

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

Kanichi KUSANO<sup>1\*</sup>, Masahiko YAMAZAKI<sup>2</sup>, Masataka KIUCHI<sup>3</sup>, Kouki KANEKO<sup>4</sup> and Katsuhiko KOYAMA<sup>5</sup>

<sup>1</sup>Miho Training Center, Racehorse Hospital, Japan Racing Association, Ibaraki 300-0493, Japan

<sup>2</sup>Yamazaki Horse Clinic, Ibaraki 300-0415, Japan

<sup>3</sup>Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi 400-8510, Japan

<sup>4</sup>Wismerll Company Limited, Tokyo 113-0033, Japan

<sup>5</sup>Graduate School Department of Interdisciplinary Research, University of Yamanashi, Yamanashi 400-8510, Japan

---

*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 ( $3.43 \pm 1.10$  (mean  $\pm$  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

---

**J. Equine Sci.**  
**Vol. 27, No. 3**  
**pp. 125–129, 2016**

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,  $\alpha$ -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 H<sub>2</sub>-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.

---

Received: March 22, 2016

Accepted: June 10, 2016

\*Corresponding author. e-mail: Kanichi\_Kusano@jra.go.jp

©2016 Japanese Society of Equine Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/4.0/>>.

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 ( $3.43 \pm 1.10$  (mean  $\pm$  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 ( $\geq 38.5^\circ\text{C}$ ) 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 ( $1,500 \times g$  for 10 min). Separated plasma was stored at  $-80^\circ\text{C}$  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  $[\text{A-NH}_2^-]^+$  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  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  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:  $\text{d-ROMs/BAP} \times 100$ ).

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  $\chi^2$  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  $47.4 \pm 12.0$ , female  $47.0 \pm 12.1$  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  $\pm$  SD values in Table 2. Although the d-ROMs level in females ( $161.0 \pm 28.5$  U.CARR) was significantly higher than that in males ( $148.2 \pm 23.5$  U.CARR), BAP ( $2,520 \pm 228 \mu\text{mol/l}$ ) was significantly lower compared with that in males ( $2,658 \pm 184 \mu\text{mol/l}$ ). The OSI was significantly lower in females ( $16.1 \pm 2.9$ ) compared with males ( $18.3 \pm 3.0$ ). Po *et al.* [25] reported data for OS biomarkers (d-ROMs,  $286 \pm 24$  U.CARR; BAP,  $3,403 \pm 124 \mu\text{mol/l}$ ; OSI,  $8.4 \pm 0.5$ ) 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,  $358 \pm 37$  U.CARR; BAP,  $2,533 \pm 66 \mu\text{mol/l}$  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  $\pm$  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 (μmol/l)	2,658 ± 184	2,520 ± 228	<0.001
OSI (arbitrary 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) <sup>a</sup>	156.4 (102.6–210.2) <sup>b</sup>
	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) <sup>c</sup>	2,264 (1,832–2,695) <sup>d</sup>
	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) <sup>e</sup>	14.8 (9.9–19.7) <sup>f</sup>
	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. <sup>a</sup>*P*<0.05 vs 2 and 3 years old. <sup>b</sup>*P*<0.05 vs 2 years old. <sup>c</sup>*P*<0.05 vs 2, 3 and 5 years old. <sup>d</sup>*P*<0.05 vs 2, 3 and 4 years old. <sup>e</sup>*P*<0.05 vs 2 and 5 years old. <sup>f</sup>*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.

## References

1. Alberti, A., Bolognini, L., Macciantelli, D., and Caratelli, M. 2000. The radical cation of N, N-diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. *Res. Chem. Intermed.* **26**: 253–267. [CrossRef]
2. Andriichuk, A., Tkachenko, H., and Kurhaluk, N. 2014. Gender difference of oxidative stress biomarkers and erythrocyte damage in well-trained horses during exercise. *J. Equine Vet. Sci.* **34**: 978–985. [CrossRef]
3. Bangert, S.K., Marshall, W.J., and Leonard, M.W. 2008. p. 19. *In: Clinical Biochemistry: Metabolic and Clinical*

