—ORIGINAL—

A Study on the Presence of Ferritin-binding Proteins in Fetal Horse Plasma

Masafumi HASHIMOTO[1], Yasuo NAMBO[2], Takashi KONDO[3], Kiyotaka WATANABE[1] and Koichi ORINO[1]*

[1]Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Kitasato University, Aomori 034-8628, [2]Equine Science Division, Hidaka Training Research Center, Japan Racing Association, 535–13 Nishicha, Urakawa-cho, Urakawa-gun, Hidaka, Hokkaido 057-0171, [3]Epizootic Research Center, Equine Research Institute, Japan Racing Association, 1400–4 Shiba, Shimotsuke-shi, Tochigi 329-0412, Japan

J. Equine Sci.
Vol. 22, No. 1
pp. 1–7, 2011

In mammal circulation, ferritin-binding proteins (FBPs) are thought to be involved in clearance of circulating ferritin after complex formation with it through receptor-mediated uptake. However, there is no report on fetal FBP in fetal circulation. Although iron concentrations of fetal horse plasma were higher than those of adult horse plasma, plasma ferritin concentrations and ferritin-binding activities were found to be significantly lower in fetus than in adult. FBPs were purified from fetal or adult horse plasma on horse spleen ferritin-Sepharose 4B affinity column. Partially affinity-purified fetal horse plasma FBPs were mainly separated into 65 and 41 kDa bands in addition to minor bands with higher molecular masses ranged from 102 to 140 kDa on SDS-PAGE under reducing condition. The adult horse plasma FBPs were separated into 74, 54 and 28 kDa bands, and the 74 and 54 kDa bands reacted with antibodies specific for horse IgM and IgG heavy chains, respectively, by immunoblotting analyses. On the other hand, no antibodies to horse immunoglobulin classes detected any bands in fetal horse plasma FBPs. The affinity-purified adult and fetal horse plasma FBPs did not contain fibrinogen as a plasma specific FBP, probably due to its lower affinity to the ligand ferritin. These results demonstrate the presence of FBPs which are different from adult horse plasma FBPs including anti-ferritin autoantibodies in fetal plasma.

Key words: *adult horse*, *autoantibody*, *fetal horse*, *ferritin*, *ferritin-binding protein*

Ferritin is an iron storage protein with a molecular mass of 500,000 Da to accommodate up to maximum 4,500 iron atoms and is found in all tissues [6, 21, 27]. It has dual function to store iron and to segregate iron from iron-catalyzed reactive oxygen species production [6, 21]. Tissue ferritin is a 24-mer protein composed of various proportions of two types of subunits termed H (heart type) and L (liver type) chains [6, 21, 27]. H and L chains have distinct physiological properties [6, 11, 12, 21]; the H chain has ferroxidase essential for iron uptake, while the L chain does not have ferroxidase, but promotes iron core growth inside the ferritin shell by providing iron nucleation sites [6, 17, 21].

This article was accepted October 6, 2010
*Corresponding author. e-mail: orino@vmas.kitasato-u.ac.jp

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 [2–4, 25, 26, 29]. The iron/protein ratios of serum ferritin in human (0.02–0.07)[5, 33], canine (0.03–0.116)[31] and rat (0.02 ± 0.008)[32] are much lower than those of tissue ferritins with mean value of 0.2 [6, 31]. A variety of ferritin-binding proteins (FBPs) in mammalian serum and/or plasma have been described: H-kininogen in human serum [28], alpha-2-macroglobulin in rabbit [24] and horse [14] sera, autoantibodies in horse [16], bovine [18], canine [30] and feline [23] sera, and fibrinogen in horse plasma [20]. These FBPs may be involved in the clearance of circulating ferritins following complex formation with

it [14, 21, 24]. In mammals, although alpha-2-macroglobulin is known to be a common FBP [14, 24], we proposed that anti-ferritin autoantibodies are mammalian common FBP [21].

