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Cow metabolic status assessed from fat/protein ratio in milk affected ovarian response and number of transferable embryos after superovulation

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Abstract: This work aimed to evaluate the quantity and the quality of flushed embryos based on the metabolic status of dairy cows, lactation number, and size of the ovaries. Fifty-nine Holstein cows on 1st to 5th lactation were enrolled in the experiment. Monitoring took place during the period from October to November and from March to June. Cows with corpus luteum were included for the hormonal treatment – superovulation and timed insemination. The cow was inseminated, resp. re-inseminated, during the induced heat with insemination doses from one bull from the same batch. Embryo flushing was performed on the 7th day after the first insemination. We isolated individual embryos after flushing, and morphologically evaluated them under a stereo microscope. The metabolic status of tested cows was determined based on the ratio between fat and protein in milk around the period of embryo flushing (< 1.1; 1.1–1.3; > 1.3). Data about fat and protein content were taken from milking parlour records. Data were evaluated in SAS v9.4 with GLM procedure. The results of our study showed that there is a significant relationship between the fat/protein ratio and the total number of flushed embryos, resp. the number of transferable embryos. The highest number of flushed and transferable embryos were collected from the group of cows with fat/protein ratio between 1.1–1.3. The fat/protein ratio within these values represents cows in an optimal metabolic state. We also observed a significant positive relationship between the size of the ovaries and the number of flushed and transferable embryos. Lactation number did not significantly affect monitored parameters. The assessment of the fat/protein ratio might become a useful tool for the evaluation of cows selected for embryo transfer. Our findings could be used to improve the efficacy of the superovulation system, with the aim to extract the maximum number of transferable embryos.

Keywords: corpus luteum; Holstein; embryo flushing; lactation number; ovary size

Dairy cows get into a negative energy balance (NEB) after calving, which not only increases the metabolic burden for the organism of dairy cows but also increases the susceptibility to metabolic diseases and infections (Gross and Bruckmaier 2019). Diseases are most common during the NEB period, and they cause a significant increase in veterinary cost, milk losses, need for labour, and

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stress to the animal. Studies show that excessive stress is often associated with fertility disorders (Becker et al. 2012). During the period of deepest NEB, the concentration of unesterified fatty acids in milk and blood is increased (Duchacek et al. 2020) and luteinizing hormone secretion is disrupted, thus the ovulation is prevented (Jolly et al. 1995). According to various authors, the peak for increased energy demand (nadir) occurs around the 2nd week after calving (Jolly et al. 1995), or even between the 4th and 8th week after calving (Walsh et al. 2011). It was also reported that follicles, which emerge after the nadir showed faster growth, larger diameter, increased oestradiol production, and were more likely to ovulate (Beam and Butler 1997).

There are several indicators for the energy balance of dairy cows. One of the most commonly used indicators is the body condition score (BCS) (Walsh et al. 2011). However, Beam and Butler (1999) found that the resumption of follicular growth occurs regardless of the animal's energy balance. On the other hand, multiple studies had confirmed that thin animals have a prolonged onset of ovarian function after calving. In these animals, the consequences of NEB did manifest by irregular pulses of luteinizing hormone, the insufficient response of follicles to gonadotropin stimulation, and reduced follicle function (Chagas et al. 2007), as well as lower quality of preimplantation embryos (Makarevich et al. 2016). Changes in milk composition, like increased content of fat, protein and their ratio, occurred as the result of the increased mobilization of body fat reserves and greater accumulation of triacylglycerol in the liver after calving (Gross et al. 2011). In particular, the fat/protein ratio in milk (F/P ratio) above 1.35 to 1.5 was associated with a large energy deficit (Heuer et al. 1999; Gross et al. 2011) and thus a decrease in BCS. Bezdicek et al. (2020) in their study also confirmed the importance of BCS and its changes in relation to reproduction, health, and production. Furthermore, studies confirmed the relationship between negative energy and results of embryo transfer (Bezdicek et al. 2015), *in vitro* fertilization (Chrenek et al. 2015), and ovarian activity (Kuznicka et al. 2016). Skilful management and adequate nutrition during a post-partum period are the key factors to the growth and the maturation of the follicles (Armstrong et al. 2001).

