the broiler diet resulted in worse feed conversion (P=0.0393) and viability (P=0.0313) from 1 to 42 days of age, when compared with broiler performance that did not receive the additive in the diet (Table 10). There was no effect of treatments (P>0.05) for carcass yield.

DISCUSSION

In the present study was evaluated the chondroitin sulfate, glucosamine sulfate and vitamin C *in o*vo feeding and used the same additive in the birds diet for evaluation during rearing. Femur, tibia and joint cartilages were analyzed with to understand the action of GAGs and vitamin C on bone and cartilage development.

Macroscopic deformations and mineral composition alterations and bone formation usually are result from chondrogenesis abnormalities and compromise the performance and animal welfare of birds, because they prevent these animals from walking to the feeder and drinking fountain (Sanotra *et al*., 2001; Ogunwole, 2015). Our data show that both for *in ovo* feeding and feed nutrition during broilers rearing, the chondroitin sulfate, glucosamine sulfate and vitamin C influenced the characteristics evaluated, resulting in increase of the proximal femoral epiphysis cartilage and the tibia proximal epiphysis thickness at 43 days of age.

The GAGs and vitamin C can influence the cartilage macroscopy and long bones formation, inasmuch as these substances stimulate the synthesis of the proteoglycans and collagen and are capable of increasing the proliferation of the chondrocytes and the biosynthesis

of the matrix (Clark, 1991), they are essential in the endochondral ossification process, responsible for the longitudinal growth of long bones, due to epiphyseal disk calcification (Gerber & Ferrara, 2000; Anderson *et al*., 2005).

The bone longitudinal growth occurs through the precise balance between chondrocyte proliferation, cartilage matrix production and mineralization, hypertrophy and hypertrophic chondrocyte invasion (Farquharson & Jeferreis, 2000; Gerber & Ferrara, 2000; Jeferreis *et al*., 2000). The chondrocyte hypertrophy is essential for vascular invasion and subsequent replacement of the calcified matrix by boné (Gerber & Ferrara, 2000), suggesting that the vascularization of the growth disc inferior region represents crucial step in the interaction between the chondrogenesis processes (cartilage production) and osteogenesis (bone tissue formation), especially during the fast growth period of the long bones or fractures repairs (Gerber & Ferrara, 2000).

In recent meta-analysis study (Zeng et al., 2015) with 54 articles, where chondroitin sulfate and glucosamine sulfate were tested in 16,427 patients, the use of chondroitin plus glucosamine or glucosamine isolated resulted in pain reduction and clinical improvement of patients bone functions compared to placebo.

For the mineral percentage in bone, bone mineral composition (BMC) and bone mineral density (BMD) our data show interesting results. *In ovo* feeding or feed nutrition in rearing with chondroitin sulfate, glucosamine sulfate and vitamin C was able to increase the percentages of ash, phosphorus, calcium, BMD and BMC values. Therefore, should choose to use the additive in one of the two phases and not in both. According to Vargas *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 the bones. Therefore, the bone mineral increase prevents the appearance of bone problems and/or diseases in broilers (Waldenstedt, 2006).

Muraleva *et al*. (2012) studied alendronate, glucosamine and dihydroquercetin effect in the osteoporosis treatment in rats and found an increase in BMD (0.24 g/cm^2) and maximum femoral breaking strength (1761 N). Henrotin *et al*. (2005) state that the use of chondroitin sulfate and glucosamine as chondroprotection promotes the improvement of symptoms such as lameness and pain on canines. Ogunwole (2015) also found no effect of increasing vitamin C levels in the diet on the tibia weight and length.

In ovo feeding with chondroitin sulfate, glucosamine sulfate and vitamin C reduced the number of chondrocytes in the tibia proximal epiphysis cartilage, in addition the additive promoted increase of the osteocytes number in the tibia diaphysis in birds with 43 days of age. To occur the calcification of the tibia proximal portion, it is necessary, the proliferation, hypertrophy and subsequent chondrocytes degeneration (cartilaginous tissue cells) (Reyes-Botella et al., 2000; Bruyere, 2004, Anderson *et al*., 2005), leaving a space or gap that must be rapidly filled by osteoblasts, which retained by the newly synthesized matrix, it is called osteocytes (Pack, 2003). Osteocytes are involved in the structural and biomechanical regulation of the bone tissue mass (Pizauro Júnior, et al., 2002).

