

Effect of Treadmill Exercise and Hydrogen-rich Water Intake on Serum Oxidative and Anti-oxidative Metabolites in Serum of Thoroughbred Horses

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The present study aimed to clarify changes of oxidative stress and antioxidative functions in treadmill-exercised Thoroughbred horses (n=5, 3 to 7 years old), using recently developed techniques for measurement of serum d-ROMs for oxidative stress, and BAP for antioxidative markers. Also, the effect of nasogastric administration of hydrogen-rich water (HW) or placebo water preceding the treadmill exercise on these parameters was examined. Each horse was subjected to a maximum level of treadmill exercise in which the horses were exhausted at an average speed of 13.2 ± 0.84 m/sec. Blood samples were taken 4 times, immediately before the intake of HW or placebo water at 30 min preceding the treadmill exercise, immediately before the exercise (pre-exercise), immediately after the exercise (post-exercise) and at 30 min following the exercise. In all horses, both d-ROMs and BAP values significantly increased at post-exercise. The increase in d-ROMs tended to be lower in the HW trial, as compared to the placebo trial at pre-exercise. The increase in BAP was considerable at approximately 150 % of the pre-exercise values in both the HW and placebo treatment trials. The BAP/d-ROMs ratio was significantly elevated at post-exercise in both treatment trials, while a significant elevation was also observed at pre-exercise in the HW trial. BAP, d-ROM, and the BAP/d-ROM ratio tended to decline at 30 min after the exercise, except BAP and BAP/d-ROMs in the placebo trial. These results demonstrate that the marked elevation of oxidative stress and antioxidative functions occurred simultaneously in the intensively exercised horses, and suggest a possibility that HW has some antioxidative efficacy.

Key words: BAP, d-ROMs, exercise, hydrogen-water, Thoroughbred

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Exercise training is known to produce oxidative stress, which is possibly derived from mitochondria functions and the other cellular metabolisms [3, 6, 9, 14, 20, 24, 31–34, 38], through the increase of reactive oxygen species (ROS) with free radicals in extracellular space in skeletal muscles, blood and the other organs. The excessive increment of

free radicals is considered to induce tissue damage such as inflammation, necrosis and apoptosis, and may delay the recovery from fatigue of skeletal muscle [3]. Recently, a new technique for indirect measurement of ROS has been developed in clinical medicine, and it is a more convenient technique than the method utilizing electron spin resonance (ESR) that serves as a direct measurement of free radicals. To examine ROS in humans, instead of ESR, Diacron-Reactive Oxygen Metabolites (d-ROMs) are used as an indicator of the total amount of free radicals in ROS and

non-ROS free radicals in the blood and cerebrospinal fluid [8, 10, 15, 17, 19, 21, 23, 37, 42]. Although many reports regarding the correlation between d-ROMs changes and diseases or physical stress in humans have been published, there have been few reports about d-ROMs in experimental animals [22] or domestic animals [13]. Fazio, F. *et al.* [13] reported a significant linear regression between d-ROMs and homocysteine values in Thoroughbred horses after a 2,100-meter race. The possibility of increment of anti-oxidative capacity by exercise training and the existence of daily rhythms of anti-oxidative parameters has been suggested in horses [30].

It is known that the antioxidative system functions to protect the living body from oxidative stress. Recent advances have also provided a fast and convenient method for measuring the antioxidative potency in the blood by analysis of its reduction ability. The BAP (Biological Antioxidant Potential) test, which has been recently developed in the clinical and sports science fields [16, 23, 28], reflects the reduction ability, the amount of electron (e^-) supply, of the blood.

