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PREGNANCY AND STILLBIRTH LOSSES IN DAIRY COWS WITH SINGLETON AND TWIN PREGNANCIES

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The aim of this study was to examine the effect of twin pregnancy, fetal laterality, the number of corpora lutea (CL) and cavitary CL on pregnancy losses in Holstein-Friesian cows with a positive pregnancy diagnosis based on ultrasonography between days 29–42 after AI. Pregnancy was confirmed by transrectal palpation between days 57–70 after AI and at the time of drying-off as well. Twin pregnancy rate was 8.4% at the time of the early pregnancy examination. Pregnancy loss did not differ between singleton- and twin-carrying animals either between days 57–70 of gestation or at drying-off. More losses occurred in singletons between days 29–42 and 57–70 in cows with cavitary than in cows with non-cavitary CL (12.1% vs. 3.6%; $P < 0.05$) and in cows with double CL than in cows with single CL (7.3% vs. 3.6% %; $P < 0.05$). Between days 57–70 of gestation and drying-off this difference was still significant (20.7% vs. 3.7%; $P < 0.001$), while it was non-significant between cows with one CL (5.7%) vs. double CL (3.7%). Cavity occurrence was not affected by hormone therapy prior to AI (either $PGF_{2\alpha}$ or OvSynch; 4.4% vs. 5.4%, respectively); however, the number of CL was reduced by the treatments (11.6 vs. 19.6%; $P < 0.0005$). In twin pregnancies there was no difference in the pregnancy losses between bilateral and unilateral pregnancies at any time point. The length of gestation was 278.2 ± 10.5 (singleton) and 267.4 ± 31.2 (twin) days, respectively ($P < 0.01$). The stillbirth ratio was higher in twin carriers than in singleton carriers (19.5% vs. 5.3%; $P < 0.001$).

Key words: Dairy cow, single and twin gestation, pregnancy loss, corpus luteum, corpus luteum with cavity

Reproductive efficiency is severely affected by embryonic and fetal mortality on intensive dairy farms. The rising percentage of pregnancy losses has been attributed to the increasing milk production (Silva-Del-Río et al., 2009; Hoedemaker et al., 2010; Andreu-Vázquez et al., 2012; Gábor et al., 2016), heat stress (Humblot, 2001; López-Gatius et al., 2009), timing of insemination (Hum-

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blot, 2001) and other factors such as compromised oocytes, embryo quality, inadequate uterine environment and infectious agents, metabolic and health status of the animal as well as some dietary ingredients (Saacke et al., 2000; Santos et al., 2004; Gábor et al., 2016). Twin pregnancy, as an economically unwanted phenomenon in dairy cattle, was also demonstrated to increase losses either in the late embryonic and/or the early fetal period (Day et al., 1995; López-Gatius et al., 2004; Silva-Del-Río et al., 2009; Mur-Novales et al., 2018).

The prevalence of losses during pregnancy decreases with the progress of gestation, and the majority of losses may occur in the early embryonic period (Diskin and Morris, 2008; López-Gatius et al., 2009). Under clinical circumstances, transrectal ultrasonography (TRUS) represents an accurate method for pregnancy diagnosis in the late embryonic and early fetal period (Pieterse et al., 1990; Szenci et al., 1998; Fricke, 2002). Because of the occurrence of late embryonic/early fetal losses, primary pregnancy diagnoses must be confirmed (Santos et al., 2004) in order to achieve reliability and to manage herd health issues. This confirmation is usually performed around Day 60 of pregnancy when placentation is completed (Ball, 1997). At that time pregnancy is considered to be firmly established and the chances of losses are greatly reduced. Most of the losses in singleton and twin pregnancies used to occur between the time of early pregnancy diagnosis and the confirmation of gestation (Szenci et al., 2000; López-Gatius et al., 2004), but early pregnancy diagnosis is still not a common practice in Hungary. Twin pregnancy also shortens the length of gestation (Echternkamp and Gregory, 2002; Norman et al., 2009).

In cattle, luteal structures containing cavities are also well described in the literature (Okuda et al., 1988; Balogh et al., 2014). Based on serum progesterone measurements these structures are known to have non-pathologic origin (Perez-Marín, 2009); however, differences in their reaction to treatment may occur (Hatvani et al., 2013) or different levels of some metabolic or endocrine parameters can be measured (Balogh et al., 2012). The function and the progesterone production of a corpus luteum (CL) with a cavity are not affected, and these biological entities act as normal CL; however, their possible effects on pregnancy and on the maintenance of gestation are still in the focus of research (Hatvani et al., 2013). Besides pregnancy loss, stillbirth also has a major effect on bovine reproduction (Szenci, 1985; Meyer et al., 2000). Several reports confirm that twin pregnancy increases the stillbirth rate (Lombard et al., 2007; Hossein-Zadeh et al., 2008).

