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Glutamine in ovo - Effects on the development and health of the gastrointestinal tract, antioxidant status and immune response in chickens

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Thamírys Vianelli Maurício de Souza[1], Matheus Faria de Souza[2], José Henrique Stringhini[3], Fernanda Alves Duarte[4], Laura Alves Duarte[5], Renata Rodrigues Gomes[6], Kalebe de Oliveira Maia[7].

ABSTRACT

We observed that in birds there is an extensive embryonic mortality at the end of the incubation period, a period in which events related to hatching occur, such as the opening of the membrane and the eggshell, the beginning of pulmonary respiration and the emergence of the egg itself. The frequent activity of embryos implies a large consumption of energy, which together with the accelerated intestinal development, observed especially at the end of incubation, causes a very high energy demand on their part. At this time, birds rely on the residual yolk sac as their only supply of nutrients to provide energy. Which, generally, does not constitute meeting the high nutritional requirements of the moment, causing catabolism of body resources such as muscles and immune cells, causing a decrease in body weight, a reduction in pectoralis and immunity, in addition to a decline in the weight of the organs. On the other hand, we observed that the development of the small intestine can also be improved before hatching through nutritional stimulation through in ovo feeding (IOF). Glutamine is a conditionally essential amino acid that is strongly linked to the antioxidant status of chicks and their immune defenses. It also has well-documented stimulatory effects on the proliferation of epithelial cells of the small intestine that lead to better morphology of the small intestine in several species. This set of potential benefits of glutamine in addition to the need to study and better understand the biological mechanisms involved in the relationships between nutraceutical additives inoculated via in ovo (IOF) and poultry, explain the choice of this amino acid for this study, as well as describe the objective of the study.

Keywords: Amino acids, Industrial Poultry, Performance, Immunocompetence.

[1] Master of Science in Veterinary Sciences
Federal University of Goiás – GO
[2] Doctor in Monogastric Nutrition
Federal University of Viçosa – MG
[3] Doctor in Animal Science
Federal University of Goiás – GO
[4] Master's student in Animal Science
Federal University of Goiás – GO
[5] Master's student in Animal Science
Federal University of Goiás – GO
[6] Master in Animal Science
Federal University of Goiás – GO
[7] Graduating in Animal Science
Federal University of Goiás – GO

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INTRODUCTION

It has been observed that in birds there is extensive embryonic mortality towards the end of the incubation period, a period in which events related to hatching take place, such as the opening of the egg membrane and shell, the start of pulmonary respiration and the actual emergence of the egg.

The frequent activity of the embryos means that they consume a lot of energy, which, together with the accelerated intestinal development seen especially towards the end of incubation, results in a very high energy demand on their part.

In addition, after hatching, most neonates are often subject to a delay in feed intake, which can take between 24 and 72 hours. This is due to differences in hatch times within the hatch window, as well as transportation in commercial practice.

During this period of delayed feed intake, the birds rely on the residual yolk sac as their only nutrient supply to provide energy. However, the remaining yolk sac appears to make an insufficient contribution to the nutritional needs for both maintenance and growth in today's newborn poultry, which in turn drives the mobilization of critical body resources (mainly muscle) to provide the energy needed for maintenance, causing a decrease in body weight, a reduction in chest size and a decline in organ weight.

For all these reasons, the last days of incubation, as well as the first days after hatching, are a critical period for the survival and development of late embryos and neonates due to the considerable catabolism of energy.

On the other hand, we have observed that the development of the small intestine can also be improved before hatching by nutritional stimulation through in ovo feeding (IOF). In ovo feeding is a method of supplying nutrients to the small intestine of poultry embryos by injecting a nutrient solution into their amniotic fluid, prior to amniotic ingestion, which occurs from the 17th day of incubation until hatching.

In the small intestine of birds, glutamine and fructose-6-phosphate are known to be substrates for the synthesis of glucosamine-6-phosphate and therefore glycoproteins (including mucins and membrane receptors). Glutamine is therefore strongly linked to the antioxidant status of chicks and their immune defenses. It also has well-documented stimulatory effects on small intestinal epithelial cell proliferation via the mitogen-activated protein kinase (MAPK) and mechanistic target of rapamycin (mTOR) signaling pathways, resulting in improved small intestinal morphology in various animals and humans.

