

## CHRONIC AMYLOID ARTHROPATHY AND INCREASED SERUM AMYLOID LEVELS IN BROWN LAYERS

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### Abstract

Serum amyloid-A (SAA) levels were investigated in chickens with experimentally induced amyloid arthropathy in comparison with healthy counterparts. Forty-eight 5-week-old chickens were allocated into two equally numbered groups. *Enterococcus faecalis* was injected intraarticularly at concentrations of  $10^9$  cfu/ml, to induce amyloid arthropathy in one of the groups, whereas the other one was kept as a control and injected intraarticularly only with 0.9% NaCl (1 ml). All the chickens were necropsied at the 13<sup>th</sup> week after the injections. Joint sections were examined histopathologically and immunohistochemically. Blood samples were collected and SAA levels were determined by ELISA. Amyloid accumulation in joints was only seen in the experimental group (18/24). The SAA levels found were  $154 \pm 20$  ng/ml and  $419 \pm 27$  ng/ml in the control and experimental groups, respectively, and the differences were highly significant ( $P < 0.001$ ). In conclusion, SAA plasma concentrations are influenced by amyloid arthropathy. Consequently, SAA may be a sensitive variable to assess the physical welfare in chicks; and increases in these values can be suggestive of chronic inflammatory processes, including amyloid arthropathy.

**Key words:** chickens, amyloid, amyloid arthropathy.

The acute-phase response has been shown to be a part of systemic and non-specific defence mechanism; known to occur during infection, inflammation, tissue injury caused by trauma or surgery, neoplastic growth, or immunological disorders, including a change in the concentration of many plasma proteins (10, 19), known as acute-phase proteins, whose synthesis is altered in the liver (17, 34). Acute-phase proteins are commonly defined as positive proteins, whose concentrations increased by at least 25% during inflammatory state. In addition, a number of negative acute-phase proteins, whose concentrations decreased significantly under these circumstances, have been reported (34). One of the very important positive acute phase proteins is the serum

amyloid-A (SAA), a precursor protein in secondary or reactive amyloidosis (type AA) (4, 12, 31, 34, 38). Many reports in this area have emphasized the importance of SAA in numerous pathological disorders, including inflammation, atherosclerosis, thrombosis, AA-amyloidosis, rheumatoid arthritis, and neoplasia in man (7, 15, 26, 30, 36, 44). Elevation of SAA levels has also been shown to be a useful parameter to distinguish non-healthy animals from healthy ones (1, 2, 15). Although well documented in several mammalian species (1, 2, 8, 16), little is known about SAA in chickens (6, 21, 27). The SAA levels were recently measured in this species (21, 35), and the type of protein was identified as the precursor of amyloid-A protein deposited in avian amyloid arthropathy (19, 20, 29), and amyloidosis in ducks (6, 25). In recent years, acute-phase proteins have received increasing attention in human and veterinary medicine; as a tool to measure the health status in various animals (14, 18, 27, 33). Their concentration pattern has been demonstrated to reflect the overall activity of the disease process (1, 36). The altered health status in birds often resulted in measurable changes in blood chemistry (11, 28). Therefore, measuring the plasma levels of certain acute-phase proteins (18, 33, 37, 39-41), including SAA protein (6), could be useful for monitoring the poultry's health. It is suggested that an increase in plasma SAA levels may potentially contribute to the development of AA amyloid deposition in joints (26, 35, 42). Some authors (23) have suggested that orally administered AA amyloid can enhance the experimentally induced amyloidosis in mice, and some other authors (9) indicated that like prions, this pathological material should be banned for consumer risk groups. Therefore, to make the consumer aware of such a risk, the measurements of SAA in chickens and other animals could be considered in routine diagnostics. In a previous *in vivo* study, we have detected significant elevation in SAA values in chickens with amyloid arthropathy induced by intra-articular injections of Freund's adjuvant (35), and to our best knowledge, this was the only report dealing with the

SAA concentrations in amyloidotic chickens. The main aim of this study is to test our preliminary results concerning the role of SAA protein in poultry amyloidosis, and its use in diagnostics.

## Material and Methods

**Ethics.** The experimental protocols were approved by the Animal Care and Use Committee at Uludag University; and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

**Animals and experimental design.** Forty-eight 5-week-old brown-layer chicks were purchased from a commercial breeder. The chickens had been vaccinated against infectious bronchitis, and Marek's, Newcastle, and Gumboro diseases. A commercial layer pellets and water were provided *ad libitum*. The light schedule was 14 h light and 10 h dark. After one week of acclimatisation, the chicks were allocated into two equally sized groups. In order to induce amyloid arthropathy, one of the groups was intraarticularly injected with 1 ml of inoculum containing  $10^9$  colony forming units (cfu/ml) of an arthropathic and amyloidogenic *Enterococcus faecalis* strain (6085.94), into the left tibio-metatarsal joint (22), whereas the other group was injected with 1 ml of sterile 0.9% NaCl and was kept as a control. The bacterial suspension was prepared as described by Landman *et al.* (22), and the cfu were determined by using McFarland method. All the pullets were necropsied at the end of the 13<sup>th</sup> week after the injections.

**Tissue sampling and processing.** Joint samples were collected during the necropsy. The samples were fixed in a 10% phosphate buffered formalin solution for 24 h and processed routinely; embedded in paraffin, sectioned at 5 $\mu$ m and stained with haematoxylin and eosin (H&E) and Congo red (24). Congo red stained sections were examined in polarized light for the characteristic green birefringence of amyloid. The blood samples were collected during necropsy, and the serum was obtained and stored in the freezer till use.