The aim of the present study was to evaluate the effects of twin pregnancies on the prevalence of pregnancy loss as well as stillbirth. It was also examined whether laterality or CL with a cavity affect the prevalence of losses in cases of singleton pregnancies.

Materials and methods

Experimental design and pregnancy diagnoses

This study was carried out on three large-scale Hungarian dairy farms during a 13-month period between November 2012 and December 2013. The 305-day milk production was 9,300 kg (Farm A), 8,700 kg (Farm B) and 11,000 kg (Farm C) in the year before the study, respectively. Nutritional supplementation was in line with the NRC recommendations (National Research Council, 2001) on each farm. Farms were visited once a week (Farm A) or every second week (Farms B and C) to diagnose early pregnancy by means of TRUS using a 4.5–8 MHz linear-array rectal transducer (BCF Technology Ltd., Livingstone, United Kingdom) as described previously (Szenci et al., 1995), as part of the reproductive management of each farm, between days 29 and 42 after AI. Parity number and milk yield did not differ significantly between singleton- and twin-carrying animals.

The criteria for a positive pregnancy diagnosis were as follow: (1) at least one viable embryo with a detectable heartbeat, (2) clear fluid in the allantoic and amniotic vesicles (with complex integrity of both membranes), and (3) at least one corpus luteum (CL: minimum 2 cm in diameter) on one of the ovaries. By scanning both uterine horns the content of each horn (one or two embryos, embryo with or without heartbeat, right or left uterine horn) and the structures on the ovaries (number of CL, CL with a cavity at least 5 mm in diameter) were also recorded. The diagnosis of singleton pregnancy was established when one live embryo and one or more CLs were detected. The criterion for twin pregnancy was two viable embryos with at least one CL. In total, 1253 positive pregnancy diagnoses were followed up until calving.

After early pregnancy diagnoses, pregnancy was confirmed between days 57 and 70 of gestation by 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 TRP at the time of drying-off between days 221 and 227 of gestation. The number of fetuses was recorded at calving.

All reproductive interventions before AI [single prostaglandin treatment (0.5 mg cloprostenol im., $PGF_{2\alpha}$: Cyclix, Virbac, France) or OvSynch (day 0: GnRH 0.1 mg gonadorelin im., Gonavet 50, Veyx Pharma, Austria), Day 7: $PGF_{2\alpha}$ (0.5 mg cloprostenol im.), Day 9 p.m.: GnRH, Day 10 a.m.: AI protocol or spontaneous oestrus] were also recorded.

The care of the animals and the experimental design of this study were approved by the Local Animal Ethics Committee in Budapest, Hungary.

Statistical analysis

Statistical analysis was performed with Stata 15 MP (StataCorp. 2017 Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). The chi-squared test was used to test the independence of two categorical variables in a contingency table. Fisher's exact test was used to test whether the relative proportions of one variable were independent of the second variable. The independent group *t*-test was used to compare the means of the same variable between two groups. Welch's *t*-test was used assuming unequal variances. The equality of variances was tested by Levene's test to see if the samples had equal variances. ANOVA was used to test for differences in the means of the dependent variable broken down by the levels of the independent variable.

Logistic regression analysis was used to model the expected value of the binary response variables as a linear function of the explanatory variables. In three-dimensional tables when controlling for the third variable, the homogeneity of odds ratios was assessed with the Mantel-Haenszel estimate and a chi-squared test.

The P value was the two-tailed probability computed using the corresponding distribution. If the P value associated with the test was small, this was regarded as an evidence to reject the null hypothesis in favour of the alternative. For statistical testing two-tailed tests with a 5% level of significance were used.

Results

Evaluation of twin pregnancies

In total, 1253 positive pregnancy diagnoses [Farm A: n = 304 (24.3%), Farm B: n = 674 (53.8%), and Farm C: n = 275 (21.9%)] were made between days 29 to 42 and followed up until calving. The prevalence of twin gestations diagnosed between days 29–35 (73/866, 8.4%) and days 36–42 (32/387, 8.3%) was similar; therefore, the dates of pregnancy diagnoses were not used further as an independent factor in the analysis. Most of the animals (n = 1148) carried singleton pregnancies, while there were 105 (8.4%) twin pregnancies. Twin pregnancy rates were also similar for the three farms [Farm A: 7.6% (23/304), Farm B: 8.6% (58/674) and Farm C: 8.7% (24/275; $P > 0.05$, respectively] at the time of the early pregnancy diagnoses; therefore, the different farms were also not used further as an independent factor in the analysis.

All twin and singleton pregnancies were evaluated according to the laterality of gestation. Among the 1148 cows carrying singletons, 670 pregnancies (58.3%) were located in the right uterine horn and 478 pregnancies (41.6%) in the left uterine horn. Twin pregnancies were located either unilaterally (n = 57; 54.3%) or bilaterally (n = 48; 45.7%).

