

Szent István University  
Doctoral School of Veterinary Sciences

**STUDY OF THE HEALTH STATUS  
OF EQUINE FETUSES  
IN LIPIZZANER MARES**

Theses of Doctoral (PhD) Dissertation

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## Introduction and objectives

The basis of horse breeding is the birth of a healthy foal. Since the 1970s, veterinary researchers have been trying to find methods that can tell whether the fetus is healthy, i.e. the birth of a healthy and viable neonate can be expected. During gestation, we can learn relatively little about fetal health. Therefore, it is very difficult to determine the well-being of fetuses as such; it is more useful to study the dam–fetus unit, the two components of which cannot be separated either in space or in any other dimension. In my dissertation I tried to study equine fetal well-being as thoroughly as possible, using the tools and methods available today, with the objective of obtaining scientific results that can be utilised for decreasing fetal morbidity and mortality.

The following objectives were set for my studies:

1. To search for and find veterinary methods suitable for the study of fetal well-being in horses.
2. To study the haematological and blood biochemistry parameters of pregnant mares depending on the mare's age, fetal age, the stage of pregnancy (early-mid or late pregnancy) and to reveal differences, if any, between pregnant and non-pregnant mares in this respect.
3. To study the concentration of *alpha-fetoprotein* (AFP) in maternal blood and its significance for fetal well-being, and to determine reference values in mares with a normal pregnancy and with pregnancy loss.
4. To monitor heart rate variability (HRV) in pregnant mares and in their fetuses in association with fetal well-being, and to compare HRV parameters in pregnant and non-pregnant mares.
5. To assess the applicability and importance of transabdominal fetal ultrasound examinations in the equine species under field conditions.
6. To develop a rapid examination protocol for transabdominal ultrasound examinations to assess the health status of equine fetuses.

## **Materials and methods**

### ***Horse population used for the studies***

For all five research projects (haematology, blood biochemistry, AFP detection, heart rate variability measurement, transabdominal ultrasound), the examinations and samplings were performed in the Hungarian National Stud of Szilvásvár. At the start of the work (in November 2013) the horses included in the studies were clinically healthy, 6–24 years old (mean age:  $13 \pm 3$  years) Lipizzaner broodmares kept in loose housing. Their health status was determined on the basis of the history, the clinical base values and the physical examination performed on the spot. Both pregnant and non-pregnant mares were available for the samplings. Fetal age, i.e. the stage of gestation was calculated from the last artificial insemination (AI, Day 0), as described by Bucca et al. (2005): first month = days 1 to 30.

### **Studies no. 1–3: Laboratory blood and serum analyses from maternal blood**

#### ***Method of blood sampling from horses***

Between November 2013 and April 2014, blood samples were always taken from the external jugular vein according to the rules of the profession. The blood tubes were labelled immediately, placed in a cooling box and transported, within 6 hours, to the Laboratory of the Department and Clinic of Food Animal Medicine in Üllő, where they were stored in a deep freezer until their processing was started.

#### ***Haematological and biochemistry analyses from maternal blood***

Visibly haemolysed samples were excluded from the analysis.

Using the *Abacus Junior Vet* haematology analyzer the following haematological parameters were determined: white blood cell count (WBC), lymphocyte count (LYM), monocyte count (MID), granulocyte count (GRA), red blood cell count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean

corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), mean platelet volume (MPV).

Using an Olympus AU640 Chemistry Immuno Analyzer the values of the following biochemical variables were determined in serum samples: albumin (ALB), total protein (TP), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glutamate dehydrogenase (GLDH), glucose (GLUCOSE), triglycerides (TG), creatine kinase (CK), lactate dehydrogenase (LDH), urea (UREA) and creatinine (CREA).

#### ***Determination of fetal alpha-fetoprotein concentration in maternal blood***

For the determination of equine alpha-fetoprotein (AFP) concentrations, the frozen serum samples as well as the ELISA test reagents and tools were allowed to thaw at room temperature before performing the ELISA test according to the manufacturer's instructions (Mybiosource eqAFP Instruction Manual, 11<sup>th</sup> edition, 2014) in the Poultry and Swine Virology Laboratory of the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. The ELISA test required a 37 °C incubator (Stuart incubator SII19, Merck) and a 450 nm ELISA reader (Multiscan EX, Interlabsystems Ltd., Budapest).

#### ***Statistical analysis of haematological and biochemical test results***

The data were recorded in the Microsoft Excel 2010 (Microsoft, California, USA) program in all cases. All statistical analyses were performed using the freely available, commercial statistical program R (R Core Team, Vienna, Austria). Normal distribution of the data was checked using the Shapiro-Wilk or the Anderson-Darling test. The possible impact of maternal and fetal age was studied by correlation calculation. Whenever a correlation was established, the correlation between the variables was checked by linear regression. If the distribution was Gaussian, the Pearson correlation test, while in other cases the Spearman rank correlation test was

used. Reference values were determined by the use of the freely available Reference Value Advisor (<http://www.biostat.envt.fr/spip/spip.php?article63>) program, which gives the statistical methods to be used for reference values in the veterinary field, as a function of sample numbers, on the basis of the most up-to-date currently valid recommendation (International Federation of Clinical Chemistry, American Society of Veterinary Clinical Pathology).

