AGE-related differences in the hematological profile of Andalusian broodmares of Carthusian strain

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ABSTRACT: Normal hematological values need to be defined for each equine breed and age in order to increase diagnostic precision. No published data on hematology exist for Carthusian horses. This research compares the hematological characteristics of pregnant Carthusian broodmares of different ages. Forty-four pregnant Carthusian broodmares were divided into three age Groups: A (4–7 years; n = 18), B (8–12; n = 15) and C (13–17; n = 11). Jugular blood samples were taken every 14 days during pregnancy and data were pooled for each animal. The following hematological variables were determined: red blood cells (RBC), hemoglobin (HB), hematocrit (HCT), volumetric indices, white blood cells (WBC) and platelets (PLT). Furthermore, the numbers and percentages of lymphocytes (LYMP), band (BNL) and total neutrophils (NL), eosinophils (EOS), monocytes (MON), basophils (BAS) and the neutrophil/lymphocyte ratio (N/L) were counted on blood smears. Total serum protein concentrations (TSP) were also measured. The lower values of RBC, WBC, LYMP and PLT in the older broodmares (Group C) possibly reflected a decline in bone marrow activity. The lower RBC of these mares was compensated by an increased MCV. The higher NL values in Group C, both BNL and NL, could have represented subclinical infections, since these animals also presented the highest TSP. Likewise, the animals of Group C showed the highest EOS counts. This research demonstrated that ageing significantly influences the hematological values of Carthusian broodmares, with the most marked differences in mares older than 13 years and that these physiological variations must be taken into account in a clinical context.

K**eywords**: age; broodmares; hematology; horses

Hematological analysis in horses is an important aid for clinical diagnosis of systemic, infectious and some parasitic diseases. It can also provide significant information about the response to treatment, the severity and the systemic effects of a disease, and the metabolic state of a single animal or herd (Ricketts, 1987; Lassen and Swardson, 1995; Messer, 1995). Despite the extended use of hematology in equine medicine, interpretation may be a challenge in some cases, because it can be significantly influenced by a great number of factors. Hematological parameters may vary according to breed, sex, age, reproductive status, fitness and training levels, exercise, feeding, circadian variations, handling procedure of the animals during blood withdrawal, degree of excitement and health state (Jain, 1993; Rose and Hodgson, 1994; Messer, 1995; Kramer, 2000).

The horse is unique in comparison to most other mammalian species in that the spleen is a very capacious organ, storing up to one third of the RBC, and this reserve can by mobilized by exercise, stress or excitement (Persson, 1967; Schalm et al., 1975; Rose and Hodgson, 1994; Lassen and Swardson, 1995). The degree of excitement during blood withdrawal depends on the temperament and the breed (Rubio et al., 1995). According to our experience, Carthusian horses are less nervous than other warm blooded breeds such as Arabians and Thoroughbreds. For that reason, several hematological variables in Carthusian horses might be lower than the reference values described for other equine breeds, making diagnosis of slight anemia difficult in some cases. This fact must be emphasized, since middle anemia is common in horses in Spain, because of some parasitic (*Babesia ca-*

balli and *Theileria equi*) and infectious (*Anaplasma phagocytophilum*) diseases which are considered endemic and have high prevalence in our country.

The influence of age on the hematological parameters has been evaluated in different equine breeds (Ralston et al., 1988; McFarlane et al., 1998, 2001; Cebulj-Kadunc et al., 2003; Satue et al., 2008). In this way, the importance of appropriate ranges of reference for accurate interpretation of clinico-pathological data is well recognized in equine medicine. Most of the studies concerning the hematological parameters focused on foals from birth to four years of age (Stewart et al., 1970; Harvey et al., 1984; Jain, 1993), although recently old horses have received more attention, probably because the increase in the population of geriatric animals (McFarlane et al., 1998; Paradis, 2002).

It is acknowledged that for comparisons between individuals and with reference data in a clinical diagnostic situation, it is necessary to consider normal variations due to age, sex and breed, in order to increase diagnostic precision. Likewise, although the normal range of hematological parameters for the equine species falls into a broad range, the physiological variations for a determined breed are usually quite narrow (Schalm et al., 1975; Rose and Hodgson, 1994).

