The effect of threonine on mucin2 gene expression, intestinal histology and performance of broiler chicken

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Abstract

Threonine needs based on Ross manuals might be 14% of the National Research Council (NRC) recommendation, thus the effect of threonine on mucin2 gene expression, histological characteristics and performance were evaluated in Ross male broilers fed diets containing 0.8% (NRC requirement), 0.87% (average of NRC and Ross requirement), 0.94% (Ross requirement) and 1.01% (more than Ross requirement) total threonine. The dietary treatments consisted of an isonitrogenous corn-soybean meal-based diet with incremental levels of threonine. At the first day of chicken age, 24 pens were equalized to 12 birds per pen, in a completely randomized design and dietary treatments were randomly distributed for the 14-d period. Live performance measurements improved (P<0.05) as dietary threonine increased from 0.8% to 0.87%. The least performance was related to diet containing 1.01% threonine. Histological assays showed that villi height, crypt depth and villi surface increased as dietary threonine increased from 0.8% to 0.87% and decreased in 0.94% and 1.01% threonine. There was no effect of threonine on mucin2 gene expression and goblet cell density. According to these results, threonine needs in starter period in Ross (308) broilers is more than NRC recommendation.

Introduction

Dietary amino acid concentration should closely meet maintenance and tissue growth requirements of commercial broilers and under- and over- formulation of amino acids will decrease performance and increase nitrogen excretion, respectively (Kidd et al., 2004). Threonine (Thr) was discovered over 60 years ago and is considered to be the third limiting amino acid for broilers fed corn-soybean meal (Waldroup et al., 2005). The essential amino acid Thr may be of particular interest in terms of intestinal growth and development due to its importance in the structure of mucins (Horn et al., 2009). The nutrient Thr must be considered in dietary formulations for commercial broilers because its excess is costly and its deficiency will decrease the sufficiency of total sulfur amino acid (TSAA) and lysine (Lys) use (Kidd, 2000).

The intestinal tract epithelium is covered by a mucus layer composed predominantly of mucin glycoproteins, which are synthesized and secreted by goblet cells distributed along the villi and goblet cells utilize substantial amounts of nutrients for the synthesis of mucins (Uni et al., 2003). Mucin affect protecting the gut from acidic chime, digestive enzymes and pathogens, filtering nutrient in the gastrointestinal tract, nutrient digestion and absorption (Horn et al., 2009). Proteins and specific amino acids have been shown to alter mucin secretion and may interact directly with goblet cells or with the enteric nervous system to elicit changes in mucin secretion (Montagne et al., 2000; Claustre et al., 2002; Faure et al., 2005). Studies in piglets showed between 80% and 90% to dietary Thr is used by the intestine (Schaart et al., 2005). The portal-drained viscera (PDV) has a high obligatory visceral requirement for Thr and the high rate of intestinal Thr utilization is due mainly to incorporation into mucosal proteins (Van Klinken et al., 1995; Lien et al., 1997).

Intestinal mucin synthesis is sensitive to dietary Thr supply, which suggests that the gut’s requirement for Thr may comprise a significant proportion of the whole body requirement (Nichols and Bertolo, 2008). De novo synthesis of mucosal and mucin proteins is sensitive to luminal Thr concentration, which demonstrates the importance of dietary amino acid supply to gut protein metabolism (Nichols and Bertolo, 2008). The structure of Mucin gene (MUC2) is composed by 11% Thr (Gum, 1992). The hydroxyl group of Thr and serine is necessary for ester linkages on the mucin amino acid backbone to carbohydrate groups that make up the majority (50% to 80%) of the molecular weight of mucin (Montagne et al., 2004). There is a lack of information about the effects of Thr levels more than NRC (1994) on performance and mucin dynamics in poultry, especially for starter period. However, Ross requirement (0.94%) is 14% more than NRC (1994). Thus, this experiment was conducted to determine whether providing dietary Thr higher than NRC (1994) can effect on performance, type of gut mucins, goblet cell density, and mucin2 (MUC2) mRNA abundance in growing broiler chicks.

Materials and methods

Experimental design

One-day-old chicks were feather sexed, and male birds were selected. Chicks were then transported to a research facility and placed in pens at this age. The pens were 0.8x1.2 m and contained a tube feeder and nipple water line. Birds were reared at 32°C and 29°C from days 1 to 7 and 8 to 14, respectively. Birds had access to continuous lighting and unrestricted access to feed and water. Management and husbandry practices were in accordance with current standards.

