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The effect of age and sex on serum proteins in the Pega donkey (*Equus asinus*)

A.M. Girardi*, C.Z.P. Toledo, P.C. Silva, L.C. Marques

School of Agrarian and Veterinary Sciences, Sao Paulo State University, Jaboticabal, Brazil

*Corresponding author: annitamgirardi@gmail.com

ABSTRACT: In this study sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the influence of age and sex on serum proteins in 110 Pega donkeys, 79 females and 31 males, classified into three age groups (under one year – eight animals; one to three years – 33 animals and over three years old – 69 animals). SDS-PAGE allowed identification of 10 serum proteins, some with unknown functions. No age-related differences were observed ($P > 0.05$) for haptoglobin, α_1-acid glycoprotein and 23 kDa molecular weight protein (MWP_{23}) and no sex-related differences ($P > 0.05$) for immunoglobulin A, albumin, MWP_{23} and haptoglobin. With advancing age, immunoglobulin A levels decreased ($P < 0.001$), whereas immunoglobulin G levels increased ($P < 0.001$). The youngest donkeys showed the lowest albumin ($P < 0.001$) and 33 kDa molecular weight protein (MWP_{33}) ($P < 0.05$) means. The oldest group showed a higher transferrin mean ($P < 0.05$) than the youngest one. Animals between one and three years had the highest mean ($P < 0.001$) for 138 kDa molecular weight protein (MWP_{138}). Animals over three years showed higher ceruloplasmin levels ($P < 0.05$) than the one-to-three year old group. Males showed greater means of MWP_{138} ($P < 0.05$), ceruloplasmin ($P < 0.05$), α_1-acid glycoprotein ($P < 0.001$) and MWP_{33} ($P < 0.01$). Females had higher transferrin ($P < 0.001$) and immunoglobulin G ($P < 0.001$) concentrations. Sex and age influenced the levels of the majority of proteins in the serum profile of Pega donkeys, including some acute phase proteins. Our results differ from those of previous studies in the effects of breed and environmental factors on some of the measured variables.

Keywords: acute-phase proteins; biochemistry; blood protein electrophoresis; SDS-PAGE

The Pega donkey (*Equus asinus*), the most popular Brazilian donkey, is a large breed with Iberian origin, also popular in some other countries in South America, such as Bolivia, Paraguay and Colombia. Its breeding began in Minas Gerais State and, since then, the Pega breed has been selected for more than two centuries, mainly because of its outstanding characteristics as a saddle-type mule. Currently, the Brazilian Association of Pega Donkey Breeders (ABCJ Pega) has approximately 2000 members and about 20 000 mules and donkeys registered (Canisso and McDonnell 2010). However, only a limited amount of research has been performed on the haematological and serum biochemical variables of this breed (Campos et al. 1968; Girardi et al. 2014; Girardi et al. 2015; Girardi et al. 2016).

The lack of clearly established reference ranges often complicates the interpretation of the results of biological analysis performed in donkeys for veterinarians and owners (Pitel et al. 2006), and much necessary information is extrapolated from that which already exists for horses, which cannot provide a valid comparison (De Aluja et al. 2001). Changes in haematological variables for donkey breeds and populations may be influenced by age, sex and the time of sampling, as well as by exercise, geographical and nutritional factors (Mori et al. 2004), and should be considered in the clinical analysis of these animals.

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful technique used for separation of proteins by molecular weight: polyacrylamide fractionates proteins and small oligonucleotides, while SDS is an anionic detergent that denatures proteins, making them negatively charged (Wenk and Fernandis 2007).

Acute-phase proteins (APPs) are blood proteins of the innate immune system that change concentration in response to external or internal challenges such as infection, inflammation, surgical trauma, or stress (Murata et al. 2004; Eckersall and Bell 2010); they act as modulators of the inflammatory response by interacting with both host immune cells and pathogens (Ceciliani et al. 2012). Because circulating concentrations of APPs reflect disease severity, they may be useful for *in vivo* disease monitoring (Murata et al. 2004; Danscher et al. 2011), for assessing disease pathogenesis and animal health, including diagnosis, prognosis, response to therapy, and welfare status (Eckersall 2008; Eckersall and Bell 2010; Danscher et al. 2011; Ceciliani et al. 2012; Tothova et al. 2014). APPs are non-specific biomarkers with species-specific responses (Eckersall and Bell 2010; Tothova et al. 2014); therefore, physiological values should be established for each species. However, the influence of inflammation on APP concentrations and the usefulness of these concentrations for detection of diseases in veterinary clinical practice, particularly in farm animal medicine, are not well documented (Tothova et al. 2014).