Due to the important biology of the bone development, it can be inferred that the reduction of the cartilage chondrocytes number is due to the fact of the high apoptosis index of these cells, which in turn contributed with cartilage calcification process, and consequent increase of tibia osteocyte number. Therefore, nutrition with chondroitin sulfate, glucosamine sulfate and vitamin C favored the chondrogenesis and osteogenesis, which may contribute to the disease prevention such as tibial dyschondroplasia, which is result of a particularity in the process of differentiation of the chondrocytes, taking to formation of a prehypertrophic layer chondrocytes and a cartilage in the proximal tibia that is not calcified (Almeida Paz *et al*., 2005).

In vitro studies show that vitamin C facilitates the chondrocytes differentiation (Monsonego *et al*., 1997), increasing the alkaline phosphatase activity and the osteopontin synthesis (Beck *et al*., 2000). According to these authors, vitamin C is an important inducer of osteoblast differentiation, stimulating secretion of extracellular matrix rich in collagen and gene effectors specific such as: alkaline phosphatase, osteocalcin and osteopontin. Vitamin C also stimulates other hydroxylation reactions, including the hydroxylase that converts 25-$(OH)_2$ D_3 to 1,25-$(OH)_2$ D_3 (Farquharson & Jeffereis, 2000). The active form of vitamin D 1,25-$(OH)_2$ D_3 regulates the bone tissue development, the metabolism and the calcium homeostasis, besides participating of the cells regulation, growth and differentiation on bone tissue (Whitehead, 2004).

Bone development is stimulated by blood supply and by high oxygen tension, thus, hypoxia (pCO2 increase and pO2 drop) does not favor the cartilage production of cartilage (Kiaer *et al*., 1992; Pizauro Júnior *et al*., 2002; Greenbaum, 2004). We observed pCO2 increase, but there was no pO2 and pH drop, therefore, we cannot classify as hypoxia. Sgavioli *et al*. (2013) in similar study they observed blood pH decrease of birds at 42 days of age, through *in ovo* feeding with 2 μg of vitamin C, without change in pCO_2 and pO_2.

It is known that the pCO_2 generates bone mineral resorption and calcium excretion by the kidneys (Bushinsky, 1996). Calcium is present in three forms in the blood: bound calcium protein, complexed with calcium and organic acids or free ionized calcium, the latter being used by poultry during bone mineralization (Pizauro Junior et al., 2002; Lieben *et al*., 2012). Thus, the Ca blood increase in broilers from *in ovo* feeding with GAGs and vitamin C shows greater availability of iCa to replacement during the poultry growth. This increase in ionized calcium can be explained by the ability of this mineral complexing to glycosaminoglycans (Yamaguchi,

1993). The GAGs have strong ionization potential, facilitating the calcium release in the bone tissue formation (Yamaguchi, 1993).

During the bone matrix formation not only the calcium and phosphorus ions are found, but also the sodium ion too (Banks, 1992). The sodium ion concentration in the blood of broiler chickens at 42 days of age increased with the use of GAGs and vitamin C *in ovo* feeding, favoring its mobilization for the bone matrix formation (Vieites *et al*., 2011).

Despite the negative information on the chondroitin sulfate and glucosamine sulfate use (Glyn-Jones et al., 2015), Zeng et al. (2015) in meta-analysis they observed that the use of chondroitin and glucosamine favors the clinical evolution in the treatment of patients with osteoarthritis in the knee, with structural modification due to reduction in joint space.

The addition of 0.74 g/kg of GAGs and vitamin C in the diet worsened feed conversion (1.590) and economic viability (96.68), when compared to birds that did not receive the additive during rearing (1.531 and 98.97, respectively). According to the Cobb-Vantress (2015) broiler nutrition guide at 42 days of age, in males batches, the feed conversion should be 1.691. Thus, despite the worsening, the feed conversion verified is still below that recommended by cited guide. Feed conversion is important for broilers because it is related to the bird efficiency in converting the diets nutrients in body mass (Emmerson, 1997).