This set of potential benefits of glutamine, in addition to the need to better study and understand the biological mechanisms involved in the relationship between nutraceutical additives and poultry, explains the choice of this amino acid for the present study, as well as describing its objective.

DEVELOPMENT

INTESTINAL DEVELOPMENT X IN OVO NUTRITION (IOF)

The mucosal lining of the small intestine is a highly functional epithelium composed of absorptive, secretory and sensory cells, which are constantly renewed by multipotent intestinal stem cells (ISC). ISC reside within the crypts and constantly proliferate for self-renewal and generation of progenitor cells, which differentiate into the functional cells that line the villi facing the lumen1.

Compartmentalization of the multipotent and differentiated regions of the small intestinal epithelium occurs during villus formation, through a polarized mesenchymal bone morphogenetic protein (BMP) signaling gradient that limits all proliferative and Wnt-responsive ISCs to the lower regions of the developing villi. These regions develop into crypts as the small intestine matures, while the villi become exclusively filled with differentiated cells2.

In chicken embryos (Gallus gallus), the formation of villi occurs on the 15th day of embryonic development (embryonic 15 d, E 15), and the crypts develop on the 21st day (day of hatching) 3,4.

After hatching, initial feeding stimulates the completion of small intestinal maturation by expanding the epithelium of the crypts and villi, mediating cell proliferation and differentiation and activating nutrient transporters, digestive enzymes and mucin secretion 4,5.

On the other hand, the development of the small intestine can also be improved before hatching by nutritional stimulation through in ovo feeding (IOF). IOF is a method of providing nutrients to the small intestine of poultry embryos by injecting their amniotic fluid with a formulated nutrient solution on day 17 of incubation, prior to amniotic ingestion, which occurs up until the day of hatch 6.

Several studies have shown that IOF of specific nutrients improved perihatch gut development and functionality, expanding the dimensions of the villi and crypts and increasing the ability to digest and absorb nutrients 7,8.

Reicher et al. (2022)9 confirmed that IOF of leucine and glutamine on day 17 of incubation increased intestinal cell counts by 33% and 40%, respectively, compared to the

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control. Importantly, they explained that glutamine and leucine supplementation via the in ovo route accelerated the development and maturity of the small intestine throughout the pre-hatching phase, positively regulating the proliferation and differentiation of multipotent cells.

Therefore, the main objective of IOF is to accelerate the initial development of the gastrointestinal tract and stimulate its ability to digest and absorb nutrients in newborn chicks and, consequently, enhance their rapid growth genetics. The literature confirms that IOF of carbohydrates (Slawinska et al. 2020; Asa et al. 2022)10,11, amino acids (Nazem et al. 2019; Gonzales et al. 2022; Reicher et al. 2022)12,13,9 and probiotics (Abdel-Moneim et al. 2020a; El-Moneim at al. 2020)14,15 improves intestinal histomorphology (taller villi, higher villus-to-crypt ratio and larger goblet cells).

GUT HEALTH X IN OVO NUTRITION (IOF)

The gut microbiota plays an essential role in the function and health of the gastrointestinal tract. Therefore, modulating the microbiota of the gastrointestinal tract has been suggested as an effective strategy to improve the host's intestinal health, nutrient digestion, immunity and productivity 15.

Unlike mammals, chicks hatch with a sterile gastrointestinal tract and, consequently, any contact with pathogenic bacteria represents a risk of infection and disease. Previous colonization of this tract by useful bacteria helps the birds to cope with environmental and pathogenic stressors 14,15.

In ovo nutraceutical injection (IOF) technology is a useful tool for establishing early colonization of beneficial microorganisms in the embryonic gastrointestinal tract. Extensive evidence illustrates that IOF of probiotics (Castaneda et al. 2021)16, prebiotics (Tavaniello et al. 2018; Stadnicka et al. 2020)17,18 and symbiotics (Boguslawska-Tryk et al. 2021; Shehata et al. 2022)19,20 help maintain a healthy microbial balance by strengthening useful microorganisms such as Lactobacillus, Bacillus and Bifidobacterium.

