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Clinical evaluation of bovine twin pregnancy

PhD thesis

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1.1. General summary

Several pregnancy diagnosing methods are intensively used worldwide to diagnose whether a pregnant cattle is carrying multiple fetuses or not. The different rectal palpation techniques (palpation of the uterine fluctuation, amniotic vesicle, or fetal membrane slip) are useful and widespread however they are really dependent on the skill of the examiner. An experienced veterinarian/technician even from Day 35 with the fetal membrane slip technique is able to set up pregnancy diagnoses. However, the manual techniques are limited in diagnosing twins: usually only the bilateral twins can be diagnosed accurately. The prediction accuracy of unilateral twinning is low. Ultrasonography in the early period (around Day 30) of gestation is also widely used with high accuracy to diagnose early pregnancy. At this time usually twin pregnancies are also visible. When dealing with twin pregnancy at a herd level, we always have to take into consideration the phenomenon of late embryonic/early fetal mortality. This can influence the accuracy of our diagnostic tests. Another possibility for diagnosing early pregnancy in the field is the measurement of pregnancy specific proteins, like bovine pregnancy-associated glycoprotein-1 (bPAG-1), bovine pregnancy-specific protein B (bPSPB) or pregnancy serum protein (PSP-60), which are secreted by the binucleate cells originating from the bovine trophoblast. In contrast to progesterone (P4) measurements, they are good indicators of the presence of an alive conceptus.
The relevant literatures of clinical diagnostic possibilities of twin pregnancy in cattle are overviewed. The different methods of pregnancy diagnosis were evaluated, underlining the advantages and disadvantages of each method.
After this, in order to be able to distinguish twin pregnant carrying animals from singleton pregnant ones with the goal of setting up clinically accurate cut-offs to help clinician veterinarians was performed.
In practice, pregnancy loss due to embryonic/fetal mortality is the main factor affecting the results of pregnancy diagnoses, therefore the nature of the phenomenon must be taken into consideration when evaluating any diagnostic method. However, the measurement of pregnancy proteins either in sera or milk is an accurate method for setting up pregnancy diagnosis, at the moment it is not accurate for distinguishing between twin and singleton pregnancies. Diagnosing pregnancy from Days 24-25 by means of ultrasonography is also proper for diagnosing twins, but because of the

above mentioned effect of pregnancy loss, confirming diagnosis is required, in cases of twin pregnancies by means of ultrasonography.

1.2. Materials and methods

In Experiment 1 altogether 84 Holstein-Friesian dairy cattle were included in our study with a confirmed pregnancy by means of ultrasonography at the late embryonic period between Days 29 to 42 of gestation (time-point 1). Animals were divided into 3 groups: the first group (TWIN Group, n=29) had two viable embryos and two mature corpora lutea on the ovaries at the time of pregnancy diagnosis. Animals with one viable embryo in the pregnant uterine horn and two corpora lutea on the ovaries formed the second group (DCL Group, n=35), while animals with a singleton pregnancy (one viable embryo and one corpus luteum) served as the control group (CON Group, n=20). All animals were kept in free barns with cubicles and were fed according to the NRC recommendations.

Animals were examined four times during the study. First, at the time of early pregnancy diagnosis (time-point 1) which was performed by a real-time B-mode diagnostic ultrasound scanner (EasiScan, BCF technologies, Bellshill, UK) equipped with a 5 to 9 MHz linear-array rectal transducer, then monthly for three additional occasions (namely: between Days 57 to 70 (time-point 2), Days 85 to 98 (time-point 3), and Days 113 to 126 (time-point 4) of gestation, respectively). At each examination, the operator was required to record the number and the presence of embryos in the uterine horn and the number of the corpus luteum. At every time-point, when it was possible, the viability of the fetus was confirmed by the detection of the heartbeat. In those cases, when scanning of the entire uterus especially at time-points 3 and 4 was not feasible, the presence and the movement of the fetus was used to confirm the viable pregnancy.

Following transrectal ultrasonographic examinations blood samples were collected into vacutainer tubes (Monovette 9ml, Sarstedt, Nümbrecht, Germany) at every time-point. Plasma concentrations of P4 were estimated by validated solid-phase ^{125}I radioimmunoassay (RIA) method (Coat-A-Count TKPG; Diagnostic Products Corporation) as described by Zoli et al. (1992). Plasma bPAG-1 measurements were performed according to the method of Zoli et al. (1992) and subsequently modified by Perényi et al. (2002). A PAG I67kDa preparation, purified according to the protocol of

Zoli et al. (1991) was used as standard and tracer. The sensitivity of the bPAG-1 RIA test was 0.06 ng/mL, and the intra- and inter-assay coefficients of variation were 5.0 and 5.5%, respectively.