The purpose of this study was to determine the influence of age and sex on serum proteins in Pega donkeys. It is our hope that the obtained results will guide more accurate diagnosis, facilitate a better understanding of important physiological processes and support future scientific investigations.

MATERIAL AND METHODS

Blood samples were collected from 110 donkeys, 79 females and 31 males. All animals were apparently healthy (based on the animals' history, physical examination, haematological and biochemical analyses performed concomitantly), maintained under field conditions and bred for reproductive purposes, in three donkey herds in the states of Sao Paulo and Minas Gerais, Brazil. All farms had the same breeding system, with feeding on pasture, supply of feed and mineral mixture and appropriate health management. Blood was collected on the day of birth, on the 3rd, 7th, 15th days and monthly until 12 months of life from animals under one year of age, and the results were compared with the other age groups. The other donkeys were separated into two age groups: one-to-three years (33 animals) and over three years old (69 animals), each animal sampled only once. The collections were performed in the morning, in non-fasting animals, only under mechanical restraint, using a closed evacuated system (BD Vacutainer, BD Diagnostics – Preanalytical Systems, Sao Paulo, Brazil) and multiple sample needles and plastic tubes of 10 ml volume without anticoagulant. Collection was achieved by external jugular venipuncture after proper regional antisepsis; each tube was completely filled. The samples were homogenised after collection and packed in a cooler with reusable ice packs for temporary storage and transportation. Blood serum was separated by centrifugation after clotting and stored below freezing at –20 °C, in plastic sterile microtubes (Meyer and Harvey 1998), because the main biochemical constituents remain stable in this condition (Thrall 2007). The serum protein fractionation by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the technique described by Laemmli (1970). After fractionation, the gel was stained for 10 min in Coomassie blue solution (50% methanol, 40% water, 9.75% glacial acetic acid, 0.25% Coomassie blue), and was then placed in a solution of 7% acetic acid to remove excess dye until the fractions became clear. The concentrations of fraction were determined using a computerised scanning densitometer (Shimadzu CS 9301, Tokyo, Japan). As a reference, a marker solution (SigmaMarker wide range, Sigma-Aldrich, St Louis, USA) with molecular weights ranging from 6.5 to 200 kDa, was used. The serum protein fraction concentrations (mg/dl) were determined by multiplying the percentage of each fraction by the total protein concentrations, obtained through a semi-automatic spectrophotometer analyser (LabQuest, Labtest Diagnostica S.A., Lagoa Santa, Brazil) using biuret the method as previously described (Girardi et al. 2014). These analyses were made at the Research Laboratory of the Department of Veterinary Clinic and Surgery, School of Agrarian and Veterinary Sciences, Sao Paulo State University, Jaboticabal, Brazil.

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Data analysis was performed using statistical software (GraphPad InStat version 3.10, 32 bit for Windows, GraphPad Software Inc., San Diego, USA). Each parameter was tested for normality applying the Kolmogorov and Smirnov methods. The values were given as mean (or median, when there was not a Gaussian distribution) ± SD, and including 95% confidence limits. To determine the age effect on variables, the Tukey-Kramer multiple comparisons test was used for normally distributed data, or a non-parametric test (Kruskal-Wallis test and Dunn's multiple comparison post test) was used if the data did not seem to have Gaussian distribution. Unpaired *t*-tests were used to determine the effect of sex, for normally distributed data, and non-parametric tests (Mann-Whitney *U*-test) were performed for data not normally distributed. Unpaired *t*-tests were also used to compare the present results with those from other studies. Dixon-Reed and Tukey's tests helped the identification of outliers, using a set of macroinstructions (Reference Value Advisor V 1.4) (Geffre et al. 2011) for spreadsheets (Microsoft Excel, Microsoft, Redmond, USA). The level of statistical significance was set at $P < 0.05$.

This study was approved by the Ethics Commission for the Use of Animals of the Faculty of Agricultural Sciences and Veterinary, Sao Paulo State University, protocol No. 6369/10. All institutional and national guidelines for the care and use of animals were followed.

RESULTS

Ten protein fractions were separated by electrophoresis: immunoglobulin A (IgA – 155 kDa), 138 kDa molecular weight protein (MWP_{138}), ceruloplasmin (Cp – 113 kDa), transferrin (Tf – 80 kDa), albumin (62 kDa), immunoglobulin G (IgG – heavy chain of 55 kDa and light chain of 26 kDa), haptoglobin (Hp – 42 kDa), α_1-acid glycoprotein (α_1AG – 39 kDa), 33 kDa (MWP_{33}) and 23 kDa molecular weight protein (MWP_{23}) (Figure 1). Most variables exhibited a Gaussian distribution for all age and sex groups, except for Cp, which did not seem to be normally distributed for sexes.