The number and the quality of oocytes and embryos for superovulation did reflect the potential fertility of dairy cows (Kubovicova et al. 2013). The success of the ovarian response to super-ovulatory treatment was dependent on the cow's individuality, breed, season of the year, and nutritional status (Ammoun et al. 2006).

The literature shows that there is a connection between nutritional status, metabolism, milk composition, and ovarian function, resp. the number and the quality of produced gametes and embryos. The aim of this work was to assess the metabolic status of Holstein cows based on the F/P ratio in milk around the period of embryo flushing and to investigate the differences in the quantity and the quality of flushed embryos for given F/P groups. In addition, the effect of lactation number and size of ovaries on the number and the quality of flushed embryos were evaluated.

MATERIAL AND METHODS

Animals and farm

This experiment was carried out in accordance with Czech legislation for the protection of the animals against abuse (No. 246/1992) and with directive 2010/63/EU on the protection of animals used for scientific purposes. The study was conducted in the production environment of a commercial dairy farm with Holstein cows in the Central Bohemian Region of the Czech Republic. The farm was located in a dry, slightly warm area generally used for potato cultivation. The experiment included 59 Holstein cows, which were selected from the herd of 460 heads (22 cows on the first lactation, 15 cows on the second lactation, 13 cows on the third lactation, seven cows on the fourth lactation, and two cows on the fifth lactation). The selected cows were on average 81.05 days after calving, with a minimum of 32 days and a maximum of 132 days. The dairy cows were housed in free-stall stable bedded recycled manure solids. In addition, cows had access to a covered outdoor area. Cows on the farm were divided into several groups based on milk yield and lactation phase, with around 100 heads per group. Milking took place twice a day. The feed was given to the cows twice a day, after morning and evening milking. The feed was regularly added during the day. The composition of the feed ratio was adjusted based on daily milk yield.

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Data collection

Embryo flushing was performed during four visits on the farm, which took place from October to November and from March to June. All animals selected for embryo flushing were examined by ultrasonography (MyLab™OneVET; Esaote, Milan, Italy, with 8 MHz probe) and were included in the hormonal treatment if a corpus luteum (CL) was found. Oestrus synchronization was performed in these cows by injection of PGF2α analogue – Oestrophan (Bioveta JSC., Ivanovice na Hane, Czech Republic) at a dose of 0.5 μg (day 0). Subsequently, the development of CL was checked on the 3rd day after oestrus synchronization. Donor cows were super-ovulated on the 13th to 16th day of the oestrous cycle by the application of FSH – Pluset® (Laboratorios Callier, Barcelona, Spain). The dose of FSH was administrated every morning and evening for four days. The dose was being gradually reduced, and it was applied only in the morning on the 4th day. Oestrophan was re-administered to detect oestrus on the 15th day after the start of super-ovulatory treatment (Stadnik et al. 2013). Donor cows started to show oestrus symptoms from 16th to 17th day after the start of the treatment. All donors were inseminated 12 h after the detection of oestrus. Subsequently, each donor cow was re-inseminated by the same bull three times in 12 h intervals. The doses from the same bull and the same batch were used to re-inseminate the donor cows. Insemination and re-insemination were always performed by the same inseminationist technician.

The cranial parts of the uterine horns were flushed to collect embryos on the 7th day after the first insemination to obtain embryos. We used 300 ml of conventional flush medium supplemented with Krebs-Ringer phosphate with 1% of inactivated bovine serum (Bioniche, Ontario, Canada) to flush one uterine horn. Flushed oocytes and embryos were isolated and transferred to phosphate buffered culture medium supplemented with 20% fetal calf serum (Gibco™ BRL, Waltham, MA, USA). Subsequently, collected embryos were morphologically evaluated using a stereo microscope. The ovarian response (the number of corpora lutea on the left and right ovary), the number of flushed embryos for each phase of embryonic development were evaluated – undifferentiated (UN), fragmented (FR), morula (MO), very early blastocyst (VEBL), blastocyst (BL). In addition, the total number of flushed (all embryonic stages – UN, FR, MO, VEBL, BL) and transferable embryos (MO, VEBL, BL) were evaluated as well.

