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Theriogenology

The composition of early lactation milk in recipient dairy cows determines success in bovine embryo transfer

Toshihiro TAKANO[1,2)#], Akira GOTO[3)#], Toshihisa SUGINO[1)*]

[1)]Research Center for Animal Science, Graduate School of Integrated Science for Life, Hiroshima University, Hiroshima, Japan
[2)]Fukuoka Prefecture Dairy Cooperative Association, Fukuoka, Japan
[3)]Department of Veterinary Medicine, Faculty of Veterinary Medicine, Okayama University of Science, Ehime, Japan

ABSTRACT. To identify new criteria for selecting recipient dairy cows for embryo transfer (ET), we retrospectively examined the effects of the composition of early lactation milk on fertility risk in ET. This study investigated the association between milk fat (FAT), milk protein (PRO), and milk urea nitrogen (MUN) levels during early lactation, based on production records, and subsequent fertility risk in ET using contingency table analysis and multivariable logistic regression analysis, which included five confounding variables. The results showed that MUN levels during early lactation were negatively associated with fertility risk in ET, while FAT and PRO levels showed no clear association. A reduction in MUN levels during the peak lactation period suggests a deficiency in dry matter intake, an inadequate protein supply, and an imbalance in the ratio of proteins to fermentable carbohydrates in the rumen, which may have adversely impacted fertility risk in ET. Monitoring MUN levels is crucial for maintaining a proper protein balance. The results obtained in this study suggest that MUN levels in the early lactation phase obtained from production records can be used as a predictor of fertility in recipients to improve the fertility risk in ET. No special techniques or costs are required for using production records, making them easy to use in clinical practice. Our findings provide valuable insights for optimizing cost-effectiveness and fertility risk in ET and their clinical applications.

KEYWORDS: early lactation, embryo transfer (ET), milk composition, milk urea nitrogen (MUN), recipient

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In Japan's dairy industry, the use of embryo transfer (ET) technology is driven by farmers' needs for genetic improvements, beef cattle production using Japanese Black (JB) cow embryos, and effective interventions against summer infertility in dairy cows. The JB breed is unique to Japan and is traded at a higher price than that for other beef calves [19]. The production of JB calves using ET technology in dairy cows has emerged as an additional income source for dairy farmers in Japan. Additionally, ET is stable fertility even in hot climates [36], making it a critical technology for mitigating the decline in dairy cow fertility caused by the summer heat in Japan [15, 34, 35]. Oro [29] compared the historical ET records of various countries, as compiled by the IETS, with those of Japan. He reported that the rate of cows undergoing ET in Japan is higher than in other countries, with nearly 90% of the in vivo embryos used being JB embryos.

Recipient selection is among the most critical factors in maximizing the cost-effectiveness of ET. The criteria for selecting recipient cows vary widely, including the corpus luteum (CL) size [11] and the presence or absence of coexisting first-wave dominant follicles [28]. In addition, blood flow assessment in the CL using color Doppler ultrasonography (CDUS) has been reported to be a useful indicator for selecting recipient cows [21, 31]. However, owing to the high cost of CDUS devices, it will probably take time before it is extensively used in clinical practice. Moreover, scoring and assessing vascular perfusion in multiple animals, such as in large-scale fixed-time ET programs, is labor-intensive. Therefore, it is difficult for farmers and technicians to share the subjective criteria for selecting cows for ET. In the future, to maximize the cost-effectiveness of ET, it will be necessary to identify objective criteria that can be widely used for selecting cows for ET.

Cook *et al*. [5] reported that individual cow milk recordings, such as dairy herd improvement (DHI) records, are readily available

*Correspondence to: Sugino T: sugino@hiroshima-u.ac.jp, Research Center for Animal Science, Graduate School of Integrated Science for Life, Hiroshima University, Hiroshima 739-8528, Japan
#These authors contributed equally to this work.

to dairy farmers and technicians. These records provide a non-invasive and affordable method at the individual cow and herd level to assess and monitor their early lactation energy status and future reproductive potential [5]. Among the critical milk components during early lactation, milk fat (FAT) is an indicator of body fat mobilization [6] due to the negative energy balance (NEB) during early lactation and is negatively correlated with later reproductive performance [22]. Moreover, the NEB is most extreme during the 1st month of lactation in dairy cows [6, 30]. Therefore, if a relationship is identified between the FAT value at the first DHI test and the fertility risk in subsequent ET, the FAT value could serve as a criterion for selecting recipients for subsequent ET. A few reports have demonstrated an association between the levels of milk components, such as milk protein (PRO) [25] and milk urea nitrogen (MUN) [14] during early lactation and later fertility. Madouasse *et al.* [23] reported that PRO concentrations measured on the second post-calving test date were relatively more strongly associated with reproductive performance than those measured on the first test date. Therefore, if a relationship is identified between the PRO and MUN values obtained in the second DHI test after calving and the fertility risk in subsequent ET, these values could serve as criteria for selecting recipients for subsequent ET. However, to our knowledge, there are no reports on the relationship between the values for early lactation milk composition in dairy cow recipients and subsequent ET fertility. If we could determine the relationship between the values for early lactation milk composition in potential ET recipient cows and later their fertility risk in ET, these scores for early lactation milk composition could be a critical factor in the selection of suitable ET recipients. Additionally, milk composition values derived from production information, such as DHI records, can be inexpensively disseminated to producers and technicians as quantifiable selection criteria for dairy cow recipients.