Castaneda et al. (2021)16 observed that B. subtilis IOF (106 CFU/50 µl/egg) on day 18 of incubation reduced pathogenic bacteria, including total bacterial counts, aerobic bacteria and total coliforms in the ileum and cecum on different days of the broiler growing period.

Improving intestinal health improves the general health of the host. This can be confirmed by the work of Tavaniello et al. (2018)17, who elucidated that raffinose IOF stimulated the development of useful bacteria (lactobacilli and Bifidobacteria) and suppressed the colonization of pathogenic bacteria in the gastrointestinal tract. Similarly, Stadnicka et al.

(2020)18 found that raffinose IOF improved intestinal health by decreasing intestinal populations of Clostridium, E. coli, as well as coccidia counts and increasing populations of Bifidobacteria and Lactobacilli.

Several recent studies have postulated that amino acid IOF improved the development and health of the GIT 21,22,9. Gonzales et al. (2022)13 revealed that IOF of a combination of sulphur amino acids (5.90 mg of l-methionine and 3.40 mg of l-cysteine) plus 0.15 mg of folic acid increased ileal crypt depth in neonatal chicks.

In terms of gene expression, Gao et al. (2018b)21 elucidated that the IOF of 1% l-arginine on day 17.5 of embryonic development was involved in regulating the functions of the intestinal barrier in post-hatch broiler chickens by increasing the count of proliferating cells positive for villus nuclear antigen and gene expression of claudin-1, mucin-2, zonula occludens-1 and -2 in the jejunal mucosa of 21-day- old broilers.

We should also highlight the work of Shehata el al. (2022)20, who observed that IOF of probiotics, prebiotics and/or symbiotics increased the caecal content of acetic, propionic, butyric, pentanoic, ovaleric and isobutyric acids. These organic acids lowered the intestinal pH and consequently played vital roles in suppressing pathogens in the gastrointestinal tract, supporting structural integrity, improving the barrier function of the intestinal epithelium, physiological function, intestinal immunity and health23.

Therefore, the colonization and early establishment of beneficial bacteria in the gastrointestinal tract of chickens, as well as the use of nutraceuticals in ovo can block the colonization of pathogens, improving intestinal development and health, which translates into better performance.

GLUTAMINE - CHARACTERIZATION AND POTENTIAL FOR USE IN IN OVO TECHNOLOGIES

Amino acids are beneficial in improving the general physiological state, immunization against infectious diseases and stabilization under non-infectious or management conditions (Saleh et al. 2018)24, thus improving the productive performance of poultry 25.

Enteric infections in broilers can have a major influence on endogenous amino acid losses in the gastrointestinal tract (GIT). (2017)26 stated that supplementing the diet with amino acids (glutamine, arginine and threonine) above the levels recommended for growth may be necessary to improve the immune response against Eimeria and E. coli.

Glutamine is an important substrate for the synthesis of peptides, proteins, lipids, purines, pyrimidines, amino acids, nicotinamide adenine dinucleotide phosphate (NADPH), glucosamine, antioxidants and for many other biosynthetic pathways involved in regulating cellular function. Several enzymes are involved in the metabolism of this amino acid. Glutamine is predominantly synthesized from l-glutamate (Glu) and ammonia (NH 3) by the action of the largely cytosolic enzyme Gln synthetase (GS), while the mitochondrial enzyme glutaminase (GLS) is responsible for the hydrolysis of Gln to Glu and NH3. GS is highly expressed in skeletal muscle, while GLS is found in most cells with the small intestine, kidney, leukocytes and vascular endothelium having the highest activities 27, 28.

Glutamine is also known to provide nitrogen and an energy source for the proliferation of immune cells and intestinal mucosa, and is needed along with cysteine to synthesize antioxidants such as glutathione27,28. Its supplementation has been studied in animal diets due to its effects on intestinal structure and function 29,30.

It serves as an important source of energy for enterocytes, particularly during periods of increased proliferation. It is also a so-called non-essential nutrient, but becomes essential for animals in challenging situations such as stress, infection, injury or high temperature 31.

