Response of Biochemical Markers of Bone Metabolism to Exercise Intensity in Thoroughbred Horses

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We studied the response of biochemical markers of bone metabolism to exercise intensity in horses. Four horses were walked on a mechanical walker for one week (pre-exercise). Then they performed low-speed exercise on a high-speed treadmill in the first week and medium-speed exercise in the second week and high-speed exercise in the third week of training. We measured two indices of bone resorption, serum hydroxyproline concentration and the urinary deoxypyridinoline/creatinine ratio, and serum osteocalcin (OC) concentration as an index of bone formation. Both indices of bone resorption gradually decreased during the experiment. Serum OC concentration did not change in the first week but was significantly lower in the second and the third weeks compared to in the pre-exercise period and in the first week. These results suggest that the low-speed exercise decreased bone resorption but did not affect bone formation, which possibly results in increasing bone mineral content and strengthening of bones. The high-speed exercise decreased bone formation and bone resorption, i.e., bone turnover was suppressed. The low-speed exercise may be preferable for increasing bone mineral content.
Key words: *biochemical markers of bone metabolism, exercise, horse, intensity*

Introduction

Physical activity is an important factor affecting bone metabolism in various animals. It was reported that bone mineral content (BMC) increased in horses after several months of training [25, 26]. On the other hand, a reduction of bone mass was observed in response to intensive and/or excessive exercise, particularly in growing animals [9, 18]. The failure of skeletal adaptation to mechanical stress may induce various bone disorders including fracture, with the possibility of large economic losses in horses [8, 21]. The relationship between physical activity and bone metabolism is still not sufficiently understood [16].

Thus, it is necessary to clarify the optimal level of exercise for improving bone quality.

The rate of bone metabolism can be assessed by measuring biochemical markers of bone turnover [17, 23, 33]. The markers are enzymes expressed by osteoblasts or osteoclasts or organic components released during the synthesis and resorption of bone matrix [7]. Serum osteocalcin (OC) reflects bone formation, whereas urinary excretion of total deoxypyridinoline (DPD) and hydroxyproline (HYP) are used as markers of bone resorption in horses. OC is synthesized by osteoblasts as an abundant non-collagenous protein of the organic bone matrix and some OC is secreted directly into the circulation [1]. Thus, the serum OC level should reflect osteoblastic activity [30].

Collagens comprise the major organic matrix of

bone; 90% of the organic matrix of bone is collagens. HYP is a major amino acid in collagens and 13% of their amino-acid residues are composed of HYP. HYP is released from bone during the degradation of bone matrix and has been used as an index of bone resorption. Collagens are located ubiquitously in the body of animals. The serum concentration of HYP is also influenced by the diet and the metabolism of non-bony collagens, such as muscles, skin and liver [23]. However, Lepage *et al.* suggested that HYP is a marker indicative of bone resorption, even though it lacks specificity to bone collagens [23].

Type I collagen is distributed in bone, skin and tendon; 95% of the organic matrix in bone is composed of type I collagen. Thus, degradation products of type I collagen are more specific indices of bone metabolism.

The derivatives of collagen crosslink can be used as indices of collagen catabolism. In the crosslinking residues of collagens, DPD is the most specific crosslinking residue of bone. DPD is released from the bone matrix during its degradation and excreted in urine without being metabolized [11]. Price *et al.* and Black *et al.* reported that DPD is a potential index of bone resorption in horses [3, 31].

Several studies showed significant differences of biochemical markers of bone metabolism in horses during the course of training when compared to unexercised horses, reflecting skeletal adaptation to exercise [19, 32]. However, few studies have examined the effects of exercise intensity on bone metabolism in horses. The present study was conducted to investigate the response of biochemical markers of bone metabolism to the intensity of exercise in horses.

