Changes in Serum Biochemical Markers of Bone Cell Activity in Growing Thoroughbred Horses

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ABSTRACT : We studied the changes in biochemical markers of bone metabolism in growing Thoroughbred horses. Serum osteocalcin (OC), as a marker for bone formation, and carboxy-terminal propeptide of type-I collagen (PICP), as a marker for bone formation, carboxy-terminal telopeptide of type-I collagen (ICTP), as a marker for bone resorption, were determined in nine clinically healthy horses from 3 d to 17 mo of age. The BW and withers height (WH) increased during the study. On the other hand, a rapid reduction in body weight gain (BWG) was observed between 1 mo and 9 mo of age and a rapid reduction in withers height gain was observed between 1 mo and 5 mo of age. The serum markers decreased significantly with increasing age. In particular, dramatic changes in serum markers occurred between 3 d to 1 wk and 5 to 7 mo of age in these horses, which suggests that bone turnover rapidly decreased after birth. On the other hand, the ratio of PICP to ICTP decreased through the experiment. This result suggests that the reduction in bone formation exceeded that of bone resorption. There was a significant correlation between markers and growth parameters, except for the correlation between PICP and BWG on single linear regression analysis. Serum OC and ICTP were affected by the WH in multiple linear regression analysis. These results indicated that the age-related variation in serum biochemical markers of bone metabolism reflected bone growth, but neither BW nor BWG. Therefore, we consider that changes in bone modeling are the major factor affecting the levels of serum biochemical markers by 17 mo of age in horses. (**Key Words :** Horses, Biochemical Markers Of Bone Metabolism, Body Weight, Withers Height, Growth)

INTRODUCTION

It is necessary to ensure proper bone growth and to minimize bone problems in horses because skeletal disorders result in significant morbidity and economic loss. To reduce the incidence of skeletal injuries in young horses exercised heavily (Matsui et al., 2004), it is important to monitor bone quality with noninvasive methods. Now, we can assess bone metabolism using circulating biochemical markers of bone cell activity in real time.

Recently, some researchers investigated the potential applications of markers for bone formation including osteocalcin (OC), a noncollagenous protein produced by osteoblasts and the carboxy-terminal propeptide of type-I collagen (PICP), and that for bone resorption such as the carboxy-terminal telopeptide of type-I collagen (ICTP) in horses (Price, 1998; Lepage et al., 2001).

Understanding biochemical markers of bone cell activity related to age is important to understand bone physiology and pathology in relation to nutritional status. However, there is very little information on the patterns of change in the markers within the first 2 years of life and how they may be related to physical growth measurements. The aim of this experiment was to characterize changes in serum concentrations of three biochemical markers of bone cell activity in nine young Thoroughbred horses from birth to 17 mo of age. We also investigated the relationship among serum concentrations of the three biochemical markers.

MATERIALS AND METHODS

Animals and samples

The experimental protocols for the study were reviewed

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Table 1. Changes in growth parameters during the experiment

Item	BW (kg)	BWG (kg/d)	WH (cm)	WHG (cm/d)
1 mo	103±3.0[i]	1.17±0.04[a]	111±1.2[h]	0.21±0.012[a]
3 mo	174±3.6[h]	1.05±0.04[a]	124±0.6[g]	0.17±0.008[b]
5 mo	230±4.6[g]	0.86±0.04[b]	131±0.7[f]	0.10±0.007[c]
7 mo	277±6.4[f]	0.63±0.04[cd]	135±1.0[e]	0.07±0.004[d]
9 mo	305±8.9[e]	0.43±0.04[e]	139±0.7[d]	0.06±0.004[d]
11 mo	328±9.7[d]	0.50±0.07[de]	142±0.9[c]	0.06±0.003[d]
13 mo	364±7.6[c]	0.67±0.07[c]	146±0.7[b]	0.05±0.005[de]
15 mo	409±6.9[b]	0.61±0.05[cd]	148±0.7[a]	0.04±0.005[e]
17 mo	438±8.8[a]	0.49±0.07[de]	150±0.6[a]	0.02±0.009[e]
Effect of Time	**	**	**	**