In Experiment 2 blood sample from each cow was collected from the coccygeal blood vessels 29-35 days after insemination, and sent to a routine laboratory (Androvet, Budapest, Hungary) by overnight mail. Upon arrival at the laboratory, blood samples were centrifuged (2000 rpm for 10 minutes) and resultant sera was assayed for Pregnancy-Specific Protein B (BioPRYN™; BioTracking, Moscow, ID, USA), as described earlier (Gábor et al., 2007). Cows with serum PSPB concentrations >1.1 ng/ml were considered pregnant, those with <0.6 ng/ml were considered nonpregnant, and those with concentrations between 0.6 and 1.1 ng/ml were deemed at high risk for pregnancy loss. In Trial 1 sampling for PSPB examinations on 29-35 days after AI were performed (n=7300). In this period, cows with twin calving were registered also in those farms. Data of AIs resulted in twin calving were collected (serum PSPB concentration, date of the AI, parity, milk production and body condition score by the time of the AI, AI bull, the father of the cow, hormonal treatments before the AI, daily temperature data) in a self-developed database (Bopella). In Trial 2 blood samples were collected from 98 dairy cows on two high producing dairy farms. Cows were examined by transrectal ultrasound (EasyScan, BCF Technologies, United Kingdom) three times: between 29-35, 36-42 and 43-49 days after AI, respectively. Pregnancy loss diagnosis was based on the last two ultrasound examinations (35-41 and 43-49 days post AI).

In Experiment 3 in total 1253 positive pregnancy diagnoses were followed up until calving. After early pregnancy diagnoses, pregnancy was confirmed between Days 57 to 70 of gestation by means of transrectal palpation (TRP). If manual palpation did not confirm previous pregnancy status, TRUS examinations were repeated to confirm fetal losses. Pregnancy was also confirmed by means of TRP at the time of drying-off between Days 221 and 227 of gestation. The number of foetuses was recorded at calving. All reproductive interventions before AI (single prostaglandin treatment /0.5 mg cloporostenol im., Cyclix, Virbac, France/ or OvSynch (d 0: GnRH /0.1 mg gonadoreline im., Gonavet 50, Veyx Pharma, Austria/, Day 7: PGF2α /0.5 mg cloporostenol im./, Day 9 p.m.: GnRH, Day 10 a.m.: AI/ protocol or spontaneous oestrus) were also recorded.

1.3 Results and Discussion

In Experiment 1 the concentrations of P4 and bPAG-1 were measured in cases of singleton and twin gestations with 1 or 2 corpora lutea. Due to the fact, that almost 95% of twin pregnancies are dyzigotic (Silva del Rio et al., 2006) it was worth testing that double corpora lutea might produce more P4 at different stages of gestation. Because generally from around Day 150 of gestation the placenta provides a marked amount of P4 therefore it was important to test the animals before this time-point to achieve a clinically reliable diagnosis. Several authors agree, that more than one corpus luteum usually provides higher levels of circulating P4 (Bech-Sábát et al., 2008, Wiltbank et al., 2014), and the higher P4 levels provide better environment for the conceptus to elongate and develop (Forde et al., 2011). In contrast, when we compared the P4 levels at different time-points of gestation statistically significant differences could be found only around Day 60 of gestation and a close to significant tendency around Day 90 but we were not able to set up a threshold level to discriminate pregnancies having 1 or 2 corpora lutea based on the P4 level of the individual animal (Table 2). This finding is in agreement with an earlier study (Mann et al. 2007) whereas no difference was found between animals having single or double corpora lutea neither in the weight of the luteal tissue, nor in the P4 concentrations. The same author assumes (Mann, 2009) that circulating levels of P4 may be determined by the stage of cycle. Both studies are carried out on non-pregnant, cyclic animals. Our results are showing that even in case of pregnant animals the circulating levels of P4 cannot be determined by the number of corpus luteum. Although measuring the P4 concentrations of pregnant animals with 2 corpora lutea gave results with good accuracy, but they were not able to distinguish between animals having 1 or 2 corpora lutea. The levels of bPAG-1 are affected by several factors during gestation. Pathologic pregnancies, stillbirth and fetal well-being are mentioned among these factors (Serrano et al., 2009). Previous studies (Dobson et al., 1993, Patel et al., 1997) have suggested that the number of viable embryos/fetuses have an effect on the concentrations of the pregnancy proteins in the peripheral blood. Recent studies have confirmed this hypothesis (Lopez-Gatius et al., 2007, Serrano et al., 2009), since statistically significant differences were found in the concentrations of pregnancy proteins between singleton and twin pregnancies. However, to our best knowledge no cut-off values were determined or tested to date. To exclude confounding factors such as