### Immunohistochemistry.

Immunohistochemical techniques were used to visualise the amyloid formation in the joints. The examination was performed on paraffin sections by using the streptavidin–biotin–peroxidase method, as described previously (13). Anti-mouse Amyloid A Ab-1(mc1) antibody was used (Neomarkers-Freemont, USA) to demonstrate amyloid deposition in synovial membrane. AEC was used as chromogen in all tissue sections.

**Serological study. SAA measurement.** A commercial ELISA kit (TP-802M; Tridelata, Maynooth Co. Kildare, Ireland) was used to detect SAA. In this study, a murine kit and its standards were used because a kit for chicken SAA was not available. However, the producer of the murine kit recommended its use, since there is cross-reactivity between chicken and mouse antibody (Tridelata). The wells of the microtitre strips

were coated with a monoclonal antibody specific for SAA. Test reagents and samples were allowed to reach room temperature prior to use. Fifty microlitres of diluted biotinylated SAA antibody were added to each well. The serum samples were vortexed and diluted 1:500 in 1 $\times$ diluent buffer. Fifty microlitres of the diluted sample were added in duplicate, to each well. The plates were covered with a dust cover, and incubated for 1 h at 37°C. After incubation, aspiration was performed and the plates were washed four times with diluted wash buffer. After the last wash, the plates were dried on absorbent paper. One-hundred microlitres of streptavidin–peroxidase was added into each well. The plates were covered and incubated at room temperature in the dark for 30 min. The wells were aspirated and then washed four times. The plates were dried after the last wash and 100  $\mu$ L of tetramethyl benzidine substrate, which was provided with the kit, were added. The plates were covered and incubated in the dark at room temperature for 30 min. Fifty microlitres of stop solution was added. The absorbance of each well was read at 450 nm using 630 nm as a reference. The mean absorbance for each sample was calculated as standard. The absorbance of the standards was plotted against the standard concentration on semi-logarithmic graph paper.

**Statistical analysis.** The values of the SAA were evaluated with Mann Whitney U test (32).

## Results

**Clinical findings.** Swelling of the inoculated left tibio-metatarsal joints, resulting in lameness, and was seen 5-7 d after the injections in 18 of 24 birds that exhibited amyloid in the joints. No lameness and swelling were detected in the control group throughout the experiment.

**Necropsy findings.** The left tibio-metatarsal joints inoculated with *E. faecalis* were swollen in 18 of 24 birds in the experimental group. Periarticular irregular bulging areas representing orange coloured amyloid masses were detected in the joints of all amyloid-positive chickens (18 of 24 chicks). No amyloid or arthritis was detected in the controls.

**Microscopical findings.** Amyloid formation and signs of arthritis in the injected joint was detected in the experimental group (18/24), whereas no amyloid occurrence and no arthritis were seen in the control group. In amyloid positive chickens, the synovial cells were hyperplastic. Amyloid accumulations were observed as homogenous areas among the synovial cells in the synovial cavity and around the blood vessels in the synovial membranes. Infiltrations of lymphocytes, plasma cells, heterophils, and giant cells were observed in the synovial membranes.

**Serological findings.** The SAA levels were found to be  $154 \pm 20$  ng/ml and  $419 \pm 27$  ng/ml in the control and experimental groups, respectively, and the difference between the groups was statistically significant ( $P < 0.001$ ).

## Discussion

Secondary amyloidosis has been shown as a severe complication of chronic inflammatory diseases, and is characterised by the deposition of amyloid fibrils in various organs (12). It has been reported that the major component of amyloid fibrils derives from SAA protein during proteolysis (4, 31, 38). It has also been reported that the SAA is a major acute-phase reactant; and the levels in blood increases in response to various insults (5, 20, 43). Some authors (26) suggested that an increase in the level of circulating SAA may potentially contribute to the development of AA amyloid deposition in various tissues and organs. Some authors (6) also showed that SAA is an acute-phase protein more reliable than transferrin for diagnosing disease in chickens and could be used for monitoring poultry health, and that SAA is a rapidly changing acute-phase protein in chickens, which was not detected in healthy birds. In agreement herewith, Landman *et al.* (21) showed that SAA was found in serum samples of birds with acute diseases, while SPF chicken sera and chicken sera before infection were negative for SAA. In the present study, although SAA was detected in both healthy and amyloid arthropathic birds, SAA concentrations were significantly ( $P < 0.001$ ) higher in the amyloidotic group than those of the birds in the control group. Moreover, there were no pathological lesions in control animals. These findings are in agreement with observations of Urieli-Shoval *et al.* (43) that extrahepatic production of SAA could be found in histologically normal tissues in humans. However, Chamanza *et al.* (6) observed that overproduction of SAA is a very useful for the detection of acute lesions. These authors suggested that in chronic stages of a disease, SAA should be measured in combination with other (C-reactive protein, haptoglobulin, transthyretin) acute-phase proteins. The findings of the current study are in accordance with observations of Ray and Ray (30), who reported a persistent expression of SAA during experimentally induced chronic inflammatory condition in rabbit, and also indicated that SAA could be a significant marker in detecting the chronic stages of diseases. In a previous *in vivo* study, we detected positive correlation between the circulating SAA levels and the incidence and severity of amyloid arthropathy occurrence in Brown layers 13 weeks after intraarticular Freund's adjuvant injections (35).

In conclusion, SAA concentrations are influenced by amyloid arthropathy. Consequently, SAA may be a sensitive variable, able to assess the physical welfare in chicks and increases in these values can be suggestive of chronic inflammatory processes including amyloid arthropathy.

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