In commercial fetal bovine sera, high levels of serum ferritin and it's iron content were measured, these values being much higher than those of adult bovine serum and same level as bovine tissue ferritin, respectively [8]. Fetal bovine serum ferritin may play a physiological role as iron transporter [8], although the existence of FBPs in fetal bovine serum has been not revealed yet. However, there has been no report on fetal mammal FBPs because of the difficulties to get fetal animal blood.

Fortunately, we obtained an opportunity to get six fetal horse plasma samples between 130 and 315 days of gestation. The purpose of our study is to reveal the presence of FBPs in fetal horse plasma and examine their biochemical properties.

Materials and Methods

Blood

Peripheral blood samples were collected from the jugular vein of Thoroughbred horses kept at Kitasato University. Six male horses (age 12–23 years, body weight 480–550 kg) used in this study were housed in individual stables with grass supplemented by high-quality hay and concentrated supplement. Plasma was obtained by centrifuging heparinized blood and was kept at 4°C in the presence of 0.1% sodium azide until analysis. Fetal blood samples from 130 to 315 days of gestation were available from previous research project which was conducted from 1995 to 2000 at the Equine Research Institute, JRA. Pregnant mares were killed by intravenous administration of a normal dose of xylazine followed by an overdose of a mixture of thiopental sodium and suxamethonium chloride. The fetuses were removed after euthanasia. The fetal blood samples were collected by cardiac puncture, and infused into plastic tubes containing heparin (20 IU ml^{-1}), then kept in ice and centrifuged at 1,800 × g for 10 min at 4°C. Plasma obtained was stored at –20°C until measured. All procedures were performed with permission of the experimental animal management of the Equine Research Institute.

Horse spleen ferritin

Ferritin monomers were purified from commercial horse spleen ferritin (Sigma, St Louis, MO, USA) as described previously [19].

Protein measurement

Protein concentration was measured according to the method of Lowry *et al.* [13] using bovine serum albumin (Boeringer Mannheim, Germany) as the standard.

Plasma iron and ferritin concentrations

Plasma iron concentration was spectrophotometrically determined using ferrozine according to the method described previously [1]. The determination of ferritin was carried out by sandwich ELISA to according to the procedure described previously [19]. Briefly, the concentrations of the affinity purified anti-horse spleen ferritin antibody and the alkaline-phosphatase (ALP) labeled anti-horse spleen ferritin antibody were 100 ng ml^{-1} and 250 ng ml^{-1}, respectively. To eliminate inhibitory effect of horse plasma on ferritin immunoassay [19], adult or fetal plasma 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 was heat-treated (75°C, 15 min) followed by centrifugation (24,000 × g, 20 min), and resultant supernatant was subjected to sandwich ELISA.

Ferritin-binding activity

Plasma ferritin-binding activity was measured essentially according to the method described previously [20]. Briefly, 100 μl of horse plasma diluted 10,000-fold with PBS was added to each well of an Immuno Plate Maxisorp F96 microtiter plate (Nunc, Roskilde, Denmark) and the plate was kept overnight at 4°C. The horse plasma protein-coated plate was incubated with horse spleen ferritin (500 ng/well) in PBS containing 0.1% gelatin and 0.1% Tween 20 followed by ALP-rabbit anti-horse spleen ferritin antibody (250 ng/well) [19]. The enzyme reaction was performed as described previously [19].

Purification of plasma FBPs by affinity chromatography

Adult or fetal horse plasma (1–2 ml) was applied to a column (2 × 3.5 cm) prepared by coupling the horse spleen ferritin (10 mg) to 10 ml of CNBr-activated Sepharose 4B (Pharmacia Biotech, Tokyo, Japan) equilibrated with PBS. The column was washed with

Table 1. Iron and ferritin concentrations in fetal horse plasmas

No.	Sex	Gestational age (days)	iron ($\mu g/ml^{-1}$)	ferritin (ng/ml^{-1})
1	M	130	1.48	18
2	M	155	2.90	22
3	NE	185	3.17	37
4	F	260	1.64	16
5	F	270	2.46	35
6	M	315	2.37	13
Mean ± SD			2.34 ± 0.67	24 ± 10

NE: not examined.

