NOTE *Clinical Pathology*

Effects of ozonated autohemotherapy on the antioxidant capacity of Thoroughbred horses

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ABSTRACT. The performance of horses undergoing regular intense exercise is adversely affected by oxidative stress. Thus, it is important to increase antioxidant production in horses in order to reduce oxidative stress. Ozonated autohemotherapy (OAHT) reportedly promotes antioxidant production. This study aimed to evaluate the effects of OAHT on antioxidant capacity. Ten Thoroughbred horses were used in this study. After the OAHT, we collected serum samples and measured biological antioxidant potential (BAP). We found that BAP began to increase after the OAHT and was significantly higher in the OAHT group than at 3 ($P<0.01$) and 7 days ($P<0.05$) after OAHT than in the control group at 3 and 7 days after starting collection of blood samples. Therefore, it was shown that OAHT improved the antioxidant capacity of the horses.

KEYWORDS: horse, oxidative stress, ozonated autohemotherapy

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Performance horses need regular intense exercise, which increases oxygen consumption. Intense exercise increases oxygen consumption 10–20 times in the whole body and 100–200 times in muscle [15]. Increased oxygen consumption causes the production of more reactive oxygen species (ROS) in mitochondria [10, 15, 17]. While ROS are removed from the body by antioxidants, complete removal is not possible if there is excessive production. The remaining ROS affect various tissues, which is called oxidative stress. Oxidative stress is a major cause of muscle fiber damage [11, 15], which causes poor performance and lameness; thus, reduction of oxidative stress is essential for horses. This can be achieved by increasing antioxidant production or preventing production of ROS. However, prevention of ROS production is difficult in horses that regularly undergo intense exercise. Therefore, it is important to increase the levels of antioxidants to reduce oxidative stress in horses.

Ozonated autohemotherapy (OAHT) has been reported to increase the production of antioxidants [13]. It has been shown that there is a hormesis effect with OAHT; i.e., when a small amount of oxidative stress caused by ozone is given to a living body, it promotes the production of antioxidants [1, 3, 13]. Therefore, it is presumed that the application of OAHT prior to exercise would improve the antioxidant capacity and minimize muscle damage.

This information suggests that OAHT is effective for the prevention of muscle damage and could be useful for conditioning horses. However, little is known about the effects of OAHT in horses. Therefore, the present study aimed to evaluate the effects of OAHT on the antioxidant capacity of Thoroughbred horses.

This experiment was approved by the Animal Care and Use Committee at the Miyazaki Yearling Training Farm of the Japan Racing Association. The care and use of horses complied with local animal welfare laws, guidelines and policies. Ten gelding Thoroughbred, non-race horses, were used in this study [age, 10.8 ± 4.5 years; body weight, 510.5 ± 31.3 kg; mean ± standard deviation (SD)]. The horses were stalled throughout the study period and had an exercise routine comprised of walking and trotting for approximately 30–60 min each day. The exercise routine and feeding were not changed during the study period. In order to minimize the influence of climate on this research, the entire study was performed in the summer in Miyazaki Prefecture, Japan (7/17/2014–9/3/2014).

The same horses were used as controls and for treatment. We began to collect the control samples 1 month before the OAHT, on 7/17/2014 (pre), and then collected samples 1, 2, 3, 7 and 14 days later. We performed complete blood counts on the samples and separated the serum. Serum samples were stored at −80°C for further use.

Ozone gas was generated from medical grade oxygen using an ozone generator (Otec. Lab TK-11, Otec Lab, Ebetsu, Japan). The ozone gas was adjusted to make the ozone concentration 25 μg/m*l*. The OAHT was conducted on each horse on 8/20/2014 in the following manner. First, the contents of a 1 *l* bag of saline (Otsuka Pharmaceutical Factory,

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Tokushima, Japan) were discarded, and 50 m*l* of sodium acid citrate (Citramin, Fuso Pharmaceutical Industries, Osaka, Japan) were infused into the empty saline container. Next, we placed a 14-gauge indwelling catheter into the jugular vein and collected 400 m*l* of blood into the abovementioned container. We then infused 20 μg/kg (body weight) of ozone gas into the container, which reacted with the blood. After the reaction, we slowly reinfused the ozonated blood back into the horse (3.2 m*l*/kg/hr).