There were no age-related differences ($P > 0.05$) for Hp, α_1AG and MWP_{23}. IgA levels decreased ($P < 0.001$), while IgG increased ($P < 0.001$) with aging. The youngest donkeys showed the lowest mean of albumin ($P < 0.001$) and MWP_{33} ($P < 0.05$). The oldest group had a higher Tf mean ($P < 0.05$) than the youngest one. Animals between one and three years old had the highest mean for MWP_{138} ($P < 0.001$). Animals over three years exhibited higher Cp levels ($P < 0.05$) than the one-to-three year old group (Table 1). Mean IgA, albumin, MWP_{23} and Hp levels were not significantly associated with sex ($P > 0.05$). Males showed greater means values of MWP_{138} ($P < 0.05$), Cp ($P < 0.05$), α_1AG ($P < 0.001$) and MWP_{33} ($P < 0.01$), while females had higher Tf ($P < 0.001$) and IgG ($P < 0.001$) serum concentrations (Table 2).

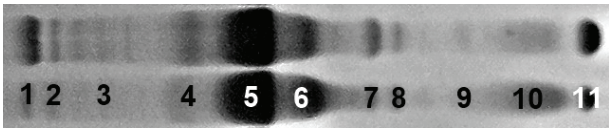

Figure 1. Serum samples of Pega donkeys after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), showing regions with the following proteins: (1) immunoglobulin A (IgA), (2) 138 kDa molecular weight protein (MWP_{138}), (3) ceruloplasmin, (4) transferrin, (5) albumin, (6) immunoglobulin G (IgG) heavy chain, (7) haptoglobin, (8) α_1-acid glycoprotein, (9) 33 kDa molecular weight protein (MWP_{33}), (10) IgG light chain, (11) 23 kDa molecular weight protein (MWP_{23})

The serum albumin concentration was lower ($P < 0.01$) than that described by Caldin et al. (2005) for animals under one year old, but higher for donkeys in the between one and three years ($P < 0.0001$) and over three-year-old ($P < 0.0001$) groups. Albumin levels in both sexes were greater ($P < 0.0001$) than those reported by Cavalcante et al. (2012).

DISCUSSION

The observation that IgG concentration increased with age is in agreement with other reports of an increasing trend of serum globulin levels in donkeys with aging (Zinkl et al. 1990; Pitel et al. 2006; Alberghina et al. 2013). This is due to the maturation of the immune system, which begins to synthesise its own immunoglobulins, with a resulting progressive increase in globulins until adulthood (Eckersall 2008). As IgA plays its predominant role in the defence of the mucous membranes (Tizard 2014), the reduced levels of this protein could be attributed to a diminished immune challenge or a greater resistance acquired by animals against antigens in these areas during their lifetime.

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Table 1. Serum proteins (g/l) in the Pega donkey (*Equus asinus*) breed subtyped by age: under one year old (A), one-to-three years old (B) and over three years old (C). Values are presented as mean ± SD and 95% confidence limits

Protein	A (n = 8)	B (n = 33)	C (n = 69)	*P*-value		
				A : B	A : C	B : C
Immunoglobulin A	2.425 ± 1.083 2.230–2.620	1.826 ± 0.619 1.591–2.061	1.205 ± 0.467 1.075–1.335	***	***	***
MWP_{138}	0.331 ± 0.149 0.304–0.358	0.473 ± 0.224 0.388–0.558	0.349 ± 0.148 0.306–0.393	***	ns	***
Ceruloplasmin	0.092 ± 0.048 0.082–0.101	0.070 ± 0.048 0.052–0.089	0.104 ± 0.062 0.086–0.121	ns	ns	*
Transferrin	3.442 ± 1.011 3.260–3.624	3.426 ± 0.506 3.217–3.635	3.879 ± 0.918 3.655–4.103	ns	**	ns
Albumin	35.514 ± 5.125 34.591–36.436	42.013 ± 5.676 39.855–44.172	43197 ± 5.550 41.842–44.551	***	***	ns
Immunoglobulin G	9.407 ± 2.798 8.779–10.035	17.929 ± 3.438 16.622–19.236	20.817 ± 4.225 19.786–21.848	***	***	***
Haptoglobin	0.649 ± 0.262 0.601–0.697	0.684 ± 0.236 0.595–0.774	0.642 ± 0.289 0.570–0.713	ns	ns	ns
α_1-acid glycoprotein	0.164 ± 0.058 0.152–0.175	0.172 ± 0.075 0.144–0.201	0.163 ± 0.066 0.147–0.179	ns	ns	ns
MWP_{33}	0.124 ± 0.044 0.112–0.128	0.170 ± 0.145 0.113–0.227	0.170 ± 0.125 0.141–0.199	*	**	ns
MWP_{23}	3.491 ± 0.981 3.315–3.668	3.702 ± 0.850 3.378–4.025	3.614 ± 0.871 3.401–3.826	ns	ns	ns