Student's *t*-test was used for analysing the variables of two groups of animals (e.g. pregnant and non-pregnant mares) and the possible differences between them. Data of more than two groups were compared by analysis of variance (ANOVA). A P value of  $< 0.05$  was considered to be significant in all cases.

### ***Statistical analysis of the results of equine alpha-fetoprotein determination***

Also here, data were recorded in the Microsoft Excel program but in this case the STATISTICA (data analysis software system, version 12) program (StatSoft Inc., USA) was used for statistical analysis. Normal distribution of the data was checked by the Shapiro-Wilk test also in this case. The data were first distributed into two groups depending on the pregnancy status: group with a normal pregnancy (pregnant mares and mares delivering a healthy foal) and mares that were bred but failed to foal (late embryo resorption or abortion). To simplify the illustrations, they depict these two groups as 'normal' and 'aborted', respectively. Data of the group with a normal pregnancy were analysed for the possible effect of the following factors: mare's age, mare's ability to conceive, fetal age. The success of conception was grouped in the individual cycles, and thus two groups were formed: (1) mares conceiving after one or two inseminations, and (2) mares conceiving after three or more inseminations. To eliminate interferences, from the AFP values determined in the laboratory we calculated, for the samples of all mares and using linear correction, the AFP values adjusted (by sample and mare) for mean age (13 years), conception rate (average number of inseminations) and fetal age (208 days) (mAFP and cAFP) and their mean as the reference value of the population (rAFP). In order to facilitate a further comparison, the differences found in the mares' AFP concentrations were converted to standard deviation units. This latter unit directly indicates the deviation of the given value from the 'usual' value. The standard deviation ratio (SDR) used by us is determined as the difference of the cAFP per mare and the

rAFP, divided by the standard deviation of the rAFP. The cAFP and the SDR were evaluated statistically by the use of univariate ANOVA, where the grouping factors were pregnancy type (normal pregnancy or abortion), gender of the fetus (male or female) and descendance (dams as well as maternal dams and sires).

#### **Study no. 4: Study of heart rate variability (HRV)**

Different leads were used for making ECG recordings from pregnant vs. non-pregnant mares, as prescribed by the instructions for use of the equipment. The use of different leads was necessary because in pregnant mares the equipment also records the fetal signs, providing a so-called fetomaternal ECG recording, which enables the subsequent evaluation of the fetal signs (heartbeats). The ECG recordings were monitored on computer using a wireless connection and were saved on the hard disk of the computer.

#### **Statistical evaluation**

The 'ecg' files recorded by the ECG equipment are stored on the hard disk of the computer. To obtain a file suitable for HRV analysis, the data had first to be extracted into a 'txt' file format in Televet's own program. Heart rate variability was analysed using the Kubios-HRV software (Biosignal Analysis and Medical Imaging Group, Kuopio, Finland). Having read the data, the program offers the possible analyses (time domain, frequency domain, non-linear analysis). The methods used for the statistical analysis of HRV parameters were the same as those described for the statistical analysis of blood parameters.

#### **Study no. 5: Transabdominal ultrasound examinations**

The examination method followed the guiding principles described by Reef (1998). For these examinations, I created a pregnant mare examination protocol and used a printout of that protocol to note down and record the data and results at the examination site.

The mares were clinically healthy, with normal history and a mean age of  $13 \pm 3$  years. All horses were placed in a stock for the ultrasound examination. Having been led into the stock, the mares were subjected to general physical examination including the measurement of rectal temperature, heart rate and respiratory rate per minute. As the results were within the physiological limits, they were not recorded. Ultrasound examinations at the stud farm were performed using a portable Sonoscape S2 Colour Doppler ultrasound machine made available to me for testing by Vet-Med Labor Ltd. The transabdominal ultrasound examinations were performed with machine's microconvex transducer no. C311 of 2–6 MHz frequency and maximum 24 cm penetration, at 2 MHz frequency. During these studies, I performed exclusively transabdominal ultrasound examinations in a triangular area bordered by the stifle, the pecten ossis pubis and the xiphoid process of the sternum, on both sides of the mare, on the ventral abdominal wall.

