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The Effect of Vitamin A on Mucin2 Gene Expression, Histological and Performance of Broiler Chicken

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Abstract: Vitamin A needs based on *Ross* manuals is 9500 IU/kg more than NRC (1994) recommendation, thus the effect of vitamin A on mucin*2* gene expression, histological characteristics and performance were evaluated in Ross male broilers fed diets containing no vitamin A supplement, 1500 IU/kg vitamin A supplement (NRC requirement), 6250 IU/kg vitamin A supplement (average of NRC and Ross requirement) and 11000 IU/kg vitamin A supplement (Ross requirement). The dietary treatments consisted of an isonitrogenous corn-soybean meal-based diet. 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 vitamin A supplement increased from 0 to11000 IU/kg. The least performance was related to no vitamin A supplement containing diet. Histological assays showed that villi height, crypt depth and villi surface increased as dietary vitamin A supplement increased .There was no effect of threonine on mucin2 gene expression and goblet cells density. According to these results, vitamin A needs in starter period in Ross (308) broilers is more than NRC (1994) recommendation.

Key words: Broiler · Mucin*2* gene expression · Histology · Performance · Vitamin A

INTRODUCTION

Retinoids (vitamin A metabolites and analogs) have important functions in vision, reproduction, hematopoiesis, differentiation of epithelial cells [1] and affect on growth, ruffled feathers, weakness, xerophtalmia, egg production and immune system [2]. Abnormal intake of dietary vitamin A causes keratinization and drying of the epithelial in the gastrointestinal tract, respiratory tract and ocular surface [3]. Vitamin A and its derivates are required for maintenance of various epithelial tissues at the appropriate differentiated stages and small intestine is one of the tissues exhibiting rapid cell proliferation and differentiation [4]. Therefore, this tissue was selected for the current study and jejunum in small intestine is particular interest because it is a major site of nutrient absorption in poultry [5]. Vitamin A deficiency in experimental animals leads to depression of synthesis in the intestinal mucosa of glycoproteins, activation of the enzymes of their catabolism, a decrease in the number of goblet cells and lengthening of the cell cycle of the epithelium of the crypts [1].

Mucus is a complex viscous adherent secretion synthesized by specialized goblet cells in the columnar epithelium such as the respiratory tract, the gastrointestinal tract, the reproductive tract and the oculo-rhino-otolaryngeal tracts [6]. Mucin is the major constituent of the mucus layer and serves a crucial role in protecting the gut from acidic chyme, digestive enzymes and pathogens. In addition to its protective function, mucin is involved in filtering nutrients in the gastrointestinal tract (GIT) and can influence nutrient digestion and absorption [7]. Currently, 17 subtypes of human mucin coreroeins have been isolated from different organs and are referred to as the MUC family. The MUC family can be classified into 2 groups . One of them is the secreted and mucus-forming mucins (MUC2, MUC5B, MUC5AC and MUC6) and the other group is the membrane-bound mucins (MUC1, MUC3, MUC7). MUC2 and MUC3 mucins are highly

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expressed in the small intestine [8]. In the eye, vitamin A deficiency is reported to be associated not only with keratinization and squamous metaplasia, but also with a reduction in the goblet cell population and an increase in conjunctival epithelial cell mitosis. Vitamin A has been shown to be involved in biosynthesis of glycoconjugates by the rat corneal epithelium, but it is not clear whether the reduction of goblet cells is due to altered glycosylation of the secretory mucins expressed by the goblet cells or to altered expression of the mucin gene [3].

In this report, NRC [9] requirement for vitamin A (1500 IU) was compared to Ross [10] recommendation for vitamin A and the more and less levels of it. More specially, our aim was to determaine the effect of these levels on performance, type of gut mucins, goblet cell density and *MUC2* mRNA abundance in growing broiler chicks.

MATERIALS AND METHODS

Experimental Design: Day-old chicks were feather sexed and male birds were selected. Chicks were then transported to a research facility and placed in pens (12 birds/pen) at this age. The pens were 0.9×1.2 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. Basal diet was formulated based on soybean meal-corn to provide a minimum of 100% of NRC [9] amino acid recommendations for 1 to 14 days of age. We used corn instead of wheat or sorghum because our aim was research about mucus in jejunum and in this way the effects of non starch polysaccharides on mucus decreased. Treatments supplemented to any vitamin A (T_1), 1500 IU/kg (T_2), 6250 IU/kg (T_3) and 11000 IU/kg (T_4). The NRC [15] recommendation is 1500 IU/kg for vitaminA and the Ross [10] requirement for vitaminA is 11000 IU/kg in corn based diet. Thus, this study was carried out to compare NRC [9] requirement to Ross [20] requirement and the less and more levels of vitamin A. The experimental protocols were reviewed and approved by the Animal Care Committee of the Ferdowsi University of Mashhad.

