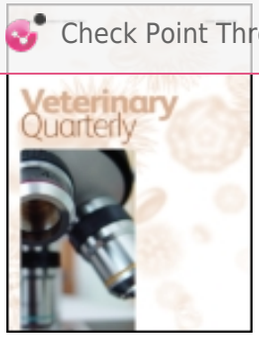

Veterinary Quarterly

ISSN: 0165-2176 (Print) 1875-5941 (Online) Journal homepage: https://www.tandfonline.com/loi/tveq20

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To cite this article: Alper Sevimli , Murat Yalcin , I. Taci Cangul & Sami Aydin (2012) Investigation of serum amino acid and serum amyloid A concentrations in chickens with amyloid arthropathy, Veterinary Quarterly, 32:1, 17-23, DOI: 10.1080/01652176.2012.689117

To link to this article: https://doi.org/10.1080/01652176.2012.689117

Veterinary Quarterly
Vol. 32, No. 1, March 2012, 17–23

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Investigation of serum amino acid and serum amyloid A concentrations in chickens with amyloid arthropathy

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(*Received 6 January 2012; final version received 24 April 2012*)

Background: Increased proteolytic cleavage of serum amyloid A (SAA) may potentially contribute to the development of AA amyloid deposition
Objective: To study the possible relationship between amyloid artropathy and expression of SAA and some serum amino acids.
Animals and methods: Values of 15 serum amino acids and SAA were investigated in chickens with experimentally induced amyloid arthropathy. Thirty-four, 5-week-old chicks were allocated into two groups: one group was injected intra-articularly with 0.25 mL complete Freund's adjuvant at the left tibio-metatarsal joint to induce amyloid arthropathy, whereas the other group served as control. All pullets were necropsied 13 weeks after injection. Collected tissue samples were examined histopathologically. Blood samples were collected and SAA concentrations were measured with enzyme-linked immunosorbent assay. High-performance liquid chromatography was used to assess the amino acid concentrations in serum.
Results: Amyloid accumulation in joints occurred only in the experimental group (89%). SAA concentrations of 166 ± 17 and 423 ± 39 (SD) ng/mL were found in the control and experimental groups, respectively ($p < 0.001$). In the experimental group, an increase was observed in all examined amino acid concentrations except for citrulline. The most significant ($p < 0.001$) increases were noticed in serine (from 159 ± 15 to 360 ± 29 μmol/L), glycine (from 151 ± 20 to 279 ± 16 μmol/L), isoleucine (from 48 ± 2 to 80 ± 6 μmol/L), and phenylalanine (from 49 ± 2 to 90 ± 3 μmol/L).
Conclusion: The results of this study suggest that there is a positive correlation between some serum amino acid values, especially serine, glycine, isoleucine, and phenylalanine, and the high concentrations of SAA in chickens with amyloid arthropathy.

Keywords: amyloid; SAA; arthropathy; chicken; amino acid

1. Introduction

Acute-phase proteins have received increasing interest in human and veterinary medicine as a tool to measure the general health status (Kushner and Mackiewiez 1987; Saini and Webert 1991; Kent 1992; Murata and Miyamoto 1993; Gruys et al. 1994). Their concentration pattern reflects the overall activity of the disease process (Alsemgeest et al. 1994; Shimetani et al. 2001).