Materials and Methods

Animals and diets

The experimental protocols for the study were reviewed and approved by the Animal Welfare and Ethics body of the Japan Racing Association. Four 4-yr-old Thoroughbred stallions were used in this study. Their initial average BW was 440 kg and their body condition scores were between 5 and 6 according to the method of Henneke *et al.* [13]. Prior to the initiation of the experiment, these horses were housed in box stalls and pastured for 4 hr on approximately 1,000 m^2 timothy pastures 3 days a week for 2 months, and were

Table 1. Treadmill exercise protocol

Gait	Duration (min)	Speed (m/s)	Slope (%)
First week			
Walk	1	1.8	0
Trot	3	3	0
	3	3	3
Canter	3	6	3
	3	8	3
Walk	5	1.8	0
Second week			
Walk	1	1.8	0
Trot	3	3	0
	2	3	7
Canter	2	6	7
	2	8	7
	2	10	7
Walk	5	1.8	0
Third week			
Walk	1	1.8	0
Trot	3	3	0
	3	3	8
Canter	2	6	8
	2	8	8
	2	10	8
	2	12	8
Walk	5	1.8	0

stabled continuously throughout the experiment except for the days of exercise. The horses consumed 10 kg/day timothy hay, 1 kg/day alfalfa hay, 2 kg/day oats grain and 1 kg/day commercially pelleted vitamin and mineral supplement (Ace ration 2; Nosan Corporation, Japan) as 2 equal meals (offered at 06:00 and 17:00 hr) daily throughout the experiment. The diet was expected to maintain or exceed the current Japanese feeding standard recommendation for exercising horses [6]. Horses were allowed free access to water from an automated watering system during the experiment and daily water consumption was not determined.

Exercise

After a 1-week adjustment period, horses were walked on a mechanical horse walker (Classic Run CR1200; Yodo Machine, Japan) at 6 km/h for 20 min a day during the first week of the experiment (pre-exercise period). Thereafter, a low-speed incremental exercise (8 m/sec) was performed in the first week of exercise, a medium-speed incremental exercise (10 m/sec) was performed in the second week and a high-

Table 2. Changes in serum and urinary biochemical markers of bone during the experiment

	Pre-exercise	Exercise-period		
		First week	Second week	Third week
Serum OC[1] (mg/l)				
Mean	14.6	12.2	8.2	6.5
s.e.	1.8[a]	1.7[a]	1.2[b]	0.5[b]
Serum HYP[2] (mg/l)				
Mean	2.00	1.84	1.71	1.28
s.e.	0.16[a]	0.14[ab]	0.19[a]	0.14[b]
Urinary DPD/Cr[3] (nM/mM)				
Mean	90.3	58.2	50.0	45.2
s.e.	8.1[a]	4.9[b]	4.3[b]	3.5[c]

Values are shown as the mean ± s.e. (n=4). [abc]; Values with different letters are significantly different (P<0.05).
1) Osteocalcin, 2) Hydroxyproline, 3) Deoxypyridinoline/Creatinine.

speed exercise program (12 m/sec) was performed in the third week using a high-speed treadmill (Mustang 2200; Kagra, Switzerland) for 5 days/week in a room with controlled environmental temperature ranging between 17.5 and 18.5°C (Table 1). The heart rate was recorded using a heart rate monitor (Bandage-XL; Pollar, Finland) during treadmill exercise. The low- and medium-speed exercise protocols were designed for aerobic training and the high-intensity exercise training protocol was designed for combined aerobic and anaerobic training.

Collection and analyses of samples

Blood was collected from the jugular vein into a plain tube before walking or exercising on the last day of each exercise period. Serum was separated by centrifugation (1,400 × g, 4°C, 10 min) and immediately placed in a deep freezer and stored at –80°C until analyzed. Total urine was collected over the 5 days of each period using a commercially available equine harness (The Horse diaper; Equisan Marketing Pty Ltd., Australia) that was reported to be useful for complete collection of equine feces and urine [24]. The concentration of OC was measured in serum samples using a commercially available RIA (Osteocalcin ^{125}I RIA Kit; DiaSorin Inc., USA) according to the manufacturer's instructions. The polyclonal antibody was obtained by immunizing rabbits against bovine OC. The antiserum against bovine OC has been shown to cross-react with horse OC [28]. The validity of this kit for horse OC was reported by Lepage *et al.* [22]. The serum HYP concentration was analyzed by the method of Fujii *et al.* [10]. Urinary DPD concentration was analyzed by an enzyme immunoassay (Quidel Corp., USA). The urinary

creatinine (Cr) concentration was analyzed by a colorimetric assay (Creatinine-Test-Wako; Wako Pure Chemical Industries, Japan). The excretion of DPD was expressed as the ratio to urinary Cr concentration. The Ca concentration in the diet was measured by inductively coupled plasma-atomic emission spectroscopy (ICPS 1000; Shimadzu, Japan) after digestion with nitric acid and perchloric acid.