CONCLUSION

We conclude *in ovo* feeding during broiler rearing, with chondroitin sulfate, glucosamine sulfate and vitamin C, act on the osseous and cartilaginous system development of birds positively, increased cartilage weight and thickness, ash content, calcium and phosphorus, bone composition and mineral density, besides to reduce the chondrocytes number in the cartilage and increasing the tibia osteocytes number in broilers. The additive with GAGs

and vitamin C brought more benefits when used *in ovo* feeding or in the rearing diet, in other words, used isolated. Future studies should be carried out for understand how this additive act molecularly in relation to genes expression involved in broilers leg problems. The data found will serve as basis for future studies with different levels of GAGs and/or combination with other compounds, and may represent the solution to broiler bone problems.

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Table 1. Ingredients and nutritional composition calculate of diets for starter phase (1-21 days of age) and grower-finisher phase (21-42 days of age).

Ingredients (%)	Starter 1-21 days of age	Grower-finisher 22-42 days of age
Corn	51.97	59.72
Soybean meal 45%	39.13	31.19
Soybean oil	4.82	5.63
Dicalcium phosphate	1.95	1.29
Limestone particle	0.79	0.87
Salt (NaCl)	0.50	0.50
Vitamin and mineral supplement*	0.40	0.40
DL- Methionine 99%	0.32	0.28
L- Lysine HCl 78,5%	0.13	0.12
TOTAL	100	100
On top additive[1]		
Nutritional composition		
Metabolizable energy (kcal/kg)	3.050	3.200
Crude protein (%)	22.0	19.0
Calcium (%)	0.90	0.75
Available phosphorus (%)	0.47	0.34
Sodium (%)	0.21	0.21
Digestible methionine+cysteine (%)	0.90	0.80
Digestible methionine (%)	0.59	0.53
Digestible lysine (%)	1.20	1.00

*Nutrients per kilogram of diet: From 1 to 21 days of age - Vit. A 7,000 U.I., Vit. D3 3,000 U.I., Vit. E 25 U.I., Vit. K 0.98 mg, Vit. B1 1.78 mg, Vit. B2 9.6 mg, Vit. B6 3.5 mg, Vit. B12 10 µg, Folic Acid 0.57 mg, Biotin 0,16 mg, Niacin 34.5 mg, Calcium Pantothenate 9.8 mg, Copper 0.12 g, Cobalt 0.02 mg, Iodine 1.3 mg, Iron 0.05 g, Manganese 0.07 g, Zinc 0.09 mg, Organic Zinc 6.75 mg, Selenium 0.27 mg, Choline 0.4 g, Growth promoter (Zinc bacitracin) 30 mg, (narasin+nicarbazin) 0.1 g, Methionine 1.68 g. From 21 to 42 days of age - Vit. A 7,000 U.I., Vit. D3 3,000 U.I., Vit. E 25 U.I., Vit. K 0.98 mg, Vit. B1 1.78 mg, Vit. B2 9.6 mg, Vit. B6 3.5 mg, Vit. B12 10 µg, Folic Acid 0.57 mg, Biotin 0.16 mg, Niacin 34.5 mg, Calcium Pantothenate 9.8 mg, Copper 0.12 g, Cobalt 0.02 mg, Iodine 1.3 mg, Iron 0.05 g, Manganese 0.07 g, Zinc 0.09 mg, Organic Zinc 6.75 mg, Selenium 0.27 mg, Choline 0.6 g, Growth promoter (avilamycin) 7.5 mg, (monensin sodium) 0.1 g, Methionine 1.4 g. **1** For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C, respectively.

Table 2. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on femur area, bone mineral content (BMC) and bone mineral density (BMD) in broiler at 43 days of age.

