Changes in Serum Protein Profiles of Chickens with Tibial Dyschondroplasia

K. S. Rasaputra[1,2], R. Liyanage[3], J. O. Lay, Jr.[3], G. F. Erf[1], R. Okimoto[4] and N. C. Rath[2,*]

[1]*Department of Poultry Science,* [2]*PPPSRU, Agricultural Research Service/ USDA,* [3]*State Wide Mass Spectrometry Facility, University of Arkansas, Fayetteville, AR 72701, USA, and* [4]*Cobb-Vantress Inc., Siloam Springs, AR 72761, USA*

Abstract: Differences in serum protein profiles were analyzed to identify possible biomarkers associated with a poultry leg problem named tibial dyschondroplasia (TD) that can lead to lameness. A bead-based affinity matrix (ProteoMiner™) containing a combinatorial library of hexapeptides was used to deplete high abundant proteins and enrich the less abundant ones to compare between the sera of six-week old normal and TD affected chickens. Equal amounts of proteins in ProteoMiner depleted serum from control and TD-affected birds were subject to 2D gel electrophoresis, image analysis, and compared to identify the differentially expressed protein spots. The protein spots were characterized using in-gel trypsin digestion followed by mass spectrometry (MS). Of 46 matched protein spots in the gels, 33 were identified by peptide mass finger printing (PMF) and tandem mass spectrometry (MS/MS). Eight spots corresponding to immunoglobulins (Ig) were up-regulated in birds with TD and two spots down regulated. The up-regulated Ig proteins belonged to IgM and IgY(IgG) classes indicated by the identification of 'mu' chain and Fc fragment associated peptides respectively. Enzyme linked immunosorbent assay corroborated an increase in serum IgM levels but not IgG. Although the significance of the increase in IgM proteins in the serum of TD-affected chickens is not understood, it is likely that IgM plays some role in the removal of apoptotic chondrocytes which abound within TD lesions.

Keywords: Chicken, serum, proteominer, proteomics, tibial dyschondroplasia, IgM.

INTRODUCTION

Commercial poultry are susceptible to metabolic skeletal problems which cause lameness and affect their productivity [1]. Tibial dyschondroplasia (TD) is one such problem in young meat-type poultry where the proximal growth plates of the tibia and tibio-tarsal bones fail to undergo endochondral ossification leading to leg bone deformation, fragility, and lameness [2,3]. Eliminating TD-susceptible birds at an early stage of disease is desirable for managing breeding pools for better genetic selection. Biomarkers are molecular indicators of pathological conditions useful as diagnostic agents to monitor both initiation and progression of diseases [4]. In animal agriculture, biomarkers are useful for monitoring health and well-being of animals, surveillance against exposure to pathogens, understanding disease mechanisms, and implementing genetic selection [5,6]. Serum and plasma are widely used for minimally invasive clinical diagnosis of diseases, since the metabolic changes in tissues under aberrant conditions are often reflected therein. The blood proteome is a promising source of biomarkers because disease-induced qualitative or quantitative changes in tissue proteins resulting from differential production, modification, or degradation leak into blood and alter their serum profiles that can potentially be useful as biomarkers [4,7-10]. However, the discovery and validation of disease specific protein biomarkers in serum have been challenging due to the presence of certain high abundant proteins which tend to mask the detection of their relatively less abundant counterparts [8,11]. Thus, methods have been developed to deplete the high abundant proteins to allow monitoring changes in low abundance proteins which may also change significantly under pathological conditions [12,13]. These methods include the use of selective affinity based elimination of high abundance proteins using specific dye ligands or species specific anti-protein antibodies [14,15]. However, these methods have seen little use in veterinary applications. Dye-based ligands are limited to some selective proteins that can also deplete other low abundance proteins and the use of the commonly available anti-mammalian antibodies may not be useful for applications in poultry. An approach involving a combinatorial library of hexapeptide ligand-conjugated beads to enrich lower abundance proteins and deplete high abundant proteins has been reported [16-18]. These beads (commercially available as ProteoMiner™) have proved effective with proteomes from nonspecific sources including sera from human as well as agricultural animals [19-22]. Although Broschetti *et al.*, [18] studied chicken egg white proteome using ProteoMiner there is no report of its application to avian serum or plasma. The objective of our study was to use ProteoMiner to pre-fractionate chicken serum and identify any protein changes associated with TD.