We conducted preliminary experiments using three non-Thoroughbred horses at the Animal Resource Science Center of the University of Tokyo, to measure serum d-ROMs, BAP and electrocardiograms of the horses before and after being exercised by a veteran rider with 5-min trotting repeated twice at 5- or 10-min intervals. We obtained valuable data indicating clear increases in serum d-ROMs and BAP after the exercise, and conducted that it would be necessary to demonstrate the oxidative and antioxidative changes in Thoroughbred horses under the controlled condition of physical exercise on a treadmill, because such horses have received regular daily trainings as athletes and the intensity of the exercise can be experimentally controlled.

The evaluation of effects of dietary supplements on human athletes and animals has been carried out through the examination of d-ROMs and BAP in the blood. The effects of vitamin E supplementation on serum d-ROMs were examined in periparturient heifers, and showed that decrease in d-ROMs was not observed after the supplementation [11]. A significant decrease in d-ROMs was recognized throughout the post-surgery period in patients with esophageal cancer who were given an immuno-enhanced diet for 5 days before surgery [1]. Recently, Aoki, K. *et al.* [4] reported there were no significant changes in d-ROMs and BAP values of two groups of young soccer players, one orally given placebo water and the other hydrogen-rich water (HW), after exercise at a load of 75% of VO_2 max, even though the oral intake of HW prevented the elevation of the blood lactate level. Only a few references are available on changes in d-ROMs and BAP induced

by physical exercise. Therefore, it is of interest to clarify, using recently developed techniques, how the oxidative stress and antioxidative capacity are changed, and whether HW affects these oxidative parameters, in Thoroughbred horses which are subjected to a high level of exercise.

The objectives of this study were to demonstrate the exercise-induced alteration of serum oxidative and antioxidative activities through measurements of d-ROMs and BAP, and the effects of HW intake on these parameters in Thoroughbred horses under strictly controlled conditions of treadmill exercise.

Materials and Methods

Horses

Five Thoroughbred horses (two females and three geldings), aged 4 to 7 years old with body weight of 493.8 ± 26.6 (mean \pm SD) kg, belonging to The Equine Research Institute, Japan Racing Association, were assigned to the experiment. These horses had been routinely grazed for 2 months and specially trained for treadmill exercise prior to this study.

Supplementation of hydrogen water

Hydrogen-rich water (HW) was made by special equipment (High Density Hydrogen Water, Melodian Co., Tokyo, Japan) for the present study. The HW consisted of filtrated water (pH=6.82) with a concentration of molecular hydrogen (H_2) higher than 1 ppm. Fresh HW that was made about 3 hr before the treadmill exercise was stored in an aluminum bag (10 l in volume) and tightly sealed in order to prevent hydrogen gas escape. Two liters of HW were administered into the esophagus via a nasogastric catheter 30 min before the treadmill exercise. For the placebo trial, the same volume of normal water without hydrogen was administered by the same method.

Experimental protocol

The overall protocol of this study is illustrated in Fig. 1. Treadmill exercise on a 6 % incline was performed by each horse. Each horse was exercised on the treadmill with stepwise increases of exercise intensity every 2 min, at treadmill running speed of 1.6, 3.6, 7, 10, 12, 13 and 14 m/sec, until the horses became thoroughly exhausted. By thoroughly exhausted, we mean that the horses were too exhausted to maintain their position at the front of the treadmill with humane encouragement. The average exhaustion speed was 13.2 ± 0.84 m/sec.

The first treadmill exercise was performed after the placebo administration, and the second treadmill exercise was performed after the HW administration, after one week interval. In order to measure serum d-ROMs and