Statistical analyses

Data were expressed as the mean ± s.e. The changes in all parameters were analyzed by the MIXED procedure of SAS (Release 6.11; SAS Inst., Inc., USA) and the differences between the means for each week were analyzed with the paired *t*-test. Differences were considered significant at P<0.05.

Results

The animals received a dietary total of 45.2 g Ca/day throughout the study period. The heart rate at the maximal exercise speed was 174 ± 5 beats/min, 205 ± 3 beats/min and 230 ± 1 beats/min in the first, second and third weeks of exercise, respectively. The serum OC concentration did not change in the first week of exercise but significantly (P<0.05) decreased in the second and third weeks of exercise (Table 2). The serum HYP concentration gradually decreased during the exercise periods and a significant (P<0.05) reduction was observed in the third week of exercise. Urinary DPD/Cr significantly (P<0.05) decreased in the first week of exercise and further reductions were observed in the following exercise periods.

Discussion

Dietary Ca intake was 45.2 g/day throughout the study, which almost met the current recommendation for exercising horses [6]. In the present study, the horses were walked on a mechanical horse walker in the pre-exercise period and trained on a treadmill in the exercise periods. Exercise speed resulting in a heart rate of 200 beats/min has been suggested as suitable for race training [12]. The blood lactate concentration is likely to start increasing in most horses at a heart rate of 180 beats/min [15]. A mean peak heart rate of 175 beats/min was reached in the first week of exercise in this study. This level of exercise is unlikely to increase the blood lactate concentration; therefore, the exercise intensity in the first week was considered as low. Additionally, the exercise intensity was considered medium in the second week since the mean peak heart rate reached 205 beats/min during this period. The maximal heart rate was reported to be 229 beats/min in adult Thoroughbred horses exercised during an incremental treadmill exercise test [36]. The mean peak heart rate was as high as 230 beats/min in the third week of exercise, which was near the maximal heart rate reported by Seeherman and Morris [36]. Therefore, the exercise intensity was high in the third week.

Of the many factors influencing bone metabolism, exercise is probably one of the most important. Burr reported that bone adapts only in response to dynamic loads, not to static loads [4]. Excessive loading or loading the bone to fatigue can produce traumatic failure or lead to progressive weakening of bone [5].

OC is synthesized by osteoblasts as an abundant non-collagenous protein of the organic bone matrix [1]. The serum OC concentration is considered to reflect osteoblastic activity [30]. The serum OC concentration did not change in the first week but a significant reduction was observed in the second and the third weeks of this study. These results suggest that the low-speed exercise did not affect bone formation but the high-speed exercise suppressed it. It is also likely that short-term exercise does not affect bone formation, but the continuation of exercise suppresses it. Jackson *et al.* reported exercise decreased serum OC by 22% from baseline at the end of 20-week exercise in 2-year-old Thoroughbred horses, and serum OC was significantly lower in exercised horses than in unexercised horses as early as 4 weeks after the onset of heavy exercise [20]. Vervuert *et al.* observed a drop in the baseline serum

OC concentration at the end of 6-week training combining low- and high-speed exercise in 2-year-old Standardbred horses [37]. In contrast, Price *et al.* observed higher levels of bone formation indices, the carboxy-terminal propeptide of type-I collagen (PICP) and bone-specific alkaline phosphatase (BAP), in 2-year-old Thoroughbred horses even at the beginning of training when compared to a no-force exercise group, reflecting a general increase in bone turnover during the early stage of exercise in horses [32]. An *in vivo* experiment indicated that OC mRNA expression decreased in rat tibial periosteum cells 4 hr after loading [34], suggesting the rapid response of osteoblastic activity to mechanical stress. Therefore, the difference in the serum OC concentration probably resulted from the difference of exercise intensity in the present study. Nielsen *et al.* also reported that the serum OC concentration increased at day 14 and declined precipitously until day 42 in 16-week race training, which comprised gradual high-intensity exercise typical for a Quarter horse aged 2-year-old [27].

