Effect of Exercise on Thyroid, Parathyroid and Bone in Very Young Thoroughbreds

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Seven horses were exercised five days a week for 12 months at different gaits on a treadmill, and eight horses were pastured freely in a paddock without any training programs. They were 60 days of age at the beginning of the experiment. We compared the number of C cells in the thyroid, ultrastructure of chief cells in the parathyroid and radiography of the third metatarsal bone of the training group with the control group. In the training group, the number of C cells was not increased and chief cells were not in a phase of secretion. In addition, the bone mineral content and the cortical area did not show high values. We considered that an additional exercise used in this study had suppressive effects on the thyroid and parathyroid. Our results also suggest an additional and/or intense exercise in early stage of life (foal to weanling) may have an adverse effect on bone growth and metabolism.

Key words: C cell, chief cell, radiography, treadmill, young Thoroughbred

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The occurrence of musculoskeletal diseases is quite frequent in racehorses because they are required to run to the limit of their ability. Therefore, there have been many reports on the effect of exercise on bone metabolism when using a treadmill for the purpose of preventing bone diseases [11, 12, 14, 15, 17, 20, 21]. They have revealed that exercise had changed the morphology of the third metacarpal bones [3, 12, 13, 19, 25]. Chiba *et al.* [7] and Yoshikawa *et al.* [24] have pointed out that the endocrine system was related to fracture in horses. Nevertheless, it was difficult to find other researches into bone disease which had been related to the endocrine systems, such as the thyroid and parathyroid. Calcitonin is secreted from the C cells in the thyroid to inhibit an increase in serum calcium by inhibiting the osteoclast activity [5, 8]. Parathyroid hormone (PTH), secreted from the parathyroid, plays an important role in bone metabolism [4]. We hypothesized that exercise would have an effect on calcitonin and PTH secretion, not only on the mechanical characteristics of

bones, in other words, we discussed the effect of very early training. Weanling to yearling horses have been used commonly to evaluate the effects of exercise [2, 16]. The mechanical or morphometrical properties of bones, such as bone mineral content, cortical area and metacarpal circumference, have usually resulted in an increase with exercise and growth. It seems that there are no reports on them conducted with very young horses (under 100 days of age) as subjects. The objective of this study was to determine the effects of exercise on thyroid C cells, parathyroid chief cells and third metatarsal bones when horses are very young.

Materials and Methods

Horses

The study used 15 Thoroughbred horses with no history of diseases of the musculoskeletal and endocrine systems. The animals were 60 days of age at the start of the study. Fifteen Thoroughbred horses were separated into a training

Table 1. Trainig program

Period (month)	Walk		Gait Trot		Canter to Gallop	
0–1	1.6 m/sec	45 sec	2.6 m/sec	180 sec	5.0 m/sec	75 sec
1–2	1.6	45	2.6	180	5.5	75
2–3	1.6	45	2.6	180	6.0	75
3–4	1.6	45	3.3	180	6.5	75
4–5	1.6	45	3.3	180	7.0	75
5–6	1.6	45	3.3	240	8.0	75
6–7	1.6	45	3.3	240	9.0	75
7–8	1.6	45	3.3	240	10.0	75
8–9	1.6	45	3.3	240	10.0–10.5	75
9–10	1.6	45	3.3	240	10.5	75
10–11	1.6	45	3.3	240	10.5–11.0	75
11–12	1.6	45	3.3	240	11.0	75

group (cases 1–7; 5 females and 2 males) and a control group (cases 8–15; 4 females and 4 males) randomly. The training group was exercised five days a week for twelve months on a flat treadmill (Mustang-2200, Kagra). The daily exercise regimen consisted of walking (1.6 m/sec), trotting (2.6–3.3 m/sec) and cantering to galloping (5.0–11.0 m/sec). Their speeds and times were increased monthly as shown in Table 1. The last month of training consisted of walking (1.6 m/sec) for 45 sec, trotting (3.3 m/sec) for 240 sec and galloping (11.0 m/sec) for 75 sec. The total training distance was 915–1,652 m a day, 5 days/week (accelerated with growth). The training group and control group were pastured in the same area (11,141 m²) for 4 hr every day. The moving distance in the pasture was investigated in 15 horses in both groups, visually monitored [10]. An observer recorded the type of exercise, direction and distance of each horse's activity on a graphic representation of the pasture. Markers were installed on the fences of the pasture at approximately 10-meter intervals. The observer confirmed and recorded the position of horse and direction of the horse's movement in relation to the markers. All horses received concentrate twice daily, and water and hay were available *ad libitum* throughout the study. The daily nutrient requirements of horses in both groups were calculated on the basis of the "Japanese Feeding Standard for Horses" (in Japanese, Equine Research Institute, Utsunomiya, Japan 1998). The mean body weight in both groups at birth, at the start of the study and just before euthanasia was, 56.1 ± 6.4 kg, 154.3 ± 12.3 kg and 264.6 ± 15.1 kg in the training group, and 53.7 ± 4.8 kg, 153.0 ± 17.1 kg and 252.4 ± 15.6 kg in the control group, respectively. There were no significant differences in the mean body weight between both groups at the start of the study and at euthanasia ($p > 0.05$). The horses were