We collected blood samples before the OAHT (pre) and on 1, 2, 3, 7 and 14 days after the OAHT (post). We performed complete blood counts on the collected blood and separated the serum. Serum samples were stored at −80°C for further use. After all the samples were collected, routine biochemical tests were conducted using the stored serum samples. Afterwards, we measured the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) in the serum samples using a free radical analyzer (Free Carpe Diem, Diacron International, Grosseto, Italy). We calculated the oxidative stress index (OSI) from the measured d-ROMs and BAP (calculation formula: d-ROMs/BAP ×100).

Some data did not show a normal distribution; thus, we used non-parametric tests for statistical analysis. All data were analyzed using Statcel2 (OMS Publishing Inc., Tokorozawa, Japan). We used the Steel-Dwass test for comparisons in each group. Differences between the OAHT and control groups were determined using the Wilcoxon signed–ranks test. The significance level was set at $P<0.05$ for all analyses.

The blood test results for one day after OAHT are presented in Table 1. No abnormal complete blood counts, biochemical tests or clinical signs were observed during the study period. The medians and ranges of the d-ROMs, BAP and OSI are shown in Tables 2, 3 and 4, respectively.

Although the d-ROMs fluctuated in both groups, there were no significant differences within each group. No significant difference in d-ROMs was confirmed between the control and OAHT groups at any time point.

The BAP fluctuated in the control group, but there was no significant difference within that group. On the other hand, the BAP of the OAHT group began to increase 2 days after the OAHT, and the value at 3 days was significantly higher than the pre, 1-day and 14-day values ($P<0.01$, $P<0.05$ and $P<0.01$, respectively). The OAHT group had a higher BAP at 3 ($P<0.01$) and 7 days ($P<0.05$) after OAHT than the control group at 3 and 7 days after starting collection of blood samples.

The OSI also fluctuated within both groups, and there was no significant difference within either group. The OAHT group had a significantly lower OSI at 3 ($P<0.05$) and 7 days ($P<0.01$) after OAHT than the control group at 3 and 7 days after starting collection of blood samples.

This study aimed to evaluate the effects of OAHT on the antioxidant capacity of Thoroughbred horses. In humans, OAHT has been performed in Europe since the 1970s and has been reported to be safe [2, 13]. No abnormal clinical signs, abnormal complete blood count values or abnormal biochemical test findings were observed in this study. Therefore, it appeared that there were no side effects of OAHT and that it would be a safe treatment for horses.

Both d-ROMs and BAP have been reported to be precise and reliable methods of assessing oxidative stress in horses [4, 12]. Tsubone *et al.* reported that hydrogen-rich water intake changed the BAP/d-ROMs value and lowered oxidative stress during exercise. They concluded d-ROMs and BAP were useful methods for determining exercise-related physiological changes in horses [16]. Therefore, we measured d-ROMs and BAP in this study.

It has been shown that d-ROMs are an index of ROS [12]. The fluctuations in d-ROMs without significant differences between the groups suggested that the fluctuations were a physiological variation. Furthermore, since there were no significant differences before and after OAHT, it was thought that the OAHT caused only mild oxidative stress and that its influence was short-lived.

The BAP is an index of antioxidant capacity [12] that fluctuated in the control group; however, there were no significant differences. Thus, this fluctuation was also thought to be due to physiological variations. On the other hand, BAP was significantly increased by 3 days after OAHT in the OAHT group compared with the value for the equivalent period in the control group. Therefore, OAHT appeared to improve the antioxidant capacity of the horses. However, the improvement in antioxidant capacity was not observed immediately after OAHT and was only confirmed 3 days later. Since OAHT uses the reaction of the living body against slight oxidative stress [5, 13], it is likely that it takes some time to start production of antioxidants after the treatment. Thus, it would be better to perform OAHT 3 days prior to exercise when using it to prevent muscle damage. Moreover, the improvement in the antioxidant potential due to the OAHT was observed until 7 days after treatment, and the effect was no longer measurable at 14 days after treatment. Therefore, we assumed that the improved antioxidant capacity due to OAHT lasted between 7 and 14 days.