A previous paper described the hematological characteristics of Andalusian horses (Rubio et al., 1995). This research evaluated the hematological parameters at rest and after a standardized exercise test in 4-year-old male Andalusian foals. As far as we are aware there are no reports concerning hematological parameters in Carthusian mares. For that reason, in the present paper, we present the hematological characteristics of Andalusian mares of different ages of the Carthusian strain used for reproductive purposes. Furthermore, the data presented here belong to animals of Carthusian strain, a well-known and highly valued strain of the Andalusian breed. We wish to verify the hypothesis that age induces some hematological modifications in Carthusian broodmares.

MATERIAL AND METHODS

Mares

Forty-four Andalusian pregnant broodmares of Carthusian strain were sampled during a total period of 11 months. According to their age, the animals were divided into three groups: A (n = 18; between 4 and 7 years), B (n = 15; between 8 and 12 years) and C (n = 11; between 13 and 17 years). The assignment of the mares in the different groups of age was based on the information provided by the clinicians of the farm. Group A included young animals, as these broodmares start their reproductive activity at four years. Group B was formed by animals of medium age and Group C was composed of animals whose reproductive function had started to decline. After 17 years of age, the animals were not used as broodmares and they were dedicated to other activities.

All the mares were subjected to a general clinical examination before the taking of blood samples and in all cases, no significant clinical findings were observed. Only mares without evidence of trauma or illness before or during the study were included.

Blood withdrawal

Venous blood samples were drawn from the jugular vein every 14 days during the 11 months of study. The study was initiated after confirming pregnancy with ultrasonography and it finished on the week of delivery. Although pregnancy might influence hematological variables, the samples of all individuals of each age group were pooled, as the main objective of this study was to analyze the effects of age.

In order to reduce the circadian variations, all the samples were taken between 9:00 and 13:00 h, before feeding the animals. Immediately after extraction, and directly from the syringe, a blood smear was made. All the smears were air-dried. The remaining blood was divided into two parts and poured into EDTA-3K tubes and glass tubes without anticoagulant. Hematological determinations were performed with the samples stored in EDTA-3K and total proteins were measured in serum. During sampling and transport, samples were kept refrigerated and blood parameters were measured within the first 12 h after extraction.

Blood parameters

Several hematological variables were analyzed with a semi-automatic cell counter (Sysmex-F 820). Measured parameters included red blood cells (RBC), hemoglobin concentration (HB), hemato-

crit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobinconcentration (MCHC), white blood cells (WBC) and platelets (PLT).

The blood smears were ethanol-fixed and stained according to the May-Grünwald-Giemsa technique. In these smears, the following parameters were determined by immersion microscopy: lymphocytes (LYMP), band neutrophils (BNL), total neutrophils (TNL), eosinophils (EOS), monocytes (MON), basophils (BAS) and neutrophil/lymphocyte ratio (N/L). The differential WBC count was expressed both in numbers and percentages. Total serum proteins (TSP) were quantified by immersion refractometry (Atago).

Statistical analysis

Data are expressed as mean±standard deviation (SD). All the data were distributed normally and the differences linked to age were analyzed by ANOVA using a statistical software program (SPSS®). When significant differences were detected, a post-hoc analysis was performed (Tukey HDS test) in order to clarify between which groups these differences existed. A P value of <0.05 was fixed as significant.

RESULTS

Mean and SD values for the blood erythrocyte parameters (RBC, HB, HCT, MCV, MCH, and MCHC), PLT and TSP concentrations are presented in Table 1 for the three age groups. RBC was lower in Group C than in Groups A and B, whereas MCV and MCH increased progressively with age. On the other hand, the PLT count was significantly lower in Groups B and C in relation to A. Finally, TSP concentrations were significantly higher in Group C than in Group A, and without any significant difference between A and B (Table 1).

Tables 2 and 3 summarize the means and SD of the WBC parameters in the three age groups, in numbers and in percentages. WBC values were significantly lower in the Carthusian broodmares older than 13 years in comparison to the other age groups. In contrast, LYMP numbers and percentages underwent a progressive decrease with age, with lower values in the Group C age category. Both BNL and NTL, in numbers and percentages, were significantly higher in Group C. These differences between groups led to a progressive increase in N/L ratio. Moreover, EOS count was significantly lower in Group A in comparison to Groups B and C (Table 2).