MWP_{23}, MWP_{33}, MWP_{138} = 23, 33, 138 kDa molecular weight proteins
ns = not significant ($P > 0.05$)
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

In agreement with what was observed in this study, Laus et al. (2015) reported no difference between genders for serum albumin levels in donkeys, but other studies, which separated serum albumin by electrophoresis, found gender differences in albuminaemia (Gacek et al. 1973; Gacek et al. 1975; Cavalcante et al. 2012). Caldin et al. (2005), Alberghina et al. (2013) and Stanisic et al. (2015) found no differences in serum albumin levels between age groups, while in this present report we observed an increasing trend with advancing age. Most donkey populations exhibit distinct albumin values due to different agro-climatic regions, management and nutritional practices (Gupta et al. 2016). In addition, different electrophoretic techniques and differences between breeds may cause such variations (Girardi et al. 2016), showing the importance of this kind of study.

Cp is part of the α_2-globulin fraction, Tf is a β-globulin and IgG is a γ-globulin (Eckersall 2008). Thus, the higher levels of Cp in males corroborates the observations of Gacek et al. (1973) and Gacek et al. (1975) of greater α_2-globulin levels for male donkeys. For females, the higher Tf and IgG levels that we observed are also in agreement with these authors, who reported greater beta and γ-globulin levels, respectively, for jennies. The higher Tf values in older animals corroborates the findings of Caldin et al. (2005), who described a higher β-globulin average for animals over three years old. The higher Cp levels in adults compared to the one-to-three-year group are not in agreement with previous studies that reported, for the α_2-globulin fraction, greater values for donkeys up to one year old (Caldin et al. 2005), and no differences between young and adult animals (Alberghina et al. 2013).

MWP_{138}, MWP_{33} and MWP_{23} are not characterised in the literature, and their functions are unknown. This finding is interesting because there is a continuous quest in veterinary science to discover new APPs and to characterise their potential applications (Murata et al. 2004).

To the best of our knowledge, this is the first report in which SDS-PAGE was used to evaluate

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Table 2. Serum proteins (g/l) in Pega donkeys (*Equus asinus*), subtyped by sex, ordered by decreasing molecular weight. Values are presented as mean ± SD and 95% confidence limits

Protein	Female (n = 79)	Male (n = 31)	P
Immunoglobulin A	2.128 ± 0.888 1.948–2.308	2.248 ± 0.973 2.052–2.443	ns
MWP_{138}	0.323 ± 0.119 0.299–0.347	0.369 ± 0.180 0.331–0.406	*
Ceruloplasmin	0.076 ± 0.066 0.077–0.101	0.093 ± 0.121 0.105–0.154	*
Transferrin	3.808 ± 0.902 3.643–3.972	3.263 ± 1.012 3.060–3.465	***
Albumin	39.531 ± 6.271 38.387–40.675	37.829 ± 6.570 36.516–39.141	ns
Immunoglobulin G	17.647 ± 5.854 16.580–18.715	13.981 ± 4.877 12.964–14.998	***
Haptoglobin	0.663 ± 0.282 0.611–0.716	0.648 ± 0.266 0.594–0.701	ns
α_1-acid glycoprotein	0.154 ± 0.054 0.144–0.165	0.190 ± 0.087 0.172–0.208	***
MWP_{33}	0.123 ± 0.067 0.110–0.136	0.150 ± 0.070 0.136–0.165	**
MWP_{23}	3.606 ± 0.859 3.449–3.763	3.459 ± 0.936 3.271–3.647	ns

MWP_{23}, MWP_{33}, MWP_{138} = 23, 33, 138 kDa molecular weight proteins; ns = not significant ($P > 0.05$); P = P-value
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

age and sex effects on serum proteins in donkeys. Unlike previous studies, which separated the serum proteins into albumin, α-globulin, β-globulin, γ-globulin fractions and their subdivisions, SDS-PAGE allowed the partition of individual proteins, making the results most accurate and meaningful.

The results indicate that age and sex influenced most of the serum proteins in Pega donkeys. The data obtained in this present study might be useful for future research on donkey physiology and metabolism.

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