The Lipizzaner mares were examined once each during their pregnancy. The examinations lasted 30–40 minutes depending on the presentation and activity of the fetus. The results measured by the ultrasound machine were recorded in millimetres or centimetres. With the help of ultrasound, first I determined the number and presentation of the fetus(es). Then I located the fetal thorax and measured the heart rate, the greatest diameter of the fetal aorta and the fetal respiratory movements, if any. Subsequently I recorded the greatest depth of the allantoic and amniotic fluids in all four ventral quadrants of the mare's abdominal cavity (right cranial, right caudal, left cranial and left caudal quadrants). Then I assessed the degree of granularity on a scale of 0 to 3, where the transparent fluid was given a score of 0 and the most granular one was scored 3. Subsequently I recorded the thickness of the uteroplacental unit that I measured along the mare's linea alba, at the midline of the distance between the xiphoid process and the mammary gland. I evaluated the continuity of the uteroplacental unit. During the examination I recorded whether or not I saw hippomanes and, summarising the findings, I described the activity and tone of the fetus. The activity of the fetus (on a scale of 0 to 3) depends on the amount of movement during the examination time. The fetus gets an activity score of 1 and 2 if it moves during 33% and 66% of the examination time, respectively, while fetuses moving continuously during the examination time are scored



3. If no fetal movement can be observed, a score of 0 is given. Subsequently I evaluated the abnormalities, if any. After the mares had foaled, I recorded the course of parturition, the health status, body weight (kg), height (cm), thoracic circumference and cannon girth (cm) of the newborn foal. During the ultrasound examination I used the pregnant mare examination form for recording the data. While the mare was prepared for examination, with the stud manager's help we recorded the mare's data: name, breed, age, reproductive history (i.e. whether or not there had been any unfavourable events during the previous deliveries). This was followed by taking the history data including the time of the last insemination, the expected foaling date and the occurrence of any problems during the current gestation or during previous pregnancies.

## Results

### *Results of Study no. 1*

During this study, a total of 121 blood samples from 30 Lipizzaner mares (23 pregnant and 7 non-pregnant) were processed: 94 out of the 121 samples originated from pregnant and 27 from non-pregnant mares.

Analysis of the obtained data revealed that the mares' age had no effect on the haematological parameters. Significant differences were found between the pregnant and non-pregnant groups in haematocrit value, haemoglobin concentration, red blood cell count and platelet count. The values of these parameters were higher in the samples taken from pregnant mares. There were significant correlations between fetal age and the values of haematological parameters: the granulocyte count increased while the MCH and MCHC values decreased with the advancement of pregnancy. Differences were also obtained by comparing two distinct stages of pregnancy (early-mid stage: gestation days 60–210 vs. late stage: gestation days >210). The granulocyte count and white blood cell count were higher and the MCH and MCHC values lower in late than in early-mid pregnancy.

### *Results of Study No. 2*

Twenty pregnant and 10 non-pregnant mares were included in this study. A total of 136 venous blood samples (105 from pregnant and 21 from non-pregnant animals) were collected and tested to determine the possible impact of fetal age, the month of gestation, the stage of pregnancy and the fact of pregnancy on the values of blood biochemical variables.

The mean age of the mares was  $13 \pm 3$  years in both groups. I found a negative correlation between the biochemical values and the mare's age for the following parameters: albumin, AST, GLDH, total protein and GGT, which were lower ( $P < 0.001$ ) in older than in younger mares. The other eight parameters (ALP, GLUCOSE, GLDH, CREA, LDH, TG, TP, UREA) did not show a correlation with the mare's age.

The mares' blood triglyceride levels showed significant differences between pregnant and non-pregnant animals. Serum TG concentration was  $0.24 \pm 0.08$  mmol/L (mean  $\pm$  SD) in pregnant mares and  $0.20 \pm 0.08$  mmol/L in non-pregnant animals (Table 1). The triglyceride concentrations started to rise from the fifth month of gestation.

Most of the parameters studied by us were correlated with fetal age, with the exception of GGT and CK. Albumin, AST, ALP, total protein and urea values showed a negative while GLDH, triglyceride, glucose and creatinine values a positive correlation with the age of fetuses.

Total protein concentration of the blood significantly decreased from the fourth month of gestation to the last prepartal blood sampling, but albumin levels remained relatively unchanged up to the beginning of the late pregnancy stage, from which time both protein levels decreased up to the end of gestation. Both total protein and albumin levels were significantly lower in late pregnant mares than in mares in earlier stages of gestation.

Urea concentrations showed a similar trend: decreasing total protein concentrations were followed by decreasing urea concentrations. Serum creatinine and GLDH were significantly higher in late pregnant mares than in early-mid pregnancy. The glucose concentrations also increased, but this increase typically occurred in the last three months of gestation.

An opposite tendency could be observed for AST and GGT values, as the activities of these two enzymes were lower in late pregnant mares than in earlier stages of pregnancy. The activity of creatine kinase were high throughout gestation in Lipizzaner mares but it was not affected either by fetal age or the stage of pregnancy.

### ***Results of Study No. 3***

In the equine alpha-fetoprotein (AFP) study a total of 111 samples from 30 mares (23 pregnant and 7 non-pregnant mares, the latter including 6 mares with late embryonic mortality and one with abortion) were assayed.

The mare's age ( $P = 0.011$ ) and her ease of conception ( $P < 0.001$ ) had a significant impact on the serum AFP concentration. The impact of fetal age on serum AFP, however, did not reach the level of significance ( $P = 0.088$ ), although the AFP

values tended to decrease with the advancement of pregnancy. The older the mare was and the more difficult it was for her to conceive, the lower AFP levels were measured. The mares were grouped according to their ease of conception: the first group included mares that were easy to get pregnant (mares conceiving after one or two inseminations), while the second group comprised less easily conceiving mares that required three or more insemination attempts to get pregnant.