Data from milk performance control of dairy cattle and "in-line real-time" milk analysers (Afilab with Afifarm 4.1, Afikim, Israel) were used for the evaluation. We monitored daily milk yield (kg), the content of fat (%), and protein (%), with subsequent calculation of the fat/protein ratio in milk (F/P ratio). The average milk yield during the embryo-flushing day was 30.44 kg, ranging from 16.7 kg to 48.3 kg. The F/P ratio was evaluated 25 days before flushing, 11 days before flushing, and three days after flushing. The F/P ratio ranged from 0.79 to 1.83, which showed that tested cows had high variability in their energy balance, resp. metabolic status. The highest mean value of 1.2 was observed 25 days before flushing. This value gradually decreased to 1.18 at 11 days before flushing, and to 1.12 at three days after flushing.

The size of ovaries was measured by ultrasonography and tested cows were divided into three groups – small (up to 2 cm, n = 18), medium (3–4 cm, n = 29), and large (5 cm and more, n = 12).

Statistical evaluation

The program SAS v9.4 (SAS/STAT®; SAS Institute, Inc., Cary, NC, USA) was used for statistical evaluation. Basic statistics were calculated by the UNIVARIATE procedure. The CORR procedure was used to determine relations among monitored parameters. We assumed that all variables have a normal distribution for this evaluation. The GLM procedure was used for the evaluation of chosen effects on the number of corpora lutea on the right ovary (CL^R), the number of corpora lutea on the left ovary (CL^L), the number of undifferentiated embryos, the number of fragmented embryos, the number of morula-stage embryos, the total number of flushed embryos, and the total number of transferable embryos. The Tukey-Kramer method was used to evaluate differences of least square means. The model equation included fixed effects of lactation number, groups based on F/P ratio at 11 days before flushing, groups based on the size of the ovaries, linear regressions on days in milk, and milk yield. The effect of lactation number was adjusted to three levels (1. lactation, n = 22;

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2. lactation, n = 15; 3. and more lactation, n = 22). Tested cows were divided into three groups based on their fat/protein ratio at 11 days before flushing (closely corresponds to the metabolic status of the animals): under 1.1 (n = 16); from 1.1 to 1.3 (n = 25) and above 1.3 (n = 18). This grouping corresponds with the definition of a negative energy balance according to Cejna and Chladek (2005).

RESULTS

In Table 1 we present the basic statistics of the ovarian response and the numbers of flushed embryos for each stage. The right ovary showed a slightly better response to superovulation compared to the left ovary. We obtained on average 0.31 UN per cow per flushing. Cows in the test yielded a much higher proportion of FR (1.14) and MO (1.97). In contrast, we found only small numbers of embryos during stages of VEBL and BL, and therefore they were excluded from further evaluation. We obtain on average 3.98 embryos per cow per flushing, while 2.78 of them were viable for embryo transfer. However, we observed a high degree of variability in the ovarian response and the number of obtained embryos. This could be explained by differences in the metabolic status of tested animals, which we further investigated based on F/P ratio.

The model equation included the effects of lactation number, groups based on F/P ratio at 11 days before flushing, groups based on the size of the ovaries, and linear regressions on days in milk (DIM) and milk yield. The model explained variability from 22% (UN) to 65.1% (CL^L) and was statistically significant for all parameters, except for UN. Lactation number was not significant for evaluated parameters. The F/P ratio groups were statistically significant ($P < 0.05$) for CL^L, MO, the total number of flushed embryos and transferable embryos. The size of the ovaries significantly affected ($P < 0.05$) all monitored parameters except UN. In addition, DIM was statistically significant ($P < 0.05$) for FR, MO, the total number of flushed embryos and transferable embryos. Milk yield significantly ($P < 0.05$) corresponded only with the number of CL^R and CL^L.