embryonic/fetal mortality, we evaluated only those animals, whose calving ended with the number of offsprings that were originally diagnosed. When comparing one embryo carrier animals (either with 1 or 2 corpora lutea) with twin carrying animals statistically significant differences were detected at each time-point regarding the pregnancy proteins. To have a diagnostic aid in the clinical practice, threshold levels should be established as early as possible to diagnose twin pregnancies which have major importance at herd level. From the third month of gestation we were able to select a cut-off value (39.4 ng/mL) which could disseminate singleton and twin pregnancies with high AUC. In our study at time-points 1 and 2 the cut-off values were almost the same, suggesting that bPAG1 concentrations are starting to elevate from the baseline value of Day 30 only at the third month of gestation. The sensitivity of the bPAG1 remained low in every time-point measurement, but we could rule out negative diagnoses with a high specificity.These results are suggesting that at the early stage of gestation, measurements of bPAG-1 as pregnancy diagnosis alone is not enough to disseminate singletons and twins. Moreover, the losses between Days 30 and 60 are increased in cases of twin pregnancies (Bech-Sábát et al., 2008), but these losses cannot be predicted with the pregnancy proteins.

In Experiment 2 it was concluded, that twinning in the dairy cattle population appears to be increasing over time (Kinsel et al., 1998, Johanson et al., 2001, López-Gatius and Hunter, 2017). If this trend continues, the dairy industry must be prepared to cope with the negative effects associated with twinning (Fricke, 2001). In the late 50's and early 60's the prevalence of twinning was approx. 1 % in dairy cattle (Noakes, 2009) and significantly increased over the past decades along with the increase in milk production (Nielen et al., 1989). Our results showed a similar trend and we registered almost 7 % twin calving rate in the 3 herds. This is definitely much higher twin calving rate than earlier (1-5 %, depending on breed). Although a common belief that hormonal treatments are major causes of twin pregnancies, our data do not support this hypotheses. The only exception is the single PGF injection and this is in agreement with findings of Andreu-Vázquez et al. (2012a) who reported that just those estrus synchronization protocol used before AI affected the twin pregnancy rate when PGF were used together with other hormones (PRID, eCG). PGF alone (Kinsel et al. 1998) or in combination with FSH/LH or GnRH (Nielen et al. 1989) has increased risk of twinning. No effect of milk production on the risk of twin pregnancy was found, in agreement with a previous study (Andreu-Vázquez et al. 2012b). Likelihood of twin

pregnancy increased with parity. Older cows have been described to be more likely to deliver twins (Cady and Van Vleck, 1978, Nielen et al., 1989, Eddy et al., 1991, Kinsel et al., 1998, Andreu-Vázquez et al., 2012b). Our results showed that AI bull, bull's sire, bull's grandfather and the cow's father affected also twin calving ($p \leq 0.02$). Johanson et al. (2001) analyzed calving data of North American Holsteins (1,324,678 births of 37,174 sires of cows from the National Association of Animal Breeders (NAAB) calving ease database). Heritability estimates for the sire of the cow effect were 2.1% by the linear model analysis and 8.7% by the threshold model analysis. Sire predicted transmitting abilities (PTA) for twinning rate ranged from 1.6 to 8.0%. They concluded that sire selection can be used to reduce the incidence of twins and also the increased cost of production associated with twins. In our study we found much higher frequency of twin calving in cows diagnosed pregnant with more than 3 ng/ml serum PSPB concentrations at 29-35 days after insemination (Table 3). Although individual differences could be detected, the trend was similar to results of others who stated that cows bearing twins showed significant higher plasma PAG-I (López-Gatius et al., 2007) or plasma PAG I, PAG-II and P4 concentrations (Garcia Ispierto et al., 2016) throughout the study period than cows bearing singletons. However, there was a significant difference in PSPB serum concentration between singleton and twin pregnancies in the first two sampling days (Table 1) in Trial 2, differences were non-significant in overall between PSPB serum concentration of singleton and twin pregnant samples (2.1 and 2.9 ng/ml). Probably the low size of the study population and the effects of milk production on PSPB values may explain this lack of significance. Daily milk production at pregnancy diagnosis slightly exceeded 40 kg and milk production correlated negatively with plasma PAG-I values in a previous study (López-Gatius et al., 2007). Although lower pregnancy loss rate (9.4 %) was recorded in twin pregnant than singleton pregnant (55.9 %) cows (in these cases small morphological abnormalities – less amount of amnionic fluid, smaller size of the embryo was seen), no doubt that twin pregnancy is a higher risk factor for terminating pregnancy in cattle (López-Gatius and Hunter, 2017, López-Gatius and Hunter, 2017). Statistical analysis showed lower risk of twin pregnancy in higher parity cows ($p=0.023$), but several other data confirm (Johanson et al. 2001, Gábor et al. 2016) that in this case it is an accidental finding probably caused by the relatively low number of cases. All other findings (open days, number of AIs and milk production had no effect on twin pregnancy) are supported by earlier researches. Not surprisingly parity has impact on