		In ovo feeding (IOF)	Feed nutrition (FN)					Probability		
			0 g/kg	0.74 [2] g/kg	P	Mean	SEM	IOF	FN	IOF x FN
Femur	Area (cm^2)	Un injected egg	16.04	16.67	0.1157	16.35	0.164	0.7285	0.0088	0.5694
		4 μg of additive[1]	15.78	16.73	0.0530	16.26				
		P	0.5181	0.8744						
		Mean	15.91 B	16.70 A						
	BMC (g)	Un injected egg	3.05 Bb	4.12 A	<.0001	3.59	0.110	0.0017	<.0001	<.0001
		4 μg of additive[1]	3.93 a	3.92	0.9211	3.93				
		P	<.0001	0.1417						
		Mean	3.49	4.02						
	BMD (g/cm^2)	Un injected egg	0.19 Bb	0.25 A	<.0001	0.22	0.007	0.0017	0.0036	0.0013
		4 μg of additive[1]	0.25 a	0.25	0.7506	0.25				
		P	<.0001	0.9308						
		Mean	0.22	0.25						

A-B; a-b: Means followed by different letters uppercase in lines or lowercase in columns differ significantly, respectively ($P<0.05$). [1] 4 μg of additive: contained 1.2 μg of chondroitin sulfate, 1.2 μg of glucosamine sulfate and 0.2 μg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 3. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on femur proximal epiphysis area, bone mineral content (BMC) and bone mineral density (BMD) in broiler at 43 days of age.

		In ovo feeding (IOF)	Feed nutrition (FN)					Probability		
			0 g/kg	0.74 [2] g/kg	P	Mean	SEM	IOF	FN	IOF x FN
Proximal epiphysis femur	Area (cm^2)	Un injected egg	3.62	4.07	0.0598	3.84 a	0.191	0.0202	0.1125	0.1150
		4 μg of additive [1]	3.50	3.50	0.9932	3.50 b				
		P	0.5429	0.0579						
		Mean	3.56	3.78						
	BMC (g)	Un injected egg	0.68 Bb	0.95 Aa	<.0001	0.82	0.044	0.4582	0.0009	<.0001
		4 μg of additive [1]	0.86 a	0.82 b	0.3159	0.84				
		P	0.0003	0.0040						
		Mean	0.77	0.88						
	BMD (g/cm^2)	Un injected egg	0.19 Bb	0.23 A	0.0003	0.21	0.005	0.0004	0.0373	0.0008
		4 μg of additive [1]	0.25 a	0.24	0.2382	0.24				
		P	<.0001	0.8330						
		Mean	0.22	0.23						

A-B; a-b: Means followed by different letters uppercase in lines or lowercase in columns differ significantly, respectively (P<0.05). [1] 4 μg of additive: contained 1.2 μg of chondroitin sulfate, 1.2 μg of glucosamine sulfate and 0.2 μg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 4. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on femur diaphysis area, bone mineral content (BMC) and bone mineral density (BMD) in broiler at 43 days of age.

		In ovo feeding (IOF)	Feed nutrition (FN)					Probability		
			0 g/kg	0.74 [2] g/kg	P	Mean	SEM	IOF	FN	IOF x FN
Diaphysis femur	Area (cm^2)	Un injected egg	3.86	3.55	0.1313	3.70 a	0.176	<.0001	0.2841	0.2742
		4 µg of additive [1]	3.01	3.01	0.9867	3.01 b				
		P	0.0503	0.0535						
		Mean	3.43	3.28						
	BMC (g/cm^2)	Un injected egg	0.73 Bb	1.09 Aa	<.0001	0.93	0.040	0.5542	<.0001	<.0001
		4 µg of additive [1]	0.89 Aa	0.89 Bb	0.9730	0.89				
		P	0.0064	0.0006						
		Mean	0.82	0.99						
	BMD (g/cm^2)	Un injected egg	0.21 Bb	0.31 A	<.0001	0.26	0.011	0.0037	0.0004	0.0003
		4 µg of additive [1]	0.30 a	0.30	0.9273	0.30				
		P	<.0001	0.4383						
		Mean	0.25	0.30						

A-B; a-b: Means followed by different letters uppercase in lines or lowercase in columns differ significantly, respectively (P<0.05). [1] 4 µg of additive: contained 1.2 µg of chondroitin sulfate, 1.2 µg of glucosamine sulfate and 0.2 µg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 5. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on femur distal epiphysis area, bone mineral content (BMC) and bone mineral density (BMD) in broiler at 43 days of age.

