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Reference range of blood biomarkers for oxidative stress in Thoroughbred racehorses (2–5 years old)

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The oxidant and antioxidant equilibrium is known to play an important role in equine medicine and equine exercise physiology. There are abundant findings in this field; however, not many studies have been conducted for reference ranges of oxidative stress biomarkers in horses. This study was conducted to determine the reference values of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) using blood samples from 372 (191 males, 181 females) Thoroughbred racehorse aged 2 to 5 (3.43 ± 1.10 (mean ± SD)) years old. There were obvious gender differences in oxidative biomarkers, and growth/age-related changes were observed especially in females. Gender and age must be considered when interpreting obtained oxidative stress biomarkers for diagnosis of disease or fitness alterations in Thoroughbred racehorses.

Key words: *oxidative stress biomarker, reference range, Thoroughbred racehorse*

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The disequilibrium between oxidants and antioxidants is closely related to a variety of diseases in horses such as recurrent airway obstruction, chronic obstructive pulmonary disease, exercise-induced pulmonary haemorrhage, equine grass sickness, equine motor neuron disease, pituitary pars intermedia dysfunction, endometritis, osteoarthritis, white muscle disease, equine post-anaesthetic myopathy, degenerative joint disease, and *Rhodococcus equi* pneumonia [8, 9, 16, 27].

The effect of exercise on the oxidant and antioxidant equilibrium has also been studied for more than 20 years [10, 14–19, 24, 32]. Interest in the role of oxidative stress (OS) status in equine medicine and exercise physiology has increased the need for development of reliable methods to quantify the biomarkers related to OS. There are a variety of direct and indirect evaluation methods for oxidants and antioxidants. However, since Alberti *et al.* [1] validated reactive oxygen metabolites (d-ROMs) using electron spin resonance (ESR), d-ROMs is now considered as 'gold standard' biomarker for measuring total systemic oxidative status. In a similar fashion, a biological antioxidant potential (BAP) test that indicates systemic antioxidative properties including uric acid, ascorbic acid, proteins, α-tocopherol, and bilirubin has been introduced [4]. Currently, both d-ROMs and BAP are regarded as quick, simple, precise, and reliable biomarkers to assess OS status in humans and animals including horses [8, 23, 25].

This background led to recent studies in which antioxidative strategies such as oral administration of micellized natural vitamin E, intravenous infusion of H2-saline, intake of hydrogen-rich water, and ozonated autohemotherapy were examined to improve antioxidant capacity in horses [5, 26, 28, 29, 32].

However, regarding the reference ranges for OS biomarkers in horses, there have been only a few preliminary studies, which were conducted in small populations of varying age and breeds [9, 25], and further research on this field is necessary to satisfy the demands of veterinarians.

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To establish reference ranges of OS biomarkers in Thoroughbreds, d-ROMs and BAP were analyzed using blood samples from 372 Thoroughbred racehorses (191 males and 181 females) aged 2 to 5 (3.43 ± 1.10 (mean ± SD)) years old. The blood samples were collected from clinically healthy Thoroughbred racehorses at the time of quarantine when entering Miho Training Center, Japan Racing Association, in January to December 2011. All horses were transported via horse van, and their transportation times were classified into 3 categories, short (approximately 1 hr, transit distance 30 km), medium (approximately 4 hr, transit distance 350 km), and long (approximately 20 hr, transit distance 690 km) durations of transportation, according to the differences in distance from their original farms. Age and number of horses in each transportation category by sex are shown in Table 1. All horses had free access to hay and water after arriving at the quarantine stable. Sampling was conducted at 10 AM, and a rest period of at least 7 hr was provided for horses travelling from a remote location (transportation time exceeding 4 hr); horses travelling from a neighboring location (transportation time less than 1 hr) were provided a rest period of 3 hr. Before sampling, body temperature was measured, and a clinical examination was performed by a veterinarian; horses presenting a high (≥38.5°C) body temperature or suspected of having an abnormal health condition were excluded from sampling. Blood samples were collected by jugular venipuncture into sterile Vacutainers containing heparin, and plasma was obtained by centrifugation (1,500 × g for 10 min). Separated plasma was stored at −80°C until analysis.

