VITAMIN D-BINDING PROTEIN ISOFORMS AND THEIR CONCENTRATION IN SERUM OF MARES AND THEIR OFFSPRING

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Abstract

The study was conducted on 22 Thoroughbred mares and their progeny for four foaling seasons. Blood from the mares was sampled in 36th-48th h after delivery. Blood of the foals was sampled in the course of delivery and in 3rd-4th week of life. Results indicate that serum vitamin D-binding protein (DBP) concentration did not differ in the mares and in their newborns and 3-4-week-old foals, whose DBP had F, and FS phenotypes. It was concluded that phenotype of DBP is not related with serum DBP concentration.

Key words: mares, foals, vitamin D-binding protein, phenotype.

Vitamin D-binding protein (DBP), also called Gc protein, exerts a pleiotropic action. It plays a role as an actin scavenger, preventing an accumulation of fibrillar actin and counteracting the formation of thrombi, is a carrier of vitamin D3 and its derivatives, and has the capacity to bind monounsaturated and saturated fatty acids (5, 7, 13, 23, 26). It also binds to the surface of several cells and enhances chemotactic activity of C5a and C5a des Arg of the complement (7, 13, 23, 26). Following removal of a galactose and sialic acid residues, it acts as a macrophage activating factor (DBP-MAF).

Horse serum contains two isoforms of DBP which could be disclosed in PAGE as fast anodal (F) and slow (S) band (24, 25). DBP types are controlled by two codominant autosomal alleles, F and S, at a single locus, therefore there are three possible phenotypes of this protein to a given horse: F, FS, or S (10). Previous studies presented by Ouragh et al. (18) showed a new variant of DBP designated as D. Authors observed six horses of the Barb breed and five of Arab x Barb crossbreeds with DF phenotype of DBP. D variant of DBP is probably controlled by codominant allele D, at the DBP locus and D allele is the least frequent in horses.

In some clinical situations in humans, DBP phenotype can be considered as a predisposition factor to some disorders. Humans express three phenotypes of DBP described as Gc1F, Gc1S, and Gc2 (13). Studies of Ishii et al. (8) and Ito et al. (9) indicate that unlike the Gc2-allele, the homozygous Gc1F-phenotype is a significant risk factor for the development of chronic obstructive pulmonary disease (COPD). Bahr et al. (1) found a strong correlation between the Gc2-allele and the development of rheumatic fever characterised by a pronounced activity of B cells. They found that Gc2 is twice as frequent in rheumatic fever cases as in the normal population is striking (relative risk 2.25).

Phenotype of DBP is sometimes linked with coat colour of the horse. A dominant tobiano gene, which is responsible for forming of a white spotting in horses called tobiano, shows phase conservation with DBP S allele (3). The chromosome containing the allele for tobiano has a haplotype with DBP S allele as well. This phase conservation can be used to identify putative homozygotes for the tobiano gene, based on homozygosity for DBP S, so that breeders can assure the production of tobiano coloured offspring (4).

Genetic variability of DBP within series of species could be used as a tool for the genetic characterisation of populations (21, 24, 27). DBP, together with other plasma proteins, enables estimation of genetic variability of the given group of animals, which is crucial to breeding progress. DBP phenotype detection has useful application as an additional parameter to paternity determination in horses. Genetic variability of horses in Poland was determined by Pikuła et al. (19, 20) based on PAGE protein pattern including DBP.
Some DBP functions are dependent on DBP phenotype, other – not. For example studies of Schellenberg et al. (22) showed that all isoforms of Gc-globulin equally enhance the chemotactic activity of C5a.

Our previous study conducted on horses showed significant differences in serum DBP concentration in mares and their foals (17). It was also stated that serum DBP level in foals rose between 36th - 48th h and 3rd-4th week of life (16). In available literature, there is no data on the relation between DBP concentration and DBP phenotype in animals. The aim of the present study was to determine the relationship between the phenotype and concentration of DBP in sera of mares and their newborn and 3-4-week-old foals.

Material and Methods

Sample collection. The studies were conducted on 22 Thoroughbred mares, aged 5–22 years, and on their progeny for four foaling seasons. The mares were kept in mothers’ stable beginning in December of the year before foaling and July-November of the next year, or from the moment of the foal weaning. Subsequently, the mares were transferred to the main stables and after becoming pregnant they returned to the mothers’ stables. In the course of the studies, the mares and their progeny were fed oat grain and field hay. All animals remained under continuous veterinary care and clinically manifested no pathological sings.