MATERIALS AND METHODS

Zoom strips pH 3-10, IEF rehydration buffer (Invitrogen, Carlsbad, CA), recombinant trypsin (Promega, Madison, WI), peptide calibration standard (Bruker Daltonics, Germany),

*Address correspondence to this author at the USDA/ARS; O-307, Poultry Science Center, University of Arkansas, Fayetteville, AR 72701, USA;
Tel: 479-575-6189; Fax: 479-575-4202;
Emails: narayan.rath@ars.usda.gov, nrath@uark.edu

1875-0397/12

OMIX® Tips (Varian, Santa Clara, CA), Coomassie® Plus protein assay reagent (Pierce, Rockford, IL), unstained protein molecular weight markers (Fermentas, Hanhover, MD), ProteoMiner™ Protein Enrichment Small-Capacity Kit (Bio-Rad, Hercules, CA), 2D cleanup kit (GE Healthcare Bio-sciences Corp., Piscataway, NJ), and chicken IgG and IgM enzyme linked immunosorbant assay (ELISA) quantitation kits (Bethyl Laboratories, Montgomery, TX) were purchased. All other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Animal experiments were approved by the University of Arkansas Institutional Animal Use and Care committee.

Serum Preparation

Blood was collected from 6-wk-old Cobb 500 male chickens. Lame birds were selected preliminarily by visual inspection based on wobbly gaits and inability to stand for more than 2-3 minutes. Both normal and lame chickens were bled *via* wing vein using Monovette® syringes (Sarstedt, Germany). Blood was allowed to clot for 2-3 h at room temperature then left overnight at 4^0C. The collected serum was centrifuged at 250 g for 20 min to remove residual cells then frozen at -20^0C for subsequent analysis. Chickens were euthanized following blood collections, and dissected to ascertain the incidence of TD by longitudinal sectioning of proximal tibial growth plates. TD was indicated by an irregular broadening of growth plates due to the presence of non-ossified cartilage [23] (Fig. **1**). Chickens with normal standing abilities and growth plate morphology were used as controls. Three replicate samples per group, each consisting of pooled sera from 3 birds, were used for analysis.

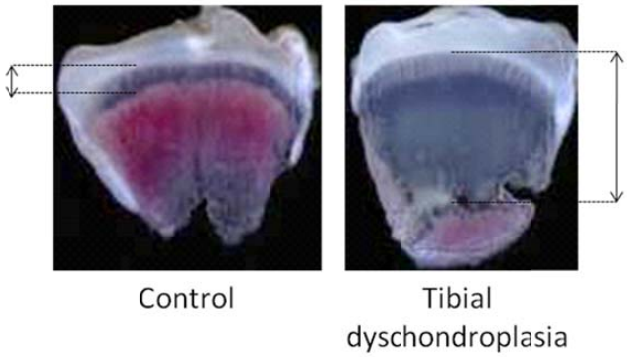

Fig. (1). Proximal growth plates of tibia from control and tibial dyschondroplasia (TD)-affected chickens showing abnormally broadened growth plate in the later.