Measurement of d-ROMs was performed using a colorimetric method of final derivatives, i.e., hydroperoxide in the serum reacts with N, N-Diethyl-p-phenylenediamine to form $[A\text{-}NH2^{-}]^{+}$ using a free radical analyzer (FREE carpediem, Wismerll, Tokyo, Japan). This d-ROMs test was invented and developed by Carratelli, M. and the validity of this method has been demonstrated by comparison with the results of the ESR method, which serves as a direct measurement of unpaired electrons [1, 30, 31]. The BAP test was simultaneously carried out using the same blood sample. BAP was determined by color reaction of thiocyanate, which reflects reduction potency from Fe^{3+} to Fe^{2+} due to electrons (e^{-}) in the blood, using the same free radical analyzer. The validity of the BAP test has been verified by comparing hydroxyl radical scavenging activity with ESR spectrometry, and various stabilities of the assay have also been assessed [11, 13]. The oxidative stress index (OSI), which gives the relationship between the level of OS and pathology [8, 25], was calculated from the measured d-ROMs and BAP (calculation formula: d-ROMs/BAP × 100).

The unpaired *t*-test was used to compare the variables between males and females, and one-way ANOVA was used to compare the variables between ages. The χ^2 test was also applied to test the differences in proportions of horses in the transportation categories. For all oxidative stress markers, a two-way ANOVA was performed to test the significance of interaction of age and transportation time and main effects of age and transportation time. All statistical analyses were performed using the JMP v.11.0.0 software (SAS Institute, Cary, NC, U.S.A.).

There was no significant difference in age by gender (male 47.4 ± 12.0, female 47.0 ± 12.1 months old) or in the proportions of horses in the transportation categories (Table 1). Differences in d-ROMs, BAP, and OSI for males and females are shown as mean ± SD values in Table 2. Although the d-ROMs level in females (161.0 ± 28.5 U.CARR) was significantly higher than that in males (148.2 ± 23.5 U.CARR), BAP (2,520 ± 228 μmol/*l*) was significantly lower compared with that in males (2,658 ± 184 μmol/*l*). The OSI was significantly lower in females (16.1 ± 2.9) compared with males (18.3 ± 3.0). Po *et al*. [25] reported data for OS biomarkers (d-ROMs, 286 ± 24 U.CARR; BAP, 3,403 ± 124 μmol/*l*; OSI, 8.4 ± 0.5) from 10 healthy Thoroughbreds foals and discussed that there were no differences between genders. Celi *et al*. [8] reported data for OS biomarkers (d-ROMs, 358 ± 37 U.CARR; BAP, 2,533 ± 66 μmol/*l* from 15 horses (10 geldings, 5 mares) but they did not discuss gender differences.

The gender differences in oxidative stress status observed in our study have also been reported in humans, bovine tissue, and Ukrainian Warmblood sport horses [2, 6, 12]. It is suggested that differences between sexes in antioxidant capabilities might be associated with regulation of the antioxidative enzymes by sex steroid hormones [22] and related to differences in antioxidant defense of XX and XY cells [18]. An obvious reason for gender disparity in OS status was not revealed in this study. However, the difference in emotional character, with female horses having a more pessimistic nature than male horses [20], might have an influence on the differences in production and deactivation of reactive oxygen metabolites.

Reference values for d-ROMs, BAP, and OSI according to age and sex are described in Table 3. The reference range for a particular measurement is generally defined as the prediction interval between which 95% of values of a reference group fall into and is used for various physiological measurements in humans and animals. Thus, the reference ranges for parameters with a normal distribution are often indicated as mean ± 2SD values [3, 21, 23]. Since the OSI in our study did not show a normal distribution, reference range values for all parameters are indicated as both mean and median values. In females, the d-ROMs for 2 years old were significantly higher compared those for 4 and 5 years

Table 1. Age and numbers of Thoroughbred racehorses in the transportation categories

	Male (n=191)	Female (n=181)
Months of age	47.4 ± 12.0	47.0 ± 12.1
Category of transportation		
Short	97 (50.8%)	96 (53.0%)
Medium	55 (28.8%)	44 (24.3%)
Long	39 (20.4%)	41 (22.7%)

Mean ± SD. Short, approx. 1 hr; medium, approx. 4 hr; Long, approx. 12 hr.

Table 2. Differences in d-ROMs, BAP and OSI for the male and female Thoroughbred racehorses

	Male (n=191)	Female (n=181)	*P*
d-ROMs (U.CARR)	148.2 ± 23.5	161.0 ± 28.5	<0.001
BAP (*μ*mol/*l*)	2,658 ± 184	2,520 ± 228	<0.001
OSI (arbitary units)	18.3 ± 3.0	16.1 ± 2.9	<0.001

Mean ± SD.