Tissue Sampling: At day 14, one chicken from each replicate of treatments 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 freezed 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 [5].

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 Rotary microtome (LEICA RM 2145) and placed on glass slides. Then, slides routinely stained with Gill's haematoxylin and eosin (HandE). 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 [11].

Neutral and Acid Mucin Staining: Neutral mucin was measured by staining 6 ìm sections with periodic acid-schiff (PAS) [12]. Briefly, procedure is:1) Deparaffinize and hydrate to water to eliminate the contribution of sialic acid residues before PAS staining; 2) Oxidize in 0.5% periodic acid solution for 15 minutes; 3) Rinse in distilled water; 4) Immerse in Schiff reagent for 30 minutes (sections become light pink color during this step); 5) Wash in warm water for 10 minutes (immediately sections turn dark pink color); 6) Dehydrate with ethanol and mounth in glass slide. The number of PAS positive (PAS+) along the villi were counted by light microscopy.

Acid mucin measured by staining 5 ìm sections with AB, pH 2.5 [7]. Briefly, methods is; 1) Dissolving dye in 3% acetic acid to provide a solution with PH 2.5; 2) Bring sections to distilled water; 3) Staining in the AB solution pH 2.5 for 15 mins; 4) Wash well in running tap water for 5 minutes; 5) Rinse in distilled water; 6) Counterstain with neutral red stain for 1 minute; 7) Rapidly dehydrate in absolute alcohol, clear and mount. The number of AB positive cells (AB+) along the villi were counted with light microscopy by using EPIX XCAP software.

Histomorphometric analysis were performed on HandE-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), villus width at midvillus height and villus surface area

$[(\pi \times mh \times h) + (\pi \times mh/2)^2]$, where mh is the width at the midvillus height. 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 (mm^2).

Reverse Transcription (RT)-PCR and Real-time PCR: Relative real-time PCR was performed to assess MUC2 gene expression in jejunum of broiler chicken. Total RNAs were extracted from jejunum using the RNX™ (-Plus) (RN7713C, Cinnagen Inc. Tehran, Iran) according to manufacturer's instructions. RNA concentration was assayed by spectrophotometer in length wave of 260/280 nm. Before RT reaction, samples of the isolated RNA was treated with 1 U/ml of RNase-free DNase I (EN0521, Fermentas, Opelstrasse 9, Germany) per 1 mg of RNA in order to eliminate residual DNA in the presence of 40 U/ml of ribonuclease inhibitor (E00311, Fermentas, Germany) and 1 × reaction buffer with $MgCl_2$ at 37°C for 30 min. To inactivate the DNase I, 1 ml of 25 mM EDTA per 1 mg of RNA was added and incubated at 65°C for 10 min. Standard RT reactions were performed with 2 mg total RNA using Random hexamer as a primer and a RevertAid™ First Strand cDNA Synthesis Kit (K1622, Fermentas, Germany) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without Revert Aid™M-MuLV Reverse Transcriptase (RT-reaction) to provide a control for DNA contamination in the subsequent PCR. To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneous. After that we applied relative real-time PCR for quantitative measurement of Gene expression. The primers for MUC2 (Intestinal mucin 2; Gallus gallus, XM_421035) were as follows: 5'- TCA CCC TGC ATG GAT ACT TGC TCA-3', forward 5'-TGT CCA TCT GCC TGA ATC ACA GGT-3' and with primers from the Gallus gallus 18S ribosomal RNAgene(GI7262899;forward:5'-CGATGCTCTTAACTGAGTGT-3',reverse: 5'CAGCTTTGCAACCATACTC-3'). All primer were confirmed in NCBI [13]. Real time PCR was executed in triplicate. Reaction conditions were 40 cycles of a two-phase PCR (denaturation at 95°C for 30 s; annealing at 62°C for 1 min.) after an initial denaturation step (95°C for 10 min.). In real-time assay, a melt curve analysis, performed at the end of the PCR cycles, will confirm specificity of primer annealing. 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 SYBR® Green PCR Master Mix (ABI, USA), 0.2 mM of each forward and reverse primers and 7.4 µl double distilled water. For each sample, we had a target gene and also a reference gene as internal control. The efficiency-calibrated model is a more generalized $\Delta\Delta C_t$ model. In this model, C_t is the sign of the first cycle that amplification curve begins to rise. In this method, we should consider both C_t of target gene and also C_t of reference gene or housekeeping gene. ΔC_t for each target gene is then calculated by subtracting the C_t number of target gene from that of housekeeping gene for each sample. $\Delta\Delta C_t$ for each gene was calculated by subtracting the ΔC_t of target sample from that of control sample [14].