Serum Fractionation

Aliquots of pooled sera from control and TD groups were subjected to fractionation by ProteoMiner beads using manufacturer's instructions with some minor modifications based on our preliminary trials. The ProteoMiner columns were first equilibrated per instructions in the kit then incubated with 1.2 ml of sera diluted 1:1 with saline for 2 h at RT with constant rocking. The column was drained and washed three times to remove unbound proteins, and the captured proteins were eluted successively (3 x 100 µl) during 15 min at RT. Protein content of the pooled eluents were measured using Coomassie plus protein assay. Eluting reagent was used to prepare bovine serum albumin (BSA) standards and to equalize the protein content of all samples *via* dilution. One hundred µg of protein from each sample was treated with 2D cleanup kit according to the manufacturer's protocol for gel electrophoresis.

Gel Electrophoresis and Imaging

For 2-D gel electrophoresis, the cleaned protein pellet was suspended in 155 µl of isoelectric focusing (IEF) rehydration buffer consisting of 8 M urea, 2% immobilized pH gradient (IPG) buffer, 2% CHAPS, 65 mM DTT, and a trace amount of bromophenol blue. The immobilized pH gradient (IPG) strips (7 cm pH 3-10) were passively rehydrated overnight followed by first dimension focusing at a gradient of 0-2,000 V for 1.2 h using Zoom IPG Runner mini-cell (Invitrogen, Carlsbad, CA). The focused proteins on the IPG strip were then reduced and alkylated for 15 min with 2% DTT and 2.5% iodoacetamide made in a solution containing 6 M urea, 50 mM Tris-HCl, 30% glycerol, and 2% SDS [24]. The equilibrated strips were run on 10% polyacrylamide gels at 12 mA constant current per gel in a Multicell Protean II BioRad apparatus (Richmond, CA). Molecular weight markers were used as reference and the gels were stained with Coomassie blue.

Each gel was scanned to acquire image with a Gel logic 2200 imaging system (Carestream Health, CT), and analyzed using Melanie software (version 5.0, Swiss Institute of Bioinformatics, Geneva, Switzerland). The feature detection algorithm was used to select the spots, match them among replicate gels from established landmarks and correct the mismatches resulting from differences in electrophoretic migration. Spot pairing was carried out using a control gel with maximum number of spots as the reference. The spots were quantified and normalized relative to total density of all spots correcting for gel-to-gel variations. Differential densitometric expression of the spots was determined using Student's t-test. A P value of ≤ 0.05 was considered significant.

Identification of Proteins and Mass Spectrometry

Individual spots were excised for in-gel trypsin digestion prior to mass spectrometric analyses. Each spot was destained by acetonitrile (ACN) treatment then dried using a Speedvac centrifuge [24]. Approximately, 200 ng of trypsin in 25 mM ammonium bicarbonate was added in a volume enough to rehydrate the gel pieces. Digestion was performed overnight at 37^0C. The tryptic peptides were eluted from the gel matrix with 60% ACN/5% formic acid, and the volume reduced to ~10 µl with a Speedvac centrifuge. The samples were desalted and eluted in 5 µl of 60% ACN containing 0.1% formic acid using C_{18} OMIX® tips. The eluant (2 µl) was mixed with an equal volume of saturated α-cyano 4-hydroxycinnamic acid made in 50:50 water/ACN, 0.1% formic acid and spotted on a Bruker MTP 384 stainless steel MALDI target. MALDI-TOF and MALDI LIFT-TOF/TOF mass spectra were obtained using an Ultraflex II MALDI TOF/TOF (Bruker Daltonics GMBH, Bremen, Germany) in its positive ion mode. The proteins were identified by MALDI mass spectrometry using peptide mass fingerprints (PMF) and by tandem mass spectrometry (MS/MS). The

combined PMF and MS/MS data were searched in a MASCOT data base using Bruker Biotools version 3.1 to identify the proteins. The NCBI database was searched under chordata taxonomy. Parameters were set to monoisotopic, peptide tolerance ± 200 ppm, MS/MS tolerance ± 0.8 Da with one missed cleavage, and carboxyamidomethylation of cysteines and methionine oxidation as fixed and variable modifications, respectively. All the identifications were based on a match of ≥ 3 peptides with a $P \leq 0.05$.

