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Original article

# Chicken amyloid arthropathy: serum amyloid A, interleukin-1 $\beta$ , interleukin-6, tumour necrosis factor- $\alpha$ and nitric oxide profile in acute phase (12<sup>th</sup> hour)

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## Abstract

Acute phase response (APR) is part of the early defense system, which is triggered by different stimuli including, infection, trauma, stress, inflammation and neoplasia. The APR complex is a reaction which induces homeostasis and recovery. In this research, serum amyloid A (SAA), interleukin (IL)-1 $\beta$ , IL-6, tumour necrosis factor alpha (TNF- $\alpha$ ) and nitric oxide (NO) levels were measured 12 hours following injection. For this purpose, Thirty-two 5 weeks old laying chicken were allocated into four groups and intra-articular injections of Freund's adjuvant were used to induce amyloid arthropathy in Groups II, III and IV. Vitamin A in group II, and methylprednisolone in group IV were added to enhance and to reduce the severity of amyloidosis, respectively. At the end of the research, it was observed that TNF- $\alpha$  and NO increased significantly ( $P < 0.05$ ) in vitamin A and methylprednisolone groups whereas SAA decreased significantly ( $P < 0.05$ ) in all groups. It was also observed that IL-6 increased ( $P < 0.05$ ) in vitamin A group and decreased in all other groups however, IL-1 $\beta$  decreased in vitamin A and methylprednisolone groups, while it was increased in the control group.

The results of this study suggest that there is a positive correlation between serum TNF- $\alpha$  levels in acute and chronic phase in chickens with amyloid arthropathy.

**Key words:** chicken, amyloid arthropathy, vitamin A, SAA, cytokines

## Introduction

The acute phase protein (APP) response is an innate reaction towards tissue injury and follows rapidly (6-12 h) after onset of any disease compromising tissue homeostasis, for example infections, trauma, inflammation and some tumors. It can also be induced by lipopolysaccharide injection and pro-inflammatory cytokines (Baumann and Gaudie 1994, Moshage 1997, Gabay and Kushner 1999) and nitric oxide (NO) (Titheradge 1999). Serum amyloid A (SAA) is a sensitive marker of the acute phase response (Steel and Whitehead 1994). Although well documented in several mammalian species (Gruys and Hol 1984; Alsemgeest et al. 1994, 1995b; Koetz et al. 1998), little is known about SAA in chickens (Landman et al. 1996, Chamanza et al. 1999, Sevimli et al. 2004, 2005, 2008). Amyloid A which is a precursor protein of SAA, is upregulated by pro-inflammatory cytokines, notably the IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Ramadori et al. 1985, Ganapathi et al. 1991, Betts et al. 1993, Hagihara et al. 2004).

Vitamin A and related retinoids has positive effects on functions of leucocytes, stimulating release of cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Dillahay et al. 1988, Turpin et al. 1990, Ross 1999) and immune system as well (Semba 1998). Katz et al. (1987) showed that retinoids stimulate both an increase in the number of macrophages *in vivo* and enhance their activity *in vitro*. Therefore, anti-inflammatory agents such as colchicine and dimethylsulfoxide are used for amyloid treatment in humans (Kisilevsky et al. 1995, Soto et al. 1996) and mice (Inoue et al. 1996). However, there is too few studies about treatment of avian amyloidosis (Sevimli et al. 2005, Sevimli et al. 2008).

To our best knowledge, there is not any research about acute phase of amyloid arthropathy model in chickens investigating SAA, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO together at the same time. In this research, it was aimed to observe inductive effect of vitamin A and suppressing effect of methylprednisolone during acute phase (12 hours) of amyloid arthropathy developing in 5 weeks-old chicks.

## Materials and Methods

**Ethics:** The experimental protocols were approved by the Animal Care and Use Committee at Afyon Kocatepe University (162/2004).