Serum amyloid A (SAA), the precursor protein in inflammation-associated reactive amyloidosis (AA type) (Anders et al. 1977; Landman et al. 1988; Rysava et al. 1992; Husby 1994; Landman et al. 1994; Strissel et al. 1997; Ovelgönne et al. 2001; Upragarin et al. 2002), is a major acute-phase reactant, the level of which increases in the blood in response to various insults (Calnek et al. 1991; Landman et al. 1994; Urieli-Shoval et al. 2000). Findings emphasize the importance of SAA in various pathological processes, including inflammation, atherosclerosis, thrombosis, AA-amyloidosis, rheumatoid arthritis, and neoplasia in man (Cortet et al. 1993; Migita et al. 1996; Ray and Ray 1999; Yavin et al. 2000; Kimura et al. 2001; Shimetani et al. 2001; Yamane et al. 2001). SAA ratio is also a useful parameter to distinguish healthy animals from animals with inflammation (Alsemgeest et al. 1994; Alsemgeest, Lambooy, et al. 1995; Kimura et al. 2001). Bacterial infections (i.e., lipopolysaccharide) induce the release of proinflammatory cytokines (IL-1, IL-6, and TNF-α), which cause fever, the hepatic secretion of acute-phase proteins, and sickness behavior such as anorexia (Johnson 1998; Luheshi 1999). 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 mediate absorption, transport, uptake and deposition of amino acids, lipids, vitamins, and minerals (Hallquist and Klasing 1994). Although well-documented in several mammalian species (Gruys and Hol 1984; Alsemgeest et al. 1994; Alsemgeest, Horadagoda, et al. 1995; Koets et al. 1998), little is known about SAA in chickens (Landman et al. 1996; Chamanza et al. 1999; Sevimli et al. 2004, 2005, 2008).

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ISSN 0165–2176 print/ISSN 1875–5941 online

http://dx.doi.org/10.1080/01652176.2012.689117
http://www.tandfonline.com

Altered health status of birds often results in measurable changes in blood biochemistry (Hurwitz and Griminger 1961; Okumura and Tasaki 1969), measuring plasma levels of certain acute-phase proteins (Kushner and Mackiewiez 1987; Singh 1988; Saini and Webert 1991; Takahashi et al. 1994; Tohyo et al. 1995, 1996), including the SAA protein also (Chamanza et al. 1999; Gruys et al. 2005), could be useful in monitoring poultry health. It has been suggested that increased circulating proteolytic cleavage of SAA may potentially contribute to the development of AA amyloid deposition (Migita et al. 1996).

Until today, studies examining amino acid composition in human rheumatoid arthritis patients (Borden et al. 1952; Peter et al. 1978) and acute-phase proteins of humans (Barker 1984, 1987) have been conducted. In animals, serum amino acid compositions of rats with arthritis (Sluka and Westlund 1992) and of chickens with AA-amyloidosis have been studied (Landman 1998). There are some studies revealing the amino acid sequence of AA amyloid in animal species including chickens (Landman 1999) and ducks (Guo et al. 1996). To the best of our knowledge, there are no data about amino acid concentrations in serum of chickens with amyloid arthropathy. For this reason it would be worth noting to measure the amino acid concentrations in blood of chicks with increased SAA concentrations. The main aim of this article is to present the possible relationship between amyloid artropathy and expression of SAA and some of the serum amino acids. The results of this study are preliminary results and can be useful for future studies on poultry diagnostics.

2. Materials and methods

2.1. *Animals and experimental design*

Thirty-four, 5-week-old brown layer chicks were used. All chicks were fed *ad libitum* with a commercially prepared feed. The light schedule was as 14 h light and 10 h dark. The chicks were allocated into two groups. In order to induce amyloid arthropathy, one group was intra-articularly injected with 0.25 mL complete Freund's adjuvant (experimental group; $n = 18$) at the left tibio-metatarsal joint, whereas the other group was injected with the same amount of 0.9% NaCl and was used as control (control group; $n = 16$). All pullets were euthanized by decapitation and necropsied 13 weeks after injection aged 18 weeks.

2.2. *Tissue sampling and processing*

Joint samples obtained during necropsy were processed routinely and stained with haematoxylin-eosin and Congo red stains (Lee and Luna 1968). Birefiringence was evaluated with the examination of slides stained with Congo red under polarized filter (Catalog no: 31-52-62-26, Bausch and Lomb, Rochester, NY, USA).