Immunoglobulin ELISA

Individual serum samples from control and TD affected birds were assayed in triplicates to determine avian IgG (IgY) and IgM concentrations using respective immunoglobulin standards by ELISA according to the manufacturer's instructions. Ninety six-well flat-bottom polystyrene plates were coated overnight at 4°C with affinity purified goat anti chicken IgG or IgM antibodies diluted to 1:100 in carbonate buffer, pH 9.6. Plates were washed with a wash buffer containing 50 mM Tris, 0.14 M NaCl, (TBS), 0.05% Tween 20, pH 8.0 and blocked with 200 µl of 1% bovine serum albumin per well in TBS for 30 min at RT. The reference standard or serum samples (100 µl) diluted 1:40,000 in TBS containing 1% BSA and 0.05% Tween 20 were added to respective wells and incubated for 1 h at RT. Following the wash steps 100 µl of diluted HRP conjugated detection antibody (1:50,000 of goat anti-chicken IgG (IgY)-Fc antibody or 1:75,000 of goat anti-chicken IgM antibody) was added to each well, and incubated at RT for 1 h. The plate was washed five times between each step with the wash buffer. The HRP enzyme activity was measured using tetramethylbenzidine (TMB) substrate solution by incubation in the dark for 15 min. The reaction was stopped by adding 0.18 M H_2SO_4 and the absorbance (O.D) were read at 450 nm within 30 min. The immunoglobulin (Ig) concentrations were calculated from their respective standard curves. The percentage changes in respective immunoglobulin (Ig) concentrations were calculated relative to the median values of sera from control birds.

Statistics

Quantitative results were expressed as mean and standard error of the mean (SEM). The results were analyzed using Student's *t*-test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Gel electrophoresis of serum treated with ProteoMiner beads showed enrichment of several protein bands as compared with equal concentrations of unfractionated whole serum (Fig. **2**). Depletions and enrichments in several protein bands were visually apparent although some high abundant proteins did not appear to deplete (Fig. **2b**, arrow). Forty-six protein spots, picked by the Melanie software were matched in all gels of which 33 proteins could be identified and 13 that were not identified included 2 down regulated spots in TD-affected chicken sera (Table **1**). A representative gel image and the areas of gels showing differential changes in protein spots are shown in Fig (**3**). Densitometric comparisons of the spots between the two groups showed quantitative increases in the intensities of 8 spots in TD affected chicken sera with *P* values corresponding to:1=0.05, 2=0.05, 3=0.01, 4=0.003, 5=0.007, 6=0.05, 7=0.03, and 8=0.04, respectively. Of 8 protein spots that were up-