Table 2 shows results for the effects of lactation number, F/P ratio, and the size of the ovaries on monitored parameters of embryo flushing. As in the model equation, the effect of lactation number did not show any significance for embryo flushing. The cows on the second and third lactation had numerically higher numbers of flushed and transferable embryos compared to cows on the first lactation, however, the differences were not significant. In addition, more MO were flushed from the cows on the second lactation compared to the cows on other lactation numbers. The highest number of FR was flushed from the cows on the third and subsequent lactation, while the most UN were observed for the cows on the first lactation.

Table 1. Basic statistics for the ovarian response, and the number of obtained embryos per cow per flushing

Variable	n	$\bar{x}$	SD	Min.	Max.	SE	V (%)
CL^R	59	3.66	2.55	0	10	0.33	69.66
CL^L	59	3.41	2.50	0	10	0.33	73.36
UN	59	0.31	0.59	0	2	0.08	194.91
FR	59	1.14	2.07	0	12	0.27	182.43
MO	59	1.97	1.99	0	9	0.26	101.27
VEBL	59	0.34	0.73	0	3	0.10	216.43
BL	59	0.24	0.80	0	4	0.10	335.13
Total number of flushed embryos	59	3.98	4.36	0	24	0.57	109.58
Total number of transferable embryos	59	2.78	2.75	0	10	0.36	98.88

BL = number of obtained blastocysts; CL^L = number of corpora lutea on left ovary; CL^R = number of corpora lutea on right ovary; FR = number of obtained fragmented embryos; MO = number of obtained morula-stage embryos; n = number of cases; SD = standard deviation; SE = standard error of arithmetic mean; UN = number of obtained undifferentiated embryos; VEBL = number of obtained early blastocyst-stage embryos; V = variation coefficient; $\bar{x}$ = arithmetic mean

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Table 2. Effect of lactation number, F/P ratio, and size of the ovaries on monitored parameters of embryo flushing

Effect	Level	CL^R	CL^L	UN	FR	MO	Total number of flushed embryos	Total number of transferable embryos
Lactation number	1	3.28 ± 0.403	2.86 ± 0.356	0.48 ± 0.126	0.85 ± 0.376	1.68 ± 0.326	3.27 ± 0.665	2.36 ± 0.440
	2	4.48 ± 0.498	3.58 ± 0.439	0.15 ± 0.156	1.11 ± 0.464	2.54 ± 0.403	4.67 ± 0.821	3.15 ± 0.544
	3+	4.22 ± 0.477	3.76 ± 0.421	0.27 ± 0.150	1.69 ± 0.445	1.85 ± 0.386	4.55 ± 0.787	3.16 ± 0.521
F/P ratio	under 1.1	4.37 ± 0.523	3.13 ± 0.461	0.29 ± 0.164	0.79 ± 0.487	2.00 ± 0.423	3.89 ± 0.862	3.32 ± 0.570
	1.1–1.3	3.96 ± 0.404	4.18 ± 0.357	0.44 ± 0.127	1.88 ± 0.377	2.77 ± 0.327^{a}	5.74 ± 0.666^{a}	3.66 ± 0.441^{a}
	above 1.3	3.63 ± 0.533	2.88 ± 0.470	0.18 ± 0.167	0.99 ± 0.496	1.30 ± 0.430^{b}	2.84 ± 0.878^{b}	1.69 ± 0.581^{b}
Size of ovaries	small	$2.08 \pm 0.497^{A,a}$	1.56 ± 0.439^{A}	0.07 ± 0.156	0.33 ± 0.463^{a}	1.13 ± 0.402^{a}	1.88 ± 0.819^{A}	1.70 ± 0.543^{a}
	medium	$3.81 \pm 0.390^{A,b}$	3.74 ± 0.344^{B}	0.27 ± 0.122	0.82 ± 0.364^{a}	2.00 ± 0.315	3.77 ± 0.643^{a}	2.75 ± 0.426
	large	6.08 ± 0.567^{B}	4.89 ± 0.500^{B}	0.57 ± 0.178	2.51 ± 0.529^{b}	2.94 ± 0.458^{b}	$6.83 \pm 0.935^{B,b}$	4.22 ± 0.619^{b}