PBS until the absorbance of the effluent at 280 nm was less than 0.01. PBS containing 3 M KSCN (pH 7.0) was used as the elution buffer and the absorbance of each fraction was measured at 280 nm. Peak fractions were dialyzed by PBS. Pooled fractions collected from fetal or adult horse plasma samples were concentrated with Centriplus YM-50 (Millipore Corp., Billerica, MA, USA) and used as partially affinity-purified samples.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Affinity-purified adult and fetal horse FBPs were separated by SDS-PAGE using a 4.5% polyacrylamide stacking gel and a 10% polyacrylamide running gel according to the method of Laemmli [10]. Immunoblot analysis was carried out to detect horse immunoglobulin classes according to a previously described procedure [16]. ALP conjugated polyclonal antibodies specific for horse IgM and IgA heavy chains (Bethyl Laboratories, Montgomery, TX, USA) and horse IgG Fc fragment (Rockland Immunochemicals, Gilbertsville, PA, USA) were used as probe. ALP-labeled antibodies bound on the membrane were detected using 100 mM Tris/HCl (pH 9.5) containing 5 mM $MgCl_2$, 0.4 mM nitro blue tetrazolium, and 0.4 mM 5-bromo-4-chloro-3-indolylphosphate disodium salt 1,5-hydrate as described previously [16].

Results

Iron and ferritin concentrations and ferritin-binding activity in adult and fetal horse plasma

Iron and ferritin concentrations of six fetal horse plasmas were measured in the gestation age ranged from 130 to 315 days (Table 1). Iron concentrations in six fetal plasmas (Mean ± SD: 2.34 ± 0.67 $\mu g\ ml^{-1}$) were higher than those of six adult horse plasmas (1.9 ± 0.30 $\mu g\ ml^{-1}$) (Fig. 1A). Remarkable lower plasma ferritin concentrations were found in fetal (24 ± 10 $ng\ ml^{-1}$) than in adult horse (239 ± 90 $ng\ ml^{-1}$) (Fig. 1B). Ferritin-binding activity was also found in both of fetal and adult horse plasma, and ferritin-binding activities of fetal plasmas were significantly lower than those of adult plasmas (Fig. 1C).

Characterization of fetal horse FBPs

For structural characterization of horse plasma FBPs, partially affinity-purified adult and fetal horse plasma FBPs were run against commercial horse fibrinogen as plasma specific FBP [20] and maker proteins on SDS-PAGE under reducing condition (Fig. 2A). Partially purified fetal FBPs were separated into 65 and 41 kDa bands in addition to several bands with higher molecular masses ranged from 102 to 140 kDa, whereas partially affinity-purified adult horse plasma FBPs were separated into 74, 54 and 28 kDa under the same condition. Horse fibrinogen was separated into 74, 60 and 54 kDa bands as Aα, Bβ and γ chains, respectively. Immunoblotting analyses showed that 74 and 54 kDa bands detected in the adult horse plasma FBPs reacted with antibodies to horse IgM and IgG heavy chains, respectively, although antibody to IgM heavy chain cross-reacted with IgG heavy chain (Fig. 2B). Anti-IgA antibody did not detect any band in adult horse FBPs in contrary to previous data [16]. On the other hand, antibodies to horse immunoglobulin classes did not react with any band of fetal plasma FBPs. In addition, the corresponding bands with all three bands (Aα, Bβ and γ chains) detected in horse plasma specific fibrinogen were not observed in fetal and adult horse plasma FBPs, suggesting that the purified fetal and adult horse plasma FBPs did not contain fibrinogen.