The mean values of corrected AFP (cAFP) and standard deviation ratio (SDR) significantly ( $P < 0.001$ ) differed in mares with a pregnancy loss ( $n = 7$ ) and in those with a normal pregnancy ( $n = 23$ ), and showed the following values:

- group with pregnancy loss (complicated pregnancy): 152.00 pg/mL (cAFP mean), standard error of the mean (SEM): 18.61, 115.52 (lower 0.95 confidence interval), 188.48 (upper 0.95 CI)
- group with normal pregnancy (i.e. the reference, rAFP, deviation 49.25): 72.93 pg/mL (cAFP mean), SEM: 10.27, 52.80 (lower 0.95 CI), 93.06 (upper 0.95 CI)
- group with pregnancy loss: 1.606 (SDR mean), SEM 0.378, 0.865 (lower 0.95 CI), 2.347 (upper 0.95 CI),
- group with normal pregnancy: 0.000 (SDR mean), SEM 0.209, -0.408 (lower 0.95 CI), 0.409 (upper 0.95 CI)

A concentration difference of 79.07 pg/mL detected in cAFP corresponded to 1.606 deviations in the standard deviation ratio (SDR) (*Figure 5*).

The gender of the fetus was recorded only after foaling. We established that the gender of the foal had no influence on AFP level ( $P = 0.396$ ). However, notable and statistically significant differences were found in AFP levels between individual mares. The mares were born to a total of 26 mares: the effect of the grandmother is conceivable ( $P < 0.10$ ). The mares were sired by 14 stallions, and we found no differences between the fathers ( $P = 0.955$ ) in terms of AFP levels.

#### ***Results of Study No. 4: Study of heart rate variability (HRV)***

The mares' basic clinical values were normal and within the reference ranges reported for adult horses. All mares delivered their foal without problems. During the

studies I analysed and compared 37 ECG recordings from 28 pregnant mares and 20 ECG recordings from 9 non-pregnant (control) mares.

### **Study of heart rate variability in pregnant mares**

Heart rate variability analysis revealed significant correlations for two parameters: the standard deviation of normal-normal intervals (SDNN) and the root mean square of successive differences (RMSSD). SDNN significantly decreased with the progression of gestation ( $P < 0.0001$ ; corr.  $-0.739$ ), and RMSSD also showed a significant decrease ( $P = 0.0004$ ; corr.  $-0.628$ ). The SDNN values decreased from  $486 \pm 270$  ms (mean  $\pm$  SD) in the fifth month of gestation to  $98 \pm 24$  ms (mean  $\pm$  SD) by month 11. The RMSSD values showed a similar decrease from  $609 \pm 402$  ms (mean  $\pm$  SD) in the fifth month to  $100 \pm 66$  ms (mean  $\pm$  SD) by the eleventh month. The other parameters studied (HR and RR intervals) did not change during pregnancy. The mean heart rate of pregnant mares was  $31 \pm 10$ /minute (mean  $\pm$  SD), while their mean RR interval was  $2212 \pm 594$  ms (mean  $\pm$  SD).

### **Study of fetal heart rate variability**

Analysis of the ECG recordings taken of 28 pregnant mares in 'Foetus' mode revealed that the day of pregnancy was negatively correlated with the mean fetal heart rate (FHR). The FHR decreased with the progression of pregnancy, and this decrease proved to be statistically significant ( $P = 0.004$ , corr.  $-0.53$ ). The decrease was the greatest between gestation months 6 and 7, followed by a slow decrease thereafter. From the initial value of  $114 \pm 3$ /minute (mean  $\pm$  SD) measured in the fifth month of gestation the fetal heart rate decreased to  $89 \pm 18$ /minute (mean  $\pm$  SD) by the prepartal period (gestational month 11). In our study, we did not find correlations between the time of gestation and the RR intervals, SDNN, RMSSD and SDHR values of fetuses.

### **Comparison of heart rate variability in pregnant vs. non-pregnant mares**

With the exception of the SDHR, all HRV parameters were significantly different in pregnant as compared to non-pregnant mares. The *t*-test demonstrated that pregnant mares had significantly lower HR values ( $P < 0.0001$ ) and significantly higher

SDNN values, RR intervals and RMSSD than did non-pregnant mares ( $P = 0.001$ ,  $P < 0.0001$  and  $P = 0.0002$ , respectively).

### ***Results of Study No. 5 (transabdominal ultrasonographic examinations)***

In the framework of this study, I performed transabdominal ultrasound examinations on a total of 29 late-pregnant Lipizzaner broodmares (between gestational days 270 and 340) in two consecutive years (2013–2014). During the examinations, on the pregnant mare examination form I recorded the measurability and the values of the different parameters. Not all parameters indicated in the literature could be measured on all occasions.