### Animals and experimental design

Thirty-two, 1-day old, ISA Brown layer chicks obtained from a commercial hatchery (Hastavuk-Bursa,

Turkey) were used. A vaccination program was designed. The animals received inactive Newcastle Disease Virus (ND) + infectious bursal disease (IBD) vaccine (Gumbopest, Merial RTA, subcutaneously), live ND vaccine (Intervet, in drinking water) and infectious bronchitis (IB) vaccine (MA5CLONE30, Intervet, in drinking water) on day 7, live IBD vaccine (Bursine Plus, Ford Dodge-Refarm, in drinking water) on day 18 and 25. The chicks were allocated into four group (n:8). The groups were arranged as follows: group I (Negative Control), group II (Severe Amyloid-Vitamin A), group III (Moderate Amyloid-Positive Control) and group IV (Mild Amyloid-Treatment). To induce amyloid arthropathy in the experimental groups II, III and IV, the birds were injected with 0.25 ml complete Freund's adjuvant (CFA) into the left inter-tarsal joint at the fifth week of the experiment as described previously (Landman et al. 1998, Sevimli et al. 2005), while group I was injected intra-articularly with the same amount of 0.9% NaCl and was kept as negative control. Group II received a commercial diet containing high levels of vitamin A (75.000 IU/kg) *ad libitum* throughout the study to enhance the severity of amyloidosis (Sevimli et al. 2004, 2005, 2008) while groups I, III and IV were fed *ad libitum* with a commercial diet containing normal levels of vitamin A (5000 IU/kg). Group IV was injected intramuscularly with 10 mg/kg methylprednisolone on the day of CFA injection (Sevimli et al. 2005). All injection were started by 8 A.M. and completed by 9 A.M. Chicks were fed a starter diet during first 4 weeks which contains 211 g/kg crude protein and 2950 kcal/kg metabolisable energy while a grower diet containing 190 g/kg crude protein and 2850 kcal/kg metabolisable energy has used during 5<sup>th</sup> week (Afyon Yem A.S., Afyon, Turkey). Composition of starter and grower diets formulated in order to meet requirements of the chicken are shown in Table 1. The nutrient composition of the starter and grower diets was determined according to the AOAC (2000). Metabolisable energy levels of each diet were estimated using the following equation devised by Carpenter and Clegg (Leeson and Summers 2001). All chicks were necropsed 12 hours after injection.

**Tissue sampling:** Samples from internal organs (liver, spleen) and left inter-tarsal joint were preserved in 10% buffered formaldehyde. Routine histopathological were used to obtain procedures, 5  $\mu$ m sections and stain them with HE.

**Serological studies:** Blood samples of 8 birds per group were collected from the carotid artery into non-heparinized tubes before necropsy and blood samples were centrifuged at 1600x g. Then SAA, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO levels of obtained serums were measured.

Table 1. Composition of the starter and grower diets (g/kg).

Ingredients	Starter	Grower
Maize	514.77	526.59
Soybean meal	245.92	221.57
Full fat soybean	137.14	21.40
Wheat	55.77	132.87
Sunflower meal	–	55.35
Dicalcium phosphate	17.41	15.74
Limestone	16.66	12.30
Rovimix 121-L	2.50	2.50
NaHCO <sub>3</sub>	2.27	1.64
Salt	2.09	1.99
DL-Methionine	1.66	1.01
L-Lysine	0.82	1.04
Toxin binder	1.00	1.00
Anticoccidial (Lasalocid sodium)	1.00	1.00
Choline clorit	0.50	0.50
Phytonine	0.50	0.50
Analyzed composition		
Dry matter	870.0	870.0
Ash	64.89	59.8
Crude protein	211.0	190.0
Crude fat	51.1	30.2
Crude fiber	31.4	36.5
Calcium	1.05	1.00
Total phosphorus	7.5	7.1
Metabolisable energy <sup>b</sup> , kcal/kg	2950	2850

<sup>a</sup> Rovimix 121-L, Provided per 2.5 kg of diet: vitamin A 12,000.00 IU; vitamin D<sub>3</sub> 2,500.000 IU; Vitamine E 20,000 mg; Vitamine K<sub>3</sub> 4,000 mg; vitamin B<sub>1</sub> 3,000 mg; vitamin B<sub>2</sub> 6,000 mg; vitamin B<sub>6</sub> 5,000 mg; vitamin B<sub>12</sub> 20 mg; niacin, 25,000 mg, Ca-D-Pantotenate, 6,000 mg; folic acid, 750 mg; choline clorit, 250,000 mg.