Among the amino acids with potential for use in in ovo nutrition, glutamine stands out for being a trophic agent, important in the process of cell proliferation, since it induces the enzyme ODC (ornithine decarboxylase), as well as producing vital polyamines for the process 32,33.

Finally, glutamine can also be hydrolyzed into glutamate, acting as an immunomodulatory agent, inhibiting pro-inflammatory cytokines, protecting the mucosa from exacerbated immune response and pH changes.

GLUTAMINE X ANTIOXIDANT STATUS OF POULTRY

Several enzymes are involved in glutamine metabolism. This amino acid is predominantly synthesized from l-glutamate (Glu) and ammonia (NH 3) by the action of the largely cytosolic enzyme Gln synthetase (GS), while the mitochondrial enzyme glutaminase (GLS) is responsible for the hydrolysis of Gln to Glu and NH3. GS is highly expressed in skeletal muscle, while GLS is found in most cells with the small intestine, kidney, leukocytes and vascular endothelium having the highest activity. Glutamine is also metabolized by glutamine: fructose-6-phosphate aminotransferase (GFAT), which condenses the amino group of glutamine and fructose-6-phosphate into glucosamine-6-phosphate, a precursor for N- and O-linked glycosylation reactions 34.

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The glutamate product is used for the synthesis of the antioxidant glutathione: a small three-amino acid peptide (Glu-Cys-Gly) that is an efficient peroxide-based free radical neutralizer. Alternatively, it can be further metabolized by Glu dehydrogenase and/or aminotransferases into α-ketoglutarate, which then enters the Krebs cycle generating ATP and serving as an anaplerotic carbon source for the formation of non-essential amino acids and lipids 35.

In addition, the production of NADPH by malate-pyruvate cycling promotes redox homeostasis, providing the reducing equivalents for glutathione reductase to regenerate glutathione. Finally, in the intestine, enterocytes convert Glu into delta1-pyrroline-5-carboxylate allowing the formation of l-proline, l-ornithine and l-citrulline. By generating l-citrulline, which is subsequently metabolized to the NOS substrate l-arginine by the concerted action of argininosuccinate synthetase and argininosuccinate lyase in the kidney, Gln also functions as a precursor of l-arginine to drive NO synthesis 36.

Confirming this, Hu et al. (2020)37 studying different levels of glutamine supplementation in the diet of chickens under cyclic heat stress, concluded that the inclusion of glutamine increased the activity of antioxidant enzymes and reduced the expression of markers related to lipid peroxidation and muscle oxidation.

In addition, Bai et al. (2019)38 in a study supplementing chicken diets subjected to different periods of heat stress with glutamine observed that Gln supplementation was associated with improvements in antioxidant capacity and lipid peroxidation in heat-stressed broilers.

Surai et al. (2019)39 also state that in the case of the antioxidant system of the developing embryo, it can be said to consist of: antioxidant enzymes (glutathione peroxidase GSH-Px, superoxide dismutase SOD and catalase CAT), water-soluble antioxidants (ascorbic acid, glutathione, taurine, carnitine, etc.), fat-soluble antioxidants (vitamin E, carotenoids, coenzyme Q) and mineral antioxidants (Se, Mn, Zn, etc.). Poultry embryos are more susceptible to oxidative stress due to their extremely rapid development, high metabolic rates and high content of polyunsaturated fatty acids in their tissues. In ovo inoculation (IOF) using natural antioxidants increases the activity of antioxidant enzymes, which play a fundamental role in the elimination and detoxification of free radicals and also decreases the concentration of malondialdehyde (MDA) as an index of lipid peroxidation 11,40.

Amino acids have a high antioxidant capacity as they maintain a balance between the synthesis and removal of free radicals.

IN OVO GLUTAMINE (IOF) X BIRDS' IMMUNE RESPONSE

The state of health of poultry is directly related to their immune system; birds with an adequate immune system grow better.