pregnancy loss (Table 5), since cows with more calving had higher pregnancy loss (20% vs. 28.6%, $p<0.05$).

Although we also have no clear explanation for the decrease of serum PSPB concentrations at the different bleeding times, an apparent decline in plasma PAG-1 values on Day 42 of gestation was previously described (López-Gatius et al., 2007). This is not surprising in view of the fact that PAG molecules are a family of closely related proteins which expression patterns vary temporarily during the different pregnancy periods (Green et al., 2000, Garbayo et al., 2008).

In Experiment 3 we compared the outcome of singleton and twin pregnancies in order to evaluate gestations in terms of pregnancy losses (late embryonic/early and late fetal mortality, stillbirth). A total of 1253 gestations were evaluated, and 8.4% twin gestations were found. The prevalence of twin gestation varies widely, because it is possible to identify herds as twinning or as non-twinning ones (Kirkpatrick et al., 2002). At the time of early pregnancy diagnoses we did not find a twinning rate as high as reported in a recent study (15%, Andreu-Vázquez et al., 2012). As the main factor responsible for the growing number of twin gestations worldwide, increased metabolic activity associated with the rising milk yield of cows has been mentioned (Lopez et al., 2005, Wiltbank et al., 2000). In an American study (Lopez, 2005) with >45 kg daily milk yield, the percentage of multiple ovulations exceeded 50%, indicating that milk yield correlates with twin pregnancy rate, which should be consider in high-producing dairy herds (Kirkpatrick et al., 2002). In contrast, some authors did not find any relationships between milk production and multiple ovulations (López-Gatius et al., 2005), which thought to be responsible for the majority of twin gestations. In accordance with this finding, the highest prevalence of twin pregnancy at the early TRUS examinations was found on the farm with the highest milk production (around 11,000 kg/lactation). Although we did not observe increased twin gestation rates in higher production herds at early pregnancy diagnosis, our results suggest that the increasing milk production may affect twin pregnancy. Usually twin gestations suffer more often from this phenomenon (Kastelic et al. 1989); however, in this study, there was no evidence for a higher prevalence of losses in twin pregnancies until pregnancy confirmation. Between Days 57–70 and drying-off there was also a non-significant difference in losses. This finding is in contrast with a study, where high rate of pregnancy loss (28.8%) was detected until Day 90 (López-Gatius et al., 2004). In accordance with our findings, it is highlighted that twin gestation management should focus on the late fetal