The foal's birth weight was recorded in the case of 21 mares examined in the stud farm, and thus the correlation between fetal aortic diameter and birth weight could be studied in 21 cases. A significant correlation ( $P = 0.004$ ;  $R^2 = 0.615$ ) was obtained between the two variables, inferring that fetal aortic diameter is a good predictor of the foal's birth weight. The regression equation is as follows:

$$y = 0.1924x + 11.056,$$

where 'y' is the fetal aortic diameter in mm and 'x' is a foal's expected birth weight in kilograms.

### **Elaboration of a rapid examination protocol for late-pregnant mares**

Based upon the data reported in the literature, my own results and the measurability of the parameters, I elaborated a rapid examination protocol which contains the following elements:

- fetal heart rate (acute hypoxia marker)
- fetal aortic diameter (chronic hypoxia marker)
- combined thickness of uteroplacental unit, CTUP (placentitis marker)

I used this examination protocol on 20 pregnant Lipizzaner mares. Each examination took less than 15 minutes ( $9.5 \pm 3.5$  minutes), and all three fetal and maternal parameters could be measured successfully. The results of the examinations ( $n = 20$ ) were as follow:

- fetal HR (mean  $\pm$  SD):  $111 \pm 12$ /minute
- fetal aortic diameter (mean  $\pm$  SD):  $20 \pm 0.8$  mm
- CTUP (mean  $\pm$  SD):  $12.4 \pm 2.6$  mm

## Discussion

The care of pregnant mares has outstanding importance in both the clinical and the ambulatory practice, as many horse owners view the foal to be born as the mare's greatest asset.

Experience shows that the values of haematological and biochemical parameters are influenced by numerous factors in addition to the species, breed, gender, keeping conditions and nutrition. During study no. 1, I collected blood samples from a total of 30 mares including 23 pregnant and 7 non-pregnant animals. Our results indicate that pregnancy has an effect on haematopoiesis, as the haematocrit level, haemoglobin concentration and red blood cell count was significantly higher in pregnant Lipizzaner mares. The platelet count also tended to increase with the progression of pregnancy. In our study, the mares' age had no effect on any of the haematological parameters, which findings is at variance with the results of previous research. From our results it can be established that the granulocyte count as well as the MCH and MCHC were associated with the age of the fetus. Mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration decreased in late pregnancy; a plausible explanation is that the bone marrow functioning with increased intensity produces red blood cells with lower haemoglobin content but the overall haemoglobin content still increases due to the higher RBC count. During pregnancy, the fetus of increasing size takes up an increasing amount of oxygen through the placenta; thus, it is conceivable that the mare adapts to the changing demands not only by her increased circulating blood volume but also by the higher RBC and platelet count and increased haemoglobin concentration.

In the second study, I analysed the correlations between the mares' blood biochemical values and the age of the mare, age of the fetus, the stage of pregnancy and the existence of pregnancy.

In that study, TP concentration and the ALB, AST, GLDH and GGT enzyme activities were found to decrease in association with the mare's advancing age, and these parameters showed lower values in older than in younger animals. For total protein (TP) and the GLDH enzyme the *P* values were close to the critical limit of 0.05;

thus, it is possible that these correlations were found to be statistically significant because of the limited number of samples tested.

In our study, the AST activities and albumin concentrations increased with the mares' advancing age. In an earlier study in the Zemaitukai horse breed the GGT activity increased with the mare's age, while we found it to decrease. Statistical analysis has revealed that in the studied population the mares' blood triglyceride concentration was the only parameter showing significant differences between the pregnant (0.24 mmol/L) and the non-pregnant (0.2 mmol/L), most likely because of the increase in the rate of lipid metabolism during gestation.

Most of the biochemical parameters, with the only exception of GGT and CK, showed some type of change in association with fetal age (i.e. with the progression of pregnancy). In contrast with the findings of earlier studies where the TP and ALB concentrations did not change during pregnancy, in our study the total protein values significantly decreased from the fourth month of gestation to the last prepartal sampling, while albumin concentration showed a substantial decrease only in late pregnancy. The decrease found in total protein and albumin concentrations in late pregnancy can probably be explained by the fact that before parturition the mammary gland of mares starts to produce colostrum, the primary source of which is the maternal blood.

The urea concentrations followed a trend similar to that shown by total protein, which can be explained by the fact that the degradation of proteins produces urea, and thus decreasing blood protein levels may result in lower blood urea concentrations. The higher creatinine concentrations found in late pregnancy in our study are probably attributable to the increased workload of the kidney characteristic of that period and the combined excretory activity of the dam and fetus.

In the last three months of gestation we measured elevated blood glucose values in the Lipizzaner mares which is at variance with the results of earlier studies but consistent with findings that demonstrated increased blood glucose levels in the last prepartal samples.