In the course of delivery, blood from a newborn foal was sampled from the pulsating umbilical vein (0 h). Blood of foals was sampled also in 3rd-4th week of life from the external jugular vein. Blood of mares was sampled in 36th-48th h after delivery from the external jugular vein. The total of 69 serum samples of mares and 49 sera (0 h) and 36 sera (3-4 week) of foals were examined. The study protocol was approved by a local Ethics Commission.

Sample treatment. The blood was allowed to clot at room temperature and then was centrifuged at 1,200 x g for 10 min. The obtained clear serum was portioned and stored at -20°C.

Western blot DBP phenotype analysis. Phenotypes of DBP were visualised using polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (6) with own modifications. Electrophoresis was conducted in 10% (w/v) polyacrylamide gel (2). A 0.187 M Tris-borate-citrate buffer, pH 8.9, was used as buffer for the separating gel. Separation was conducted in 14.5 cm long gel, in an electric field under constant 200 V voltage supply (tension gradient ca. 1.38x10³ V/m²) in concentrating gel and 600 V (tension gradient ca. 4.14x10³ V/m²) in separating gel (Biorad Protean II with Power Pac 1000, Bio-Rad, Hercules, USA). 0.062 M Tris-borate-citrate buffer, pH 8.9, was used as electrode buffer. Electrophoresis was interrupted after 4.5 h.

Transfer from gel onto nitrocellulose (Protran® – BA85, Schleicher & Schuell Biotechnology, Germany) was performed under constant tension of ca. 10 A/m² for 1.5 h using the semidyed method in a von Keutz graphite chamber. A 0.025 M Tris, 0.192 M glycine, and 20% methanol, pH 8.3, buffer was used for the transfer. The nitrocellulose membrane was blocked in 2% casein blocking solution for 0.5 h, then incubated for 2 h with the first antibody (obtained by us rabbit polyclonal IgG directed against human DBP (14, 15) in 2% casein solution. This antibody cross-reacts with horse DBP. The nitrocellulose membrane was then washed with TBS-T (0.054 M Tris, 0.145 M NaCl, HCl to pH 7.3 with an addition of 0.03% Tween 20). The next step encompassed incubation with goat anti-rabbit IgG HRP (Sigma-Aldrich®, USA), 1:1,000 dilution ratio, in 2% casein solution. After washing the membrane with TBS-T, the enzymatic reaction was developed with 1.4-chloro-naphtol; 0.3-0.4 g • L⁻¹ of 0.05 M Tris-HCl buffer, pH 7.6. The reaction was stopped by washing off the excess of substrate with distilled water. Visual immunoblotting results were scanned.

Determination of DBP concentration. Serum concentrations of DBP were measured using a self-designed ELISA. In brief, anti-human DBP IgG were obtained through the hyperimmunisation of goats and rabbits by commercial human DBP (G-C Globulin, MP Biomedicals Inc., USA). A detailed procedure was described by Madej et al. (15). ELISA-plates (F96-MaxiSorp, Nunc GmbH, Germany) were coated by rabbit IgG anti-human DBP antibodies in 0.1M carbonate acid buffer, pH 9.6, and incubated overnight at 4°C.

After each step the wells were washed 3 times with PBS-Tween 20 (0.1% v/v). Unbound sites were blocked by the casein from bovine milk for 1 h at 37°C. Then tested samples were incubated in 0.1% casein in PBS and incubated for 1 h at 37°C. A pool of horse serum in three appropriate dilutions was used as an external standard. As a secondary antibody, biotinylated goat IgG anti-DBP was added and incubated for 1 h at 37°C. Then the streptavidin-horseradish peroxidase conjugate (ExtrAvidin-HRP, Sigma-Aldrich, USA) was added and incubated for 1 h at 37°C. O-phenylenediamine was used as a substrate (0.5 g/L) in 0.05 M citrate buffer, pH 5.0. The reaction was stopped after 5 min by addition of 1 M sulphuric acid. The absorbance was measured by BioTek EL340 spectrophotometer (Winooski, USA) at λ 490 nm wavelength. Data were acquired and computed by the use of KC3 software (BioTek Instruments).

Due to the lack of purified horse DBP, pooled horse sera were used as a standard and results were expressed using arbitrary units – equine unit (EqU) in which:

1EqU = amount of DBP in 1 • 10⁻¹⁰ L horse serum pool.