2.3. *Serological study-SAA measurement*

Blood samples were collected at necropsy. A commercial SAA kit for enzyme-linked immunosorbent assay (TP-802 M, Tridelta, Maynooth Co. Kildare, Ireland) was used to detect SAA according to the manufacturer's instructions as previously described (Sevimli et al. 2005; Alasonyalilar et al. 2006). 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 was carried out. The producer of the murine kit recommended the use of the murine kit since there was cross-reactivity between chicken and mouse antibody. Test reagents and samples were allowed to reach room temperature before use. Eight-well strips were used for the assay and the remaining strips were rebagged, the bags were sealed and stored in a refrigerator. Fifty microliters of diluted biotinylated anti-SAA was added to each well. Serum samples were vortexed and were diluted 1:500 afterwards in 1× diluent buffer. Fifty microliters of diluted sample was added to each well in duplicate. Sides of the plate were gently tapped to mix. Plates were covered and incubated at 37°C for 1 h. After incubation, aspiration was done and the plates were washed four times with diluted wash buffer. After the last wash, plates were tapped to dry on absorbent paper. Hundred microliters of streptavidin-peroxidase was added to each well. The plates were covered and incubated at room temperature in the dark for 30 minutes. The plates were aspirated and the wells were washed four times. The plates were tapped to dry after the last wash. Hundred microliters of TMB substrate was added. The Plates were covered and incubated at room temperature in the dark for 30 min. Fifty microliters of stop solution was added. Absorbance of each well was read at 450 nm using 630 nm as reference. The mean absorbance for each sample was calculated as standard value. The absorbance of the standards was plotted against the standard concentration on semi-logarithmic graph paper.

2.4. *Amino acid measurement*

Concentrations of 15 amino acids in serum were measured by high-performance liquid chromatography (HPLC) system (HP 1100 series, Hewlett-Packard, Palo Alto, CA, USA) which was coupled to a post-column derivatization unit (Pickering Laboratories, Mountain View, CA, USA). This system was combined with a quaternary pump (HP, G1311A, Hewlett-Packard), a fluorometric detector (HP, G1321 A, Hewlett-Packard), and an autosampler (HP, G1329 A, Hewlett-Packard). Amino acids separated on lithium exchange column (series number 5338, Pickering Laboratories) with LI280 and LI750 eluents (Pickering Laboratories) were reacted with OPA in a post-column derivatization unit (both from Pickering Laboratories). The flow rate of the quaternary pump and post-column derivatization unit was 0.3 mL/min. Column and

post-column reaction temperatures were adjusted to 40°C and 45°C, respectively. Other chromatographic conditions, such as the gradient program of the LI280, LI750, and lithium regenerant eluents, were similar to the conditions published elsewhere (Grunau and Swiader 1992). OPA reactive compounds were detected at 330 nm excitation and 465 nm emission wavelengths, and chromatograms were analyzed with a software package (HP Chemstation, Revision A. 08.03., 847). Hundred microliters serum + 100 µL Seraprep (Pickering Laboratories) were centrifuged for 5 min in a Beckman microfuge. The supernatant (20 µL) was injected into the HPLC system without further purification. Amino acid concentrations were calculated by comparing peak heights of the samples with amino acid standards. Due to technical reasons the amino acids lysine, arginine, histidine and tryptophan could not be measured.

2.5. *Statistical analysis*

The values of SAA and serum amino acids were evaluated with variance analysis and Tukey's tests (SAS Institute Inc., 1991). Values are given as mean ± SD and *p*-values below 0.05 were regarded as significant.

3. Results

3.1. *Clinical findings*

In the experimental group, swelling of the inoculated left tibio-metatarsal joints resulting in lameness was seen 5–7 days after the injection. No lameness and swelling were detected in the control group and the right tibio-metatarsal joint of the experimental group throughout the experiment.

3.2. *Necropsy findings*

In the experimental group, the inoculated left tibio-metatarsal joints were swollen. In some cases, the size of the left tibio-metatarsal joint was a few times of the right one and the swelling ascended to the hip-bone (Figure 1). In amyloid-positive chickens, periarticular irregular bulging areas representing orange coloured amyloid masses were detected in the joint.

3.3. *Microscopical findings*

In the experimental group, amyloid formation in the injected joint was observed in 16 out of 18 (89%) animals, whereas no amyloid occurrence was seen in the control group. In amyloid-positive chickens the synovial cells were hyperplastic. Amyloid accumulations were seen as homogeneous areas among the synovial cells, in the synovial cavity, and around the blood vessels in the synovial membranes (Figures 2 and 3). Infiltrations of lymphocytes, plasma cells, heterophils, macrophages, and giant cells were observed in the synovial membranes. No amyloid formation was observed in the right tibio-metatarsal joint and the internal organs.