CL^L = number of corpora lutea on left ovary; CL^R = number of corpora lutea on right ovary; F/P ratio = fat/protein ratio at 11 days before flushing; FR = number of obtained fragmented embryos; MO = number of obtained morula-stage embryos; UN = number of obtained undifferentiated embryos

^{A,B}Different letters in columns within effects means statistical significance ($P < 0.01$); ^{a,b}different letters in columns within effects means statistical significance ($P < 0.05$)

Data are presented as least square means ± standard error of least square means

The ovarian response, resp. CL^R and CL^L parameters did not significantly differ among the F/P ratio groups. However, numerically higher values for CL^R and CL^L were achieved by cows with the F/P ratio below 1.1, resp. in the range from 1.1 to 1.3. The highest numbers of UN (0.44) and FR (1.88) were achieved at the F/P ratio of 1.1–1.3, although the results were not significantly different to the other F/P ratio groups. We observed significant differences among the F/P ratio groups for MO, the number of flushed and transferable embryos ($P < 0.05$; Table 2). Significantly highest numbers of MO (+0.77 to 1.47; $P < 0.05$) were flushed at the F/P ratio of 1.1–1.3. The highest numbers of flushed (5.74) and transferable embryos (3.66) were observed for the group with an ideal F/P ratio (1.1–1.3). These values were significantly ($P < 0.05$) higher compared to the F/P ratio group above 1.3 and non-significantly exceeded the group of F/P ratio under 1.1. To summarize, the group of F/P ratio 1.1–1.3 achieved the best results.

The size of the ovaries significantly affected the monitored parameters, except UN. Even though differences in UN were not significant, we could see a clear trend, which was observed for all monitored parameters. As the size of the ovaries increased, numbers of CL^R ($P < 0.01$), CL^L ($P < 0.01$), FR ($P < 0.05$) and MO ($P < 0.01$) increased as well. These relations were further reflected in the total number of flushed and transferable embryos. We managed to flush +4.95 more embryos from large ovaries than from small ovaries ($P < 0.01$). In addition, large ovaries also produced +2.52 more transferable embryos compared to small ovaries ($P < 0.05$). Interestingly, small ovaries showed higher proportions of transferable embryos from the total number of flushed embryos. The differences grew, as the ovary size increased.

DISCUSSION

In this study, we aimed to time the experimental period for embryo flushing into autumn and spring period. The exclusion of the hottest and coldest months helped to minimize the effect of the season. Heat stress, which occurs in Central Europe especially during the summer months, has a significant detrimental effect on reducing feed intake and deepening of negative energy balance. The physiological development of ovarian and follicular processes,

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fertilization, and successful embryo development is also negatively affected (Bezdicek et al. 2021). Significance of heat stress on the super-ovulatory response, resp. the number of transferable embryos was also confirmed in the work of Barati et al. (2006).

Ferraz et al. (2016) confirmed the effect of lactation number on embryo viability when older dairy cows had a lower number of viable embryos compared to dairy cows on the first lactation and heifers. This observation was further confirmed by the study of Sartori et al. (2009), in which they demonstrated better results for heifers compared to lactating cows. This finding was indirectly confirmed by Maillo et al. (2012), who observed poor development of oocytes, as well as embryos, in animals with high milk yield compared to animals with lower milk production. These relations were not proven in our study, and we observed contradictory results. Parity did not significantly affect embryo flushing in our study. However, we noticed a clear trend, which suggests that embryo flushing was more successful in older cows compared to cows on the first lactation. One of the reasons behind this trend could be the already mentioned milk production, which could have caused more stress and higher a burden for the organism of first-lactation cows compared to cows with higher lactations. This could be advantageous especially from the perspective of embryo transfer when we need to use already well-tested and highly productive cows, which are well suited for specific production conditions.