At day 1, 24 pens were equalized to 12 birds per pen, in a completely randomized design and dietary treatments were randomly distributed for the 14-day period. Diets were formulated to provide a minimum of 100% of NRC (1994) amino acid recommendations for 1 to 14 days of age. The dietary treatments consisted of isonitrogenous corn-soybean meal-based diets with 4 levels of total Thr (0.8%, NRC requirement; 0.87%, average of NRC and Ross requirement; 0.94%, Ross requirement; and 1.01%, more than Ross requirement). Treatments were achieved by the addition of...
crystalline L-Thr (98.5 % Thr, Degussa Co., Essen, Germany) at the expense of filler in the test diet containing 0.8% Thr (Table 1). Supplementation of L-Glutamic acid served to make the diets isonitrogenous.

Corn and soybean meal were analyzed for CP (AOAC, 1990) and amino acids (all samples were analyzed by Degussa Co., in Essen, Germany) prior to formulation. The experimental protocols were reviewed and approved by the Animal Care Committee of the Ferdowsi University of Mashhad, Iran.

**Tissue sampling**

At day 14, one chicken from each replicate was killed and intestinal segments removed. Samples (approximately 4 cm) were taken from the midpoint between the point of entry of the bile duct and Meckel’s diverticulum (jejunum). These segments were cut in half and one part was frozen in -80°C and the other part was flushed with 0.9% (wt/vol) NaCl and then was fixed in fresh 4% formaldehyde buffer. Jejunum was of particular interest because it is a major site of nutrient absorption in poultry (Horn et al., 2009).

**Analysis of histological samples**

After fixation, soaked samples were rinsed several times in absolute alcohol, and then embedded in paraffin. Serial 6-µm longitudinal sections were cut on a Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar, Germany) and placed on glass slides. Then, slides routinely stained with Gill’s hematoxylin and eosin (H&E). For the histochemical evaluation of gut mucins, other representative sections were stained with 1% Alcian blue (AB) for the demonstration of all acidic mucins (sialomucins and sulfomucins). Periodic acid Schiff was used for detection of neutral mucins (Law et al., 2007).

**Neutral and acid mucin staining**

Neutral mucin was measured by staining 6 µm sections with periodic acid-schiff (PAS) (Uni et al., 2003). Briefly, procedure steps consist of i) deparaffinize and hydrate to eliminate the contribution of sialic acid residues before PAS staining; ii) oxidize in 0.5% periodic acid solution for 15 min; iii) rinse in distilled water; iv) immerse in Schiff reagent for 30 min (sections become light pink color during this step); v) wash in warm water for 10 min (immediately sections turn dark pink color); vi) dehydrate with ethanol and mount in glass slide. The number of PAS positive (PAS+) along the villi were counted by light microscopy.

Acid mucin measured by staining 5 µm sections with AB, pH 2.5 (Lev and Spicer, 1964). Briefly, method steps consist of i) dissolving dye in 3% acetic acid to provide a solution with PH 2.5; ii) bringing sections to distilled water; iii) staining in the AB solution pH 2.5 for 15 min; iv) wash well in running tap water for 5 min; v) rinse in distilled water; vi) counterstain with neutral red stain for 1 min; vii) rapidly dehydrate in absolute alcohol, clear and mount. The number of AB positive cells (AB+) along the villi was counted with light microscopy by using EPIX XCAP software (Image pro Express, Mediacybernetic, Atlanta, GA, USA).

Histomorphometric analysis was performed on H&E-stained tissue sections. The parameters measured were as follows: villus height (measured from the tip of the villus to the villus-crypt junction), crypt depth (measured from the crypt-villus junction to the base of the crypt), and villus surface area \( \pi \times \text{midvillus height} \times \text{midvillus width} \times \text{h} \), where \( \pi \) is the width at the midvillus height and \( h \) is villus height (Law et al., 2007). Villi length and width were measured from 5 villi per bird and only complete, vertically oriented villi were measured. Goblet cell counts were taken from the same 5 villi per bird and the average value was used. Density of goblet cells was calculated as the number of goblet cells per unit of surface area (mm²).