- Aspects, Churchill Livingstone/Elsevier, Philadelphia.
4. Benzie, I.F.F., and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal. Biochem.* **239**: 70–76. [[Medline](#)] [[CrossRef](#)]
  5. Bergo, D., Miraglia, N., Schiavone, A., Polidori, M., and Prola, L. 2004. Effect on dietary polyunsaturated fatty acids and vitamin E on serum oxidative status in horses performing very light exercise. *Ital. J. Anim. Sci.* **3**: 141–145.
  6. Brunelli, E., Domanico, F., La Russa, D., and Pellegrino, D. 2014. Sex differences in oxidative stress biomarkers. *Curr. Drug Targets* **15**: 811–815. [[Medline](#)] [[CrossRef](#)]
  7. Ceballos-Picot, I., Trivier, J.M., Nicole, A., Sinet, P.M., and Thevenin, M. 1992. Age-correlated modifications of copper-zinc superoxide dismutase and glutathione-related enzyme activities in human erythrocytes. *Clin. Chem.* **38**: 66–70. [[Medline](#)]
  8. Celi, P., Sullivan, M., and Evans, D. 2010. The stability of the reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests on stored horse blood. *Vet. J.* **183**: 217–218. [[Medline](#)] [[CrossRef](#)]
  9. Crowley, J., Po, E., Celi, P., and Muscatello, G. 2013. Systemic and respiratory oxidative stress in the pathogenesis and diagnosis of *Rhodococcus equi* pneumonia. *Equine Vet. J. Suppl.* **45**: 20–25. [[Medline](#)] [[CrossRef](#)]
  10. de Moffarts, B., Kirschvink, N., Art, T., Pincemail, J., and Lekeux, P. 2005. Assessment of the oxidant-antioxidant blood balance in a field exercise test in standardbred and event horses. *Equine Comp. Exerc. Physiol.* **2**: 253–261. [[CrossRef](#)]
  11. Dohi, K., Satoh, K., Ohtaki, H., Shioda, S., Miyake, Y., Shindo, M., and Aruga, T. 2005. Elevated plasma levels of bilirubin in patients with neurotrauma reflect its pathophysiological role in free radical scavenging. *In Vivo* **19**: 855–860. [[Medline](#)]
  12. Giergiel, M., and Kankofer, M. 2015. Age and sex-related changes in superoxide dismutase activity in bovine tissues. *Czech J. Anim. Sci.* **60**: 367–374. [[CrossRef](#)]
  13. Jansen, E.H., Beekhof, P.K., Cremers, J.W., Viezeliene, D., Muzakova, V., and Skalicky, J. 2013. Long-term stability of parameters of antioxidant status in human serum. *Free Radic. Res.* **47**: 535–540. [[Medline](#)] [[CrossRef](#)]
  14. Kinnunen, S., Atalay, M., Hyyppä, S., Lehmuskero, A., Hänninen, O., and Oksala, N. 2005. Effects of prolonged exercise on oxidative stress and antioxidant defense in endurance horse. *J. Sports Sci. Med.* **4**: 415–421. [[Medline](#)]
  15. Kinnunen, S., Hyyppä, S., Lehmuskero, A., Oksala, N., Mäenpää, P., Hänninen, O., and Atalay, M. 2005. Oxygen radical absorbance capacity (ORAC) and exercise-induced oxidative stress in trotters. *Eur. J. Appl. Physiol.* **95**: 550–556. [[Medline](#)] [[CrossRef](#)]
  16. Kirschvink, N., de Moffarts, B., and Lekeux, P. 2008. The oxidant/antioxidant equilibrium in horses. *Vet. J.* **177**: 178–191. [[Medline](#)] [[CrossRef](#)]
  17. Koyama, K. 2014. Exercise-induced oxidative stress: A tool for “hormesis” and “adaptive response”. *J. Phys. Fitness Sports Med.* **3**: 115–120. [[CrossRef](#)]
  18. Marino, M., Masella, R., Bulzomi, P., Campesi, I., Malorni, W., and Franconi, F. 2011. Nutrition and human health from a sex-gender perspective. *Mol. Aspects Med.* **32**: 1–70. [[Medline](#)] [[CrossRef](#)]