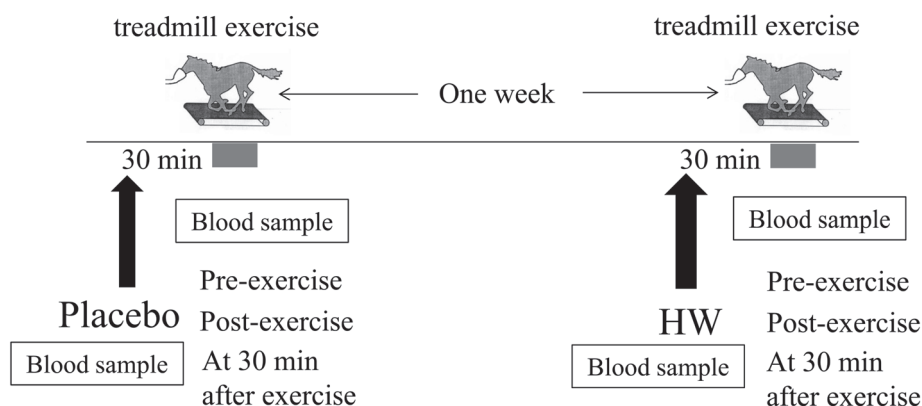


Fig. 1. An illustration of the overall experimental procedure of the present study. Horses were exercised with increment of load every 2 min at speeds of 1.6, 3.6, 7, 10, 12 and 13 m/s until they were exhausted. The first treadmill exercise was performed after placebo administration and the second treadmill exercise was performed after hydrogen-rich water (HW) administration, after one-week interval. Two liters of placebo water or HW were administered to the esophagus by a nasogastric catheter in each horse at 30 min before the treadmill exercise. Blood samples (serum) were collected 4 times: 30 min before the exercise, immediately before the exercise, post-exercise, and at 30 min after the exercise.

BAP, venous blood samples of 10 ml were collected. The blood samples were collected from the jugular vein immediately before the administration of placebo water (distilled water) or HW, 30 min before the treadmill exercise. Blood samples were also collected at pre-exercise, immediately before the onset of treadmill exercise, at post-exercise, immediately after treadmill exercise, and at 30 min after the end of treadmill exercise. In addition to the samples, blood was collected at 10:00 and 13:00 on a day without treadmill exercise from all five horses to determine their background levels of d-ROMs and BAP.

Measurements of d-ROMs and BAP

The measurement of d-ROMs was performed using a colorimetric method of final derivatives, i.e., hydroperoxide produced by free radicals, in which hydroperoxide in the serum reacts with N, N-Diethyl-p-phenylenediamine to form $[A-NH_2^-]^+$ using a free radical analyzer (FREE carpe diem, WISMERLL, Tokyo, Japan). This d-ROMs test was invented and developed by Carratelli, M. and the validity of this method has been demonstrated by comparisons with the results of the electron spin resonance (ESR) method, which serves as a direct measurement of unpaired electrons [2, 39, 40]. 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.

Statistical analysis

The d-ROMs, BAP and BAP/d-ROMs values were statistically evaluated using two-way repeated-measures analysis of variance (two-way repeated-measures ANOVA). In addition, the statistical significance of differences was tested by two-factor ANOVA with only one observation in each cell at pre-exercise, post-exercise and 30 min after exercise for each of the placebo and HW groups, and the Dunnett test was used to compare post-exercise and 30 min after exercise data with that at pre-exercise. Wilcoxon's signed-rank test was also used to examine the data of the blood samples at 30 min before treadmill exercise, before the placebo or HW administration, in order to examine if the basal conditions were the same. For all the data, differences were considered significant if the P value was less than 0.05.

Results

On the days of placebo and HW administration, the mean values of d-ROMs immediately before administration of placebo or HW at 30 min preceding the treadmill exercise were 148.3 ± 15.3 (placebo) and 152.8 ± 9.4 (HW), respectively. Likewise, the mean values of BAP at 30 min before treadmill exercise were $2,555.9 \pm 92.5$ (placebo) and $2,774.1 \pm 32.7$ (HW), respectively. There were no significant d-ROMs or BAP between the placebo and HW treatment trials.