examined or sacrificed with an overdose of intravenous barbiturate at the end of the experiment. The protocols in this study were approved by the Animal Experiment Committee of the Equine Research Institute, Japan, where the horses were reared, run on the treadmill and necropsy was carried out. All procedures were conducted on the basis of the "Guidelines for Animal Welfare and Animal Rights" established by the Equine Research Institute, which encompasses all aspects of animal well-being, including proper housing, management, nutrition, disease prevention and treatment, responsible care, humane handling and, when necessary, humane euthanasia.

Number of C cells in the thyroid gland

Annular sections of the thyroid gland, which had the maximum surface area, were prepared. They were fixed into a 10% neutral buffered formalin, embedded in paraffin, cut to 4 µm thick sections and stained with haematoxylin and eosin. Immunohistochemistry with rabbit polyclonal antibody to human calcitonin (ZYMED Laboratories Inc, 1:50 in PBS) was also conducted. Sections were deparaffinized and incubated with 3% H₂O₂ in methanol to inhibit the endogenous peroxidase. After incubation with the primary antibody overnight at 4 °C, the slides were treated with peroxidase- and Fab' fragments of secondary antibody- conjugated amino acidic polymer (HISTOFINE SIMPLESTAIN MAX PO, Nichirei Co.) for 30 min at room temperature. Positive reaction was visualized with diaminobenzidine solution and the sections were counterstained with haematoxylin. The number of C cells that bound specifically with calcitonin were counted according to the method of Yoshikawa *et al.* [23]. The mean values per unit area (/mm²) of each horse were evaluated statistically by the Mann-Whitney analysis.

Fig. 1. Schematic illustration of radiographic examination. X_1 : lateromedial diameter, X_2 : lateromedial medullary diameter, Y_1 : dorsoplantar diameter, Y_2 : dorsoplantar medullary diameter.

Ultrastructure of chief cells of the parathyroid gland

The parathyroid glands were removed at autopsy and trimmed to 1-mm cubes. The small tissues were quickly prefixed in 2% glutaraldehyde buffered (pH 7.4) with 0.05 M cacodylate, and postfixed in 1% OsO_4 solution. Tissues were dehydrated via a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut on an ultratome (ULTRACUT N, Reichert-Nissei), stained with uranyl acetate and Sato's basic lead citrate, and examined with a Hitachi H-7000 transmission electron microscope.

Radiography of the third metatarsal bone

The left or right third metatarsal bone was removed and examined radiographically. After dissecting the soft tissues, the metatarsi were cut into transverse slabs 3-mm thick at the middle of the diaphyses. The radiographs (Fuji sftexfilm FG) of these slabs were taken (39 kV, 5 mA, 2 min) with an

X-ray source (SRO-M50, SOFTRON Co.). An aluminum step wedge with 20 steps ranging from 1 to 20 mm was exposed simultaneously and used as reference material. Radiographs were scanned (Scanmaker 9600XL, MICROTEK Co.) and digitized with a Windows computer so that X-ray density could be converted to pixel values. The bone mineral content (BMC) was represented as the equivalent aluminum thickness value (mmAl) using an image analysis program (NIH Image). Measurement sites are schematically shown in Fig. 1.

The cortical cross-section area (CA) was estimated from the formula:

$$CA = \pi (X_1 \cdot Y_1 - X_2 \cdot Y_2) / 4$$

where X_1 and Y_1 represent the mid-diaphyseal lateromedial and dorsoplantar bone diameters, respectively, and X_2 and Y_2 represent the lateromedial and dorsoplantar medullary diameters, respectively.

BMC and CA were statistically analyzed by the Mann-Whitney analysis.

Results

Amount of activity

Exercise distance during pasture and treadmill running is summarized in Table 2. The net exercise distance per a day in both groups was approximately 3,000–4,700 m (increased with growth) in the training group, and 2,200–3,000 m in the control group, respectively. The total canter distance was approximately 900–1,500 m (increased with growth) in the training group, and approximately 600 m throughout the experiment in the control group, respectively.

Number of C cells in the thyroid gland

C cells were clearly identified by the immunohistochemistry for calcitonin. The training group had a significantly ($p < 0.05$) smaller number of C cells than the control group ($39.42 \pm 9.2 / \text{mm}^2$ versus $64.8 \pm 24.1 / \text{mm}^2$) (Fig. 2).