<sup>b</sup> Metabolisable energy content of diets was estimated using the equation devised by Carpenter and Clegg (Leeson and Summers, 2001).

### SAA measurement

An ELISA kit (TP-802M; Tridelata, Maynooth Co., Kildare, Ireland) was used to detect SAA according to the manufacturer's instructions as was described previously (Sevimli et al. 2005, Alasonyalylar et al. 2006, Sevimli et al. 2012). In this study, a murine kit and its standards were used as there was no kit for chicken SAA available at the period this study. The producer of the murine kit recommended the use of the murine kit since there was a cross-reactivity between chicken and mouse antibody.

### IL-1 $\beta$ , IL-6, TNF- $\alpha$ measurements

Commercial sandwich type ELISA kits (KMC0011-m IL-1 $\beta$ , KMC0061-m IL-6 and KMC3011-m TNF- $\alpha$ ) (Biosource International, California, USA) were used according to the manufacturer's instructions.

### NO measurements

Plasma nitric oxide concentrations were determined according to the procedure of Miranda et al. (2001). Nitrate was reduced to nitrite with vanadium (III) and then nitrite level measured by using Griess reagents. Serial dilutions 0.5-200  $\mu$ M of Na nitrate (Merck, Germany) were used as standards. The results were expressed as  $\mu$ M.

### Statistical analysis

One-way Analysis of Variance (ANOVA) test was used to compare differences between groups (mean values of SAA, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO). Tukey test was applied to observe significance level of between groups (SPSS 13.0). The significance level was set at  $P < 0.05$ .

### Results

**Clinical and necropsy findings:** In all experimental groups, left inter-tarsal joint and internal organs were devoid of any lesions.

**Microscopical findings:** There were no lesions in the synovial membrane and internal organs.

### Serological findings

#### SAA levels

The SAA levels were decreased in all groups when compared with group I (Negative Control: 15220 $\pm$ 4183 ng/ml) ( $P < 0.05$ ). The lowest levels were observed in group IV (Mild Amyloid-Treatment: 2246 $\pm$ 458 ng/ml), followed by groups III (Moderate Amyloid-Positive Control: 2908 $\pm$ 770 ng/ml) and II (Severe Amyloid: 5669 $\pm$ 760 ng/ml), respectively (Table 2).

#### IL-1 $\beta$ levels

The IL-1 $\beta$  levels were increased in group III (54.5 $\pm$ 4.47 pg/ml). The IL-1 $\beta$  levels were decreased in group II and IV. The differences between the groups were not significant ( $P > 0.05$ ) (Table 2).

#### IL-6 levels

The IL-6 levels were increased in group II (27.9 $\pm$ 6.17 pg/ml) when compared with group I (5.38 $\pm$ 2.65 pg/ml)

Table 2. Serum amyloid A (ng/ml), serum cytokine (pg/ml) and serum nitric oxide ( $\mu\text{mol/L}$ ) levels in four groups (mean $\pm$ SE).

Parameters	Group I (Negative control)	Group II (Severe Amyloid- -Vitamin A)	Group III (Moderate Amyloid- -Positive Control)	Group IV (Mild Amyloid- -Treatment)
SAA (ng/ml)	15220 $\pm$ 4183 <sup>a</sup>	5669 $\pm$ 760 <sup>b</sup>	2908 $\pm$ 770 <sup>b</sup>	2246 $\pm$ 458 <sup>b</sup>
TNF $\alpha$ (pg/ml)	14.0 $\pm$ 10.1 <sup>b</sup>	55.9 $\pm$ 7.67 <sup>a</sup>	48.7 $\pm$ 6.26 <sup>a</sup>	23.1 $\pm$ 6.97 <sup>ab</sup>
IL-1 $\beta$ (pg/ml)	49.1 $\pm$ 3.79 <sup>ab</sup>	33.1 $\pm$ 8.06 <sup>b</sup>	54.5 $\pm$ 4.47 <sup>a</sup>	46.4 $\pm$ 3.44 <sup>ab</sup>
IL-6 (pg/ml)	5.38 $\pm$ 2.65 <sup>b</sup>	27.9 $\pm$ 6.17 <sup>a</sup>	5.04 $\pm$ 3.20 <sup>b</sup>	2.88 $\pm$ 1.66 <sup>b</sup>
NO ( $\mu\text{mol/L}$ )	4.70 $\pm$ 2.13 <sup>c</sup>	15.2 $\pm$ 1.39 <sup>a</sup>	12.2 $\pm$ 1.25 <sup>ab</sup>	7.74 $\pm$ 1.09 <sup>bc</sup>