Statistical Analysis: Data were analyzed using GLM procedures of SAS software [15] in a completely randomized design. Differences between means were tested using Duncan 's test [16]. Differences were considered significant at $P < 0.05$.

RESULTS

Performance Measurements: Performance measurements of male broiler presented in table 2. In T_1 , foodstuff contained sufficient vitamin A and diet contained no vitamin A supplement. Body weight was the lowest in $T_1$when compare to 1500, 6250 and 11000 IU/kg supplemental dietary vitamin A. There was a tendency for an increase in body weight as vitamin A levels in supplement increased however it was not significant. The highest body weight was related to T_4 (11000 IU/kg vitamin A supplement). Feed intake was not affected by vitamin A levels and there was no significant difference in feed intake. Broilers fed no vitamin A supplement had a poorer feed to gain ratio response (1.71) vs. broilers fed with vitamin A supplement. However, there was no significant difference in feed : gain ratio, the best amount (1.61) was belonged to 11000 IU/kg supplemental dietary vitamin A.

Histomorphometry and Histochemistry: Data for intestinal villus height, crypt depth and villus surface area are presented in Table 3. Villus height increased to 618.53 im in broilers fed with 11000 IU/kg vitamin A supplement in comparison to the other treatments. The lowest villus height (600.14 im), crypt depth (139.03 im), and villus surface (0.603 im^2) observed in broilers fed no supplemental dietary vitamin A. Villus height, crypt depth and villus surface was numerically, but not significantly, increased when vitamin A dietary increased to 11000 IU/kg.

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

Ingredients	Amount (%)
Corn	60.31
Soybean meal	28.3
Gluten meal	5.79
Poultry oil	1.22
Calcium carbonate	1.7
Dicalcium phosphate	1.19
Sodium chloride	0.23
Sodiun bicarbonate	0.26
Vitamins and minerals1	0.4
DL-Met	0.25
L-Lys HCl	0.25
L-Glutamic acid	0.1
Calculated composition	
CP, %	21.22
MEn, kcal/kg	2952.95
Ca, %	1
Available P, %	0.45
Lys, %	1.1
TSAA, %	0.83
Thr, %	0.76
Leu,%	2
Val, %	0.9
Trp, %	0.23
Arg,%	1
IIe,%	0.8
His,%	0.5
Phe,%	1

[1]Permix provided the following per kilogram of diet: cholecalciferol 2,200 IU; vitamin E (source unspecified) 10 IU; menadione, 0.9 mg; B12, 11µg; choline, 780 mg; riboflavin, 5 mg; niacin, 33 mg; D-biotin, 1 mg; pyridoxine, 0.9 mg; ethoxyquin, 28 mg; manganese, 55 mg; zinc, 50 mg; iron, 28 mg; copper, 7 mg; iodine, 1 mg; selenium, 0.2 mg.

The histochemical staining results were interpreted as follows: with PAS, neutral mucins were staining red and AB 2.5, stained all acidic mucins (sialomucins and sulfomucins) blue. There was a tendency for an increase in goblet cells density from 11.001 cells/µm^2 to 11.383 cells/µm^2 by staining with PAS (periodic acid-schiff) and from 11.101 cells/µm^2 to 11.416 cells/µm^2 by staining with AB (alcian blue), as vitamin A supplement increased however it was not significant (Table 3). Most of the goblet cells numbers that contained neutral mucins were equal with numbers of acidic mucin-containing goblet cells in all treatments. Goblet cells in all treatments randomly distributed along the villus-crypt axis.