BW = Body weight, BWG = Body weight gain, WH = Withers height, WHG = Withers height gain.
** $p < 0.001$. Values are shown as the means and s.e.
[a, b, c, d, e, f, g, h, i] Values with different letters are significantly different ($p < 0.05$).

and approved by the Animal Welfare and Ethics body of the Japan Racing Association. Nine Thoroughbred horses (six colts and three fillies) were used to collect serum samples from 3 d to 17 mo of age. All animals were clinically healthy during the experiment. All horses were raised with their mothers until weaning at 6 mo of age. Additionally, creep feed, composed of 0.70% Ca and 0.38% P, was supplementary fed to all foals after 2 mo of age. After weaning, they were fed a ration providing 0.41% Ca and 0.32% P at 6 mo of age, 0.41% Ca and 0.28% P at 9 mo of age, 0.40% Ca and 0.28% P at 12 mo of age, 0.43% Ca and 0.29% P at 15 mo of age, respectively. The composition of diet was expected to maintain or exceeded the current recommendation (NRC, 1989). The sampling times were at 3 d, 1 wk, and 1 mo of age and thereafter bi-monthly. Blood samples were collected at the same time of day (09:00) from the external jugular vein into a plain tube and serum was separated by centrifugation within 2 h of sampling and stored at -80°C until assayed. Animals' BW and withers height (WH) were measured throughout the experiment. WH was measured using an altitude stick from the ground to the withers point. BW was measured on a scale (Rodeo Tech, Kubota Corporation, Japan). The body weight gain (BWG) (kg/d) and withers height gain (WHG) (cm/d) were calculated as the difference between the data obtained 2 mo before and 2 mo after except for the first month and the last month. The gains in the first month and the last month were calculated as the difference between the data obtained on the corresponding month and 2 mo before and 2 mo after, respectively. WH reflects bone length and WHG is a useful marker of skeletal development in horses (Ott and Kivipelto, 2002).

Determination of biochemical markers of bone metabolism

The concentration of OC was measured in serum samples using a commercially available RIA (Osteocalcin ^{125}I RIA Kit, DiaSorin Inc., Stillwater, MN, 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 (Patterson-Allen et al., 1982). The validity of this kit for horse OC was reported by Lepage et al. (1990). The intra- and inter-assay coefficients of variation are 5.2% and 5.9% for OC.

The concentration of PICP was measured in serum samples using a commercially available RIA (Procollagen PICP, Orion Diagnostica, Oulunsalo, Finland) with a polyclonal rabbit antibody against PICP extracted from purified human skin fibroblasts (Melkko et al., 1990), which was previously demonstrated to cross-react with horse PICP (Price et al., 1995). The validity of this kit for horse PICP was reported by Price et al. (1995). The intra- and inter-assay coefficients of variation are 4.5% and 6.6% for PICP.

The concentration of ICTP was measured in serum samples using a commercially available RIA (Telopeptide ICTP, Orion Diagnostica, Oulunsalo, Finland) with an antibody directed against ICTP extracted from decalcified human femoral bone (Risteli et al., 1993), which was previously demonstrated to cross-react with horse ICTP (Price et al., 1995). The validity of this kit for horse ICTP was reported by Price et al. (1995). The intra- and inter-assay coefficients of variation are 4.2% and 5.4% for ICTP.

Statistical analyses

Data are expressed as the means with standard errors. First, the changes in each serum biochemical marker of bone metabolism, BW, WH, BWG and WHG with age were analyzed by MIXED procedure of SAS (SAS, 1996) for a repeated measures design. The model included the repeated effect of sampling time, the effect of sex and their interaction. Because the effect of sex and their interaction were not significant ($p > 0.20$) for any parameters, the data were re-analyzed using a model including the repeated effect of sampling time alone. When the effect of sampling time was significant ($p < 0.05$), the PDIFF option (a pairwise t-test) was used to identify means that differed at $p < 0.05$.