		In ovo feeding (IOF)	Feed nutrition (FN)					Probability		
			0 g/kg	0.74 [2] g/kg	P	Mean	SEM	IOF	FN	IOF x FN
Distal epiphysis femur	Area (cm^2)	Un injected egg	4.27	4.92	0.0503	4.59	0.200	0.3425	0.0345	0.0983
		4 µg of additive [1]	4.39	4.48	0.7092	4.44				
		P	0.5962	0.0701						
		Mean	4.33 B	4.70 A						
	BMC (g)	Un injected egg	0.76 Bb	1.02 Aa	<.0001	0.89	0.037	0.8846	0.0073	0.0001
		4 µg of additive [1]	0.92 a	0.86 b	0.2267	0.89				
		P	0.0040	0.0024						
		Mean	0.84	0.94						
	BMD (g/cm^2)	Un injected egg	0.18 Bb	0.21 Aa	<.0001	0.19 b	0.005	0.0890	0.1215	<.0001
		4 µg of additive [1]	0.21 Aa	0.19 Bb	0.0053	0.20 a				
		P	<.0001	0.0069						
		Mean	0.19	0.20						

A-B; a-b: Means followed by different letters uppercase in lines or lowercase in columns differ significantly, respectively (P<0.05). [1] 4 µg of additive: contained 1.2 µg of chondroitin sulfate, 1.2 µg of glucosamine sulfate and 0.2 µg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 6. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on cartilage femur and tibia macroscopic in broiler at 43 days of age.

			Feed nutrition (FN)					Probability		
		In ovo feeding (IOF)	0 g/kg	0.74 [2] g/kg	P	Mean	SEM	IOF	FN	IOF x FN
Cartilage femur	Proximal epiphysis weight (g)	Un injected egg	2.06 a	1.97	0.6298	2.02	0.15	0.2735	0.0564	0.0213
		4 µg of additive [1]	1.51 Bb	2.21 A	0.0098	1.93				
		P	0.0273	0.2267						
		Mean	1.84	2.09						
Cartilage tibia	Proximal epiphyses thicknesses (mm)	Un injected egg	3.80 A	2.89 Bb	0.0432	3.35	0.12	0.7551	0.7296	0.0216
		4 µg of additive [1]	3.09	3.79 a	0.1165	3.44				
		P	0.1122	0.0453						
		Mean	3.45	3.34						
	Distal epiphyses thicknesses (mm)	Un injected egg	6.75 A	5.38 B	0.0095	6.06	0.44	0.9955	0.0465	0.0402
		4 µg of additive [1]	6.05	6.07	0.9490	6.07				
		P	0.1212	0.1227						
		Mean	6.40	5.73						

A-B; a-b: Means followed by different letters uppercase in lines or lowercase in columns differ significantly, respectively ($P<0.05$). [1] 4 µg of additive: contained 1.2 µg of chondroitin sulfate, 1.2 µg of glucosamine sulfate and 0.2 µg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 7. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on femur and tibia mineral content (ash, P and Ca) in broiler at 43 days of age.

		In ovo feeding (IOF)	Feed nutrition (FN)					Probability		
			0 g/kg	0.74 [2] g/kg	P	Mean	SEM	IOF	FN	IOF x FN
Femur	Ash (%)	Un injected egg	38.15 b	39.65	0.1044	38.90	0.76	0.6635	0.7594	0.0133
		4 µg of additive [1]	40.12 Aa	38.24 B	0.0448	39.18				
		P	0.0371	0.1234						
		Mean	39.14	38.94						
	P (%)	Un injected egg	4.28 b	4.43 a	0.1551	4.36	0.09	0.7313	0.0970	0.0010
		4 µg of additive [1]	4.58 Aa	4.18 Bb	0.0008	4.38				
		P	0.0078	0.0230						
		Mean	4.43	4.31						
	Ca (%)	Un injected egg	13.78 Bb	14.53 A	0.0344	14.16	0.28	0.2394	0.8880	0.0032
		4 µg of additive [1]	14.85 Aa	14.03 B	0.0226	14.44				
		P	0.0042	0.1458						
		Mean	14.32	14.28						
Tibia	Ash (%)	Un injected egg	39.09 b	41.43	0.0879	40.26	0.92	0.4828	0.9227	0.0242
		4 µg of additive [1]	42.00 a	39.84	0.1135	40.92				
		P	0.0373	0.2370						
		Mean	40.55	40.64						
	P (%)	Un injected egg	4.43 b	4.70	0.1167	4.57	0.13	0.5685	1.0000	0.0311
		4 µg of additive [1]	4.77 a	4.50	0.1167	4.63				
		P	0.0437	0.2330						
		Mean	4.60	4.60						
	Ca (%)	Un injected egg	14.12 b	14.88	0.1424	14.50	0.41	0.2114	0.9446	0.0374
		4 µg of additive [1]	15.37 a	14.55	0.1195	14.96				
		P	0.0217	0.5143						
		Mean	14.74	14.72						