Table 2. One day exercise distance of foals in the treadmill and pasture. Values for pasture are the mean $\pm$ SD

			canter (m)		total (m)	
			control	training	control	training
days from birth	60–180 (days)	pasture	619.4 ± 333.0	500.4 ± 169.8	2254.5 ± 458.7	2088.7 ± 341.4
		treadmill		375–562.5		915–1228.5
	181–360 (days)	pasture	597.2 ± 284.1	721.6 ± 264.5	2973.5 ± 809.2	3072.3 ± 682.3
		treadmill		600–787.5		1464–1651.5

Fig. 2. Number of C Cells. Bars=mean value for each group.

Ultrastructure of chief cells in the parathyroid gland

In the parathyroid glands of the training group, the chief cells were oval to polygonal in shape. The plasma membranes had many folds and interdigitations with neighboring cells. The cisternae of the endoplasmic reticulum with ribosome-studded membranes were abundant in the cytoplasm, and distributed randomly or aggregated at the apical poles (Fig. 3. a). Some of the chief cells contained electron-dense secretory granules. The granules were distributed throughout the cytoplasm and had a diameter of 180–320 nm (Fig. 3. b). The cisternae of the Golgi complexes were not so well developed.

In the control group, the chief cells had more tortuous outlines and complicated interdigitations (Fig. 4. a). Golgi complexes were well developed. Large vacuolar bodies were occasionally encountered in the peripheral cytoplasm. Secretory granules with a diameter of 140–280 nm were observed near the plasma membrane (Fig. 4. b).

Radiography of the third metatarsal bone

There was a tendency for BMC and CA to be generally

lower in the training group than in the control group (Table 3), but there were no statistically significant differences. Detailed data for individual horses are listed in Table 4.

Radiographically, the transverse sections of the third metatarsi in the control group were symmetrical ellipsoid. Subperiosteal osteogenesis, seen as low-density finger-like projections, was observed all over the circumference. In the training group, the surface of the dorsolateral cortex was expanded caused by the marked osteogenesis at that site. X-ray density was higher in the control group than in the training group.

Discussion

Thoroughbreds are commonly allowed to pasture freely for their growing period. Otherwise, there are few reports on the optimal exercise intensity or amount of activity when horses are too young. In this study, there seemed to be a significant difference in the net amount of activity throughout the experiment between training and control groups. The canter distance in the training group was nearly twice that in the control group, so we tried to evaluate the difference in the net training effect, *i.e.*, the treadmill exercise effect in very young horses by comparing it to the paddock exercise. From the investigations of the number of C cells in the thyroid, the ultrastructure of the chief cells in the parathyroid and the radiography of the third metatarsal bone, we reached the conclusion that additional exercise used in this study had a suppressive effect on the thyroid and parathyroid, and possibly on the bone. This suggests an additional and/or intense exercise in early stage of life (foals to weanlings) have an adverse effect on the growth and metabolism of bone.

Calcitonin is secreted from C cells in the thyroid to inhibit an increase in serum calcium by inhibiting the osteoclast activity [5, 8]. In horses, C cells increase remarkably in the period from 20 to 30 days after birth [23]. The increase is considered to be necessary for their growing period to obtain a proper level of calcitonin and calcium. The treadmill exercise program used in this study did not lead to an increase in the number of C cells when compared to the paddock exercise. Free pasturage in a paddock is a common condition for foals to weanling horses, and the number of C cells in the paddock group might reflect normal calcitonin metabolism. It is suspected that an additional exercise had a negative effect on the calcitonin secretion in young horses.

Electron microscopic examination revealed that the chief cells of the training group are in a synthesizing stage, while those of the control group are secreting. Morphological

Fig. 3. Chief cells of the training group. a) The cisternae of granular endoplasmic reticulum are well developed (bar=1 μ m). b) Secretory granules are distributed throughout the cytoplasm (bar=1 μ m).

Fig. 4. Chief cells of the control group. a) They have tortuous outlines and complicated interdigitations. Golgi complexes (G) are well developed (bar=1 μ m). b) Secretory granules are observed near the plasma membrane (bar=1 μ m).

Table 3. Mean $\pm$ SD of bone mineral content and cortical area

	Training	Control
Bone mineral content (mmAl)	3.13 ± 0.62	3.35 ± 0.40
Cortical area (cm ²)	7.94 ± 0.69	8.24 ± 1.07

parameters of secretory activity in the parathyroid chief cells are based on the amount of the cisternae of the granular endoplasmic reticulum, Golgi complexes, secretory granules, and tortuosity of the plasma membrane [6, 22]. The chief cells of both groups are not in a stage of resting because they have the parameters mentioned above and they

Table 4. Bone mineral content calibrated as equivalent aluminum thickness and morphometrical analysis of the third metatarsal cortex