d-ROMs values of each horse at pre-exercise, post-

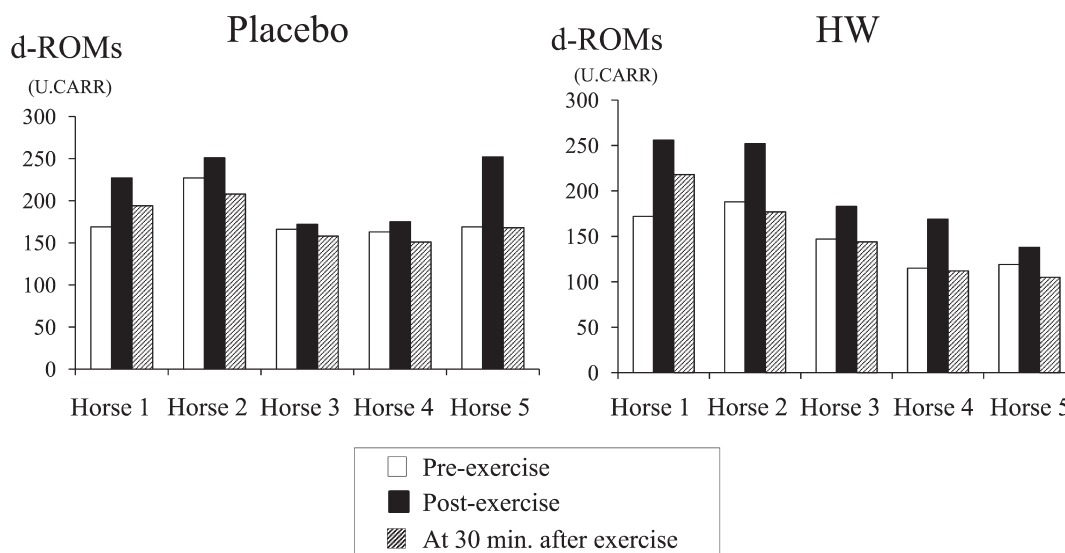


Fig. 2. d-ROMs values at pre-exercise, post-exercise and 30 min after exercise of each horse after administration of placebo and HW.

exercise and 30 min after the treadmill exercise are shown in Fig. 2. In all horses, d-ROMs tended to elevate at post-exercise, i.e., immediately after the treadmill exercise. The average changes in d-ROMs induced by the treadmill exercise in the placebo and HW trials are shown in Fig. 3. A significant difference in d-ROMs was found among the three measurement times of pre-exercise, post-exercise and 30 min after exercise in the placebo ($P < 0.005$) and HW ($P < 0.005$) trials. The d-ROMs value significantly increased at post-exercise, compared to pre-exercise, in the placebo ($P < 0.005$) and HW ($P < 0.001$) trials. No significant changes in d-ROMs were observed at 30 min after exercise in both the placebo and HW trials. The d-ROMs value at pre-exercise was lower for the HW trial (148 ± 14.3 U.CARR; mean \pm SE) than for the placebo trial (179 ± 12.1 U.CARR; mean \pm SE), but the difference was not significant.

The BAP values of each horse at pre-exercise, post-exercise and 30 min after the treadmill exercise are shown in Fig. 4. The BAP value increased in all horses at post-exercise. The average change in BAP values of the placebo and HW trials are shown in Fig. 5. There were significant differences among the three measurement times in the placebo ($P < 0.0001$) and HW ($P < 0.0001$) trials; the BAP values at post-exercise increased to 150% and 153% of the pre-exercise value in the placebo and HW trials, respectively. Significant differences from pre-exercise were found at post-exercise ($P < 0.0001$) and 30 min after exercise ($P < 0.005$) in the placebo trial, whereas a significant difference was present only at post-exercise ($P < 0.0001$) in the