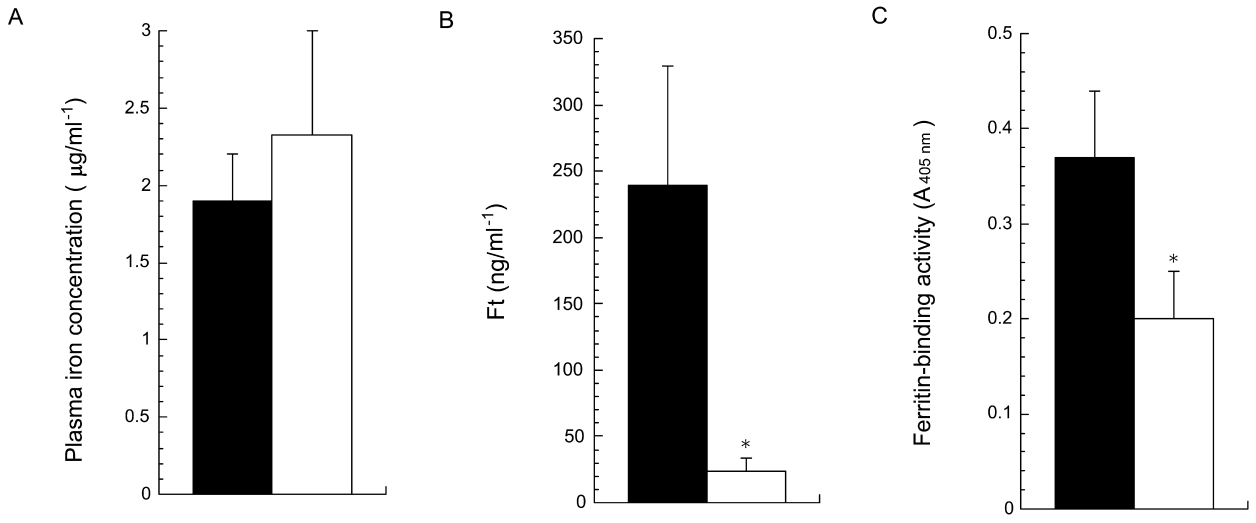

Fig. 1. Iron (A) and ferritin (B) concentrations, and ferritin-binding activity (C) in adult and fetal horse plasma.
Iron and ferritin concentrations in adult (closed bar) and fetal (open bar) horse plasma were determined by ferrozine reagent and sandwich ELISA as described in "Materials and Methods". In ferritin-binding activity, a 100 μl of 10,000-fold diluted horse plasma with PBS was added to each well of microtiter plate and kept overnight at 4°C followed by addition of 100 μl of horse spleen ferritin (5 μg ml^{-1}). The ferritin bound to the well was detected with ALP-labeled anti-horse spleen ferritin antibody. Data indicate mean ± SD of respective six horse plasma samples. *p<0.01, compared with adult horse.

Discussion

Because of high levels of ferritin and it's iron content in fetal bovine serum, fetal ferritin may be involved in the iron transporter [8]. On the other hand, various FBPs identified in mammalians provide additional means for the receptor-mediated uptake of circulating ferritin [21, 24]. However, any fetal FBP as well as fetal bovine serum and/or plasma has not been purified yet. Although it is very difficult to get fetal blood samples of large animals such as cattle and horse, we got a good opportunity to obtain valuable fetal horse plasma samples. The purpose of this study is to reveal the presence of fetal horse FBPs and their biochemical properties.