Despite this, modern chickens have become more sensitive to infectious diseases due to their high growth pattern. Therefore, much attention has been paid to improving the immune system and activating the immune response in the early stages of growth. The IOF of nutraceuticals, immune stimulants and bioactive components could be a promising tool for improving the post-hatch immune response in poultry and providing protection against diseases 41,20.

Amino acids are extremely beneficial structures in improving the general physiological state, immunization against infectious diseases and stabilization under non-infectious or management conditions (Saleh et al. 2018), thus improving the productive performance of poultry 25.

In addition, amino acid metabolism is different depending on the state of health in birds (challenge vs. non-challenge conditions) 42. digestibility, absorption and metabolism of amino acids is affected in health and disease. Their deficiency can predispose and their supplementation can prevent or alleviate diseases 42.

Amino acid supplementation resulted in cecal butyric acid production and total short-chain fatty acid production and supported growth, development, feed conversion efficiency and improved immunity 42.

An adequate supply of dietary amino acids is necessary to maintain normal immunocompetence and host protection against some diseases in all species. Dietary supplementation with free methionine attenuates intestinal oxidative stress induced by Eimeria spp. in broiler chickens 43.

In another study, Toghyani et al. (2019)44 reported that IOF of arginine (35 mg) and/or threonine (25 mg) on day 14 of incubation significantly increased antibody titers (SRBCs), while titers against Newcastle disease and avian influenza did not increase significantly. In addition, arginine IOF significantly increased the relative weights of the bursa of Fabricius and spleen compared to the simulated control at 11 days of age in broilers.

Similarly, Gonzales et al. (2022)13 revealed that IOF with a combination of sulphur amino acids (5.90 mg of l-methionine and 3.40 mg of l-cysteine) plus 0.150 mg of folic acid increased the relative weights of the thymus and spleen at 21 days of age. Furthermore, it has been shown that that IOF of 100 µg l-arginine (Subramaniyan et al., 2019)41 or 100 µg of l-arginine conjugated with 1,000 µg of silver nanoparticles (Subramaniyan et al. 2021)45 on day 14 of incubation increased serum IgM concentration in newly hatched chicks.

This amino acid plays an important role in necrotic enteritis, which causes significant economic losses in the broiler industry. L-glutamine compensates for the metabolic losses of this infection, improves intestinal development and intestinal morphology, improves growth performance and serum biochemical indices 46.

The protective effects of Gln may be the result of increased expression of junction cells (TJP) in the small intestine and decreased intestinal permeability during stress. The mechanism for these protective effects may be through Gln activation of occludin, claudin-1, zonula occludens-2 (ZO-2), zonula occludens-3 (ZO-3) and corticotrophin-releasing factor 47.

This demonstrates that Gln supplementation can regulate TJP expression, which has a beneficial effect on mucosal barrier function and health under conditions of heat stress. (2018)48 are consistent with these reports and suggest that Th1 and Th2 responses were inhibited by the presence of glutamine and that it plays a beneficial role in alleviating the intestinal inflammatory response.

In addition, in the trials conducted by Salmanzadeh et al. (2020)49 with glutamine IOF at different levels for 43-week-old layers, an increase was observed in the number of lymphocytes and heterophils, as well as in the heterophil/lymphocyte (H/L) ratios of newly hatched chicks and heterophil/lymphocyte (H/L) and 10-day-old chickens. All the Gln levels used increased the relative spleen and bursa weights of newly hatched chicks and the spleen weight of broilers at 10 days of age. On the 26th day of age, increasing the dose of Gln increased the concentration of immunoglobulins G and M.

These data and reports reinforce the potential of glutamine, especially that supplied via in ovo feeding (IOF), as an amino acid of unparalleled importance for the general health of chickens and, consequently, for broiler performance.

FINAL CONSIDERATIONS

After analyzing the reports presented, it can be concluded that glutamine has a huge range of benefits for animals and humans, many of which still need to be better studied and elucidated.

In addition, when combined with in ovo technology (IOF), it can be assumed that these effects can be taken advantage of earlier by the birds, which will have an earlier and more complete intestinal development and a modulation of the intestinal cells in order to improve its health, generating better immune responses which, in the end, will result in better all-round health for these birds.

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