Oxidative stress occurs when the balance of ROS and antioxidants favors ROS. For this reason, it is also important to evaluate the grade of oxidative stress using the OSI, which demonstrates the balance of d-ROMs and BAP [12]. The OSI was lower in the OAHT group than in the control group, indicating lower oxidative stress in the former group. The d-ROMs were not significantly different in the OAHT group compared with the control group, but BAP was significantly increased in the OAHT group. Thus, OAHT increased BAP, which suppressed oxidative stress.

Antioxidant supplements, such as vitamin C, vitamin E and polyphenol, have been used to improve antioxidant capacity [8, 14, 15]. However, sustained antioxidant supplementation inhibits mitochondrial adaptation to oxidative stress and may adversely affect training efficiency, as shown in a rat and human study [6]. Moreover, Marshall *et al.* reported [7] that sustained supplementation of highly concentrated vitamin C reduces running speed in racing dogs. Therefore, methods to improve antioxidant capacity should not be solely dependent on antioxidant supplementation to prevent muscle damage so that training efficacy and performance

Table 1. Blood test results (one day after OAHT)

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10
RBC ($\times 10^6/\mu l$)	908	835	812	842	742	904	906	893	773	879
WBC ($/\mu l$)	8,000	5,600	8,000	10,300	5,900	7,600	8,500	11,600	9,800	10,500
Hct (%)	38.3	34.7	37.2	37.9	34.4	44.2	41.3	40.3	35.6	40.7
PLT ($\times 10^4/\mu l$)	19.2	12.4	12.8	14.3	8.6	14.4	9.3	6.3	13.1	17.4
CK (U/*l*)	142	136	148	155	116	167	152	215	161	101
GOT (U/*l*)	276	236	251	226	187	352	287	273	264	202
LDH (U/*l*)	462	227	375	420	316	506	354	336	413	329
ALP (U/*l*)	166	91	139	164	182	147	118	168	146	180
γ-GTP (U/*l*)	11	9	10	10	10	10	12	13	11	12
TP (g/d*l*)	6.5	6.7	6.8	6.6	6.5	6.5	7.2	6.8	6.5	7.3
ALB (g/d*l*)	3.5	3.5	3.5	3.4	3.3	3.4	3.6	3.3	3.5	3.7
A/G	1.2	1.1	1.1	1.1	1.0	1.1	1.0	0.9	1.2	1.0
BIL (mg/d*l*)	1.7	2.7	1.6	1.8	1.7	2.0	2.8	1.2	1.8	3.3
BUN (mg/d*l*)	13.1	15.5	18.3	15.9	15.1	15.7	14.5	21.4	21.3	18.1
UA (mg/d*l*)	0.52	0.38	0.51	0.30	0.51	0.42	0.38	0.22	0.44	0.38
CRE (mg/d*l*)	1.0	1.3	1.6	1.4	1.5	1.4	1.1	1.2	1.4	1.5
Ca (mg/d*l*)	12.2	12.4	12.1	12.3	11.5	12.0	12.3	12.5	12.2	12.7
Fe (μg/d*l*)	107	119	112	112	86	139	123	179	132	154
Na (mEq/*l*)	137	140	140	139	135	138	138	136	140	139
K (mEq/*l*)	4.2	4.3	4.4	4.3	4.4	3.9	3.9	4.5	3.8	4.4
Cl (mEq/*l*)	102	105	103	106	101	102	102	98	103	104

Table 2. d-ROMs values

		Pre	1 day	2 days	3 days	7 days	14 days
Control	Median	153	161	158.5	173	179	165.5
	Range	72–230	130–227	129–226	79–236	135–235	59–206
	Mean ± SD	153.3 ± 41.2	165.7 ± 26.7	160.8 ± 27.1	167.8 ± 45.6	181.2 ± 32.9	159.2 ± 42.6
OAHT	Median	149	146.5	151	152	145	137
	Range	126–187	123–185	115–184	125–203	120–206	120–232
	Mean ± SD	153.8 ± 20.7	151.7 ± 22.9	155.1 ± 24.4	157.4 ± 24.3	151.6 ± 25.8	149.8 ± 35.1

Unit: U.CARR.