Although plasma iron concentrations of six fetal horse plasmas between 130 and 315 days of gestation age were higher than those of adult horse plasmas, the differences were not significant. This may explain that iron is actively transported from the mother to the fetus [9]. Although serum iron concentration was markedly higher at birth, but rapidly decreases at normal level of adult at 3 days old [7]. However, plasma ferritin concentrations in fetal horses were much lower than those in adult horses ranged from 141 to 340 ng ml^{-1} (239 ± 90 ng ml^{-1}). Serum ferritin concentrations (85 ± 8 ng ml^{-1}) for foals were lower than those for adult at birth, but transiently increases near normal levels (159 ± 11 ng ml^{-1}) of adult after taking colostrums with high levels of ferritin (354 ± 44 ng ml^{-1}) [7], although biochemical characterization of colostrum ferritin has not been performed yet. The lack of correlation between serum iron concentration and storage iron was observed in foal and adult horses [7], and serum ferritin level can be good indicator of iron stores from fetal stage to adult stage including foal development.

We developed ferritin-binding assay using plasma protein-coated microtiter plate as described previously [20]. Ferritin-binding activity was observed in both of fetal and adult horse plasmas, and the binding activities were significantly lower in fetal than in adult. The levels of plasma iron and ferritin concentrations and the ferritin-binding activities showed similar predictive biphasic changes between 130 and 315 days of gestation with concomitant sharp drop in around 250 days of gestation (data not shown), suggesting increasing growth of horse fetus. Eventually, fetal growth in maternal thoroughbred horse is about double size between 220 and 279 days of gestation [22]. However,

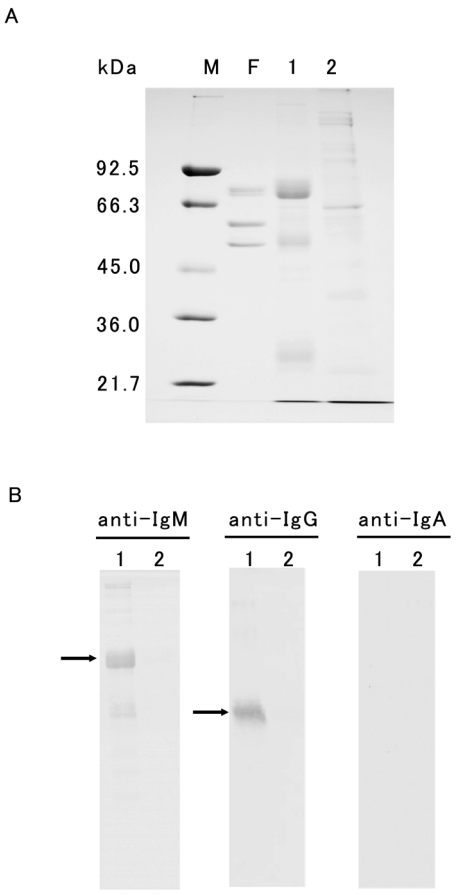

Fig. 2. Separation by SDS-PAGE (A) and immunoblotting (B) of partially purified adult and fetal horse plasma FBPs. A) Partially purified adult (5 μg, lane 1) and fetal (5 μg, lane 2) horse plasma FBPs were run against commercial horse fibrinogen (F, 3 μg) and marker proteins (M, 1 μg each): phosphorylase b (92.5 kDa), serum albumin (66.3 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (36.0 kDa) and adenylate kinase (21.7 kDa). B) Partially purified adult horse plasma FBPs (lane 1, 2 μg) and fetal horse plasma FBPs (lane 2, 2 μg) were detected with ALP-conjugated polyclonal antibodies specific for horse IgM, IgG and IgA heavy chains. Arrows indicate bands detected with the specific antibodies used.

this study provides only preliminary data, and further study is needed on larger group to examine correlation between fetal development and growth and iron metabolism.