DISCUSSION

This research assesses the hematological differences when comparing pregnant Carthusian broodmares of different ages. We found that mares older than 13 years (Group C) presented lower RBC, WBC, LYMP and PLT and higher MCV, MCH, TNL, BNL, NL, N/L, EOS and TSP values in com-

Table 1. Mean ± SD of the erythrocyte variables, platelet count (PLT) and total serum protein concentrations (TSP) in Carthusian broodmares of three different age groups (A, B, and C)

Group of age	A (n = 18)		B (n = 15)		C (n = 11)	
	samples (n)	mean ± SD	samples (n)	mean ± SD	samples (n)	mean ± SD
RBC (10^6/mm^3)	379	9.111 ± 1.565^{AC}	331	8.832 ± 1.619	181	8.595 ± 1.716
HB (g/dl)	379	12.27 ± 1.601	331	12.50 ± 1.434	181	12.54 ± 1.615
HCT (%)	379	43.35 ± 5.259	331	43.60 ± 4.584	181	43.42 ± 5.079
MCV (fl)	379	48.11 ± 4.338$^{AB, AC}$	331	50.14 ± 5.346^{BC}	181	51.44 ± 5.705
MCH (pg)	379	13.7 0 ± 1 .909$^{AB, AC}$	331	14.39 ± 1.756^{BC}	181	14.91 ± 2.282
MCHC (g/dl)	379	28.53 ± 3.680	331	28.82 ± 3.057	181	29.08 ± 3.674
PLT (10^3/mm^3)	379	212.4 ± 115.6$^{AB, AC}$	331	189.6 ± 92.58	181	188.6 ± 93.99
TSP (g/dl)	379	6.844 ± 0.496^{AC}	331	6.927 ± 0.463	181	7.035 ± 0.444

AB = differences between groups A and B; AC = differences between groups A and C; BC = differences between groups B and C; P < 0.05

Table 2. Mean ± SD of the leukogram in absolute values in Carthusian broodmares of three different age groups (A, B, and C)

Group of age	A (n = 18)		B (n = 15)		C (n = 11)	
	samples (n)	mean ± SD	samples (n)	mean ± SD	samples (n)	mean ± SD
WBC (10^3/mm^3)	378	10.36 ± 2.753^{AC}	331	10.21 ± 2.230^{BC}	181	9.630 ± 2.129
LYMP (10^3/mm^3)	378	4.865 ± 1.277$^{AB, AC}$	331	4.342 ± 1.114^{BC}	181	3.412 ± 0.999
BNL (10^3/mm^3)	360	0.183 ± 0.097$^{AB, AC}$	316	0.216 ± 0.116	171	0.218 ± 0.105
NL (10^3/mm^3)	360	4.740 ± 1.544$^{AB, AC}$	316	5.011 ± 1.405^{BC}	171	5.393 ± 1.488
EOS (10^3/mm^3)	360	0.540 ± 0.377$^{AB, AC}$	316	0.633 ± 0.375	171	0.695 ± 0.475
MON (10^3/mm^3)	360	0.228 ± 0.133	316	0.231 ± 0.104	171	0.234 ± 0.117
BAS (10^3/mm^3)	360	0.035 ± 0.024	316	0.002 ± 0.007	171	0.005 ± 0.036
N/L ratio	360	0.988 ± 0.278$^{AB, AC}$	316	1.195 ± 0.402^{BC}	171	1.686 ± 0.636

AB = differences between groups A and B; AC = differences between groups A and C; BC = differences between groups B and C; $P < 0.05$

Table 3. Mean ± SD of the leukogram in percentages in Carthusian broodmares of three different age groups (A, B, and C)

Group of age	A (n = 18)		B (n = 15)		C (n = 11)	
	samples (n)	mean ± SD	samples (n)	mean ± SD	samples (n)	mean ± SD
LYMP (%)	378	47.43 ± 5.830^{AB,AC}	331	42.90 ± 6.295^{BC}	181	35.47 ± 8.137
BNL (%)	360	1.737 ± 0.733^{AB,AC}	316	2.032 ± 1.008	171	2.211 ± 0.935
NL (%)	360	45.30 ± 5.893^{AB,AC}	316	48.59 ± 6.772^{BC}	171	55.23 ± 7.526
EOS (%)	360	5.048 ± 2.984^{AB,AC}	316	6.202 ± 3.421^{BC}	171	7.100 ± 4.358
MON (%)	360	2.205 ± 1.183	316	2.288 ± 1.010	171	2.411 ± 1.116
BAS (%)	360	0.033 ± 0.223	316	0.021 ± 0.054	171	0.049 ± 0.390