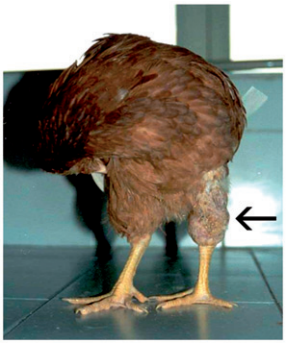

Figure 1. Enlargement of the left tibio-metatarsal joint after complete Freund's adjuvant injection.

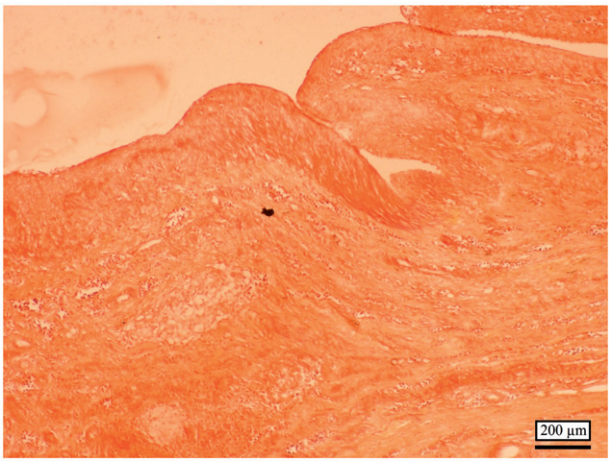

Figure 2. Amyloid deposition in synovial membrane: Congo red staining, ×10.

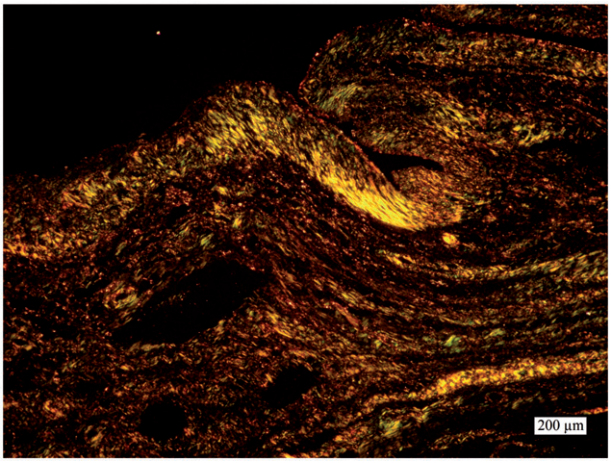

Figure 3. Amyloid deposition in synovial membrane: Congo red staining, polarized light ×10.

3.4. *Serological findings*

The SAA concentrations in serum were 166 ± 17 and 423 ± 39 ng/mL in the control and experimental

Table 1. Values of serum amino acid concentrations (μmol/L) in 18-week-old chicks shown as mean ± SD.

Amino acid	Control group	Experimental group
Taurine[a]	226 ± 16	306 ± 26
Aspartic acid[b]	23 ± 6	44 ± 4
Threonine[b]	128 ± 20	187 ± 13
Serine[c]	159 ± 15	360 ± 29
Glutamic acid[b]	162 ± 42	203 ± 21
Glutamine[a]	135 ± 26	263 ± 16
Glycine[c]	151 ± 20	279 ± 16
Alanine[a]	172 ± 10	193 ± 13
Citrulline[c]	45 ± 7	11 ± 1
Valine[b]	64 ± 7	132 ± 13
Methionine[ns]	47 ± 5	48 ± 4
Isoleucine[c]	48 ± 2	80 ± 6
Leucine[b]	95 ± 10	179 ± 11
Tyrosine[b]	54 ± 8	88 ± 6
Phenylalanine[c]	49 ± 2	90 ± 3

Notes: In order to induce amyloid arthropathy, one group was intra-articularly injected with 0.25 mL complete Freund's adjuvant (experimental group; $n = 18$) at the left tibio-metatarsal joint, whereas the other group was injected with the same amount of 0.9% NaCl (control group; $n = 16$).
[a]The difference between the groups is significant at $p < 0.05$.
[b]The difference between the groups is significant at $p < 0.01$.
[c]The difference between the groups is significant at $p < 0.001$.
[ns]The difference between the groups is not significant at $p > 0.05$.

groups, respectively, and this difference between the groups was statistically significant ($p < 0.001$).