The activity of the GLDH enzyme was higher in late than in early-mid pregnancy and exceeded the reference values used for adult horses all over the world. In this study, AST activity was lower in late pregnancy than in earlier stages of gestation



but higher than the reference value established for adult horses; however, it was not influenced either by maternal and fetal age or the stage of pregnancy. Earlier, elevated CK activities were reported in the peripartal period and attributed to injuries of the birth canal during parturition. As in our study such traumatic injuries did not occur, the elevated levels may have been due to moving the mares to the pasture daily. Such exercise may have resulted in elevated concentrations of the isoenzyme of muscle origin in the blood of Lipizzaner mares, which is the known to be most common cause of CK activity increase in horses.

LDH enzyme activity was not influenced either by the age of the dam or fetus or by the stage or presence of pregnancy.

In agreement with the findings of earlier studies, in the mares studied by us we measured higher ALP activities than the reference value for adult horses. The activity of the ALP enzyme may increase during pregnancy when anabolic processes and osteoclast activity tend to increase, and the ALP enzyme can be found in the placenta as well.

From the above it can be seen that while there may be pregnancy-associated changes in the values of haematological and biochemical parameters in the Lipizzaner breed, these changes cannot be associated with fetal well-being, i.e. are not diagnostically useful in this regard, although they well reflect the health status of the dam.

Taking up the thread of observations reported in the human and partially in the veterinary literature, in our third study we investigated the potential diagnostic importance of alpha-fetoprotein (AFP) in pregnant and non-pregnant mares. To the best of my knowledge, our study was the first in the world to determine AFP serum concentrations in pregnant Lipizzaner mares ( $72 \pm 10$  pg/mL) and Lipizzaner mares with a pregnancy loss ( $152 \pm 18$  pg/mL). We successfully proved the correlation of AFP concentrations with some reproductive conditions. Although our knowledge of the equine AFP molecule is very limited, our study has demonstrated that mares with late embryonic mortality or abortion have significantly higher AFP concentrations than mares with a normal pregnancy. This observation is consistent with the results of an earlier US study on English Thoroughbred mares.

In seven out of the 30 mares examined in this study late embryonic mortality is likely to have occurred, as these mares had been pregnant but did not abort and did not deliver a foal either. There has been only one publication reporting AFP concentrations measured in the blood of mares; in that study, the AFP values measured in the blood of five mares higher (approx. 240–660 pg/mL) than those found in the Lipizzaner mares in this study (32–205 pg/mL). Unfortunately the breed of the mares examined in that study was not specified and we can only assume that they must have been warm-blooded horses but probably of a breed other than the Lipizzaner. In the earlier study (Sorensen et al., 1990) unfortunately not a single specific blood concentration was given but at least we know that they examined English Thoroughbreds.

In humans, AFP concentration keeps decreasing with the progression of pregnancy and its measured values can be used for estimating gestational age. Only one of the two works investigating AFP in horses studied the correlation between gestational age and AFP concentration, and found that the AFP level continuously decreased towards the end of pregnancy. As, however, the majority of samples tested in that study originated from the first 100 days of pregnancy, the few higher AFP values found by those authors does not unambiguously prove the trend of AFP changes in the second and third trimesters. The samples tested in our study showed roughly uniform distribution throughout pregnancy (originating from different periods of pregnancy from day 60 to day 325), and the AFP values measured in them showed a significant decrease as parturition approached. However, whether or not this is a general trend in horses is still questionable and requires confirmation.

Based upon the data obtained in this study we could not establish a statistically significant correlation between the gender of fetuses and the AFP levels of the dams.

In our study we did not find correlations between the body measurements studied (birth weight, withers height, thoracic circumference and cannon girth) and fetal AFP concentrations. A possible explanation may be that in our study and also in other previous equine studies the samples were obtained from the dams and not the fetuses.

From our results it can be concluded that both the age of the fetus and the reproductive status and age of the dam are correlated with the AFP concentrations. In older mares lower AFP values were measured, which will have to be taken into account

when determining reference values for a given population. In addition, we found substantial differences in AFP levels between individual mares.

Telemetric fetomaternal ECG is an examination modality that can record both the maternal signals and the fetal heartbeats and, thus, it provides an excellent opportunity for studying fetal well-being. In the fourth study of my PhD work, I performed telemetric fetomaternal ECG examinations, for the first time in Hungary, on 28 pregnant Lipizzaner broodmares in months 5 to 11 of gestation, as well as on 9 non-pregnant mares. This noninvasive method provides useful information on the well-being and health status of equine fetuses and their dams. In contrast to earlier reports, in two of our mares we successfully recorded fetal signals as early as day 121 of gestation (at the start of our study only two mares were at that stage of pregnancy).

In agreement with the previously reported findings, the fetal heart rate significantly decreased with the progression of pregnancy but the increase of the RR intervals did not reach the level of significance.