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

Age of animal	OC (ng/ml)	PICP (ng/ml)	ICTP (ng/ml)	PICP/ICTP ratio	OC/ICTP ratio	PICP/OC ratio
3 d	63.6 ± 12.5^a	$6,130\pm442^a$	41.4 ± 4.2^a	164 ± 27^{bc}	1.71 ± 0.40^b	130 ± 23^a
1 wk	70.2 ± 9.3^a	$6,816\pm400^a$	32.6 ± 2.3^b	222 ± 25^a	2.28 ± 0.36^a	113 ± 17^{ab}
1 mo	38.3 ± 3.5^b	$4,486\pm559^b$	26.7 ± 2.0^c	174 ± 23^b	1.47 ± 0.13^b	129 ± 22^a
3 mo	34.3 ± 3.1^{bc}	$3,575\pm318^c$	23.7 ± 1.6^c	156 ± 15^{bc}	1.47 ± 0.10^b	107 ± 9^{abc}
5 mo	22.0 ± 1.0^c	$2,318\pm117^d$	18.1 ± 0.6^d	130 ± 8^{cde}	1.23 ± 0.06^b	107 ± 8^{abc}
7 mo	17.8 ± 1.3^c	$1,963\pm193^d$	13.9 ± 0.4^{de}	140 ± 12^{bcd}	1.28 ± 0.08^b	115 ± 14^{ab}
9 mo	20.0 ± 1.7^c	$1,733\pm109^{de}$	13.9 ± 0.6^{de}	126 ± 9^{cdef}	1.43 ± 0.10^b	91 ± 8^{bcd}
11 mo	20.9 ± 1.5^c	$1,621\pm102^{de}$	15.7 ± 0.9^{de}	106 ± 9^{def}	1.36 ± 0.11^b	81 ± 9^{bcd}
13 mo	18.2 ± 1.8^c	$1,597\pm158^{de}$	15.1 ± 0.5^{de}	105 ± 8^{def}	1.19 ± 0.09^b	93 ± 11^{bcd}
15 mo	18.1 ± 0.9^c	$1,200\pm78^e$	13.6 ± 0.8^e	91 ± 8^f	1.35 ± 0.07^b	68 ± 5^d
17 mo	14.6 ± 1.0^c	$1,093\pm69^e$	11.7 ± 0.6^e	95 ± 8^{ef}	1.28 ± 0.12^b	76 ± 5^{cd}
Effect of time	**	**	**	**	**	**

OC = Osteocalcin, PICP = Carboxy-terminal propeptide of type-I collagen, ICTP = Carboxy-terminal telopeptide of type-I collagen.
Values are shown as the means and s.e. ** $p<0.001$.
[a, b, c, d, e, f] Values with different letters are significantly different ($p<0.05$).

Multiple linear regression analysis was performed by GLM procedure of SAS (SAS, 1990) using the data between 1 mo and 17 mo of age. The different biochemical markers were considered as dependent variables and WH, BW, WHG and BWG were considered as covariates in the analysis. Additionally, the effect on each animal was treated as a fixed effect. It must be noted that this regression analysis was approximate because the independent variables were related to each other.

RESULTS

Growth

The changes in BW, WH, BWG and WHG are shown in Table 1. The BW and WH indicated a gradual increase during experiment ($p<0.05$).

The BWG rapidly decreased between 1 mo and 9 mo of age ($p<0.05$), then partly recovered at 13 mo of age ($p<0.05$). Thereafter, BWG decreased again ($p<0.05$).

The WHG rapidly decreased between 1 mo and 5 mo of age ($p<0.05$), then the reduction in WHG was slow and almost stable between 7 mo and 13 mo of age. Thereafter, the WHG decreased again ($p<0.05$). The WHG was extremely low (0.02 cm/d) in the last month; 10-fold less than the gain in the first month.