3.5. *Amino acid concentrations*

All examined serum amino acid concentrations, except citrulline, were higher in chickens with amyloid arthropathy and the elevation in all amino acid concentrations except methionine was found significant in varying degrees. Citrulline was significantly decreased ($p < 0.001$) in the experimental group when compared with the control group. Most significant increases ($p < 0.001$) were observed in serine (from 159 ± 15 to 360 ± 29 μmol/L), glycine (from 151 ± 20 to 279 ± 16 μmol/L), isoleucine (from 48 ± 2 to 80 ± 6 μmol/L), and phenylalanine (from 49 ± 2 to 90 ± 3 μmol/L) concentrations (Table 1).

4. Discussion

Secondary amyloidosis is a serious complication of chronic inflammatory diseases and is caused by the deposition of amyloid fibrils in various organs (Husby 1994). The major component of amyloid fibrils is derived from SAA protein by proteolysis (Rysava et al. 1992; Strissel et al. 1997). SAA is a major acute-phase reactant in the blood and its level increases after various insults to the body (Calnek et al. 1991; Landman et al. 1994; Urieli-Shoval et al. 2000). Increased circulating proteolytic cleavage of SAA has been suggested to contribute to the development of AA amyloid deposition (Migita et al. 1996). However, SAA is not responsible for the formation of amyloidosis on its own (Migita et al. 2001) as neutrophils and macrophages also play important roles (Skogen et al. 1980; Zekerias et al. 2000; Sevimli et al. 2005). In the experimental group of this study, intense lymphocyte, heterophil, and macrophage infiltrations were observed, a finding in agreement with previous data.

Some studies revealed the importance of SAA in monitoring poultry health (Chamanza et al. 1999). 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. Landman et al. (1996) reported that SAA is the precursor of amyloid A protein deposited in avian amyloid arthropathy and these authors also suggested that specific pathogen-free chicken sera and chicken sera before infection were negative for SAA.

This study investigated the effects of amyloidosis on the acute-phase protein SAA and serum amino acids in brown layer chicks with amyloid arthropathy compared to their healthy counterparts. Although SAA was detected both in the sera of healthy chicks and chicks with amyloid arthropathy, SAA concentrations were significantly ($p < 0.001$) higher in the amyloid arthropathy group. In addition, no pathological findings were observed at postmortem examination of control animals. These finding are in agreement with our previous results (Sevimli et al. 2005; Alasonyalilar et al. 2006). Chamanza et al. (1999) observed that SAA is a more useful protein for detecting acute lesions. These authors suggested SAA to be measured in combination with other acute-phase proteins in chronic stages of disease. The findings of this study indicate that SAA could also be a significant marker in detecting chronic stages of disease. These findings are similar to those of Ray and Ray (1999) who reported a persistent expression of SAA during experimentally induced chronic inflammatory condition in rabbits. In an experimental study by Upragarin, Asten, et al. (2005), SAA concentration was 200 ng/mL at 24 h and 400 ng/mL at 48 h post-LPS administration in parallel to the dose of lipopolysaccharide. In another study by Upragarin, Toussaint, et al. (2005), SAA was induced by turpentine and *Staphylococcus aureus*. While SAA concentration was 20 ng/mL in the negative control group, in the experimental group the concentrations were 28.9 μg/mL at 12 h and 84.6 μg/mL at 72 h following induction. In this study, mean SAA concentrations 13 weeks after the injection were 423 ng/mL in the experimental group and 166 ng/mL in the control group. The varying values obtained at different studies may be related to the difference between the injected inocula, individual immune variations, different experimental durations, and the kits (cross-reactivity in our kit) used. Differences in serum amino acid concentrations between the groups were also detected in this study. Konashi et al. (2000)

