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SHORT COMMUNICATION

# Effect of in ovo injection of threonine on immunoglobulin A gene expression in the intestine of Japanese quail at hatch

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## Summary

The objective of this study was to investigate the effect of in ovo injection of threonine (THR) on immunoglobulin A (IgA) gene expression of Japanese quail on hatch day. A total of 540 Japanese quail eggs were assigned into nine groups of 60 each and were set in a single-stage incubator. Treatments were as follows: non-injected (control), two diluent levels (0.05 or 0.1 ml saline), two sites of injection (in or under the air sac) and with or without nutrients (0.5 mg/ml THR). Eggs were injected on d 11 of incubation. On hatch day, after euthanizing hatched quail chicks, the intestine was removed and the jejunum was separated. The relative mRNA expression of jejunal IgA increased ($p < 0.05$) by the injection of 0.05 ml THR under the air sac when compared to the control group or other treatments of injection. Compared to the control group, no differences were imputable to treatments of 0.1-ml injections on IgA gene expression. Differences with other injected groups were not significant. It was concluded that injection of 0.05 ml saline containing 0.5 mg THR/ml under the air sac can improve jejunal IgA mRNA expression in newly hatched Japanese quail chicks.

**Keywords** Japanese quail, in ovo injection, threonine, gene expression, immunoglobulin A

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## Introduction

The immune system of birds is complicated and contained several cells as well as soluble factors that must work together to produce a shielding immune response. A properly functioning immune system is vital for poultry because most commercial flocks are raised under non-conventional rearing conditions.

The gut lining of birds forms the junction between harmful microflora and the enterocytes. Secretion of immunoglobulin A (IgA), which is synthesized in the gut-associated lymphoid tissue (GALT), is one of the main specific strategies to counteract with such pathogenic microflora; IgA is secreted in a dimeric form and screened from proteolysis by a secretory component. Thus, the secretory component enhances the biological activity of IgA *in vivo* (Parry and Porter, 1978). The presence of IgA in avian secretions was first reported by Lebacq-Verheyden et al. (1972) and Orlans and Rose (1972). The principal function of secretory IgA is to impede the penetration of micro-organisms by obstruction the cohesion to mucous membranes, improve antibacterial function and nullify bacterial toxins (Mestecky and McGhee, 1987; Bai et al., 2000).

As the immune system has not been well developed and there is a lack of immunocompetence, the digestive mucosa of newly hatched chicks is often exposed to dietary, bacterial, viral and parasitic antigens (Strobel, 1986). From a nutritional point of view, substrates such as amino acids (AA), carbohydrates, vitamins, enzymes and cofactors are needed to support the clonal proliferation of antigen-driven lymphocytes (Kidd, 2004; Klasing, 2007). Besides dietary supplementation during the post-hatch growth, efforts have also been made to achieve higher protein synthesis during the embryonic period by administering AA directly into the egg (Ohta et al., 2001; Bhanja et al., 2004). Uni and Ferket (2003) patented 'In Ovo Feeding' and proved that this method can beneficially affect hatchability (Uni et al., 2005), intestinal mucosa, body weight at hatch and at 35 days of age (Uni and Ferket, 2004). Uni et al. (2003) reported that the gastrointestinal functionality of in ovo fed chicks was the same as that of 2-day-old birds fed immediately after hatch. Fast enteric development and improved nutritional

status obtained by in ovo feeding improved hatching weight, growth rate (Al-Murrani, 1982; Ohta et al., 1999) and immune responses (Konashi et al., 2000; Bhanja and Mandal, 2005). In this case, much research has been made with different species and nutrients (Zhai et al., 2011).

It is demonstrated that the threonine (THR) represents up to 30% of total essential amino acids content of the intestine (Neutra and Forstner, 1987), suggesting that THR affects the intestinal functionality and maintenance. In healthy rats, THR restriction reduced small and large intestine mucin synthesis (Faure et al., 2005). In addition, it has been shown that threonine, proline and serine are the main components of IgA (Feehally, 1997). It has also been reported that the concentration of IgA antibody in the ileum increased linearly at 0.4% L-threonine in laying hens when supplemented into the diet (Azzam et al., 2011b). In the next study at the same libratory, they showed that adding 0.2 and 0.3% of L-threonine improved egg production and serum Ig G, respectively, and beyond 0.1% L-threonine, the level of serum-free THR increased significantly (Azzam et al., 2011a). More recently, the results of experiment in our laboratory (Kermanshahi et al., 2015) showed that the in ovo injection of threonine in the quail eggs increased mucin2 gene expression, while this supplementation had no significant effects on digestive enzymes. As different poultry models were employed to examine such effects, to our knowledge, there has been rare research evaluating the effect of in ovo administration on immunity in quail. Thus, the objective of this study was to evaluate the effect of in ovo injection of threonine on IgA gene expression of Japanese quail on hatch day.