a, b, c – Different letters in the same line are statistically significant (Tukey test,  $p < 0.05$ ).

( $P < 0.05$ ). The IL-6 levels were decreased in group IV (2.88 $\pm$ 1.66 pg/ml) and group III (5.04 $\pm$ 3.20 pg/ml), respectively. The differences between the groups were not significant ( $P > 0.05$ ) (Table 2).

### TNF- $\alpha$ levels

The TNF- $\alpha$  levels were increased in all groups when compared with group I (14.0 $\pm$ 10.1 pg/ml). Increases in group II (55.9 $\pm$ 7.67 pg/ml) and group III (48.7 $\pm$ 6.26 pg/ml) were statistically significant ( $P < 0.05$ ) while it was not in group IV (23.1 $\pm$ 6.97 pg/ml) ( $P > 0.05$ ) (Table 2).

### NO levels

The NO levels were increased in all groups when compared with group I (4.70 $\pm$ 2.13 pg/ml). Increases in group II (15.2 $\pm$ 1.39 pg/ml) and group III (12.2 $\pm$ 1.25 pg/ml) were statistically significant ( $P < 0.05$ ) while it was not in group IV (7.74 $\pm$ 1.09 pg/ml) ( $P > 0.05$ ).

### Discussion

It has been widely recognized that amyloid arthropathy, characterized by the accumulation of amyloid in joints, is a common pathological disorder in certain kinds of birds (Landman 1999). Pathogenesis of amyloidosis in hens can not be fully explained yet (Landman et al. 1998b). SAA is a major acute phase reactant in the blood and its level increases after various insults to the body (Landman et al. 1994, Urieli-Shoval et al. 2000). Some studies in recent years revealed the importance of SAA in monitoring poultry health (Chamanza et al. 1999, Upragarin et al. 2005b). Chamanza et al. (1999) suggested that SAA is a rapidly changing acute phase protein in chickens, and that SAA was not detected in healthy chickens. In an experimental study by Upragarin et al. (2005a), SAA level was 200 ng/ml at 24<sup>th</sup> hour and 400 ng/ml at

48<sup>th</sup> hour after the dose of lipopolysaccharide. In another study by Upragarin et al. (2005b), SAA was induced by turpentine and *Staphylococcus aureus*. While SAA level was 20 ng/ml in the negative control group, in the experimental group the level was 28.93  $\mu\text{g/ml}$  at 12<sup>th</sup> hour and 84.56  $\mu\text{g/ml}$  at 72<sup>nd</sup> hour. Increased serum levels of SAA may potentially contribute to the development of AA amyloid deposition in joints of mammals and chickens (Migita et al. 1996, Sevimli et al. 2005, Alasonyalylar et al. 2006, Sevimli et al. 2008, Sevimli et al. 2012). In the current study, SAA level 12<sup>th</sup> hour after the injection was 15220 ng/ml in Group I, 5669 ng/ml in Group II, 2908 ng/ml in Group III and 2246 ng/ml in Group IV. The varying values obtained in different studies may be related to the difference between the injected inocula, individual immune variations and kits (cross-reactivity in our kit). In the present study SAA decreased in all groups when compared with Group I. SAA results was decreased opposing the same study during chronic period (Sevimli et al. 2008). The reason of that is blood sampling and evaluation after 12 hours of injection. Some researchers suggest that overproduction of SAA alone is not responsible for amyloidosis (Migita et al. 2001) and that neutrophils and macrophages play a very important role in the formation of amyloid fibrils (Skogen et al. 1980, Zekerias et al. 2000, Sevimli et al. 2005). In this research, there was no amyloid deposit and cell infiltration observed at histopathological examination of joints and internal organs.

