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Change of Ferritin-binding Activity in the Serum of Foal after Birth

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In mammal circulation, various ferritin-binding proteins (FBPs) are thought to be involved in the clearance of circulating ferritin after complex formation with it. However, horse FBPs are known to cause inhibitory effects on ferritin immunoassay due to the concealment of the ferritin molecule to anti-ferritin antibodies used in the ferritin immunoassay. These inhibitory effects are eliminated by heat treatment of horse serum at 75℃ for 15 min. The inhibitory effects on ferritin immunoassay in the sera of ten foal sera (5 females and 5 males) from 1 to 18 months were detected during all periods, and ferritin concentrations of the foal sera increased 20–100% as compared with those of untreated sera by same heat treatment. Ferritin concentrations of heat-treated foal sera increased after birth, reaching to ferritin levels of adult horse at 9 months of age. Thereafter, although serum ferritin concentrations fell down at 12 months of age, these concentrations increased to adult levels at 15 months of age again. The ratio of ferritin concentration of heat-treated serum to that of the untreated serum was regarded as an apparent ferritin-binding activity. Ferritin-binding activities in the sera of foals showed peak at 2 and 4 months of age in females and males, respectively. These results suggested that horse FBPs were heat unstable, and FBPs may play an important role in iron metabolism at early developmental stage.

Key words: *foal, ferritin, ferritin-binding protein, heat treatment, serum ferritin concentration*

Ferritin is a ubiquitous and conserved iron storage protein with a molecular mass of 500 kDa to store maximum 4,500 iron atoms [4, 16, 22]. It has dual function to store iron in bioavailable and non-toxic forms because iron produces a highly toxic hydroxyl radical through Fenton reaction [16, 22]. Tissue ferritin is composed of 24 subunits of distinct types of subunits termed H (heart type) and L (liver type) chains [4, 16, 22]. H and L subunits have different physiological properties [4, 6, 11, 16, 18]; the H subunit has ferroxidase essential for iron uptake, while the L subunit does not have ferroxidase, but is involved in more iron uptake by providing iron nucleation, and physiochemical stability [4, 6, 11, 16, 18].

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In normal human, equine, bovine, porcine, canine and feline sera, ferritin is found in relatively low concentrations (< 1 μg ml^{-1}), and ferritin levels are positively correlated with body iron reserves [1–3, 9, 20, 21, 24]. A variety of ferritin-binding proteins (FBPs) in mammalian serum and/or plasma have been described: H-kininogen in human serum [23], alpha-2-macroglobulin in rabbit [19] and horse [8] serum, autoantibodies in horse [10], bovine [12], canine [25] and feline [17] serum, and fibrinogen in horse plasma [15]. These FBPs may be involved in the clearance of circulating ferritins following complex formation with it [8, 16, 25].

Inhibitory effects of horse and bovine sera on ferritin immunoassay have been reported, suggesting that FBPs conceal epitopes of the ferritin molecule to anti-ferritin antibodies used in ferritin immunoassay [12, 13]. These inhibitory effects were eliminated by heat

treatment (75°C, 15 min) or by an increase in ionic strength of the serum, probably due to dissociation of FBPs from ferritin molecules, leaving ferritin intact [12, 13]. Furthermore, these treatments resulted in increase of serum ferritin concentrations and improvement of recovery of ferritin added to serum [12, 13]. Horse fibrinogen is a plasma specific FBP which binds ferritin and inhibits ferritin immunoassay [15]. Horse serum also contains alpha-2-macroglobulin [8] and anti-ferritin autoantibodies (IgG, IgM, and IgA) [10] as FBPs. However, affinity-purified anti-ferritin autoantibodies did not cause inhibitory effect on ferritin immunoassay [10] due to lower affinity for ferritin of them than that of anti-ferritin antibody used in ferritin immunoassay. At present, although FBPs were shown to be heat unstable as described in [13], it remains to be clarified how FBPs form complex with circulating ferritin mutually or alone in blood circulation.