**RNA extract, reverse transcription and real-time PCR**

Relative real-time PCR was performed to assess MUC2 gene expression in jejunum of broiler chicken. Total RNA was extracted from jejunum samples using the RNXTM (+=Plus) (RN7713C, Cinnagen Inc., Tehran, Iran) according to manufacturer’s instructions. The quantity and integrity of isolated RNA were determined by using UV absorbances (260/280). To remove residual DNA, was used RNase-free DNase I (EN0521, Fermentas, Opelstrasse 9, Germany), ribonuclease inhibitor (E00311, Fermentas, Germany) and buffer with MgCl2. DNase I was inactivated by EDTA and was incubated at 65°C for 10 min. Reverse transcription was performed by using a RevertAid™ First Strand cDNA Synthesis Kit (K1622, Fermentas) according to the manufacturer’s instructions and RT reaction was used of Random hexamer. We provided a control (one RNA sample without cDNA) for DNA contamination in PCR. All RNA samples were reversed transcribed simultaneously for minimize variations. We applied real time PCR for measurement mucin 2 gene mRNA abundance. The primers for MUC2 (Horn et al., 2009) (Intestinal mucin 2; Gallus gallus, XM_A21035) were as follows: forward 5′-TCA CCC TGC ATG GAT ACT TGC TCA 3′, reverse 5′-TGT CCA TCT GCC TGA ATC ACA GGT 3′ and with primers from the Gallsus gallus 18S ribosomal RNA gene (Smirnov et al., 2005) (GI 7262889; forward 5′-CGATGTCCTTAACTGATT-GT-3′, reverse 5′-CAGTTCGACCATACTC-3′). Real time PCR was executed in triplicate. Initial step contained 10 min at 95°C, and followed by 40 cycles of a two-phase PCR (denaturation at 95°C for 30s; annealing and extension at 62°C for 1 min). To confirm PCR product from misannealed primer was runned a dissociation curve following the real time PCR. The thermal profile for dissociation curve is, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s. Reaction mixtures for real-time PCR included 2 µL cDNA as template, 10 Power SYBRR Green PCR Master Mix (ABI, USA), 0.2 mM of each forward and reverse primers and 7.4 µL double distilled water. We used of ΔΔCt model. In this model, Ct value or threshold cycle is the first

### Table 1. Composition of basal diet fed to male broilers of 1 to 14 day of age.

| Ingredient   | %    |
|--------------|------|
| Corn         | 60.31|
| Soybean meal | 27.45|
| Gluten meal  | 5.79 |
| Poultry oil  | 1.27 |
| Calcium carbonate | 1.70 |
| Dicalcium phosphate | 1.19 |
| Sodium chloride | 0.23 |
| Sodium bicarbonate | 0.26 |
| Vitamins and minerals* | 0.40 |
| DL-Met       | 0.25 |
| L-Lys HCl    | 0.25 |
| Filler+      | 0.85 |
| L-Glutamic acid | 0.10 |

Calculated composition

| C%          | 21.22 |
|-------------|-------|
| ME, kcal/kg | 2852.95|
| Ca, %       | 1.00  |
| Available P, % | 1.45 |
| Lys, %      | 1.10  |
| TSAA, %     | 0.73  |
| Thr, %      | 2.00  |
| Leu, %      | 0.90  |
| Val, %      | 0.50  |
| Trp, %      | 0.50  |
| Arg, %      | 0.50  |
| Ile, %      | 0.50  |
| His, %      | 0.50  |
| Phe, %      | 1.00  |

*Premix provided the following per kilogram of diet: vitamin A (vitamin A acetate) 7718 U; cholecalciferol 2200 U; vitamin E (source unspecified) 10 U; menadione, 0.9 mg; B12, 1.1mg; choline, 750 mg; riboflavin, 5 mg; niacin, 15 mg; D-biotin, 1 mg; pyridoxine, 0.9 mg; ethoxyquin, 28 mg; manganese, 55 mg; zinc, 59 mg, iron, 28 mg; copper, 7 mg; iodine, 1 mg; selenium, 0.2 mg.

†The dose titration was achieved by addition of L-Thr at the expense of filler (sand).
cycle in PCR reaction in which the fluorescence signal increases significantly. \( \Delta \Delta C_t \) was calculated by subtracting the \( C_t \) amount of mucin 2 gene from Ct of 18sRNA for each sample. Then \( \Delta \Delta C_t \) was calculated from subtract \( \Delta C_t \) each treatments of \( \Delta C_t \) control. We selected the diet containing 0.8% Thr as control and then \( 2^{-\Delta \Delta C_t} \) is a good method to analyze the relative changes in gene expression (Bustin et al., 2009).

### Statistical analysis

Data were analyzed using GLM procedures of SAS software (SAS, 2006) in a completely randomized design. Differences between means were tested using Duncan’s test (1955). Differences were considered significant at P<0.05.