Biochemical markers of bone metabolism

The changes in serum markers of bone metabolism are shown in Table 2. The serum OC concentration was unchanged between 3 d and 1 wk of age and rapidly decreased between 1 wk and 1 mo of age ($p<0.05$). Then, the reduction in the OC level became slow, but significant between 1 mo and 5 mo of age. Thereafter, no significant changes in the concentration of serum OC were seen.

The changes in serum level of PICP were almost the same as those of OC. The serum PICP concentration was unchanged between 3 d and 1 wk of age and rapidly decreased between 1 wk and 5 mo of age ($p<0.05$). Then, the reduction in the PICP level became slow, but significant between 5 mo and 15 mo of age. Thereafter, no significant changes in serum PICP concentration were seen.

The serum ICTP concentration started to decrease rapidly from 1 wk of age ($p<0.05$) and continuously decreased to 5 mo of age ($p<0.05$). Then, no significant reduction in ICTP level was observed between 5 mo and 13 mo of age, but the ICTP level was significantly ($p<0.05$) lower at 15 mo of age than at 5 mo of age.

Next, we determined the ratios among serum bone markers. The ratio of bone formation markers, i.e., OC and PICP to the bone resorption marker, ICTP, increased at 1 wk of age because the bone formation markers did not change, but ICTP decreased between 3 d and 1 wk of age. Thereafter, the OC ratio decreased rapidly ($p<0.05$) up to 5 mo of age and then the ratio was almost stable between 5 mo and 17 mo of age. The PICP ratio also decreased rapidly ($p<0.05$) up to 5 mo of age and was almost stable between 5 mo and 13 mo of age. The PICP ratio slowly decreased and a significant difference was observed between 5 mo and 15 mo of age ($p<0.05$). The PICP ratio to OC was almost stable between 3 d and 7 mo of age, but significantly decreased thereafter.

Relationship between biochemical markers of bone metabolism and growth parameters

The results of multiple linear regression analysis are summarized in Table 3.

The multiple regression of OC was significant ($r^2 = 0.62$, $p<0.01$). The standard partial regression coefficient of OC to the WHG had a markedly larger value ($p = 0.08$) than the coefficient to the other parameter ($p>0.43$).

The multiple regression of PICP was significant ($r^2 = 0.81$, $p<0.01$). The standard partial regression coefficient of

Table 3. Summary of multiple linear regression analysis of serum biochemical markers of bone metabolism for growth parameters

	BW	BWG	WH	WHG
OC ($r^2 = 0.62$, p<0.01)				
Partial regression coefficient	-0.02	1.70	0.05	36.39
Standard partial regression coefficient	-0.27	0.10	0.07	0.49
p	0.441	0.432	0.877	0.078
PICP ($r^2 = 0.81$, p<0.01)				
Partial regression coefficient	-3.68	85.46	4.86	5,900.4
Standard partial regression coefficient	-0.30	0.04	0.05	0.58
p	0.230	0.680	0.885	0.004
ICTP ($r^2 = 0.73$, p<0.01)				
Partial regression coefficient	-0.01	1.91	0.05	25.26
Standard partial regression coefficient	-0.22	0.20	0.11	0.57
p	0.46	0.077	0.761	0.015

BW = Body weight; BWG = Body weight gain; WH = Withers height; WHG = Withers height gain.
OC = Osteocalcin, PICP = Carboxy-terminal propeptide of type-I collagen, ICTP = Carboxy-terminal telopeptide of type-I collagen.

PICP to the WHG had a markedly larger value (p<0.01) than the coefficient to the other parameter (p>0.23).

The multiple regression of ICTP was significant ($r^2 = 0.73$, p<0.01). The standard partial regression coefficient of ICTP to the WHG had a markedly larger value (p<0.05) than the coefficients to BW and WH (p>0.46). The regression of ICTP to BWH showed a tendency (p = 0.08), but the standard partial regression coefficient to BWH was 3-fold smaller than the coefficient to WH.