## Materials and methods

A total of 540 Japanese quail eggs were purchased from a commercial quail breeder and set in a single-stage incubator which had the relative humidity and temperature of 68% and 37.8 °C for the hatchery (0–14 days) stage and 78% and 36.8 °C for the setter (15–17 days) stage respectively. On day 11 of the embryonic stage (E11), eggs were injected with different volumes of solutions containing physiological saline with or without THR. Insulin needles (gauge 31 mm) were used to inject all solutions (except to intact control) which contained saline (0.9 g NaCl/litre of distilled water) as sham control and/or the carrier of threonine (L_Threonine, Sigma, Saint Louis, MO, USA) to the egg cavity. The experiment was conducted in a factorial arrangement of two injection sites (in the air sac (IAS): depth of injection: 5 mm or under the air sac (UAS): depth of injection 8 mm) × 2 injecting volume (0.05 ml or 0.1 ml) × 2 threonine levels (0 or 5 mg/ml) with a non-injected group as intact control. Each treatment had four replicates of 15 quail eggs. On hatch day, five hatched quail per replicate of each treatment (i.e. 20 quail from each treatment and a total of 180 chicks from all treatments) were euthanized by $CO_2$ asphyxiation, the intestine of each chick was detached, all connective tissue and fat were removed, and the jejunum were separated. The jejunal samples were stored at −70 °C until analysis. The experimental protocols were reviewed and approved by the Animal Care Committee of the Ferdowsi University of Mashhad, Iran.

Approximately 50 mg of total RNA was extracted from jejunal samples using Trizol procedure (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of total RNA was checked by native RNA electrophoresis on 1.0% agarose gel in 1 × TAE buffer (Tris-Acetate-EDTA Buffer, Sigma) (Tris 2.0 M, acetic acid 1.0 M and EDTA 0.1 M, pH 8.0). The quantity and purity of extracted RNA were assessed by the spectrophotometry method by measuring the absorbance in 260 nm and calculating the ratio of 260–280 nm respectively. Next, 2 $\mu$g of total RNA was used to synthesize the complementary DNA by MMLV (Moloney murine leukaemia virus) reverse transcriptase (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's recommendation. The abundance of IgA was determined on a Real-Time PCR system (ABI 7300, Applied Biosystems, Foster City, CA, USA), and chemicals were obtained from Thermo Fisher Scientific (SYBR PrimeScript RT-PCR kit).

The primer details are shown in Table 1. Each reaction was performed in a total volume of 20 $\mu$l in triplicate in addition to the negative control according to MIQE guidelines (Bustin et al., 2009). Product specificity was confirmed by plots of the melting curve derived by the 7300 software system and agarose gel electrophoresis of PCR products. The relative mRNA abundance was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gene expression data were normalized by beta-actin as a housekeeper gene, because previous experiments (Ubuka et al., 2005; Singh et al., 2011) demonstrated the stability of beta-actin mRNA expression in the intestine of Japanese quail and this gene was also stable in this study. The values are represented as an n-fold difference relative to the calibrator. The fold changes in the experimental groups were compared to the non-

**Table 1** Sequences of primer pairs used for amplification of target and reference genes

| Gene | Primer sequence | Amplification size | Annealing temperature (°C) | Accession number |
|---|---|---|---|---|
| Immunoglobulin A | Forward 5′ GAGGAGAGCATCAGGAAGGAGAC-3′<br>Reverse 5′ GAGGAGAGCATCAGGAAGGAGAC-3′ | 139 | 60 | S40610 |
| Beta-actin | Forward 5′ CTGGCACCTAGCACAATGAA-3′<br>Reverse 5′ CTGCTTGCTGATCCACATCT3′ | 123 | 60 | AF199488 |

injected control group, and so, the control group was considered as one in all comparisons.