In this study, we aimed to identify new, quantifiable, and widely applicable criteria for selecting dairy cow recipients by analyzing past DHI and clinical ET records. We focused on FAT levels in the first DHI test after calving and PRO and MUN levels in the second DHI test after calving as early lactation milk composition indicators in cows that later transferred embryos. We hypothesized that these milk composition values could predict subsequent ET fertility and tested this hypothesis through a retrospective cohort study, using fertility risk as the dependent variable and the FAT, PRO, and MUN values as independent variables.

MATERIALS AND METHODS

This is a retrospective study based on past clinical records and not a study involving live animals. Therefore, ethics approval was not required for this study.

In this study, we analyzed clinical and DHI records from 484 transfers of fresh or frozen embryos performed in Fukuoka Prefecture, Japan, on several commercial dairy farms by a skilled only one technician between January 1, 2017, and December 31, 2020. Fukuoka, in southern Japan, has a subtropical climate with humid summers and comparatively moderate winters. Among them, data from cows transferred multiple times during the lactation period, data from cows whose first DHI test was more than 35 days after calving, and data from cows with missing values in their records were excluded from the analysis. Additionally, to minimize the influence of reproductive disorders, ET records for cows exceeding 200 days in milk (DIM) were excluded from the analysis [3]. As a result, a total of 291 ET records were included in the final analysis. All recipients were primiparous or multiparous Holstein–Friesian dairy cows, and their average parity at the time of transfer was 2.5 ± 1.5, and the average number of DIM at transfer was 128 ± 36.

Recipient cows were prepared by natural estrus or synchronization using hormone drug treatment before transfer. Synchronized hormonal treatments were performed with the insertion of an intravaginal controlled internal drug release (CIDR) device releasing progesterone (CIDR1900; Zoetis Japan, Tokyo, Japan) for 8–9 days. In addition, an intramuscular injection of estradiol (2 mg; OVAHORMON, estradiol benzoate; ASKA Animal Health, Tokyo, Japan) was administered at the time of progesterone device insertion. Later, these cows received an intramuscular injection of prostaglandin $F_{2\alpha}$ (0.5 mg; Resipron-C, cloprostenol; ASKA Animal Health) when the CIDR device was removed from these cows. The CL of these cows was assessed using transrectal ultrasound imaging (iMAGO; IMV Imaging, Angouleme, France) before ET, and cows with a CL diameter of at least 20 mm, including the CL cavity, were selected. Subsequently, 9–10 days after CIDR removal, the embryos were transferred using a disposable catheter gun (YT gun; Yamanetech, Nagano, Japan) into the deep uterine horn, ipsilateral to the CL. Pregnancy was diagnosed 33 days after the transfer using ultrasonography.

Both *in vitro* and *in vivo* embryos were used in this study. The *in vivo* embryos used in this study were obtained from super-ovulated cows by non-surgical flushing on day 7 (day 0 =estrus) following artificial insemination (AI). The retrieved embryos were assessed using the classification criteria of the International Embryo Technology Society (IETS). Among them, embryos with quality code 1 (IETS code 1) and 2 (IETS code 2) were classified as “excellent,” “good,” or “fair.” Fresh embryos were loaded in a straw and immediately transferred to a recipient. A few embryos classified as “excellent” were cryopreserved within 3.0 hr of their retrieval using the method described in Aoyagi *et al*. [1] and later transferred non-surgically to the recipients directly after thawing. The *in vitro* embryos used in this study were fresh and frozen embryos produced by the Livestock Improvement Association of Japan. The embryos were cultured *in vitro* following the procedures described by Hamano *et al.* [16].

The early lactation milk composition was determined using the DHI test records obtained after calving. The milk compositions used as indices were FAT, PRO, and MUN, which were extracted from the monthly records of the DHI. The FAT values were extracted from the first DHI test after calving (DIM; minimum 6, maximum 35, and median 21), and the PRO and MUN values were extracted from the second DHI test after calving (DIM; minimum 36, maximum 70, and median 52). Each was used as a representative value.