The increase of ferritin concentrations may depend on the amount and nature of FBPs. In this study, the changes of ferritin-binding activities of foals sera after birth were examined without the effect of fibrinogen as a plasma specific FBP because fibrinogen changes into fibrin at blood coagulation and fibrin has no longer ferritin-binding activity [15].

Ten foals used in this study were housed in individual stables with grass supplemented by high-quality hay and concentrated supplement and kept at Taihei farm (Hachinohe-city, Japan). Peripheral blood samples were collected from the jugular vein of horses. Ten foals (5 females and 5 males) were drawn blood at 1, 2, 3, 4, 5, 6, 9, 12, 15, and 18 months of age except for one female and 2 males at 12 months of age. Serum was obtained by centrifuging coagulated blood and was kept at 4°C in the presence of 0.1% sodium azide until analysis.

Ferritin monomers were purified from commercial horse spleen ferritin (Sigma, St Louis, MO, USA) as described previously [13]. Ferritin protein determination was carried out according to the method of Lowry *et al.* [7] using bovine serum albumin (Boeringer Mannheim, Germany) as the standard.

The measurement of ferritin concentration in serum was carried out by sandwich ELISA to according to the procedure described previously [13]. The concentrations of the affinity purified anti-horse spleen ferritin antibody and its alkaline-phosphatase (ALP) conjugate were 100 ng ml^{-1} and 250 ng ml^{-1}, respectively. To eliminate inhibitory effect of horse serum on ferritin immunoassay [13], serum diluted 11-fold with phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) containing 0.1% Tween 20 and 0.1% gelatin (ELISA buffer) was heat-treated (75°C, 15 min) followed by centrifugation (24,000 × g, 20 min), and resultant supernatant was subjected to sandwich ELISA with horse spleen ferritin standards (1.6–100 ng ml^{-1}) in ELISA buffer. Data are expressed as the mean ± SE of four measurements. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's test. A p-value below 0.05 was considered statistically significant.

Apparent serum ferritin concentrations increased 20–100% by heat treatment (Fig. 1B). In this study, the ratio of ferritin concentration of heat-treated sera to those of untreated sera was considered as the total degree of ferritin-binding activities depending on the amount and nature of FBPs. Ferritin concentrations of heat-treated sera in 10 foals (5 females and 5 males) increased until 9 months of age, both reaching adult horse level (Fig. 1A). Serum ferritin concentrations of female and male foals decreased at 12 months of age, but returned to adult level at 15 months of age. Ferritin-binding activities in foal sera increased after birth, showing peak at 2 and 4 months of age in female and male foals, respectively (Fig. 1B) although those of male sera showed a biphasic peak pattern at an early stage of development. From 12 months of age, ferritin-binding activities of foal sera are constant, those of male foals being a bit higher than those of female foals. Additionally, after reaching adult level of ferritin concentrations, clearance rate of circulating ferritin may be constant regardless of ferritin levels. Further study is necessary to clarify this hypothesis.

No significant sex difference was observed in ferritin concentrations. A drop of ferritin concentrations in female and male sera may be due to a demand of iron to meet body development [5]. No significant sex difference was observed in ferritin-binding activities. The highest ferritin-binding activity of male and female foal sera was observed at early developmental stage. Although serum ferritin is relatively present at low concentration (<1 μg ml^{-1}) [1–3, 9, 20, 21, 24], circulating ferritin is most likely to be rapidly removed from the circulation ($T_{1/2}$<10 min) [14, 16]. There are two cellular ferritin uptake pathways; direct uptake by ferritin receptors and indirect uptake of ferritin-FBPs complex through receptors for FBPs [8, 16, 25]. High serum ferritin and iron concentrations and total iron binding capacity (TIBC) from at 1 to 3 days of age