It has been shown that the type AA amyloid-related precursor protein (SAA) of amyloid fibrils is increased by cytokines such as IL-1 (Ramadori et al. 1985), IL-6 (Marinkovic et al. 1989), TNF- $\alpha$  (Betts et al. 1993). Retinoids have been shown to influence many aspects of immunity including the function of leucocytes and the expression of cytokines (IL-1, IL-2, IL-6, IL-3, TNF- $\alpha$ ) (Dillahay et al. 1988, Turpin et al. 1990, Göttingens and Green 1995, Ross 1999). In our study about chronic period (Sevimli et al. 2008) it was concluded that especially IL-1 $\beta$  and TNF- $\alpha$  plays an important role in AA amyloidosis development and the role of IL-6 needed to be investigated. Sevimli

et al. (2008) reported that vitamin A increases SAA, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels significantly. It was observed in this research that SAA decreased in all groups whereas vitamin A increased IL-6 and TNF- $\alpha$ .

In chickens, proinflammatory cytokines diminish food intake, muscle deposition and growth (Klasing 1994). Disease-related changes in nutrient metabolism occur as a consequence of the acute phase response, which is characterized by changes in the production of hepatic proteins (i.e. acute phase proteins) that mediated absorption, transport, uptake and deposition of amino acids, lipids, vitamins and minerals (Hallquist and Klasing 1994). 75.000 IU/kg vitamin A was supplemented to the diets of the animals in Group II in order to increase amyloidosis. At the end of the research, it was observed that vitamin A increased TNF- $\alpha$ , IL-6 and NO significantly while it decreased IL-1 $\beta$ .

Methylprednisolone, an anti-inflammatory and immunosuppressive agent, is a synthetic adrenocortical derivative, which inhibits TNF- $\alpha$  (Xu et al. 1998) and IL-6 (Stanton et al. 1999). Methylprednisolone (Laato et al. 1989) has inhibitory effects on the synthesis of proteoglycans, glycosaminoglycans, collagen IV and fibronectin determined in amyloid (Skogen et al. 1980, Lyon et al. 1991, Landman et al. 1998b). It was observed in this research that methylprednisolone suppresses all of them except TNF- $\alpha$  and NO. In this study SAA was not suppressed by methylprednisolone. This result shows that methylprednisolone is more effective in suppressing amyloid during acute phase. Further researches are needed about dosis for suppressing amyloid formation during chronic period (i.e. 2 or 3 doses).

In the context of mediating an inflammatory response, chicken IL-1 $\beta$  appears to function like its mammalian counterpart. Studies have shown that the *in vivo* levels of IL-1 increase upon administration of lipopolysaccharid (LPS) (Klasing et al. 1987). IL-6 has been suggested to be important for the induction of immune effector responses in chickens (Kaiser et al. 2000). Information about TNF in chickens is limited. Rautenschlein et al. (1999) demonstrated for the first time that chicken TNF has several functions homologous to mammalian TNF- $\alpha$  and the authors concluded that chicken macrophages indeed secrete a TNF-like factor, comparable in its activities with mammalian TNF- $\alpha$ . Gehad et al. (2002) observed an increase in TNF- $\alpha$  release following the injection of LPS in chickens.

To the best of our knowledge, there is not any research about acute phase of amyloid arthropathy model in chickens investigating SAA, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO together at the same time. In this research, acute phase (12<sup>th</sup> hour) levels of SAA,

IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO in amyloid arthropathy development in chickens were investigated. As a result, it was observed that SAA is suppressed in amyloid arthropathy model during acute phase whereas TNF- $\alpha$  and NO increased in all study groups. Moreover, it was also observed that vitamin A increases IL-6 and TNF- $\alpha$  while suppresses IL-1 $\beta$  whereas methylprednisolone suppresses all of them except TNF- $\alpha$  and NO. Depending on those results, it was concluded that TNF- $\alpha$  plays an important role in amyloid arthropathy development in acute period. The mentioned parameters needed to be observed daily and distinctly. Besides, we believe that methylprednisolone which is used in therapy is effective but multiple administrations are better than single dose administration.

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