AB = differences between groups A and B; AC = differences between groups A and C; BC = differences between groups B and C; $P < 0.05$

parison with younger mares (Groups A and B). The lower RBC values could have been compensated by means of an increased erythrocyte size, which caused higher MCH values, and therefore, HCT did not differ between the groups. Despite the hypothesized decline in the regenerative capacity of the bone marrow (McFarlane et al., 1998), the oldest broodmares presented higher numbers of NL, including immature forms. The TSP concentrations achieved the highest values in the oldest broodmares. The LYMP counts were progressively lower with age and the lower EOS counts in the youngest group of mares might have represented a lesser exposure to allergens and/or parasites.

The reduction in RBC with ageing has been described already in Standard-bred trotters (Stewart et al., 1970; Jain, 1986; Ralston et al., 1988), Lipizzaner (Cebulj-Kadunc et al., 2002), Spanish Purebred (Satue et al., 2008) and feral horses (Plotka et al., 1988). However, the research performed by McFarlane et al. (1998) only found a trend of falling RBC when geriatric horses were compared to a control group. The results obtained in the Carthusian broodmares are in agreement with the data presented by Plotka et al. (1988), Ralston et al. (1988) and Cebulj-Kadunc et al. (2002), since Group C presented significantly lower RBC values than Group A. This fact could have been related to

a reduced regenerative capacity of the bone marrow, the presence of subclinical chronic diseases and/or pituitary-dependent hyperadrenocorticism (pars intermedia dysfunction). There were no clinical or clinicopathological findings compatible with chronic diseases in any of the studied Carthusian broodmares. In the same way, none of the animals of Group C was reported to develop clinical signs consistent with pituitary dysfunction, such as laminitis, hirsutism, polyuria and polydipsia during the first six months after the study. Therefore, we consider that the main reason for the lower RBC in older broodmares was the degree of activity of the bone marrow.

Increased erythrocyte size seems to be a frequent finding associated with ageing in horses (Ralston et al., 1988; McFarlane et al., 1998; Satue et al., 2008). Similarly, a significant progressive rise in MCV from Group A to B and to C was observed in the Carthusian broodmares. Increased MCV has been described in adult horses in response to severe anemia with an intense bone marrow regenerative response (Easley, 1985; Radin et al., 1986). Anemia was not detected in any of the Carthusian broodmares studied. Therefore, in the absence of anemia in an adult horse, the larger size of the erythrocytes might reflect changes derived from RBC maturation dynamics, as suggested by McFarlane et al. (1998).

This change of MCV with ageing has also been reported in elderly human patients with low concentrations of serum vitamin B12 or erythrocyte folate but a normal blood count (Matthews et al., 1988). These patients were treated with vitamin B12 and folic acid for three months and as a consequence, MCV decreased. According to these authors, the increased MCV and its response to vitamin B12 and folate supplementations reflect a true deficiency of these vitamins. Stillions et al. (1971) reported that horses do not have an absolute dietary requirement for these vitamins because they are produced by microbial fermentation via microbes present in the large intestine. There are some situations that might cause a reduction in these vitamins, such as intensive training, exercise or confinement (Roberts, 1983). None of these scenarios can be applied to the older Carthusian broodmares, since they were kept most of the time at pasture, and did not perform intense exercise. It might be suggested that the higher MCV in the Group C could be due to the lower digestive capacity of the older animals.

The progressive increase in MCH in the Carthusian broodmares probably was the result of the larger size of the erythrocytes. This age-dependent change has also been observed in old horses of various breeds (McFarlane et al., 1998; Satue et al., 2008). The lack of significant differences in HCT and HB in Group C in relation to the other age groups could have resulted from the lower RBC and the higher MCV and MCH. This age-related change in erythrocyte number has been observed in human (Takubo and Tatsumi, 2000, Martin et al., 2001) and other animal species, such as cattle (Monke et al., 1998), sheep (Ramos et al., 1992), cats (Nakai et al., 1992), dogs (Harper et al., 2003) and donkeys (Zinkl et al., 1990).