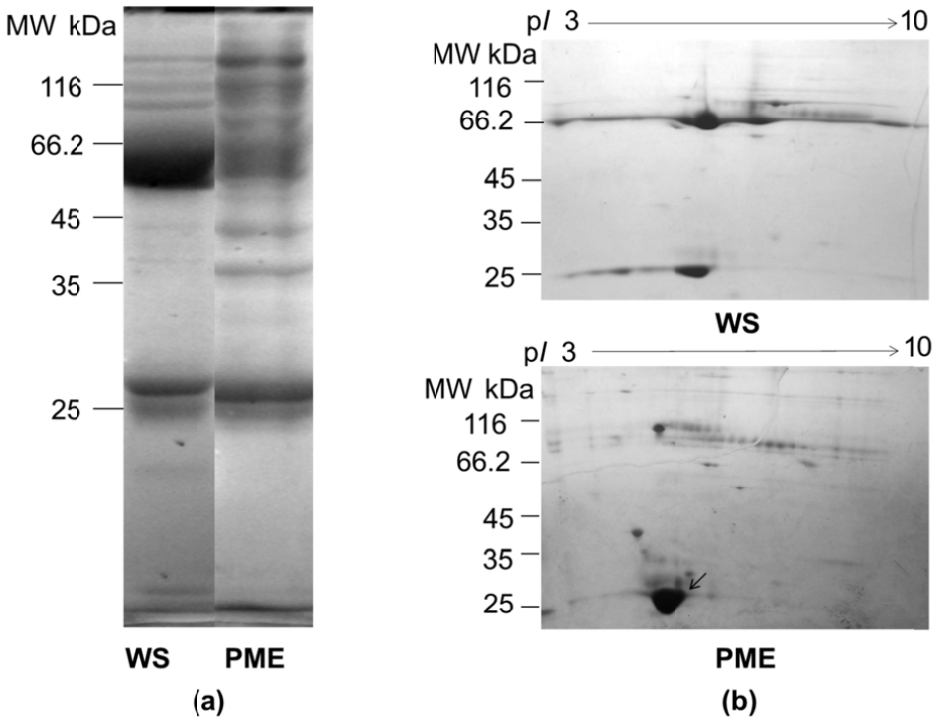

Fig. (2). Comparative profiles of (**a**) 1-D (**b**) 2-D gel images of normal control serum proteins before and after treatment with ProteoMiner beads. MW- molecular weight, WS- whole serum, PME- ProteoMiner fractionated serum. Arrow shows the protein spot that did not deplete by Proteominer treatment.

Table 1. Chicken Serum Proteins Identified by Tandem Mass Spectrometry

Spot #	Protein	Accession #	Score	No. of Peptides Matched
*1-5, 13-15	Chain A, structure of an avian IgY-Fc 3-4 fragment	gi|220702235	74	3
*6-8, 9-12	Ig mu chain C region	gi|127513	99	8
16-19	Ig gamma chain (clone 36)	gi|86318	118	18
20	Apolipoprotein A-IV	gi|45384392	97	14
21	Apolipoprotein A-I	gi|45382961	86	17
22-25	Apolipoprotein A-I	gi|227016	79	6
26-27	Albumin precursor	gi|45383974	117	9
28	Vitamin D-binding protein	gi|45382425	101	5
29	β- Actin	gi|47550655	117	7
30	Enolase 3-1	gi|213511756	70	4
31	Predicted: similar to complement regulator factor H	gi|118094043	80	22
32	Predicted: hypothetical protein	gi|118085512	58	4
33	Apolipoprotein A-IV	gi|45384392	327	11

*Corresponds to high abundant protein spots in TD.