Case	Bone mineral content (mmAl)				Morphometry				
	dorsal	planter	lateral	medial	X ₁ (cm)	X ₂ (cm)	Y ₁ (cm)	Y ₂ (cm)	CA (cm ²)
1	3.55	3.44	3.71	3.44	3.5	3.1	1.6	1.5	6.63
2	2.68	1.91	2.94	2.23	3.9	3.5	1.7	1.6	8.58
3	2.65	2.13	2.61	2.16	3.7	3.4	1.4	1.5	8.23
4	3.52	3.47	3.76	3.40	3.8	3.2	1.5	1.4	7.90
5	3.57	2.85	4.02	2.59	3.6	3.4	1.2	1.2	8.48
6	3.48	2.68	3.16	3.07	3.6	3.4	1.2	1.4	8.29
7	3.20	2.31	2.58	2.77	3.4	3.5	1.5	1.6	7.46
8	3.72	3.37	3.51	3.45	4.2	3.7	1.6	1.4	10.44
9	3.93	3.37	3.59	3.56	3.5	3.3	1.3	1.3	7.74
10	4.20	3.88	4.15	3.85	3.6	3.2	1.2	1.2	7.91
11	NE	NE	NE	NE	NE	NE	NE	NE	NE
12	2.98	2.35	3.01	2.86	3.8	3.7	1.7	1.7	8.77
13	3.40	3.01	3.53	3.08	3.6	3.3	1.7	1.5	7.32
14	3.34	3.03	3.65	3.04	3.9	3.4	2.0	1.7	7.74
15	3.19	2.84	2.88	3.12	3.7	3.2	1.4	1.4	7.76

X₁; lateromedial diameter, Y₁; dorsoplantar diameter, X₂; lateromedial medullary diameter, Y₂; dorsoplantar medullary diameter, CA; cortical area, NE; not examined. Case 1–7; training group and 8–15; control group.

do not contain storage granules. Secretory protein is produced by ribosomes and transported via the endoplasmic reticulum to the Golgi complex, where it is condensed, modified, and packaged to form secretory granules [9]. Well developed granular endoplasmic reticula observed in the training group may reflect the fact that the chief cells have been synthesizing secretory protein. In the control group, findings such as well developed Golgi complexes, tortuous plasma membrane, and secretory granules at the periphery, suggest that the chief cells have been secreting. Large vacuolar bodies encountered occasionally near the plasma membrane are considered to be the manifestation of exocytosis which had been reported by Setoguchi *et al.* [18]. Parathyroid hormone (PTH), secreted from the parathyroid, plays an important role in mineral metabolism and bone remodeling [4]. Activation of the parathyroid, as observed in the control group, may be necessary for bone growth and development. It is suspected that an additional exercise would suppress PTH secretion in young horses.

There are many researches into the effect of exercise on bone metabolism in young horses, and they reported that the mechanical properties of the third metacarpal bones have increased [2, 16]. In this study, the third metatarsal bones of the training group did not show significantly high BMC and CA values ($p > 0.05$), in other words, the exercise program used in this study did not cause a positive reaction in bone morphology. The net exercise amounts in studies conducted by Bell *et al.* [2] and Raub *et al.* [16] seem to be closer to

that of the control group than the treadmill group. In addition, the canter distance in the treadmill group was nearly twice that in the control group. It is suspected that a proper amount of exercise may have a positive effect on bone metabolism, whereas additional exercise may have a negative or no effect on bone morphology. Unfortunately, we could not examine the third metacarpal bones. Generally, they have been selected as an indicator of systemic bone metabolism. It may still be difficult to discuss systemic bone metabolism only studies of the hindlimbs. Nevertheless, according to the report of Barneveld and von Weeren [1], which was studied the changes in the third tarsal bone (T3) of Dutch Warmblood foals, compact bone density of T3 was significantly lower in the box-rested foals than in both the trained and pastured group. Although they did not strongly stress the difference between the trained and pastured group, compact bone density of T3 was found to be less in the trained foals than in the pastured group. In conclusion, we consider that an additional exercise had a suppressive or no effect on bone metabolism in young horses.

We also found an interesting result in the training group. The thickness of the dorsolateral cortex was increased as a result of the osteogenesis on the transverse section of the third metatarsal bone. In addition, BMCs were higher in the dorsal and lateral cortices than those in the plantar and medial cortices (Table 4). These findings are consistent with the observations on the equine third metacarpal bone of

McCarthy and Jeffcott [12] and Sherman *et al.* [19]. Moreover, Boston *et al.* [3] reported that horses that trained in a mode resembling racing became adapted to racing without the horse developing bucked shins (periostitis of the dorsal surface of the third metacarpal bone). If the horses used in this study were adult, they would have the ability to undergo bone adaptation to high speed exercise by providing a structure more capable of resisting exercise-induced injury (*e.g.* bucked shins or fatigue fractures).

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