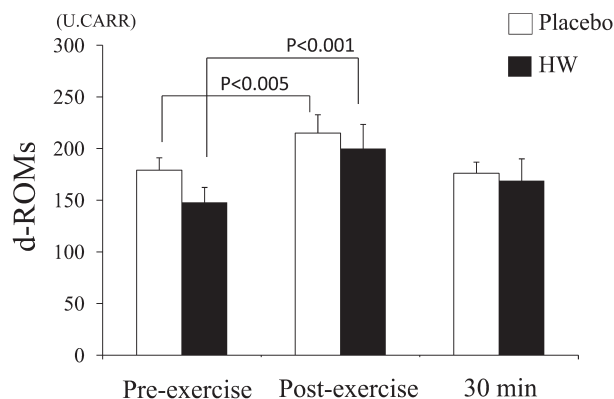


Fig. 3. Average changes of d-ROMs of all horses after administration of placebo and HW.

“30 min”: at 30 min after the treadmill exercise.

Data are shown as means \pm SE.

HW trial.

The value of the BAP/d-ROMs ratio is shown in Fig. 6. Significant differences from pre-exercise were found in the placebo ($P < 0.005$) and HW ($P < 0.05$) trials; the differences were greatest at post-exercise in the placebo ($P < 0.001$) and HW ($P < 0.01$) trials, and they were also significant at 30 min after the exercise in the placebo ($P < 0.001$) and HW ($P < 0.05$) trials. As for comparisons between the placebo and HW trials on d-ROMs, BAP and BAP/d-ROMs ratio, a significant difference ($P = 0.04$) was observed only for BAP/d-ROMs (two-way repeated-measures ANOVA) without interaction between substance (placebo or HW)

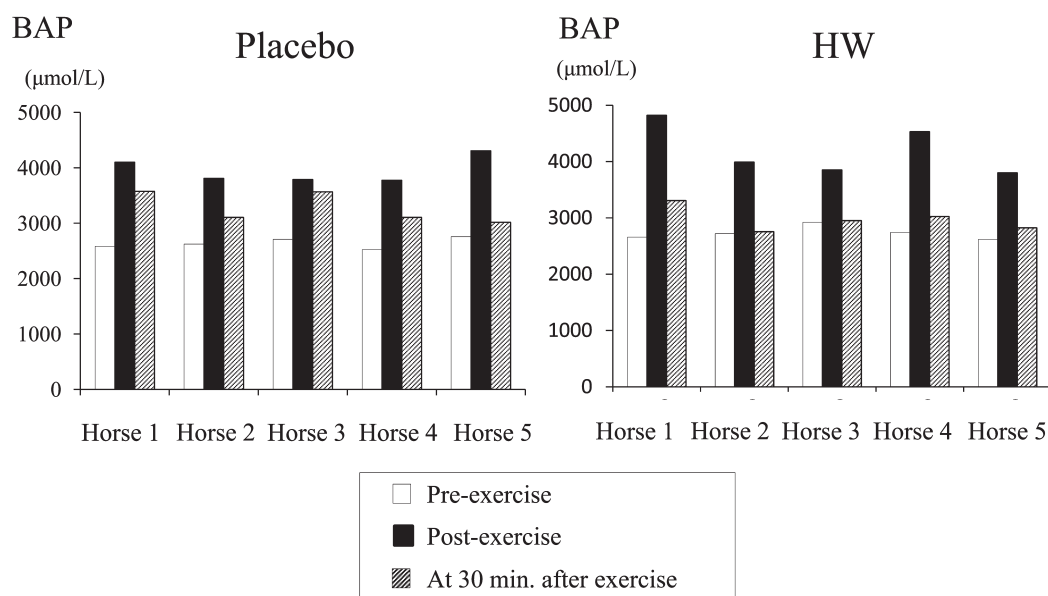


Fig. 4. BAP values at pre-exercise, post-exercise and 30 min after exercise of each horse after administration of placebo and HW.