To our knowledge, although it is the first report that horse plasma fibrinogen was identified as an FBP which bound ferritin and inhibit ferritin immunoassay, neither affinity-purified fetal nor adult horse plasma FBPs contain fibrinogen, probably due to it's lower affinity to affinity column as in alpha-2-macroglobulin [16]. However, further study need to examine whether fetal fibrinogen has ferritin-binding activity and inhibitory effect of ferritin immunoassay or not, using the alternative method. Additionally, immunoblot analysis showed that adult horse plasma FBPs contained IgM and IgG autoanibodies to ferritin, but not IgA contrary to previous data [16]. Eventually, IgA heavy chain as anti-ferritin autoantibody was only faintly detected by immunoblot analysis as described before [16] because of the low content in horse serum. This can explain differences of IgA content depending on individual condition. In this study, the attempt to identify fetal horse FBPs was unsuccessful, and anti-ferritin autoantibodies were not detected in fetal horse plasma FBPs although anti-ferritin autoantibodies are thought to be mammalian common FBP [21]. However, some FBPs are species-specific as in H-kininogen, fibrinogen, and unidentified FBP found in human [28], horse [21], and feline [23], respectively. Fetal horse FBPs might be species-specific and fetal specific FBP. It also remains to elucidate physiological role of FBSs in fetal circulation.

Acknowledgements

This study was partially supported by a Grant for Scientific Research (No. S1007) from the School of Veterinary Medicine, Kitasato University, Japan.

References

1. Abe, T., Kinda, T., Takano, Y., Chikazawa, S., Higuchi, M., Kawasaki, N., Orino, K., and Watanabe, K. 2006. Relationship between body iron stores and diquat toxicity in male Fischer-344 rats. *Biometals* **19**: 651–657.
2. Addison, G.M., Beamish, M.R., Hales, C.N., Hodgkins, M., Jacobs, A., and Llewellin, P. 1972. An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. *J. Clin. Pathol.* **25**: 326–329.
3. Andrews, G.A., Chavey, P.S., and Smith, J.E. 1994. Enzyme-linked immunosorbent assay to measure serum ferritin and the relationship between serum ferritin and nonheme iron stores in cats. *Vet. Pathol.* **31**: 674–678.
4. Andrews, G.A., Smith, J.E., Gray, M., Chavey, P.S.,

and Weeks, B.R. 1992. An improved ferritin assay for canine sera. *Vet. Clin. Pathol.* **21**: 57–60.