period, however our study did not report the time period between the first pregnancy diagnosis and the confirmation crucial. It has also been reported (López-Gatius and Hunter, 2005, Lopez Gatius et al., 2010) that in some cases only one embryo undergoes this partial loss of pregnancy. Our study did not contain data to analyse partial losses, further evaluation is required. More pregnancy losses occurred in singleton pregnancies when gestation was maintained by a cavitary CL ($p < 0.05$) between Days 29-42 and 57-70 with an increased prevalence of pregnancy losses until drying-off. Although the presence of a cavity in a mature CL was not found to be associated with a reduced ability of progesterone production (Okuda et al., 1988; Perez-Martin, 2009, Balogh et al., 2012, Balogh et al., 2014), in the present study pregnancy losses were associated with the existence of a cavitary CL. Although detailed evaluation of the role of CL with cavity in the maintenance of bovine pregnancy was not the main focus of this study, it seems that medical therapy might be required to maintain the affected gestations, because almost one third of singleton pregnancies with a cavitary CL were lost. Significantly more losses were found in singleton pregnancies with a cavitary than in those with a non-cavitary CL with an OvSynch treatment before AI, due to the limited number of our cases it needs further confirmation. At the same time, difference was not found in cases of prostaglandin treatments. Earlier studies (Bech-Sábat et al., 2009; López-Gatius et al., 2002) and a recent review (Szenci, 2015) recommend to induce secondary CL by pharmacological treatments to maintain pregnancy in the early stage of gestation. Surprisingly, more losses occurred for singleton pregnancies with two CL than those having one CL in the first two months of gestation, while there was no difference between them until calving. It is presumable that those cows carried twins however one of them was lost before our TRUS and according to López-Gatius et al. (2002) these pregnancies were not likely to be maintained however this finding needs further confirmations. The laterality of singleton pregnancy did not show any effect on pregnancy losses until Days 57–70 but after that period left horn pregnancies were more likely to be lost. In twin pregnancies, an increased laterality-associated mortality rate is known from previous studies (López-Gatius and Hunter, 2005), especially in the case of unilateral twins. The development of two conceptuses in the same uterine horn is known to be uncomfortable for the dam, but in our study, there was no difference in pregnancy losses between unilateral and bilateral twin pregnancies. Further studies are needed to confirm these findings. The stillbirth rate was four times higher for twin than for

singleton pregnancies, which points out the importance of the accurate diagnosis of twin gestations before calving in order to reduce stillbirth rate. If it is not possible, at that time after each singleton calving the possible presence of a second foetus in the uterus has to be excluded (Niles, 2016).

In conclusion, when analysing twin pregnancies in dairy cattle pregnancy loss did not differ between singleton- and twin-carrying cows at the confirmation of pregnancy between Days 57–70 of gestation, moreover, at drying-off also a non-significant difference was detected between singleton and twin carrying groups. In singleton pregnancies, presence of a cavity in the corpus luteum effected pregnancy loss. Between Days 57–70 of gestation and drying-off this difference between cavitary vs. non-cavitary CL was still significant, while it was non-significant between cows with one CL vs. double CLs. The occurrence of cavities in cases where a single CL was present was not affected by hormone therapy prior to AI, however, the number of CL was reduced by pharmacological treatments. The stillbirth ratio was also higher in twin carriers than in singleton carriers. Although the role of the number of CL and cavitary CL in maintaining pregnancies requires further evaluation, our study highlights the importance of follow-up twin pregnancies to decrease stillbirth rate.

4. Summary of main scientific results

In the first study we tried to achieve clinically applicable cut-off values in cases of measurement of pregnancy proteins (bPAG-1) and progesterone to discriminate between singleton and twin pregnancies. In second study PSPB concentration measurement was also used for the dissemination of twin and singleton pregnancies. In the third study we evaluated the losses in cases of twin and singleton gestations with a special regard to the number of corpora lutea.

- Progesterone concentrations –however statistically significant differences could be obtained- did not give clinically applicable cut-off values. At the same time bPAG-1 as a diagnostic test for twin pregnancy, with both high sensitivity and specificity, was applicable from Day 85 after AI.
- Clinically acceptable diagnoses could be achieved only at the late fetal stages of gestation (time-points 3: from Day 85 after AI) Our data suggest, that other tools -such as ultrasonography- are required to identify twinning at the late embryonic/early fetal stage (time-points 1 and 2: between Days 28-42 and 57-70 after AI).
- There was a significant difference in PSPB serum concentration between singleton and twin pregnancies in the first two sampling periods (Days 29-35 and Days 36-42 after AI). As a result of our measurements no real predictive value of PSPB was found for twin pregnancy or pregnancy loss, probably due to the relatively low number of experimental animals and the negative effect of high milk production on PSPB values in Trial 2.
- We found that lower PSPB serum concentration at Days 29-35 after AI represented a higher risk for pregnancy loss. There was lower risk of twin pregnancy in higher parity cows ($P = 0.023$), although further studies are needed to confirm this finding.
- We could not detect higher pregnancy losses with these methods between twin and singleton pregnancies, therefore ultrasonography is advised to perform for the confirmation of pregnancy in order to be able to find the differences mentioned in the literature.
- In our study stillbirth significantly decreased the number of alive calves as an outcome of twin pregnancies.

- The number of the corpora lutea and the cavities in the corpora lutea also affected pregnancies: singleton pregnancies with cavitary corpora lutea suffered higher pregnancy loss than those ones without having cavities. In twins, the lower incidence of cavities in pregnant animals did not let us to confirm this result.

6. Publications in peer-reviewed journals related to the thesis

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