The urinary DPD/Cr ratio decreased in the first week of exercise, and further decreased thereafter. In addition, the serum HYP concentration gradually decreased during the exercise periods, and a significant reduction was observed in the third week of exercise. DPD is a reliable marker of bone degradation [35]. Lepage *et al.* suggested HYP is a bone resorption marker, even though it lacks specificity to bone collagen [23]. Therefore, the results of the present study suggest that exercise suppresses bone resorption irrespective of its intensity. Jackson *et al.* reported that the serum concentration of DPD, which was significantly correlated with urinary DPD, was not significantly lower in an exercised group than in a sedentary group in 2-year-old Thoroughbred horses at the end of 20-week exercise, during which exercise intensity was moderate in the first half and extremely high in the latter half [20]. Jackson *et al.* also reported a decrease in the carboxy-terminal telopeptide of type-I collagen (ICTP) [20], which is another marker for bone resorption [23]. Hiney *et al.* also reported the reduction of ICTP during the early stage of 16 week training of gradual high-intensity exercise, compared to a sedentary state in yearling horses, indicating reduced bone resorption [14]. These results are in accordance with those of the present study. In contrast, Price *et al.* observed higher levels of ICTP in 2-year-old Thoroughbred horses 2 months after the initiation of

exercise compared to a no-force exercise group, but the difference disappeared thereafter [32]. Their results suggest a general increase in bone resorption during the early stage of exercise in horses. Porr *et al.* showed that exercise did not affect the serum concentration of HYP in mature horses when they exercised at a relative high intensity for 12 weeks [29].

It is possible that the length of the exercise period and/or the growth stage of horses affects the response of bone resorption to exercise. The adaptive response of bone to exercise may depend on several factors including growth, the intensity of training, and type of loading [2]. The changes in bone mass and BMC are due to the balance between bone formation and bone resorption. As mentioned above, the results of the present study suggest that the low-speed exercise did not affect bone formation, but that high-speed exercise decreased bone formation and bone resorption, indicating a reduction of bone turnover. It must be noted that the intensity of exercise was increased with each week in the present study. Therefore, we could not distinguish the effects of exercise intensity and exercise period on bone formation in the present experiment. Therefore it is also possible that the continuation of low-speed exercise suppresses bone formation. In contrast to this negative effect, low-speed exercise had positive effects on bone mass in growing and mature animals; however, reduced bone mass was observed in response to intensive and/or excessive exercise, particularly in humans during growth [9] and in growing rats [18]. The increase in bone mass is considered to result from a reduction in bone resorption without change in bone formation in low-speed exercised animals. Furthermore, the decrease in bone mass is probably due to greater reduction of bone formation than increase of bone resorption in intensively exercised animals.

BMC was reported to decrease in horses during 60 to 120 days of training [27]. It was suggested that the decrease in bone density after the onset of training resulted from a transient change in bone remodeling in order to remove damaged bone tissue. The removal of bone tissue is induced by bone resorption. Therefore, the decrease in BMC would be due to the stimulation of bone resorption. Many studies including the present study suggest that bone resorption decreases in exercised horses. Therefore, a reduction in bone formation probably contributes to the reduction of BMC in exercised horses.

The majority of bone-related injuries occur during a period when BMC is reduced [26]. Therefore, low-speed exercise is preferable for increasing BMC and preventing bone-related injuries in growing horses because it decreases bone resorption without affecting bone formation. Furthermore, the appropriate level of exercise can be assessed by the serum OC concentration.

It was also reported that exercise increased BMC in horses after a temporal reduction [27]. Therefore, extended exercise differently affects bone metabolism and thus BMC. Further study is necessary to clarify the effect of exercise period on bone metabolism in horses. The optimal intensity, frequency of activity, and number of repetitions are not yet fully understood in detail. In future, biochemical markers of bone metabolism should be useful markers for monitoring changes in skeleton in relation to exercise.

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