The higher PLT count in Carthusian broodmares of Group A in relation to B and C could indicate that the bone marrow has a more marked regenerative activity in horses younger than seven years of age. The results of previous research on this subject are controversial. Some authors documented a reduction in PLTs in horses (Ralston et al., 1988; Jain, 1993; Satue et al., 2008) while others researchers did not find any significant change connected with ageing in horses (McFarlane et al., 1998) and human beings (Takubo and Tatsumi, 2000).

Our results showing an increasing TSP in older horses are in contrast with several previous studies, that reported that TSP are kept within the physiological range during most of the life of a healthy animal, up to senility, where a reduction of TSP has been presented in horses, mainly due to hypoalbuminemia, because of decreased food intake, reduced digestibility and less resistance to environmental factors, such as cold weather (Kaneko, 1997; Siciliano, 2002). TSP concentrations in Carthusian broodmares achieved the highest values in Group C. Hyperproteinemia in the absence of hemoconcentration in geriatric horses is most often the result of hypergammaglobulinemia indicating chronic antigenic stimulation (Dickinson and Lori, 2002). In our study, the different protein fractions were not determined, and therefore, the influence of chronic infections in the higher TSP concentrations in the broodmares of Group C could not be confirmed.

The leukogram significantly differed between the three age groups. Our data were in agreement with those reported for warm-blooded (Schalm et al., 1975; Lassen and Swardson, 1995), Lipizzaner (Cebulj-Kadunc et al., 2003), Spanish Purebred

(Satue et al., 2008) and aged healthy horses (McFarlane et al., 2001).

The higher NL count in the older broodmares was an unexpected finding, since a common circumstance described in association with ageing is a reduction in the ability of cells to regenerate (Fermaglich and Horohov, 2002). It could be speculated that this increase represents a predisposition to subtle infections in the oldest Carthusian broodmares.

Decreasing LYMP values with age have received considerable attention in human geriatric medicine (Negoro, 1992; Globerson, 1995; Pawelec et al., 1997). These studies documented that the progressive trend to lymphopenia in aged people is a consequence of the reduction in the number of B cells and CD4+ and CD8+ T cells because of a decrease in thymus functionality and therefore, some progressive derangement of a variety of immune parameters occurs. In fact, the most prominent immunological abnormality in the elderly individual is the reduced immune response against foreign antigens (Negoro, 1992) and the same appears to be true in horses (Horohov et al., 2002; Smith et al., 2002). Despite these LYMP changes reflecting an overall immunological senescence in the old horses, McFarlane et al. (2001) did not document differences in the concentrations of immunoglobulin with ageing. These ideas are consistent with the results obtained for the Carthusian broodmares of Group C in the present research.

The significantly higher EOS count in the Carthusian broodmares of Groups B and C compared to A could be attributed to changes in the immunological response to ageing, and/or after prolonged exposure to allergens or parasites (Jain, 1993). This trend of higher circulating EOS has been presented for different breeds of horses (Harvey et al., 1984; Jain, 1993; Lassen and Swardson, 1995) and donkeys (Zinkl et al., 1990). In contrast to this, the reports of McFarlane et al. (1998), Cebulj-Kadunc et al. (2003) and Satue et al. (2008) failed to detect any significant difference in EOS counts between young and old horses. On the other hand, Cebulj-Kadunc et al. (2003) detected an increase in BAS and a decrease in MON with ageing in Lipizzaner horses, due to an age-related decline in immunity and a normal response found in adults of most species for both cell types respectively. We did not detect any significant difference between the three age groups of Carthusian broodmares in MON and BAS numbers and percentages.

CONCLUSIONS

In conclusion, it appears that age significantly influences the hematological values of Carthusian broodmares. The most prominent changes were detected in mares older than 13 years of age. Therefore, it is important to take into consideration this normal variability when hematological analysis is applied in a clinical context. The results of this investigation show that the variations associated with age in horses occur regardless of the physiological state of the mare. In this way, pregnancy did not mask the changes promoted by the age.

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