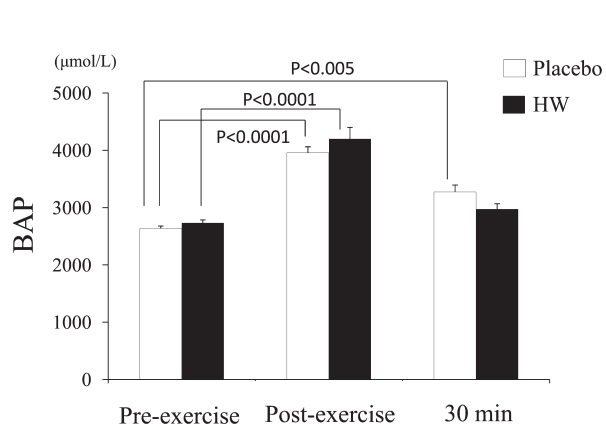


Fig. 5. Average changes of BAP of all horses after administration of placebo and HW. “30 min”: at 30 min after the treadmill exercise. Data are shown as means ± SE.

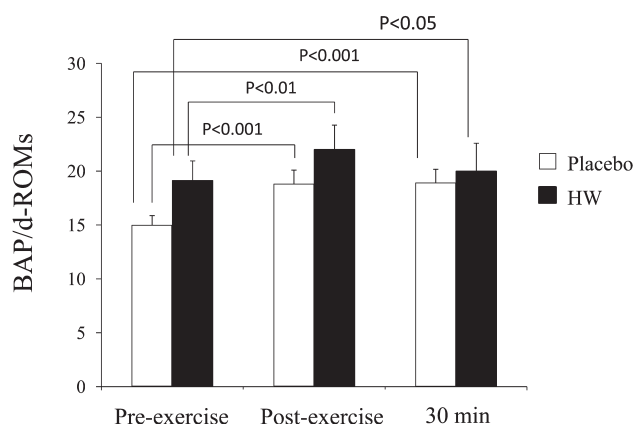


Fig. 6. Average changes of BAP/d-ROMs of all horses after administration of placebo and HW. “30 min”: at 30 min after the treadmill exercise. Data are shown as means ± SE.

and measurement time points ($P=0.87$).

Discussion

The excessive production of intramuscular or extramuscular ROS induced by intensive exercise such as supra-maximum exercise might play an important role in enhancing inflammation of the muscle. However, concurrent action of protective factors, represented by various antioxidative substances such as super oxide dismutase

(SOD), catalase, peroxidase, glutathione (GSH), homocysteine, ascorbic acid (vitamin C), α -tocopherol (vitamin E) and some minerals, might be enhanced or recruited against the oxidative stress. Therefore, the balance of oxidative and antioxidative functions in tissues and blood are thought to be more important than the production of oxidative substance alone. In the present study, significant increases in d-ROMs and BAP were induced by the intensive treadmill exercise (Figs. 3 and 5), and were accompanied by elevation of the BAP/d-ROMs ratio (Fig. 6).

There are several reports on the association of exercise with oxidative stress in equine. It has been suggested that biochemical parameters such as lipid hydroperoxides, which are indicative of oxidative stress, are changed by exercise, and that the changes are exacerbated during exercise at high temperature and humidity [25]. Also, the exercise-induced increase in plasma lipid peroxide was reduced by allopurinol-induced inhibition of xanthine oxidase, which resulted in the formation of ROS during exercise [26]. Changes in blood contents of malondialdehyde (MDA) and GSH were evident in race horses (ten 3 year-old stallions) subjected to physical exercise with a progressive strength of training [7], where the plasma MDA and GSH significantly increased after the ride and the increase in MDA was still present at 18 hr after the exercise.

The present study demonstrated that the oxidative parameter (d-ROMs) and antioxidative parameter (BAP) in the blood were clearly and simultaneously elevated by intensive treadmill exercise of Thoroughbred horses. The finding of increase in d-ROMs may be supportive of the finding of increased lipid hydroperoxidase in horses after exercise [25, 26]. Moreover, in the present study the marked increase in BAP observed at post-exercise and rapid return to the pre-exercise level at 30 min after the exercise is worthy of note. This evidence implies that antagonistic action to strong oxidative stress is designed to onset in rapid time-course and BAP recovery to the pre-exercise level occurs within a short period after the exercise. This antioxidative function may be reinforced by exercise training of Thoroughbred horses because the antioxidative capacity has been suggested to be improved by exercise training [18].