Because of different protein concentration in each sample, the obtained values of DBP, expressed in EqU • L⁻¹ were divided by total protein concentration (TPC) in g per liter (g • L⁻¹) obtaining DBP/TPC coefficient in (EqU • g⁻¹). This coefficient enables comparison levels of DBP in serum of mares and foals, which contain different protein concentrations.
Statistical analysis. The results obtained by ELISA were subjected to statistical analysis using Statistica 8.0 software (StatSoft Polska Sp., Poland). Significance of differences between the obtained results was appraised using the Mann-Whitney U test. It was assumed that differences between groups are statistically significant when \( P<0.05 \), where: \( P \) – the probability of obtaining a result at least as extreme as the one that was observed, given that the null hypothesis is true. The groups of measurements were described as the mean ± standard deviation (SD).

Results

Two DBP phenotypes (F and FS) were present in the examined sera (Fig. 1) while S phenotype was absent. In mare sera, F and FS phenotypes were detected in 84.1% and 15.9% (\( n=69 \)), respectively, while in newborn sera, F and FS phenotypes were observed in 46.9% and 53.1% (\( n=49 \)), respectively. In some cases which demonstrated FS pattern, the immunoreactivity of bands was different. However, no rule, which line was stained stronger was observed.

No significant differences were demonstrated between DBP levels found in serum of mares with F (\( n=58 \)) (1.292 ±0.486) and FS (\( n=11 \)) (1.263 ±0.535) phenotypes (Fig. 2).

There was no significant difference between DBP levels found in serum of newborns (0 h) presenting F (\( n=23 \)) (1.115 ±0.55) and FS (\( n=26 \)) (1.044 ±0.56) phenotypes (Fig. 3).

No significant differences have been demonstrated between DBP levels found in serum of 3-4-week-old foals presenting F (\( n=18 \)) (1.415 ±0.470) and FS (\( n=18 \)) (1.189 ±0.367) phenotypes (Fig. 4).

Fig. 1. Different phenotypes of horse DBP disclosed by Western-blotting: F – “fast” band, S – “slow” band.

Fig. 2. DBP levels expressed as DBP/TPC coefficient in (EqU · g⁻¹) in sera of mares with F (\( n=58 \)) and FS (\( n=11 \)) phenotypes.
Fig. 3. DBP levels expressed as DBP/TPC coefficient in (EqU \cdot g^{-1}), in sera of newborns (0 h) presenting F (n=23) and FS (n=26) phenotypes.

Fig. 4. DBP levels expressed as DBP/TPC coefficient in (EqU \cdot g^{-1}), in sera of 3-4-week-old foals presenting F (n=18) and FS (n=18) phenotypes.

**Discussion**

In the presented study we observed F and FS phenotypes in mares and foals. We did not find the S phenotype. This is in agreement with other studies showing that the S phenotype is rarely detected in horse population (10, 25).

Previously, we revealed that serum DBP concentration is significantly higher in mares-mothers (36-48 h after delivery) then in non-pregnant mares (15). We also disclosed no significant differences in serum DBP level in foals before (0 h) and after (36-48 h of life) colostrum feeding, therefore the second group was not included in the investigations (17). Our previous studies also revealed that serum DBP level in foals at 36th-48th h of life was significantly lower than in 3rd-4th week of life (16). Based on the presented results, we conclude that phenotype of DBP has no influence on the concentration of this protein in serum of mares-mothers, nor in foals during the first weeks of life.
Our results revealed that the relationship between the phenotype and DBP plasma concentration in horses is inconsistent with that observed in humans. In fact, Lauridsen et al. (11, 12) stated that the circulating level of DBP in Danish Caucasian postmenopausal woman population depends on the phenotype of this protein. They reported, that the plasma concentration of DBP, 25-hydroxy vitamin D, and 1,25-dihydroxy-vitamin D in humans is significantly higher in DBP1-1, intermediate in DBP1-2, and the lowest in DBP2-2 phenotypes.

During presented studies it was observed that colour intensity of the bands in heterozygotes was not equal. Probably, heterozygotes express each isotype in different way. Interestingly, this inequality in expression of both forms of DBP seems to have no effect on general serum DBP concentration.

In conclusion, presented results indicate the presence of F and FS DBP phenotypes in studied horses. Because of the rarity of the S phenotype, we did not find this phenotype in the present work performed on a finite horse population. We also showed that the phenotype of DBP has no influence on serum DBP concentration in mares-mothers and in newborn and 3-4-week-old foals.

References