DISCUSSION

We investigated the changes in serum concentrations of biochemical markers of bone formation (OC and PICP), and of bone resorption (ICTP) in Thoroughbred horses from birth to 17 mo of age.

The concentration of OC was stably high between 3 d and 1 wk of age. Reller et al. (2003) showed that plasma OC levels increased during the first week of postnatal life in foals. We did not measure serum OC just after birth. Therefore, we might not have detected the increase in OC in the early postnatal period in this experiment. To our knowledge, this is the first report demonstrating the changes in PICP and ICTP in the early postnatal life of foals. The concentration of PICP was also stably high between 3 d and 1 wk of age, which was a similar change to the OC concentration. Davicco et al. (1994) reported that plasma OC and insulin-like growth factor-I (IGF-I) increased between birth and 1 week of age in horses and they suggested that the increase in IGF-1 stimulated bone formation in this period.

The ICTP concentration decreased between 3 d and 1 wk of age. However, the level of each bone marker in this period was the highest during the experiment, which suggests that the rates of bone formation and bone resorption are higher in the early postnatal period than in the later period.

In general, the markers of bone formation and bone resorption change in parallel during growth in horses (Price et al., 2001). However, the ratios of formation markers to resorption marker in this period were the highest during the experiment.

The highest ratios of bone-formation markers to bone-resorption marker with the highest concentration of bone markers in this period indicated the largest difference between bone formation and bone resorption. Bone formation exceeds bone resorption during longitudinal bone growth, allowing for bone modelling (Fraher, 1993). Kajantie et al. (2001) reported the ratio of ICTP to amino-terminal propeptide of type I procollagen, another serum marker of bone formation, had some potential as a clinically useful indicator of growth velocity in human children. These results suggest rapid longitudinal bone growth and bone modeling in the early postnatal period of horses.

The serum markers of bone formation and bone resorption rapidly decreased between 1 wk and 5 mo of age. Price et al. (2001) also reported a marked decrease in either serum bone formation markers, i.e., the bone-specific isoenzyme of alkaline phosphatase and OC, or a bone resorption marker, ICTP between 0 and 6 mo of age. Other studies also showed that the circulating OC level rapidly decreased during the fist few months of postnatal life (Davicco et al., 1994; Lepage et al., 1990). Black et al. (1999) reported that serum OC and urinary excretion of deoxypyridinoline, a bone resorption marker, were higher in weanlings than adult horses. The present experiment supported these previous reports. The rapid reduction in serum markers of bone formation and resorption suggests a reduction in bone turnover during this period.

A histological study showed that the rate of periosteal bone apposition was high, but that a large portion of the cortical bone consisted of primary bone, while remodeling of primary bone was not observed in horses aged less than 6 mo (Stover et al., 1992). The present experiment indicated that the WHG was rapidly decreased between 1 mo and 5 mo of age, then the rate of gain reduction slowed, which

was consistent with the report of Hiney (2004) indicating the rapid growth of the skeleton during the early stage of life and a gradual slowing thereafter as horses mature.

The present experiment showed that the ratios of OC and PICP to ICTP rapidly decreased between 1 mo and 5 mo of age, which was a similar change to the WHG. These results indicated that the difference between bone formation and bone resorption became smaller in this period, which reflected the reduction in bone modeling.

The decrease in PICP level became slow but significant between 5 mo and 15 mo of age. Additionally, the ICTP level was significantly (p<0.05) lower at 15 mo of age than in 5 mo of age. These results suggested a continuous reduction in the bone turnover rate. Horses aged 1 yr showed remodeling of the bone cortex, a marked increase in cortical porosity, and a slow periosteal bone apposition rate (Stover et al., 1992). We considered that the bone turnover rate was lower in remodeling bone than in modeling bone. Leapage et al. (1998) showed that the high ratio of OC and ICTP reflects the positive remodeling status of bone. The present experiment indicated low ratios of OC and PICP to ICTP between 5 mo and 17 mo of age, which suggested a reduction in bone remodeling. Additionally, the balance of bone formation and bone resorption probably decreases with aging in horses, which reduces the calcium requirement per kg of BW in this period.