There was one CL in 957 (83.4%) singleton pregnancies and two CL were detected in 191 (16.6%) singleton pregnancies. In the latter group, three of the cows had three CL. In twin-carrying cows only one CL was found in three cases (2.9%), and all other twin-pregnant cows had two (n = 99) or three CL (n = 3). Cavitary CL occurred in one twin-carrier cow (1.0%) and in 58 singleton pregnancies (5.1%).

The majority of the cows (n = 761; 62.3%) were inseminated without hormonal treatment prior to AI. Altogether 472 (37.7%) animals had pharmacological treatment. Three days prior to AI prostaglandin was administered to 334 animals (26.6%) and an OvSynch protocol was used in another 138 animals (11%). Out of the 472 hormonal treatments, 431 resulted in singleton (91.3%) and 41 (8.7%) in twin pregnancies. When using prostaglandin, 300 (89.8%) treatments resulted in singleton and 34 (10.1%) in twin pregnancies, while with the use of the OvSynch protocol 131 (94.9%) singleton and 7 (5.1%) twin pregnancies were obtained. Overall, the hormonal treatment before AI did not influence the twin pregnancy rate at the time of pregnancy diagnosis ($P = 0.182$). When analysing the data by calculating the Mantel-Haenszel estimate of the odds ratio controlled for farm, the OR was 0.94 ($P = 0.67$), indicating that the ORs were equal to one and there was no treatment effect on the twin pregnancy rate in the different farms. The test of homogeneity of ORs gave a P value of 0.36, indicating that the odds ratios were not different from each other.

Pregnancy losses in singleton and twin pregnancies

The confirmation of TRUS pregnancy diagnoses was carried out by TRP at days 57 to 70 of gestation. The rate of pregnancy loss diagnosed between days 29–42 and 57–70 was altogether 4.6% (53/1148) in singleton and 4.8% (5/105) in twin pregnancies ($P = 0.95$) (Table 1). Differences in pregnancy loss at drying-off were also not statistically significant between animals with singleton and twin pregnancies ($P = 0.99$). Based on logistic regression analysis, total losses were not different between singleton and twin pregnancies at any time point ($P = 0.94$, OR = 1.04 and $P = 0.96$, OR = 0.98, respectively), and we could not detect any farm effect ($P = 0.36$, OR = 0.83 and $P = 0.08$, OR = 0.79, respectively).

Pregnancy loss was also evaluated on the basis of laterality in cases of singleton and twin pregnancies. In singleton gestations, the rate of right-side pregnancy losses (35/670; 5.2%) did not differ significantly ($P > 0.05$) from that of the left-side pregnancy losses (18/478; 3.8%) between days 29–42 and 57–70. This difference was also not significant at the drying-off pregnancy check ($P > 0.05$). Based on logistic regression analysis in twin gestations neither the difference of the pregnancy losses at days 57–70 (4/57; 7% vs. 1/48; 2.1%) nor the differences at the time of drying off (4/57; 7% vs. 2/48; 4.2%) were significant ($P > 0.05$) between unilateral and bilateral pregnancies.

Table 1

Pregnancy losses and stillbirths in cases of singleton and twin pregnancies

Reproductive status	Singleton pregnancy (n = 1148) (%)	Twin pregnancy (n = 105) (%)
Pregnant, diagnosed between days 29–42 and 57–70, n (%)	1095 (95.4)	100 (95.2)
Pregnant, diagnosed between days 57–70 and drying-off, n (%)	1025 (89.3)	94 (89.5)
Dam with stillbirth, n (%)	54 (5.3)	16* (19.5)
Total number of live calves, n (%)	971 (84.6)	144 (68.6)

*both calves were stillborn in four cases

Singleton pregnancies were also evaluated based on the number of CL and the presence of a cavity in the CL diagnosed by TRUS (Table 2). Pregnancy losses occurred more often in singleton pregnancies with a cavitary CL than in those with non-cavitary ones at the time of the confirmation of pregnancy (days 57–63, P = 0.015). At the time of the confirmation of pregnancy, for cows with a cavitary corpus luteum the odds of pregnancy loss were 2.73 times higher than for those without a CL with a cavity (P = 0.02). At the time of drying-off, the odds of pregnancy loss were 2.18 times higher for cows with a cavitary corpus luteum than for those which did not have a CL with a cavity (P = 0.02) in singleton pregnancies. However, the number of CL did not have any effect on pregnancy loss either at the first time point or at the second time point of pregnancy confirmation (P = 0.9 and P = 0.45, respectively) (Table 3). There was one CL in two twin-pregnant dams and one CL with a cavity in one twin-pregnant cow; however, their pregnancies were not lost.