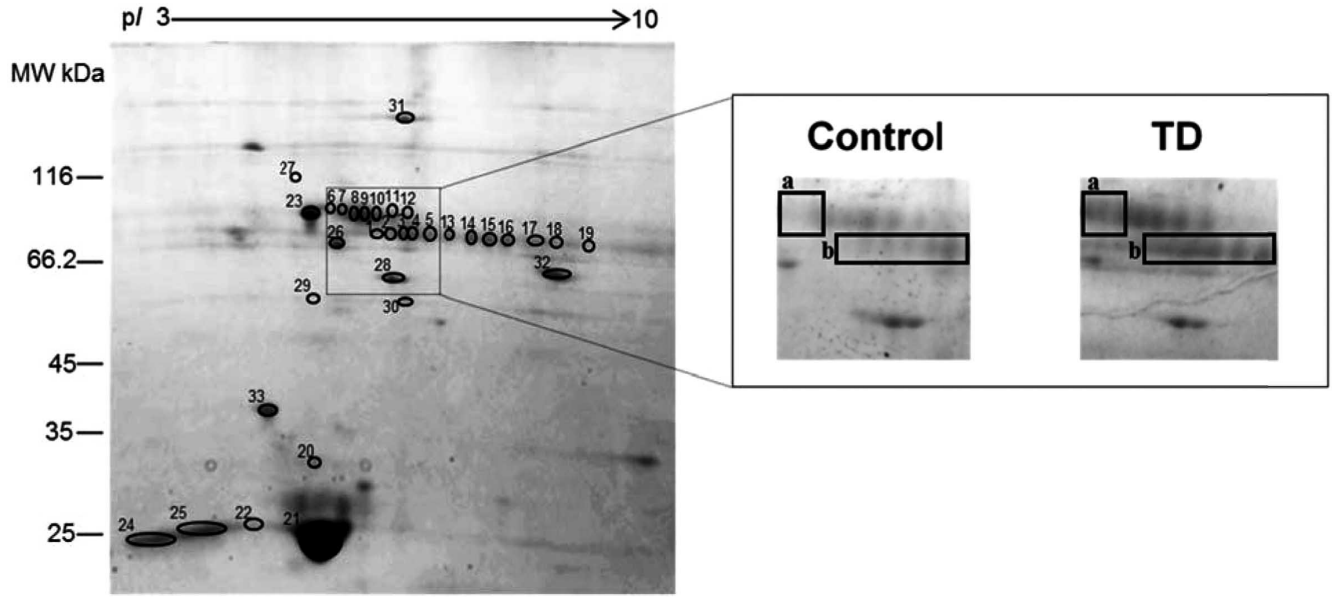

Fig. (3). Coomassie blue stained 2-D gel of proteins from sera of TD affected chickens with protein spots identified by Melanie software and characterized by PMF and tandem MS. The region in the rectangular box highlights the same areas of gels of control and TD affected chicken serum proteins. a. Ig mu chain C region, b. chain A, proteins from avian IgY (IgG) Fc 3-4 fragment. MW-molecular weight.

regulated in TD affected chicken sera 5 were identified as Fc 3-4 fragments of IgY (IgG) based on 24% sequence corresponding to 3 peptides. The peptide sequences of the fragments were as follows: R.AVPATEFVTTAVLPEER.T, R.FTCTVQHEELPLPLSK.S, and R.NTGPTTPPLIYPFA-PHPEELSLSR.V. The rest 3 were identified using 8 tryptic peptides, K.VISGPPYR.A, R.EDFEGPFR.N, R.RPTEVTWYK. N, R.RRPTEVTWYK.N, R.MECGLEPVVQQDIAIR.V + Oxidation (M), R.LSVTCMAQGFNPPHLFVR.W + Oxidation (M), K.SATLTCRVSNMVNADGLEVSWWK.E, and K. SATLTCRVSNMVNADGLEVSWWK.E + Oxidation (M), as "mu chain C-region" with 18% sequence coverage.

ELISA measurements showed statistically significant elevation in the serum levels of IgM ($P < 0.05$), but not IgG in TD-affected chickens (Fig. **4**).

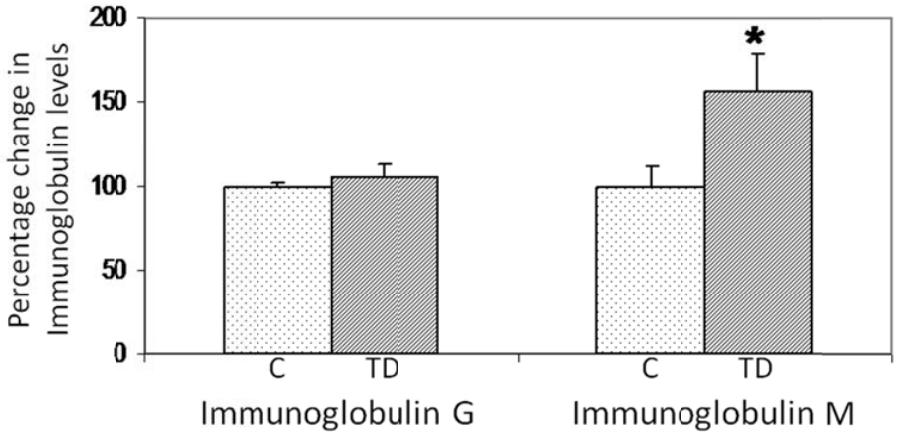

Fig. (4). Comparison of serum IgG and IgM levels of control and tibial dyschondroplasia (TD) affected chickens determined using ELISA. The percentage change in the concentrations of Ig's are shown as mean ± SEM. Control (C), Tibial dyschondroplasia (TD), (n=9 each). **P* < 0.05.