Some evidence of the efficacy of dietary supplementation with antioxidants has previously been presented. The antioxidative influence of vitamin E and selenium supplementation in 3-year-old stallions has been reported [5]. The antioxidant capacity, total antioxidant activities and thiobarbiturate reactive substances in Thoroughbred race horses were elevated after a race, and the intravenous administration of ascorbate reduced the oxidative stress, although the creatine kinase activities were not influenced by the administration [41].

In recent years, possible therapeutic effects of HW, which contains molecular hydrogen (H_2), have been noticed in experimental studies with animals. In rats with periodontitis, the intake of HW for 4 weeks lowered serum levels of ROS and oxidised low-density lipoprotein-cholesterol [12]. In mice subjected to physical restraint stress for 10 hr per day for 6 days each week, the enhanced oxidative stress in the brain was inhibited by drinking HW [27]. In addition, the inhalation of H_2 gas protected the brain from ischemia and reperfusion-induced damage in the rat [29].

Brain slices derived from mice administered hydrogen-rich pure water for 33 days showed significantly less superoxide formation than the control [35]. In a rat model of sepsis, the intraperitoneal administration of hydrogen-rich saline inhibited the increase of oxidative responses, such as ROS and malondialdehyde, in the hippocampus in a dose-dependent manner [43]. Also, drinking HW significantly suppressed intimal hyperplasia of the inferior *vena cava* in the rat [12]. All these findings suggest that HW has an antioxidative property. It is of interest to understand the biochemical mechanism of the antioxidative action of HW. The antioxidative action of molecular hydrogen (H_2) has been described in detail [29]. H_2 dissolved in culture medium of PC12 cells significantly decreased the level of hydroxyl radicals ($\cdot OH$) without decreasing the level of in super oxide anion radicals ($O_2^{\cdot -}$) or hydrogen peroxide (H_2O_2) derived from mitochondria. Accordingly, it is plausible that HW inhibits the formation of hydroxyl radicals.

The absorption of HW from the digestive organs and its time-course change in the body was described in a rat study [27]. Hydrogen in the blood was detected at 3 min after the direct instillation of saturated HW into the stomach, and the half-life of hydrogen in the muscle was estimated to be approximately 20 min after the instillation. Therefore, it is assumed that the molecular hydrogen is rapidly absorbed by the digestive organs and most of it can be metabolized within one hour in the living body. In the present study, the d-ROMs value showed a tendency to decrease already at pre-exercise after the intake of HW, compared to the placebo intake. In addition, there were significant increases in the BAP/d-ROMs ratio in the HW trial, compared with the placebo trial, at pre-exercise, post-exercises and 30 min after exercise (two-way repeated-measures ANOVA), while no significant difference was found in the same horses at 30 min before the treadmill exercise. This finding may indicate that the HW administered into the digestive tract of the horse is rapidly distributed throughout the body after administration and acts to reduce a part of free radicals in the blood. In addition, the earlier recovery of the BAP/d-ROMs ratio in the HW trial, compared to the placebo trial, may reflect lesser amounts of free radicals (d-ROMs) during and immediately after the exercise, as compared to the placebo trial.

In conclusion, the present study demonstrated that oxidative and antioxidative changes in the blood are significantly induced by treadmill exercise of Thoroughbred horses, and that the recent developed measurements, i.e. d-ROMs and BAP tests, are useful and convenient methods for determining exercise-related physiological changes in horses. Furthermore, we suggested a possibility that the supplementation of hydrogen-rich water has efficacy in lowering oxidative stress in horses.

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