## Results

### Performance measurements

The corn-soybean meal basal diet was formulated to contain 21.16% CP. Analyzed diet showed 21.15% CP. Growth performance measurements as affected by dietary Thr presented in Table 2. Broilers fed 1.01% Thr had a poorer (P<0.05) feed to gain ratio response vs. broilers fed 0.8, 0.87 and 0.94% Thr. There was not any significant difference between feed intake. The lowest and the highest feed intake and body weight gain observed in birds fed 1.01% and 0.87% Thr, respectively. Body weight of chickens decreased (P<0.05) in diets containing 1.01% Thr when compared with those of 0.8, 0.87 and 0.94% Thr.

### Histomorphometry

Data for intestinal villus height, crypt depth and villus surface area are presented in Table 3. Dietary treatments affected villus height, crypt depth and villus surface area in jejenum of broiler chicks fed levels of Thr for 14 days. Villus height increased (P<0.05) to 634.09 µm of broiler chicks fed levels of Thr for 14 days. Crypt depth and villus surface area in jejunum for broiler chickens. 3. Dietary treatments affected villus height, crypt depth and villus surface area in jejunum for broiler chickens.

### Real-time PCR

Table 5 shows the average CT results for treatments and how these CTs are manipulat-ed to determine \( \Delta \Delta C_t \) and the relative amount of MUC2 mRNA. There was not any significant effect of the different levels of Thr on MUC2 gene mRNA abundance in the jejunum for broiler chickens. MUC2 gene mRNA abundance in chicks fed 1.01% Thr decreased in comparison to the others, however it was not significant.

### Discussion

The essential amino acid Thr is typically the third limiting amino acid behind TSAA and Lys in commercial broiler diets composed of corn or sorghum, soybean meal and meat meal (Kidd and Kerr, 1996; Kidd, 2000). Thr is not only an essential amino acid for growth in young chicks, but also its preferential utilization by the gut for mucus synthesis makes it disproportionally essential for maintenance requirements. Up to 90% of dietary Thr is extracted by the portal-drained viscera (versus only about a third for other essential amino acids) (Stoll et al., 1998; Van Goudoever et al., 2000; Van Der Schoor et al., 2002; Schaart et al., 2003). Thr’s requirement for maintenance functions in the gut would be particularly sensitive to Thr supply. The protective mucous layer in the gut predominantly consists of mucins, glycoproteins that are particularly rich in Thr. More ever, mucins are continuously synthesized and very resistant to small intestinal proteolysis and hence recycling; therefore, mucin
synthesis is largely an irreversible loss of Thr (Van Der Schoor et al., 2002). As a result, a substantial and constant supply of Thr is necessary to maintain gut function and structure (Law et al., 2007). It is important to note that Ross (2007) has estimated Thr requirements 0.14% more than NRC (1994). To our knowledge, previous studies have been published examining the effects of the less Thr levels on the different characteristics. As a result, in the current experiments, dietary Thr levels increased to 0.07, 0.14, and 0.21% more than NRC (1994) recommendation. In the study presented herein and in others evaluating Thr needs of starting (Horn et al., 2009), growing (Kidd et al., 2004) and finishing (Kidd et al., 1999) male broilers, diets containing surfeit Thr allowed for better growth however performance decreased in 1.01% Thr.

Goblet cells secrete mucins (Horn et al., 2009) and mucins are polymeric glycoproteins representing an important component of the mucus layer that covers the epithelium of the gastrointestinal tract (Montagne et al., 2004). The mucin is an important component of the mucus and contributes to the lubrication of the gut epithelium, the protection of the intestinal lumen from an acidic environment and bacterial protease, colonization resistance, and the repair of the epithelium (Montagne et al., 2004). The mucin is an important component of the mucus that covers the epithelium of the gastrointestinal tract (Montagne et al., 2004). The mucin is an important component of the mucus and contributes to the lubrication of the gut epithelium, the protection of the intestinal lumen from an acidic environment and bacterial protease, colonization resistance, and the repair of the epithelium (Montagne et al., 2004). The mucin is an important component of the mucus that covers the epithelium of the gastrointestinal tract (Montagne et al., 2004). The mucin is an important component of the mucus that covers the epithelium of the gastrointestinal tract (Montagne et al., 2004).