observed that the branched-chain amino acids isoleucine, leucine, and valine have the greatest potential to modulate immune responses in chickens. It is well-known that the acute-phase response is the general non-specific mechanism induced by noxious stimuli which precedes the specific defence mechanism of the immune response (Alsemgeest, Horadagoda, et al. 1995; Gruys and Toussaint 2001). This could explain the elevation of isoleucine, leucine, and valine in chicks with amyloid arthropathy which also showed an increased serum SAA ratio. Serine concentration was found to be higher in human patients with chronic renal failure than in healthy patients (Litwin et al. 2001). A relationship between the elevation of glycine and serine has also been reported in Bobwhites (Boren et al. 1996). Serine and glycine were the other two elevated amino acids in the experimental group in this study. The elevation of serine could be explained by the chronic disease status in the amyloid arthropathy group chicks given the relation of serine to the immune system. Elevated glycine concentrations might be related to the elevated concentrations of serine.

Phenylalanine is an amino acid which promotes alertness and is known to help in controlling pain, particularly in arthritis (Peter et al. 1978). The pain associated with arthritis in the experimental group chicks is thought to have resulted in the elevation of phenylalanine in this study. Borden et al. (1952) observed an increase in lysine, phenylalanine, and tyrosine, and a decrease in arginine and histidine concentrations in human rheumatoid arthritis patients. In this study, increases in phenylalanine ($p < 0.001$) and tyrosine ($p < 0.01$) were observed in the experimental group. Sluka and Westlund (1992), on the other hand, monitored an increase in glutamine, glycine, serine, and taurine concentrations in experimentally induced arthritis in rats. In this study, there was a more significant ($p < 0.001$) increase in glycine and serine concentrations than in taurine and glutamine ($p < 0.05$). Barker (1984, 1987) studied the gross amino acid composition of human positive acute-phase and skeletal muscle proteins. Phenylalanine was the amino acid with most elevated concentration in 4 out of 6 acute-phase proteins, tryptophan in 5, and tyrosine in 3. Arginine and alanine were the other two amino acids which showed highest increase. Tryptophan and arginine could not be evaluated in our study, but a significant increase was observed in phenylalanine, alanine, and tyrosine. In his study on chickens, Landman (1998) examined the amino acid composition of AA protein and reported that the composition of tryptophan was also not evaluated. Amino acid concentrations in Landman's study were lower than those observed in our study; the discrepancy may be due to the method (direct measurement of amino acids from the serum in our study).

In this study, 15 amino acids were evaluated as the amino acids lysine, arginine, histidine, and tryptophan could not be measured. All the examined serum amino acid concentrations except citrulline were higher in chicks with amyloid arthropathy and the elevation in all amino acids except methionine was significant in varying degrees. The most significant increases were observed in serine, glycine, phenylalanine, and isoleucine.

In this study, the effect of amyloid arthropathy on serum concentration of an acute-phase protein (SAA) and of serum amino acids was studied in brown layer chicks. SAA and serum amino acid concentrations differed significantly between the control and the experimental groups. From the results presented in this study it can be concluded that SAA and serum amino acid concentrations are influenced by amyloid arthropathy. Consequently, SAA and serum amino acids, particularly serine, glycine, isoleucine, and phenylalanine, can be sensitive markers to assess the physical welfare in chicks, and increase in these values may be suggestive of amyloid arthropathy. However, it should be realized that changes in the amino acid concentrations can also be the result of the acute-phase response against arthritis itself. Further investigations are needed to get more insight into the relationship between amyloid arthropathy, SAA, and serum amino acid concentrations. To the best of our knowledge, there are no data on the amino acid concentrations in serum of chickens with amyloid arthropathy. We are convinced that the data obtained in this study may be useful for future studies to be carried out on poultry diagnostics in the future.

Acknowledgments

This work was supported by the Commission of Scientific Research Projects of Uludag University, project number: 2001/42. We thank to Prof. Dr I. Hakki ULUS, Prof. Dr M. Mufit Kahraman and Prof. Dr Deniz Misirlioglu for their scientific help.

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