DISCUSSION

The ProteoMiner as a prefractionating technique to deplete high abundance proteins and increase the dynamic range of proteins has been appealing because of its potential for application with samples from various nonspecific sources [18,25]. It has been used with serum and plasma from different species showing fair to good enrichment of less abundant proteins [19,20,22,26-28]. Our results also showed ProteoMiner treatment resulting in the depletion of several high MW proteins making the presence of less abundant proteins more apparent. However, some high abundant protein bands particularly the apolipoproteins did not appear to deplete efficiently. These results are at par with the observations of Keidel *et al*., [29] who reported the ineffectiveness of ProteoMiner to interact and deplete all proteins. Nonetheless, the broad specificity of ProteoMiner makes it an attractive platform for fractionation of complex mixtures of proteins particularly when immunodepletion is not feasible.

With respect to chicken serum, several proteins identified in this study, were similar to proteins reported in white Leghorn chicken serum by Huang *et al*., [30]. While there were no qualitative differences between normal and TD-affected chicken sera, the later had an increased level of immunoglobulin (Ig) associated 'mu' chain and Fc 3-4 fragment suggesting that these birds had elevated levels of antibodies belonging to IgM and IgG classes. The ELISA measurement confirmed increased levels of serum IgM but not the IgG antibodies in TD-affected chickens. It is not clear whether the Fc region of avian IgM contain paralogous sequences similar to IgY (IgG) Fc 3-4 region. Comparative sequence homology studies of Fc region peptides in humans have shown substantial identities among different Igs' [31]. Nonetheless, the cause of increased IgM levels in TD affected birds is intriguing. In degenerative joint diseases such as rheumatoid arthritis the serum immunoglobulin levels are often elevated due to immune dysfunction [32, 33]; but there is no report of any change of immunity associated with TD which is regarded as a metabolic disease associated with growth [34]. IgM are spontaneously produced natural antibodies which have broad specificities against microbial pathogens, autoreactive IgGs, neoepitopes in cancer cells, and apoptotic cells [35-37]. They bind to lysophospholipids in apoptotic cells and activate complement pathway leading to the deposition of C3 components of complement which mark those cells for clearance by macrophages [38]. Removal of apoptotic cells suppresses inflammation and the development of autoimmunity [37, 39, 40]. In TD the broadening of growth plate is caused by an aberrant accumulation of prematurely dead or dying chondrocytes in the maturing zone which do often resolve by a process of sequestration [23,41-43]. Autoimmunity is not associated with TD which would lead to the elevations in acute phase proteins [44]. Thus, it is possible that the elevation in serum IgM in TD affected chickens is triggered by the lesion associated apoptotic cells for their removal as has been noted in a variety of disease conditions [45-48]. The elevated IgM levels in the serum during the development of TD lesion may be a mechanism to protect against the development of autoimmunity. Nonspecific and transient nature of IgM elevation may preclude its use as a biomarker for TD.

In conclusion, our results show that ProteoMiner treatment can enrich low abundant proteins in chicken serum and the birds with tibial dyschondroplasia show increased serum IgM levels.

CONFLICT OF INTEREST

Declared none.

ACKNOWLEDGEMENTS

The authors thank Scott Zornes, Sonia Tsai for their technical assistance. This study was partly supported by a grant from Cobb-Vantress Company Siloam Springs, AR and a P20 RR15569 grant from the National Center for Research Resources (NCRR), NIH to the University of Arkansas. The authors have no conflicts of interest.

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Received: October 06, 2011 Revised: November 23, 2011 Accepted: November 26, 2011