A factorial arrangement of 2 (injection sites) × 2 (injecting volumes) × 2 (threonine levels) along with non-injected group was used to explore main effects and their interactions. Significant differences among means were determined by Tukey's honestly significant difference (HSD) using SAS (2004), and the difference less than 0.05 was defined as significant.

## Results and discussion

Table 2 shows the results of in ovo injection of threonine on the expression of jejunal IgA mRNA. These data indicated that 0.05 ml of THR when injected under the air sac (5TUAS) significantly increased ($p < 0.05$) gene expression in comparison with intact control and other injection treatments. However, when THR was injected in the air sac at 0.1 ml volume (1TIAS), the IgA gene expressed higher than intact control group (2.58 vs. 1.00, respectively), but differences compared with other injected groups were not significant. This suggests that THR may have a direct effect on immunity in the intestine. The B cells activated in mucosal-associated lymphoid tissues differentiate to plasma cells, which are inhabitant of the lamina propria and produce secretory IgA. Inhibiting the penetration of micro-organisms is the role of secretory IgA, which is carried out by blocking the adhesion to mucous membranes, to improve bactericidal function and to thwart bacterial noxious products (Walker, 1976; Mestecky and McGhee, 1987; Bai et al., 2000).

Bhanja and Mandal (2005) reported a significant difference in cell-mediated immunity with in ovo injection of AA combinations (Lys + Met + Cys), (Thr + Gly + Ser) and (Ile + Leu + Val) in broiler eggs. Azzam et al. (2011b) showed that at 0.4% L-threonine supplementation into the layer diets, the concentrations of IgA antibody in the ileum increased linearly. In their study, L-threonine supplementation modulated the concentration of IgA mainly in the ileum rather than the jejunum. However, we found that more expression of the IgA gene took place in the jejunum. This inconsistency between results can be attributed to the differences in species and age and to the fact that the ileum may be the more suitable domain for bacterial colonization in aged birds, whereas in newly hatched chicks (present study), microbial establishment has not taken place in the ileum yet.

Threonine content is relatively high in immunoglobulins (Tenenhouse and Deutsch, 1966), and thus, in

**Table 2** Effect of in ovo injection of threonine on immunoglobulin A gene expression in the jejunal samples of newly hatched quail*

| | | Serum | | | | Threonine | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | In the air sac† | | Under the air sac | | In the air sac | | Under the air sac | | | |
| Single effects | Control | 0.05 ml | 0.1 ml | 0.05 ml | 0.1 ml | 0.05 ml | 0.1 ml | 0.05 ml | 0.1 ml | SEM | p-Value |
| Relative IgA gene expression | $1^{c}$ | $1.16^{bc}$ | $1.92^{bc}$ | $2.66^{bc}$ | $1.16^{bc}$ | $1.38^{c}$ | $2.58^{bc}$ | $5.10^{a}$ | $1.64^{bc}$ | 0.482 | <0.001 |

| | V | | S | | T | | p-Value | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Interaction effects | 0.05 | 0.1 | IAS | UAS | 0 | 5 mg/ml | V | S | T | V*S | V*T | S*T | V*S*T | SEM |
| Relative IgA gene expression | $2.95^{a}$ | $1.55^{b}$ | $1.17^{b}$ | $3.33^{a}$ | $0.67^{b}$ | $3.83^{a}$ | 0.0031 | <0.0001 | <0.0001 | <0.0001 | 0.0009 | <0.0001 | <0.0001 | 0.2 |

V, volume of injection; S, site of injection; T, with or without injecting threonine; SEM, standard error of means.

a,b,c Means sharing a different superscript in a row differ significantly ($p < 0.05$).

*Results are given as means of 20 birds per treatment.

†In the air sac: 5 mm depth of injection; under the air sac: 8 mm depth of injection.

ovo administration of threonine may have caused greater immunoglobulin synthesis. In the current study, it is agreed that jejunum IgA has a specific requirement for THR; this means that a very high THR is required for gut function which was also observed in previous work (Kermanshahi et al., 2015) in our laboratory where in ovo injection of threonine in quail eggs increased mucin2 gene expression, while had no effects on digestive enzymes.

In conclusion, our study demonstrated that injection of 0.05 ml THR under the air sac of quail eggs increased IgA gene expression in the intestine of newly hatched quail and might improve the immune potency in mature quail, while more investigations on the efficacy of in ovo injection on various parameters such as hatchability, the intestinal morphology and growth performance of quail are needed.

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