The relationship between metabolic status and reproduction is not straightforward, and the biological competition for energy and nutrients between functions could be one of the reasons. Our findings regarding the effect of the metabolic status were indirectly confirmed by the studies, which focused on the evaluation of BCS as one of the indicators of the negative energy balance. Chrenek et al. (2015) reported that the quality of aspirated oocytes was better in Holstein cattle with an average body condition score of 3 compared to animals with a lower score. Similarly, Kasimanickam et al. (2020) observed more FR for cows with an ideal BCS compared to animals that were too thin, or too fat – therefore with inadequate energy balance. In agreement, we observed the best results for embryo flushing for the group with F/P in the range from 1.1 to 1.3. Cows are in a deep negative energy balance when the F/P ratio increases above 1.35 (Heuer et al. 1999). Cows with a low F/P ratio, which were in our study represented by the group of F/P ratio below 1.1, could be according to Kleen et al. (2003) characterized as a group with subclinical acidosis due to low production of free fatty acids in the rumen, which are precursors for fat in milk. These differences in metabolic status could be the reason behind better results for the group with a low F/P ratio than for the group above 1.3, but worse compared to the group with an ideal F/P ratio of 1.1 to 1.3. The optimal timing of embryo flushing seems to be outside of the deep NEB period, as was also confirmed by Stadnik et al. (2017), when they observed that timing of embryo transfer in the later postpartum period resulted in greater ovarian activity and embryo yield compared to early lactation periods. Noya et al. (2020) made the opposite finding in their nutritional experiment. Authors observed better functioning ovaries in the group of cows fed with energy-deficient feed and therefore experiencing deeper negative energy balance compared to the overfed group.

Multiple factors can influence the number of flushed embryos and overall ovarian response for cows with a high F/P ratio, including inflammatory diseases of the reproductive tract as described by Kasimanickam et al. (2020). Our hypothesis was also confirmed by studies that used other indicators of NEB, such as changes in the concentration of non-esterified fatty acids, β-hydroxybutyrate, glucose, or IGF-1 (Noya et al. 2020). Leroy et al. (2017) showed a negative effect of deep NEB on the number of embryonic cells and the overall quality of embryo maturation. Leroy et al. (2017) used high NEFA concentration and low glucose concentrations as indicators of deep NEB, and concluded that these concentrations of substances were toxic to the oocyte and strongly impaired its ability to mature and correctly develop in subsequent stages. Makarevich et al. (2016) and Bezdicek et al. (2020) reported similar findings for the quality of embryos depending on body condition.

In our opinion, using the F/P ratio to assess metabolic status is the most viable way from the practical standpoint, because values for fat and protein in milk are routinely collected through performance control of dairy cattle, or could be automatically collected by milking parlours, milking robots, and "in line" milk analysers. This accessible information will allow the farmer to make a better decision about the selection of animals for superovulation and the timing of embryo flushing.

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CONCLUSION

The size of the ovaries positively affected the total number of flushed embryos, as well as transferable embryos in our study. We also observed a significant effect of the cow's metabolic status, when the cows in deep negative energy balance yielded the lowest number of flushed and transferable embryos. The ideal metabolic state was in our study represented by a group of cows with the F/P ratio between 1.1 and 1.3, and this group produced significantly more embryos than cows with the F/P ratio above 1.3. Our results had proven the need to closely monitor the energy balance of dairy cows that were selected for embryo flushing, because optimal results can only be achieved by cows that are no longer in negative energy balance. Even though lactation number did not significantly affect our results, we noticed a clear trend, which suggests that the older cows are more suitable for purposes of embryo flushing, because of their higher number of flushed and transferable embryos. Our findings indicate the need to choose donors in a good metabolic state for the preparation and realization of embryo flushing on an individual basis, and using the F/P ratio to make this determination is a viable approach.

Conflict of interest

The authors declare no conflict of interest.

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