  19. Mills, P.C., Ng, J.C., Thornton, J., Seawright, A.A., and Auer, D.E. 1994. Exercise-induced connective tissue turnover and lipid peroxidation in horses. *Br. Vet. J.* **150**: 53–63. [[Medline](#)] [[CrossRef](#)]
  20. Momozawa, Y., Terada, M., Sato, F., Kikusui, T., Takeuchi, Y., Kusunose, R., and Mori, Y. 2007. Assessing equine anxiety-related parameters using an isolation test in combination with a questionnaire survey. *J. Vet. Med. Sci.* **69**: 945–950. [[Medline](#)] [[CrossRef](#)]
  21. Murray, W., Peter, T.A., and Teclaw, R.F. 1993. The clinical relevance of assay validation. *Comp. Cont. Educ. Pract. Vet.* **15**: 1665–1675.
  22. Pajović, S.B., and Saicić, Z.S. 2008. Modulation of antioxidant enzyme activities by sexual steroid hormones. *Physiol. Res.* **57**: 801–811. [[Medline](#)]
  23. Pasquini, A., Luchetti, E., Marchetti, V., Cardini, G., and Iorio, E.L. 2008. Analytical performances of d-ROMs test and BAP test in canine plasma. Definition of the normal range in healthy Labrador dogs. *Vet. Res. Commun.* **32**: 137–143. [[Medline](#)] [[CrossRef](#)]
  24. Pazzola, M., Pira, E., Sedda, G., Vacca, G.M., Cocco, R., Sechi, S., Bonelli, P., and Nicolussi, P. 2015. Responses of hematological parameters, beta-endorphin, cortisol, reactive oxygen metabolites, and biological antioxidant potential in horses participating in a traditional tournament. *J. Anim. Sci.* **93**: 1573–1580. [[Medline](#)] [[CrossRef](#)]
  25. Po, E., Williams, C., Muscatello, G., and Celi, P. 2013. Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of Thoroughbred foals. *Vet. J.* **196**: 269–271. [[Medline](#)] [[CrossRef](#)]
  26. Rey, A.I., Segura, J., Arandilla, E., and López-Bote, C.J. 2013. Short- and long-term effect of oral administration of micellized natural vitamin E (D- $\alpha$ -tocopherol) on oxidative status in race horses under intense training. *J. Anim. Sci.* **91**: 1277–1284. [[Medline](#)] [[CrossRef](#)]
  27. Soffler, C. 2007. Oxidative stress. *Vet. Clin. North Am. Equine Pract.* **23**: 135–157. [[Medline](#)] [[CrossRef](#)]
  28. Tsubone, H., Hanafusa, M., Endo, M., Manabe, N., Hiraga, A., Ohmura, H., and Aida, H. 2013. Effect of treadmill exercise and hydrogen-rich water intake on serum oxidative and anti-oxidative metabolites in serum of Thoroughbred horses. *J. Equine Sci.* **24**: 1–8. [[Medline](#)] [[CrossRef](#)]
  29. Tsuzuki, N., Endo, Y., Kikkawa, L., Korosue, K., Kaneko, Y., Kitauchi, A., Katamoto, H., Hidaka, Y., Hagio, M., and Torisu, S. 2016. Effects of ozonated autohemotherapy on the antioxidant capacity of Thoroughbred horses. *J. Vet. Med. Sci.* **77**: 1647–1650. [[Medline](#)] [[CrossRef](#)]

30. Vassalle, C., Boni, C., Di Cecco, P., Ndreu, R., and Zucchelli, G.C. 2006. Automation and validation of a fast method for the assessment of in vivo oxidative stress levels. *Clin. Chem. Lab. Med.* **44**: 1372–1375. [[Medline](#)] [[CrossRef](#)]
31. Verde, V., Fogliano, V., Ritieni, A., Maiani, G., Morisco, F., and Caporaso, N. 2002. Use of N,N-dimethyl-p-phenylenediamine to evaluate the oxidative status of human plasma. *Free Radic. Res.* **36**: 869–873. [[Medline](#)] [[CrossRef](#)]
32. Yamazaki, M., Kusano, K., Ishibashi, T., Kiuchi, M., and Koyama, K. 2015. Intravenous infusion of H<sub>2</sub>-saline suppresses oxidative stress and elevates antioxidant potential in Thoroughbred horses after racing exercise. *Sci. Rep.* **5**: 15514. [[Medline](#)] [[CrossRef](#)]