![Figure 1. View of goblet cells of the proximal jejunum on day 14. Each photo became disposition of goblet cells with related treatments. (0.8% = NRC requirement, 0.87% = average of NRC and Ross requirement, 0.94% = Ross requirement, and 1.01% = more than Ross requirement).](image)

| Treatment | MUC2 (Average Ct) | 18s RNA (Average Ct) | ΔCt (MUC2-18s RNA) | ΔCt (ΔCt-18s RNA) | MUC2 to NRC Rel. (%) |
|-----------|-------------------|----------------------|--------------------|-------------------|----------------------|
| 0.8       | 25.74625          | 5.09322              | 20.65303           | 0                 | 1                    |
| 0.87      | 26.56885          | 6.130465             | 20.41835           | -0.234645         | 1.176612             |
| 0.94      | 25.73105          | 6.010605             | 19.72045           | -0.932585         | 1.9066929            |
| 1.01      | 26.74109          | 6.099423             | 20.641667          | -0.011363         | 0.992257             |
| SEM       | -                 | -                    | -                  | -                 | 0.079                |

*The ΔCt value is determined by subtracting the average 18s RNA value from the average MUC2 CT value; the calculation of ΔCt involves subtraction by the ACT calibrator value; the range given for MUC2 relative to NRC is determined by evaluating the expression.*

(Deplancke and Gaskins, 2001). The physiological relevance of distinct mucin subtypes is not well understood. It has been suggested that acidic mucins protect against bacterial translocation as sulfated mucins appear to be less degradable by bacterial glycosidases and host protease (Fontaine et al., 1996; Robertson and Wright, 1997). Mucin-producing cells were observed in the small intestine from 3 days before hatch, and at this time contained only acidic mucin. After hatch and until day 7 posthatch, the proximal, middle, and distal segments of the small intestine contained similar proportions of goblet cells producing acidic and neutral mucins (Uni et al., 2003). These observations suggested that, adequate Thr intake supported the production of mixtures of neutral and acidic mucins in the small intestine. Amino acids maintain intestinal viability and mass, in addition to providing energy for normal intestinal function. As gastrointestinal tissues have relatively high protein turnover rate, high protein diets provide nutrients for basal metabolism and causes a developed small intestine. On the other hand, Thr is of vital nutritional importance, because it is the single most used essential amino acid by the metabolism of portal-drained visera (Schaart et al., 2005). Villus surface area was numerically decreased from 0.628 mm² to 0.42 mm² as the Thr levels increased from 0.87% to 1.01% Thr. There was a tendency for a decrease in crypt depth when dietary Thr increased from 0.80% to 1.01%. These results were in consis-
tent with the report by Law et al. (2007) who found that villus height in piglet receiving Thr deficient diet decreased compared to those of receiving the Thr adequate diet.

More than 20 human mucin genes have been catalogued by the National Library of Medicine. Mucins are usually subdivided into two groups, the secreted mucins (gel-forming and non-gel-forming) and the membrane-anchored mucins. The second group consists of the two large mucins MUC3 and MUC4, containing EGF-like motifs, and the small mucin MUC1, MUC6, MUC2, MUC5AC and MUC5B are the secreted gel-forming mucins (Desseny et al., 2000). MUC2 in gallus gallus is on chromosome 5 and encodes a gel-forming protein and is expressed in the small intestine and other mucus membrane-containing organs. Intestinal loss of MUC2 affects the protective capacities of the mucus layer and leads to colonic inflammation in mice (Sluis et al., 2009). The inflammation in these mice is characterized by a thickening of the mucosa, loss of epithelial integrity, and an increase in infiltrating cells (Sluis et al., 2009). Each subunit of a mucin protein backbone contains a central domain that is rich in serin, Thr, proline, alanine, and glycine, and two extending peptides (N and C terminal) that contain cystein. The splanchnic tissues of preterm infants use more than three quarters of the total dietary Thr intake, irrespective of the amount of enteral Thr delivered (Sluis et al., 2009).

In this experiment, there was no effect of diet on MUC2 mRNA abundance. Smirnov et al. (2006) reported increase in mucin mRNA expression from day 17 of incubation to 3 days of posthatch. There was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks and there was no relationship between crude mucin mRNA abundance in chicks and there was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks and there was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks and there was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks and there was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks and there was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks and there was no change of diets containing 3.3 and 8.2 g of Thr/kg for 7 days on mRNA abundance in chicks.

Conclusions

The aim of this study was to investigate the effect of threonine levels on mucin2 gene expression, intestinal histology and performance of broiler chickens. Body weight, villus height and villus surface was affected (P<0.05) in chicks fed with the various levels of threonine in this experiment. Mucin2 cDNA in jejunum tissue in broiler chicken was characterized. Mucin2 gene expression was not affected in chicks, whereas there was a tendency for a decrease in broilers fed diet containing 1.01% threonine. Additional studies need to investigate the role of threonine on mucin 2 gene expression and intestinal histology in broiler chickens.

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[page 70] [Ital J Anim Sci vol.10:e14, 2011]
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