Table 3. Reference values for d-ROMs, BAP and OSI according to age and sex in Thoroughbred racehorses

	Age (years)	2	3	4	5
Male (n=191)		*48*	*50*	*47*	*46*
Months of age	Mean ± SD	31.5 ± 1.2	42.9 ± 2.1	53.3 ± 2.4	62.8 ± 5.0
d-ROMs (U.CARR)	Mean (± 2SD)	152.3 (98.3–206.3)	144.9 (101.5–188.3)	143.7 (100.6–186.3)	152.6 (107.5–197.6)
	Median (10–90 pc)	147.5 (120.8–195.3)	146.0 (114.6–168.9)	145.0 (113.0–169.6)	152.5 (121.4–184.6)
BAP (mmol/*l*)	Mean (± 2SD)	2,691 (2,246−3,136)	2,669 (2,340−2,998)	2,616 (2,262−2,971)	2,653 (2,330−2,976)
	Median (10–90 pc)	2,680 (2,440−2,972)	2,649 (2,461−2,931)	2,601 (2,387−2,899)	2,654 (2,485−2,832)
OSI (arbitrary units)	Mean (± 2SD)	18.1 (11.9–24.3)	18.8 (12.8–24.8)	18.6 (12.7–24.6)	17.8 (12.0–23.5)
	Median (10–90 pc)	18.3 (14.4–21.6)	18.1 (15.5–23.5)	17.8 (15.6–23.8)	17.3 (14.3–22.3)
Female (n=181)		*50*	*49*	*46*	*36*
Months of age	Mean (± SD)	31.7 ± 1.2	43.1 ± 2.0	54.7 ± 2.3	63.6 ± 5.0
d-ROMs (U.CARR)	Mean (± 2SD)	171.7 (116.9–226.5)	167.9 (107.9–227.9)	145.5 (102.2–188.7)[a]	156.4 (102.6–210.2)[b]
	Median (10–90 pc)	173.5 (127.4–207.8)	164.0 (132.0–209.0)	146.5 (119.1–176.3)	150.5 (124.7–197.2)
BAP (mmol/*l*)	Mean (± 2SD)	2,630 (2,329–2,932)	2,630 (2,233–3,027)	2,483 (2,176–2,791)[c]	2,264 (1,832–2,695)[d]
	Median (10–90 pc)	2,646 (2,383–2,816)	2,636 (2,328–2,882)	2,518 (2,221–2,655)	2,224 (1,987–2,560)
OSI (arbitrary units)	Mean (± 2SD)	15.7 (10.2–21.2)	16.1 (10.5–21.7)	17.4 (11.7–23.2)[e]	14.8 (9.9–19.7)[f]
	Median (10–90 pc)	15.8 (12.8–20.0)	15.8 (12.8–20.0)	17.0 (14.2–21.3)	14.6 (11.3–19.0)

SD, standard deviation; 10 pc, the upper limit of the 10th percentile; 90 pc, the lower limit of the 90th percentile. [a]$P<0.05$ vs 2 and 3 years old. [b]$P<0.05$ vs 2 years old. [c]$P<0.05$ vs 2, 3 and 5 years old. [d]$P<0.05$ vs 2, 3 and 4 years old. [e]$P<0.05$ vs 2 and 5 years old. [f]$P<0.05$ vs 4 years old.

old, and the value for 3 years old was significantly higher compared with that for 4 years old. For BAP, the values for 4 and 5 years old, were significantly lower compared with those for other ages. The OSI for 4 years old was significantly higher compared with those for 2 and 5 years old. Furthermore, that for 5 years old was significantly lower compared with that for 4 years old. The OS biomarkers in males did not differ among ages. A significant decrease was observed in BAP with increasing aging in females.

It should be noted that the transportation time prior to blood sampling might affect the level of BAP. Indeed, our two-way ANOVA revealed a slight tendency for the BAP values to rises as the transportation time increased. To date, the effect of age on the antioxidant defense status has only been investigated in humans [7], and bulls [12]. Since the reason for the growth/age-related phenomenon has not yet been elucidated, additional study is required to clarify the defense mechanism against oxidative stress associated with growth/aging.

This study established reference values for common OS biomarkers, d-ROMs, BAP, and OSI, from blood samples of 372 Thoroughbred racehorses aged 2 to 5 years old. There were gender differences in oxidative biomarkers, and growth/age-related changes were observed especially in females. Gender and age should be considered when interpreting obtained OS biomarkers for diagnosis of disease or fitness alterations.

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