Real-time PCR: Table 4 shows the average C_T results for treatments and how these C_Ts are manipulated to determine ΔC_T, $\Delta\Delta C_T$ and the relative amount of *MUC2* mRNA. Before using the $\Delta\Delta C_T$ method for quentitation, a validation experiment was performed to determine the PCR efficiencies of the target and the reference gene. The absolute value of the slope of log ng total RNA vs. ΔC_T was < 0.1. A melting curve analysis, performed at the end of the PCR cycles, to confirm specificity of primer annealing. The melting curve displied a single sharp peak. There was not no significant effect of the different levels of Thr on *MUC2* gene expression.

Table 2: Live performance measurements of male broiler fed incremental levels of vitaminA from 1 to 14 days of age

Treatments[1] (IU/kg)	Body Weight (g/bird)	Feed Intake (g/bird)	Feed : Gain (g/g)
T_1	279.7	482	1.71
T_2	290.3	483	1.66
T_3	295.1	484	1.63
T_4	298	482	1.61
P-value	0.12	0.75	0.26
SEM	7.894	14.102	0.091

Means with no common superscripts differ significantly (P<0.05).

[1].vitamin A supplement (T_1: no vitamin A supplement, T_2: 1500 IU/kg vitamin A supplement, T_3: 6250 IU/kg vitamin A supplement, T_4: 11000 IU/kg vitamin A supplement).

Table 3: Effect of vitamin A on histomorphological and goblet cell density parameters in 14 day of age

Treatments[1](IU/kg)	Villus Height[2] (µm)	Crypt Depth[3] (µm)	Villus Surface[4]	Goblet cell density PAS[5]	Goblet cell densityAB (2.5)[6]
T_1	600.14	139.03	0.603	11.001	11.101
T_2	608.76	141.79	0.615	11.358	11.363
T_3	611.01	145.51	0.62	11.372	11.376
T_4	618.53	151.11	0.624	11.383	11.416
P-value	0.36	0.47	0.58	0.43	1.47
SEM	17.261	4.73	0.694	0.597	0.618

Means with no common superscripts differ significantly (P< 0.05).

[1].vitamin A supplement (T_1: no vitamin A supplement, T_2: 1500 IU/kg vitamin A supplement, T_3: 6250 IU/kg vitamin A supplement, T_4: 11000 IU/kg vitamin A supplement).

[2]. Villus height Measured from the tip of the villus to the villus-crypt junction.

[3]. Crypt depth is from the crypt-villus junction to the base of the crypt.

[4]. Villus surface was calculated as follows: $[(\pi \times mh \times h) + (\pi \times mh/2)^2]$, where mh is the width at the midvillus height.

[5]. Periodic acid-schiff stained neutral mucins.

[6]. Acid mucins was measured by staining sections with Alcian blue.

Table 4: Relative quantitation using the comparative C_T method

Treatments[1]	MUC2 (average C_T)	18s RNA (average C_T)	ΔC_T (MUC2-18s RNA)	$\Delta\Delta C_T$(ΔC_T-ΔC_T, T_1)	$2^{-\Delta\Delta C_T}$
T1	26.2034	5.2583	20.941	0	1
T2	25.90723	5.2146	20.69263	-0.25247	1.191244869
T3	25.76568	5.0816	20.68408	-0.26102	1.198325634
T4	25.74625	5.09322	20.65303	-0.29207	1.224395799
P-value					0.67
SEM					0.08

Means with no common superscripts differ significantly (P< 0.05).

[1]. vitamin A supplement (T_1: no vitamin A supplement, T_2: 1500 IU/kg vitamin A supplement, T_3: 6250 IU/kg vitamin A supplement, T_4: 11000 IU/kg vitamin A supplement).

[1].The ΔC_T value is determined by subtracting the average 18s RNA value from the average MUC2 C_T value.

[2]. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value.

[3]. The range given for MUC2 relative to NRC is determined by evaluating the expression: $2^{-\Delta\Delta C}{}_T$

DISCUSSION

Numerous reports have documented, in both *in vivo* and *in vitro* studies, that vitamin A deficiency or excess affect on the morphology and mucin production in the small intestine. There are so many differences between NRC [9] recommendation for vitamin A in comparison to the requirement of Ross [10]. It is 1500 IU/kg in NRC [9] and 11000 IU/kg in Ross [10]. The main purpose of this study was to compare NRC [9] requirement with Ross [10] requirement and the less and more levels of vitamin A supplement. In this experiment, body weight decreased in broilers fed T_1 (no vitamin A supplement) and when the vitamin A supplement level increased, body weight increased too, but feed intake was not affected by treatments. The final difference in weight, despite equivalent intake, may have been due to impaired nutrient utilization, as has been previously suggested [17]. There was a decline in weight, villus height, number of mucosa secreting goblet cells and total entrocytes, number of proliferating cells found along the crypt and anzimatic activity in wealing male rats fed a vitamin A deficient diet [18]. To our knowledge, studies have been published examining the effects vitamin A deficiency and excess and no studies have compared different requirements.