Comparing the values of broodmares with data reported in the literature, it can be established that the decrease of RR intervals reported to be significant in earlier studies from Austria did not prove to be significant in our study, although the tendency was the same. The heart rate of mares seems to decrease during pregnancy but the rate of that decrease did not reach the level of statistical significance in any of the studies conducted. At the same time, the SDNN and RMSSD values of broodmares decreased significantly with the advancement of gestation. This allows us to conclude that the standard deviation of normal-normal intervals (SDNN) of RR decrease (from  $280 \pm 91$  to  $89 \pm 34$ ); thus, it is possible that the increased workload and adaptation of the cardiovascular system due to fetal development causes a physiological stress in the mares. Approaching the end of pregnancy, the standard deviation of the heart rate (SDHR) also significantly decreased in the mares; according to the literature, this may occur in the case of stress. In accordance with this, the decrease of RMSSD from ( $296 \pm 96$  to  $96 \pm 57$ ) indicates the weakening of parasympathetic tone and the intensification of sympathetic processes in the regulation of the heart rate.

In addition to pregnant mares, I also examined a so-called control group consisting of non-pregnant mares. Comparing the pregnant mares' data with those of the

control group comprising non-pregnant mares, I found significant differences between the two groups in all parameters with the only exception of SDHR. The HR of non-pregnant mares was significantly lower ( $P = 0.0006$ ) while the SDNN, the RR intervals and the RMSSD were significantly greater in this group than in the pregnant mares. As the two groups were balanced in terms of breed, age, gender, keeping conditions and nutrition, the differences found in the results can perhaps be attributed to the different reproductive status of the two groups, i.e. the presence or absence of pregnancy. As the obtained results allow me to conclude that there is a difference in heart rate variability between pregnant mares being at day 121 to 330 of gestation and non-pregnant mares, it may be assumed that pregnancy as a physiological state requires substantial adaptation by the dam's organism, which process is reflected also in the change of the HRV values.

Fetomaternal ECG examination can provide values that are very useful for the assessment of fetal well-being, the most important of which is perhaps the fetal heart rate. Although this variable can be measured also by fetal ultrasonography, the values obtained by this latter modality reflect only the momentary status of the fetus, and heart rate counting during ultrasound examination has its subjective errors. Clinical experience shows that HRV analysis cannot replace transabdominal ultrasonography, as in late pregnant mares at risk immediate results are needed to assess the condition of the fetus.

While the blood samplings were performed between days 60 and 325 of pregnancy, HRV examinations between days 121 and 330, the last (fifth) transabdominal ultrasound examinations were carried out in late pregnancy, between days 270 and 340 in the Lipizzaner broodmares. I performed the ultrasound examination of a total of 29 mares at the Hungarian National Stud of Szilvásvárad. Although not all parameters could be measured at all ultrasound examinations, the importance of transabdominal ultrasonography is demonstrated by the fact that even twin pregnancy and placentitis could be detected, in one case each, with the help of this examination. I found a close correlation between fetal aortic diameter and birth weight also in Lipizzaner broodmares, which suggests that in warm-blooded breeds the developmental status of the fetus can indeed be predicted from the transabdominal ultrasound examination results of late pregnant mares.

Transabdominal ultrasonography is an excellent method that allows the veterinarian to determine the presence of pregnancy and the number of fetuses in a painless manner and even in the stable, without placing the mare in a stock. The detection of twin pregnancy allows us to inform the owner in time and get prepared for the further necessary veterinary interventions. Fetal activity and fetal heart rate are perhaps the two most important parameters indicating the viability of the fetus. During my studies, fetal activity could be observed and evaluated easily, and all of the equine fetuses achieved good activity scores.

During the examination of Lipizzaner mares, the mean heart rate of the 28 fetuses was  $91 \pm 10$ /minute, which appears to be normal for the given stage of pregnancy. It must be emphasised, however, that these measured values represent momentary values that reflect the condition of the fetus at the given moment in time.

Most specialists doing fetal ultrasonography agree that the routine examination procedure should include the examination of the maternal organs directly surrounding the fetus. Measurement of the combined thickness of the uteroplacental unit (CTUP) and the quantitative and qualitative evaluation of the fetal fluids informs the examiner about the possible presence of pathological processes (e.g. placentitis). During my studies I measured greater CTUP ( $14.3 \pm 6$  mm) values in the Lipizzaner mares than those reported in the literature earlier (12 mm).

The equine fetus is surrounded by two types of fluid, the amniotic and the allantoic fluid. Not only the quality but also the quantity of these fluids may be pathological. Although I did not see examples of this during my examinations, in two cases I observed the formation of a cyst-like structure between the uterus and the placenta. Observation of the fetal movements and tone seems to be rather simple but it is highly subjective. In late pregnant mares the fetus can be visualised along the ventral wall of the abdomen; however, regarding the dimensions the currently available ultrasound machines and transducers cannot provide more than 25–30 cm ultrasound penetration depth into the abdominal cavity and, thus, it is difficult to observe complex fetal movements using this modality.

Based upon my results and in harmony with the opinions described in the literature earlier I think that the biophysical profile of the equine fetus, which was

originally described in the 1990s, is a promising diagnostic method which, however, requires further modification and fine-tuning because of the measurement difficulties of certain parameters. This is why during my studies I made the decision to select, from the original six parameters, those parameters that are the most informative regarding fetal hypoxia, can be examined relatively easily and can be measured with high reliability during a rapid examination (lasting maximum 15 minutes).