A-B; a-b: Means followed by different letters uppercase in lines or lowercase in columns differ significantly, respectively (P<0.05). [1] 4 μg of additive: contained 1.2 μg of chondroitin sulfate, 1.2 μg of glucosamine sulfate and 0.2 μg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 8. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on femur rigidity, proximal epiphyses cartilage tibia chondrocyte number and diaphysis tibia cartilage osteocyte number in broiler at 43 days of age.

Treatments	Rigidity femur (N/mm)	Tibia cartilage	Tibia bone
		Proximal epiphysis Chondrocyte number	Diaphysis Osteocyte number
In ovo feeding (IOF)			
Un injected egg	164.63	2.16 a	49.17 b
4 μg of additive[1]	168.80	0.89 b	64.54 a
Feed nutrition (FN) g/kg[2]			
0	190.68 a	1.56	51.24 b
0.74	142.76 b	1.43	63.45 a
Probability			
IOF	0.8342	0.0008	<.0001
FN	0.0376	0.6617	0.0004
IOF x FN	0.3463	0.3163	0.0803
SEM	15.09	0.26	3.37

a-b: Means followed by different letters differ significantly (P<0.05). [1] 4 μg of additive: contained 1.2 μg of chondroitin sulfate, 1.2 μg of glucosamine sulfate and 0.2 μg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 9. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition on blood parameters: pressure of carbon dioxide (pCO_2), sodium (Na^+), ionized calcium (Cai) in broiler at 42 days of age.

Treatments	pCO_2 (mmHg)	Na^+ (mM)	Cai (mM)
In ovo feeding (IOF)			
Un injected egg	51.34 b	147.45 b	1.01 b
4 μg of additive[1]	56.22 a	150.10 a	1.15 a
Feed nutrition (FN) g/kg[2]			
0	54.48	148.36	1.08
0.74	52.76	149.10	1.08
Probability			
IOF	0.0211	0.0143	0.0114
FN	0.2993	0.5289	0.8741
IOF x FN	0.2296	0.9863	0.2447
SEM	1.47	0.69	0.03

a-b: Means followed by different letters differ significantly ($P<0.05$). [1] 4 μg of additive: contained 1.2 μg of chondroitin sulfate, 1.2 μg of glucosamine sulfate and 0.2 μg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.

Table 10. Effect of chondroitin and glucosamine sulfates and vitamin C *in ovo* feeding and feed nutrition feed intake, weight gain, conversation rate and viability in total rearing period of broilers.

Treatments	Feed intake (g)	Weight gain (g)	Conversion rate	Viability (%)	*Production efficiency index*
	1 to 42 days of age				
In ovo feeding (IOF)					
Un injected egg	4,820	3,090	1.561	97.91	464.17
4 μg of additive[1]	4,790	3,050	1.560	97.80	452.37
Feed nutrition (FN) g/kg[2]					
0	4,740	3,070	1.531 b	98.97 a	470.46
0.74	4,870	3,070	1.590 a	96.68 b	446.08
Probability					
IOF	0.7135	0.3387	0.9409	0.9837	0.3854
FN	0.1019	0.8502	0.0393	0.0313	0.0549
IOF x FN	0.2716	0.9661	0.5340	0.3512	0.2628
SEM	0.06	0.02	0.02	0.53	9.70

a-b: Means followed by different letters differ significantly ($P<0.05$). [1] 4 μg of additive: contained 1.2 μg of chondroitin sulfate, 1.2 μg of glucosamine sulfate and 0.2 μg mg of vitamin C. [2] For each 100 kg of feed was used 30 g of glucosamine sulfate, 24 g of chondroitin sulfate and 20 g of vitamin C.