Losses at calving

The length of singleton and twin gestation was 276.4 ± 22.8 and 270 ± 23.2 days, respectively. Comparing the length of singleton and twin pregnancies with the Student's *t*-test with unequal variances, neither twin and singleton, nor unilateral and bilateral (268.1 ± 31.1 vs. 272.9 ± 8.5 days) twin pregnancies showed statistically significant differences.

Eventually, 1025 singleton-pregnant animals and 94 twin-pregnant animals gave birth. A stillbirth event occurred in 53 cases (5.3%) in singleton calvings, whereas twin calvings suffered from one or two stillborn offspring in 11 cases (11.7%) (P < 0.01). It is important to mention that in four twin-pregnant cows both calves were stillborn.

Table 2

Pregnancy losses in cases of singleton pregnancies (n = 1148) according to the number of corpus luteum (CL) and the presence of a cavity in the CL

Pregnancy loss	Singleton pregnancy with a compact CL (n = 899)	Singleton pregnancy with a CL with a cavity (n = 58)	Singleton pregnancy with two CL without a cavity* (n = 191)
Diagnosed between days 29–42 and 57–70, n (%)	32 (3.6)[a]	7 (12.1)[b]	14 (7.3)[b]
Diagnosed between days 57–70 and drying-off, n (%)	51 (5.7)[a]	12 (20.7)[b]	7 (3.7)[a]
Total, n (%)	83 (9.2)[a]	19 (32.8)[b]	21 (11.0)[a]

[a,b]$P < 0.05$: within the same row; *n = 3: presence of 3 CL

Discussion

In this study, 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 a 8.4% twin gestation rate was 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 (Wiltbank et al., 2000; Lopez et al., 2005). In a study conducted in the United States (Lopez et al., 2005) on cows 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 considered in high-producing dairy herds (Kirkpatrick et al., 2002). In contrast, some authors did not find any relationship between milk production and multiple ovulations (López-Gatius et al., 2005), which are 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-producing herds at the time of early pregnancy diagnosis, our results suggest that the increasing milk production may affect twin pregnancy.

Table 3

Evaluation of losses in cases of cavitary, non-cavitary and double corpora lutea (CL) in singleton pregnancies using either OvSynch or $PGF_{2\alpha}$ treatment prior to AI

Pregnancy loss	Singleton pregnancy with a CL without a cavity (n = 362)	Singleton pregnancy with a CL with a cavity (n = 19)	Singleton pregnancy with two CL without a cavity* (n = 50)
		Prostaglandin treatment prior to AI (n = 300)	
Diagnosed between days 29–42 and 57–70, n (%)	6 (2.4)	1 (7.7)	2 (5.1)
Diagnosed between days 57–70 and drying-off, n (%)	14 (5.6)	2 (15.4)	1 (2.6)
Total, n (%)	20/248 (8)	3/13 (23.1)	3/39 (7.7)
		OvSynch treatment prior to AI (n = 131)	
Diagnosed between days 29–42 and 57–70, n (%)	4 (3.5)[a]	2 (33.3)[b]	1 (9.1)[a]
Diagnosed between days 57–70 and drying-off, n (%)	11 (9.6)[a]	2 (33.3)[b]	1 (9.1)[a]
Total, n (%)	15/114 (13.1)[a]	4/6 (66.6)[b]	2/11 (18.2)[a]

[a,b]$P < 0.05$: within the same row; *n = 3: presence of 3 CL

Twin gestations usually suffer more often from losses (Kastelic et al., 1989); however, in this study there was no evidence of 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 the results of a study where a 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 find the time period between the first pregnancy diagnosis and the confirmation to be crucial. It has also been reported (López-Gatius and Hunter, 2005; López-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 and, therefore, further evaluation is required in this regard.

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-Marín, 2009), in the present study pregnancy losses were associated with the existence of a cavitary CL. Although a 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; however, due to the limited number of our cases this needs further confirmation. At the same time, no difference was found in cases of prostaglandin treatments.

Earlier studies (López-Gatius et al., 2002; Bech-Sábat et al., 2009) and a recent review (Szenci, 2015) recommend to induce a 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 for 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 the TRUS examination. According to López-Gatius et al. (2002), these pregnancies are not likely to be maintained; however, this finding needs further confirmation.

The laterality of singleton pregnancy did not have 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 underlines the importance of the accurate diagnosis of twin gestations before calving in order to reduce the stillbirth rate. If this is not possible, then the possible presence of a second fetus in the uterus has to be excluded after each singleton calving (Niles, 2016).

In conclusion, when analysing twin pregnancies in dairy cattle, the 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 cow groups. In singleton pregnancies, the presence of a cavity in the CL affected the 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 CL. 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 rate 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, the present study highlights the importance of following up twin pregnancies to decrease the stillbirth rate.

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