Although the OC level did not significantly changed between 5 mo and 15 mo of age, the decrease in the PICP level became slow, but significant, in this period. Additionally, the ICTP level was significantly (p<0.05) lower at 15 mo of age than at 5 mo of age. These results suggested a continuous reduction in the bone turnover rate. Horses aged 1 yr showed remodeling of bone cortex, a marked increase in cortical porosity, and a slow periosteal bone apposition rate (Stover et al., 1992). WHG was observed, but gradually decreased after 5 mo of age in the present experiment. Therefore, the factor affecting bone marker levels probably changes from bone modeling to bone remodeling in this period.

The bone-formation markers and bone-resorption marker were unchanged between 15 mo and 17 mo of age. Additionally, the ratios of bone-formation markers to bone-resorption marker were also unchanged in this period. Because WHG was slight in this period, it is considered that bone remodeling dominantly affects the levels of bone-metabolism markers. Leapage et al. (1998) showed that the high ratio of OC and ICTP reflects the positive remodeling status of bone. It appears that the rate of bone remodeling is not largely changed unchanged between 9 mo and 17 mo of age. Lepage et al. (1998) reported that the ratio of OC to ICTP was 1.3 in Warmblood mature horses. This ratio was almost the same as the ratio in horses aged 17 mo in the present experiment. It is unlikely that bone remodeling at this age is largely different from that of mature horses.

The ratio of PICP to OC did not change between 3 d and 7 mo of age, but the ratio significantly decreased thereafter. It is not clear why these markers of bone formation showed the different changes during growth. Osteoblasts secrete PICP and OC into the circulation during bone formation (Allen, 2003), and PICP is a fragment of type I procollagen, which is removed from the carboxy-terminal end of the molecule during post-translational modification (Melkko et al, 1990). On the other hand, OC is a bone-specific protein, which is considered to regulate bone mineralization (Stein et al., 1990), though the precise role of this protein is not known (Allen, 2003).

As mentioned above, bone remodeling became dominant in the later period. The reduction in the ratio of PICP to OC suggests that the mineralization preferably induced collagen formation during bone remodeling, which was supported by the fact that remodeling makes bone compact; the organic content is smaller and ash content is higher with age (Hammett, 1925). It must be noted that type I collagen is not only found in bone, therefore, the serum level of PICP partly reflects its formation in other tissues such as skin, tendon and ligaments (Parfitt et al., 1987), which might affect the ratio of PICP to OC.

We examined the potential influence of growth parameters on serum bone markers using multiple linear regression analysis. The standard partial regression coefficient of each bone marker to the WHG was a negative value and its absolute value was markedly larger than the absolute value of the coefficient to the other parameters. Additionally the partial regression to the WHG showed a tendency (in OC) or significance (in PICP and ICTP), but the partial regression to the other parameter was not significant. These results indicated that the changes in bone markers depended on longitudinal bone growth, while BW, bone length and BWG had only a minor effect on bone markers.

BW is known to affect bone metabolism. Hassager and Christiansen (1989) reported that bone resorption showed a significant negative correlation with body weight in postmenopausal women. Ravn et al. (1999) reported that the body mass index showed a significant negative correlation with the plasma concentrations of OC and PICP in early postmenopausal women. Price et al. (2001) also reported that marker concentrations of bone formation and bone resorption decreased with increasing body weight in horses from birth to 18 mo of age, and that heavier horses had lower concentrations of the markers at any given age. Although they mentioned the possible effect of bone growth on bone markers, they did not measure any bone growth parameters in their experiment. The present experiment suggests that bone growth is the major factor affecting bone metabolism in horses aged between 1 mo and 17 mo, which means that bone turnover decreases with bone growth. The present experiment indicated the changes in serum

concentrations of the biochemical markers in healthy horses during growth. We consider that the determination of these markers contributes the prediction and the diagnosis of bone diseases in growing horses by comparison with the data indicating the present experiment.

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