There was a tendancy for an increase in villus height and goblet cell density in broilers fed with incremental vitamin A supplement level but it was not significant. The lowest villus height (600.14 ìm), crypt depth (139.03 ìm), and villus surface (0.603 ìm^2) observed in broilers fed no supplemental dietary vitamin A and the highest villus height (618.53 ìm), crypt depth (151.11 ìm) and villus surface (0.624 ìm^2) was belonged to 11000 IU/kg vitamin A supplement. Vitamin A deficiency caused a decreased in the number of goblet cells and villus height [4,17].

There are some doubt about the formation vitamin A from beta-carotene perfectly. Hansen and Maret [19] reported that it is necessary to reevaluate the existing theory of conversion of beta-carotene into vitamin A. For producing vitamin A from beta-carotene needs some intermediates [20] and most of the beta-carotene produced in corn is converted into the other carotenoids, which make less or no vitamin A [21]. In turn, Van Vliet *et al.* [22] found in mammalian tissues in vitro and Barua *et al.* [2] in vivo, provitamin A carotenoids are converted mainly into vitamin A by central oxidative cleavage, which is catalyzed by the enzyme carotenoid 15,159-dioxygenase (EC 1.13.11.21).

Vitamin A supplement is important and although in the current study, corn and gluten meal provided NRC [9] requirement for vitamin A, but it was not sufficient and there was a poorer performance in broiler fed to no vitamin A supplement. There was a tendency for improvement in performance, histomorphometry and histochemistry measurements as the level of vitamin A supplement increased. Also, several factors, such as mycotoxins, reoviruses, coccidial infections and nutritional imbalances can cause vitamin A depletion and consequently affect growth rate.

In this study, no significant difference between neutral and acidic mucin-containing goblet cells was seen in all treatments and the number of goblet cells was almost equal. This finding is similar to observations in mammals [23] in which acidic mucin appears to predominate throughout fetal stages and clear developmental patterns of an increasing ratio of neutral to acidic mucins were found between birth and weaning neonatal piglets [24]. Acidic mucins (oligosaccharide chains terminated with sialic acid or sulfate groups) protect against microbial penetration and translocation because these mucins are more resistant to bacterial mucolytic activities [25].

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 [26,27]. 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 [12]. These observations suggested that, adequate vitamin A intake supported the production of mixtures of neutral and acidic mucins in the small intestine.

Tracheobronchial mucin production is regulated by vitamin A, most likely via its conversion to retinoic acid [28]. Retinoic acid, bound to a nuclear receptor and the retinoic acid receptor complex acts as a ligand-mediated transcription factor that interacts with the regulatory sequence in the mucin gene [28].

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 [29]. *MUC2* in gallus gallus is on choromosom 5 and encods 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 [30]. The inflammation in these mice is characterized by a thickening of the mucosa, loss of epithelial integrity and an increase in infiltrating cells [30]. In this experiment, there was no effect of diet on *MUC2* mRNA abundance, however, in T_1 was the least. Systemic vitamin A depletion results in keratinization and drying of the epithelium in the gastrointestinal tract, respiratory tract and ocular surface. membrane-spanning mucin ASGP (rMuc4) and secretory mucin rMuc5AC are directly or indirectly regulated by vitamin A in ocular surface epithelium [3].

Conclution:In according to our results, Ross recommendation made the best performance. Body weight significantly ($P<0.05$) decreased in broilers fed with no vitamin A supplement. There is a tendency for an increase in villus height, crypt depth, villus surface, goblet cell density and MUC2 gene expression when broilers fed with 11000 IU/kg (Ross requirement [10] in comparison to 1500 IU/kg (NRC[9]) vitamin A supplement. These observations suggested that vitamin A supplement is necessary for providing vitamin A requirement and has an important role in the integrity and functionality of the intestinal epithelium.

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