5. Cragg, S.J., Wagstaff, M., and Worwood, M. 1981. Detection of a glycosylated subunit in human serum ferritin. *Biochem. J.* **199**: 565–571.
6. Harrison, P.M., and Arosio, P. 1996. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta* **1275**: 161–203.
7. Harvey, J.W., Asquith, R., Sussman, W.A., and Kivipelto, J. 1987. Serum ferritin, serum iron, and erythrocyte values in foals. *Am. J. Vet. Res.* **48**: 1348–1352.
8. Kakuta, K., Orino, K., Yamamoto, S., and Watanabe, K. 1997. High levels of ferritin and its iron in fetal bovine serum. *Comp. Biochem. Physiol.* **118A**: 165–169.
9. Kumar, A., Rai, A.K., Basu, S., Dash, D., and Singth, J.S. 2010. Cord blood and breast milk iron status in maternal anemia. *Pediatrics* **121**: e673–e677.
10. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
11. Levi, S., Salfeld, J., Franceshinelli, F., Cozzi, A., Dorner, M.H., and Arosio, P. 1989. Expression and structural and functional properties of human ferritin L-chain from *Escherichia coli*. *Biochemistry* **28**: 5179–5184.
12. Levi, S., Yewdall, S.J., Harrison, P.M., Santambrogio, P., Cozzi, A., Rovida, E., Albertini, A., and Arosio, P. 1992. Evidence that H- and L-chains have co-operative roles in the iron-uptake mechanism of human ferritin. *Biochem. J.* **288**: 591–596.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
14. Massover, W.H. 1994. α_2-Macroglobulin: a ferritin-binding protein. *Ann. N. Y. Acad. Sci.* 737: 468–471.
15. Miyata, Y., and Furugouri, K. 1987. The relationship between serum ferritin concentration and tissue non-heme iron or tissue ferritin in dairy cattle. *Jpn. J. Vet. Sci.* **49**: 1157–1159.
16. Orino, K., Hamada, S., Hashimoto, M., Nambo, Y., Kondo, T., and Watanabe, K. Identification of horse anti-ferritin antibody. *J. Equine Sci.* **17**: 113–119.
17. Orino, K., Harada, S., Natsuhori, M., Takehara, K., and Watanabe, K. 2004. Kinetic analysis of bovine spleen apoferritin and recombinant H and L chain homopolymers: iron uptake suggests early stage H chain ferroxidase activity and second stage L chain cooperation. *Biometals* **17**: 129–134.
18. Orino, K., Ishiji, T., Yamamoto, S., and Watanabe, K. 2004. Characterization of bovine serum ferritin-binding proteins. *Comp. Biochem. Physiol.* **137A**: 375–381.
19. Orino, K., Saji, M., Ozaki, Y., Ohya, T., Yamamoto, S., and Watanabe, K. 1993a. Inhibitory effect of horse serum on immunoassay of horse ferritin. *J. Vet. Med. Sci.* **55**: 45–49.
20. Orino, K., Yamamoto, S., and Watanabe, K. 1993b. Fibrinogen as a ferritin-binding protein in horse plasma. *J. Vet. Med. Sci.* **55**: 785–787.
21. Orino, K., and Watanabe, K. 2008. Molecular, physiological and clinical aspects of the iron storage protein ferritin. *Vet. J.* **178**: 191–201.
22. Platt, H. 1978. Growth and maturity in the equine fetus. *J. Royal Soc. Med.* **71**: 658–661.
23. Sakamoto, H., Kuboi, T., Nagakura, T., Hayashi, S., Hoshi, F., Mutoh, K., Watanabe, K., and Orino, K. 2009. Characterization of feline serum ferritin-binding protein as an inhibitory factor in feline ferritin immunoassay. *Biometals* **22**: 793–802.
24. Santambrogio, P., and Massover, W.H. 1989. Rabbit serum alpha-2-macroglobulin binds to liver ferritin: association causes a heterogeneity of ferritin molecules. *Br. J. Haematol.* **71**: 281–290.
25. Smith, J.E., Moore, K., Boyington, D., Pollman, D.S., and Schoneweis, D. 1984. Serum ferritin and total iron-binding capacity to estimate iron storage in pigs. *Vet. Pathol.* **21**: 597–600.
26. Smith, J.E., Moore, K., Cipriano, J.E., and Morris, P.G. 1984. Serum ferritin as a measure of stored iron in horses. *J. Nutr.* **114**: 677–681.
27. Theil, E.C. 1987. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem.* **56**: 289–315.
28. Torti, S.V., and Torti, F.M. 1998. Human H-kininogen is a ferritin-binding protein. *J. Biol. Chem.* **273**: 13630–13635.
29. Walters, G.O., Miller, F.M., and Worwood, M. 1973. Serum ferritin concentration and iron stores in normal subjects. *J. Clin. Pathol.* **26**: 770–772.
30. Watanabe, K., Hayashi, K., Miyamoto, T., Tanaka, M., Okano, S., and Yamamoto, S. 2000a. Characterization of ferritin and ferritin-binding proteins in canine serum. *Biometals* **13**: 57–63.
31. Watanabe, K., Muranishi, N., Murata, Y., Orino, K., Okano, S., and Yamamoto, S. 2000b. Biochemical properties of canine serum ferritin:

iron content and nonbinding to concanavalin A. *Biometals* **13**: 319–324.

32. Watanabe, K., Yamashita, Y., Ohgawara, H., Sekiguchi, M., Satake, N., Orino, K., and Watanabe, K. 2001. Iron content of rat serum ferritin. *J. Vet. Med. Sci.* **63**: 587–589.
33. Worwood, M., Dawkins, S., Wagstaffs, M., and Jacobs, A. 1976. The purification of ferritins from human serum. *Biochem. J.* **157**: 97–10.