Table 3. BAP values

		Pre	1 day	2 days	3 days	7 days	14 days
Control	Median	2,788	2,946.4	2,714.5	2,723.9	2,518.0	2,683.3
	Range	2,489.4–3,207.0	2,489.1–3,503.7	2,301.9–3,343.1	2,299.7–3,704.4	1,629.6–3,416.6	1,615.6–3,332.8
	Mean ± SD	2,797.0 ± 225.6	2,980.8 ± 399.2	2,758.4 ± 349.9	2,809.2 ± 394.4	2,518.3 ± 523.6	2,655.3 ± 438.3
OAHT	Median	2,735.0 [a]	2,965.3 [b]	3,029.6	3,272.4 **[a,b]	3,097.7 *	2,838.4 [a]
	Range	2,663.3–3,053.1	1,949.3–3,184.2	2,663.3–3,659.2	2,911.4–4,540.5	2,502.2–3,926.9	2,648.2–3,113.8
	Mean ± SD	2,814.8 ± 141.0	2,772.7 ± 421.0	3,055.9 ± 289.0	3,437.7 ± 473.6	3,116.2 ± 450.8	2,849.7 ± 145.7

*Significant difference between the OAHT and control groups (P<0.05), **Significant difference between the OAHT and control groups (P<0.01), a) Values with the same letters are significantly different (P<0.01), b) Values with the same letters are significantly different (P<0.05), Unit: μmol/*l*.

Table 4. OSI values

		Pre	1 day	2 days	3 days	7 days	14 days
Control	Median	5.4	5.3	5.6	6.0	7.8	6.3
	Range	2.6–8.2	4.7–7.2	4.8–8.6	2.6–9.7	5.2–11.2	2.3–8.8
	Mean ± SD	5.5 ± 1.5	5.6 ± 0.8	5.9 ± 1.1	6.2 ± 2.2	7.5 ± 2.1	6.1 ± 1.8
OAHT	Median	5.5	5.5	5.1	4.4 [a]	4.9 [b]	5.0
	Range	4.5–6.5	4.2–7.2	3.6–6.6	3.4–6.2	3.7–7.1	4.1–8.4
	Mean ± SD	5.5 ± 0.8	5.6 ± 1.0	5.1 ± 1.0	4.7 ± 0.9	4.9 ± 1.0	5.3 ± 1.3

a) Significant difference between the OAHT and control groups (P<0.05), b) Significant difference between the OAHT and control groups (P<0.01).

will not be decreased. Ozonated autohemotherapy confers a hormesis effect that improves antioxidant capacity as a result of a reaction to slight oxidative stress. Therefore, it appears that OAHT does not inhibit adaptation to oxidative stress, but may improve antioxidant capacity without affecting the training efficacy or performance of horses.

In this study, the dose of ozone (20 μg/kg) was determined with reference to levels previously reported in cattle [9]. However, Bocci *et al.* reported that 20 mg/kg of ozone showed a therapeutic effect but that this effect was weaker than that with a higher dose (40–80 μg/kg) [1]. Therefore, it was considered that the higher dose was more effective. However, since the appropriate dose of ozone was not determined in horses, a dose study is required in the future.

It was more appropriate to use a crossover design to minimize the influences of factors other than OAHT, such as individual differences and differences in climate. However, we could not clarify how long the effect of OAHT continued in horses before we started this study. Therefore, we could not determine the wash out period and were unable to use a crossover design. This study revealed that the OAHT effect lasted for approximately 7–14 days and examination by a crossover design will now be possible in the future. To further clarify the effects of OAHT, a study with a crossover design is required in the future.

In conclusion, it was confirmed that OAHT improved the antioxidant capacity of horses and that this effect lasted for 7 to 14 days in this study. Thus, it is possible that OAHT could be useful for conditioning of horses. However, the Paris Agreement prohibits the re-administration of blood in racehorses; thus, application of OAHT to this group is currently limited. The development of a substitute method to produce an effect like OAHT without the use of blood would be necessary to overcome this problem in the future. In addition, it would be necessary to examine the influence of exercise on the effects of OAHT.

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