The ‘rapid examination protocol’ (REP) thus developed comprises the following parameters:

- fetal heart rate (acute hypoxia marker)
- fetal aortic diameter (chronic hypoxia marker)
- CTUP (placentitis marker)

I tried out this examination protocol on 20 mares. None of the examinations required more than 15 minutes and I could measure all the parameters. All of the examined mares were healthy and the examinations did not reveal any pathological abnormalities. In my opinion, this protocol may be suitable for detecting major abnormalities, and even veterinarians with average experience can perform it under stud farm or riding school conditions.

Reviewing all five studies performed in the framework of my doctoral research I can conclude that I could successfully prove and characterise the effect of pregnancy on the dam’s haematological and blood biochemical variables, this alone does not provide information about fetal well-being. The studies performed so far suggest that the measurement of alpha-fetoprotein may become a diagnostic method suitable for detecting pathological features of pregnancy in the future. As, however, this method is not routinely available in Hungary and is very expensive, currently it has importance for research studies only. If we need immediate information about the health status of an equine fetus, transabdominal ultrasound examination can absolutely be recommended, complemented with fetomaternal ECG examination, as in this case we may be able to draw long-term conclusions and get a better knowledge of effects exerted by internal and external factors on fetal health status.

## **New scientific results**

- The haematological and blood biochemical values of pregnant Lipizzaner mares differ from those of non-pregnant mares
- I have obtained data on equine alpha-fetoprotein concentrations and their changes during pregnancy in Lipizzaner mares with normal pregnancy and with pregnancy loss, and determined a reference range of alpha-fetoprotein for that population
- The heartbeats of equine fetuses by telemetric ECG can be detected as early as on day 121 of pregnancy, as opposed to day 173 reported previously
- I have determined the basic data of heart rate variability parameters in Lipizzaner broodmares and their fetuses
- I have demonstrated that the values of SDNN and RMSSD parameters decrease with the progression of pregnancy
- I have proved that because of pregnancy the heart rate variability (HRV) parameters of pregnant mares (the means of HR, SDNN, RMSSD and RR intervals) differ from those of non-pregnant mares
- I have developed a three-component rapid examination protocol for the examination of equine fetuses using transabdominal ultrasonography

## **List of publications forming the basis of the PhD dissertation**

Vincze B., Gáspárdy A., Baska F., Bálint Á., Hegedűs Gy. T., Szenci O. (2015): Equine alpha-fetoprotein (eqAFP) levels in Lipizzaner mares with normal pregnancies and with pregnancy loss. *THERIOGENOLOGY*. Accepted for publication. IN PRESS. (IF 2014: 1.789)

Vincze B., Kutasi O., Baska F., Szenci O. (2015): Pregnancy-associated changes of serum biochemical values in Lipizzaner broodmares. *ACTA VETERINARIA HUNGARICA* 63:(3) 303–316. (IF 2014: 0.65)

Baska-Vincze B., Baska F., Szenci O. (2015): Fetal heart rate and fetal heart rate variability in Lipizzaner broodmares. *ACTA VETERINARIA HUNGARICA* 63:(1) pp. 89–99. (IF 2014: 0.65)

Baska-Vincze B., Baska F., Szenci O. (2014): Transabdominal ultrasonographic evaluation of fetal well-being in the late-term mare and cow. *ACTA VETERINARIA HUNGARICA* 62:(4) pp. 439–451. (IF 2014: 0.65)

Vincze B., Baska F., Szenci O. (2015): A vemhesség hatása a hematológiai paraméterekre lipicai kancákban [Pregnancy-associated changes in haematological parameters in Lipizzaner broodmares]. *MAGYAR ÁLLATORVOSOK LAPJA* 137:(4) pp. 197–202. (IF 2014: 0.2)

Baska-Vincze B., Rózsás J., Baska F., Szenci O. (2014): A transabdominalis ultrahangvizsgálat szerepe a lómagzat életképességének elbírálásában. Előzetes eredmények [Role of transabdominal ultrasonography in assessing the viability of equine fetuses: Preliminary results]. *MAGYAR ÁLLATORVOSOK LAPJA* 136:(4) pp. 195–204. (IF 2013: 0.2)



Baska-Vincze B., Baska F., Szenci O. (2014): A magzati és anyai szívfrekvencia és szívfrekvencia-változékonyság vizsgálata magyar lipicai kancákban [Study of fetal and maternal heart rate and heart rate variability in Hungarian Lipizzaner broodmares]. *MAGYAR ÁLLATORVOSOK LAPJA* 136:(7) pp. 387–394. (IF 2013: 0.2)

Vincze B., Rózsás J., Baska F., Burg A., Szenci O. (2013): A csikómagzat egészségi állapotának vizsgálati lehetőségei az állatorvosi gyakorlatban (Irodalmi összefoglaló) [Possibilities of examining the health status of equine fetuses in the veterinary practice: Review of the literature]. *MAGYAR ÁLLATORVOSOK LAPJA* 135:(6) pp. 323–331. (IF 2013: 0.2)

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