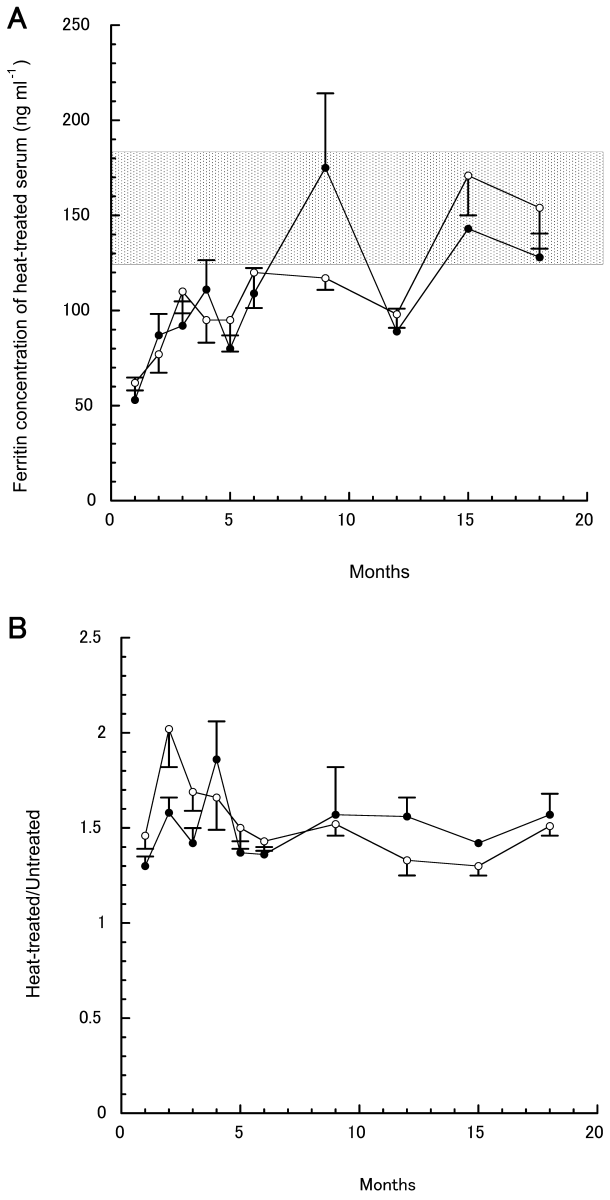

Fig. 1. Change of ferritin concentrations (A) and ferritin-binding activities (B) in ten foal sera (5 females: ○; 5 males: ●) after birth. A) Foal serum diluted 11-fold with ELISA buffer was heat-treated at 75°C for 15 min, and centrifuged at 12,000 × g for 15 min. Resultant supernatant was subjected to Sandwich ELISA. Shaded area represents mean ± SE for ferritin concentrations of healthy horses [21]. B) Ferritin concentrations of foal serum were measured by sandwich ELISA without heat treatment as described above. Individual ferritin-binding activity was measured by dividing ferritin concentrations of untreated foal serum into those of heat-treated foal serum. Each value represents mean ± SE of 5 individual samples except for data of one female serum and two males at 15 months of age.

reflect resulting from increased demand for rapid growth [5]. Transferrin turn over is important for iron supply in tissue iron uptake [14]. Additionally, canine serum ferritin has been expected to play a physiological role as an iron transporter due to its rapid clearance and higher iron content [14]. Measurements of clearance rate of circulating ferritin and serum ferritin iron content may provide indirect evidence of the involvement in iron metabolism in horse blood by ferritin receptors and/or FBPs.

Foal needs iron supply for rapid growth concomitant with increase of serum ferritin concentrations [5]. Interestingly, high ferritin-binding activity was observed in early stage of development, suggesting the existence of correlation between body growth and ferritin concentrations or ferritin-binding activity. Although further studies are necessary to clarify the involvement of iron supply by rapid clearance of circulating ferritin by FBPs, this study suggested that FBPs may play a important role in iron metabolism during foal growth. Ferritin-binding activities were still observed even after reaching to adult serum ferritin levels. Rapid clearance of circulating ferritin was also expected to contribute to the protection of oxidative stress by ferritin iron in blood stream [16].

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