The results were analyzed using contingency table analysis and multivariate logistic regression models. χ^2 and Cochran–Armitage trend tests were used to analyze categorical variables using a group contingency table analysis. In the logistic regression model, fertility risk was the dependent variable, while the levels of FAT, PRO, and MUN were the independent variables. DIM at transfer; the parity of the recipients (primiparous or multiparous); transfer season (spring, summer, autumn, or winter); embryo state (fresh or

frozen-thawed); and the embryo type (*in vivo* or *in vitro*) were included as confounding variables. In the analysis, we divided the levels of FAT, PRO, and MUN into three groups (<25%, 25 to <75%, and ≥75%) based on quartiles, and similarly divided DIM at transfer into four (<25%, 25 to <50%, 50 to <75%, and ≥75%) quartile-based groups. Additionally, there was no evidence of multicollinearity among variables. Values were considered statistically significant at $P<0.05$. All statistical analyses were performed using SAS version 9.4 (SAS Institute Japan Ltd., Tokyo, Japan).

RESULTS

The fertility risk by category for each milk composition, recipient, environmental, and embryo factor is shown in Table 1. A significant difference was observed in the variable of embryo state. In addition, a statistically significant trend was observed in the variable of MUN in the Cochran–Armitage trend test (Table 1). Tables 2–4 describe the results of the multivariate logistic regression analysis of the relationship between milk composition and fertility risk in ET, including confounding variables. As shown in Tables 2 and 3, there were no significant associations between the dependent variable fertility risk and the independent variables FAT (Table 2) and PRO (Table 3) levels. Table 4 shows a significant negative association between fertility risk and MUN levels as the independent variable. The lowest MUN category (<9.2 mg/dL) had a significantly lower odds ratio of 0.450 compared to the highest MUN category (≥13.6 mg/dL). In addition, Table 4 shows significant differences between the fertility risk and confounding variable of embryo state.

DISCUSSION

In this study, we investigated the association between the composition of early lactation milk and fertility risk in ET to determine whether milk composition can be used as a selection criterion for ET recipients. There was no significant association between the levels of FAT and PRO and fertility risk in ET in either the contingency table analysis or the multivariable logistic regression analysis; however, there was a significant association between MUN levels and fertility risk in both analyses.

Elevated FAT levels during early lactation, associated with NEB, suggest metabolic diseases [18]. Variation in FAT levels during early lactation is an important indicator of energy supply [6], and Kristula *et al*. [22] reported a negative correlation between the conception rate at first AI and FAT levels at the first DHI test. However, in this study, we did not find a significant association between FAT levels and conception success in ET. The blood levels of non-esterified fatty acids (NEFA), a measure of body fat mobilization, and FAT levels in dairy cows during early lactation have been shown to exhibit a positive correlation, and the increase in NEFA levels in the blood is concentrated at 6–7 days postpartum [8, 24]. The NEB was most extreme, on average, during the 2nd to 3rd

Table 1. Difference in fertility status in various affecting factors

Variable	Category	*N*	Conception		*P*	*P*-trend
			n	%		
Milk fat (%)	<3.57	71	43	60.6	0.3054	0.5175
(1st test record)	3.57≤ to <4.39	147	81	55.1		
	4.39≤	73	48	65.8		
Milk protein (%)	<2.79	69	41	59.4	0.2103	0.2780
(2nd test record)	2.79≤ to <3.07	142	90	63.4		
	3.07≤	80	41	51.3		
Milk urea nitrogen (mg/dL)	<9.20	69	34	49.3	0.0738	0.0225
(2nd test record)	9.20≤ to <13.60	147	87	59.2		
	13.60≤	75	51	68.0		
Days in milk at transfer	<97	70	39	55.7	0.8269	0.7529
	97≤ to <125	75	45	60.0		
	125≤ to <157	73	46	63.0		
	157≤	73	42	57.5		
Parity of recipients	Primiparous cow	96	61	63.5	0.2802	-
	Multiparous cow	195	111	56.9		
Season of transfer	Spring (March–May)	72	43	59.7	0.7634	-
	Summer (June–August)	56	30	53.6		
	Autumn (September–November)	80	47	58.8		
	Winter (December–February)	83	52	62.7		
Embryo state	Fresh embryo	119	80	67.2	0.0191	-
	Frozen−thawed embryo	172	92	53.5		
Embryo type	*in vivo*	268	161	60.1	0.2515	-
	in vitro	23	11	47.8		

N: number of recipients, *n*: number of pregnant recipients, *P*: χ^2 test, *P*-trend: cochran–armitage trend test.

Table 2. Multiple logistic regression analysis determined the association between fertility status and the first milk fat

Variable	Category	*N*	*P*	OR	95% CL	
Independent variable						
Milk fat (%)	<3.57	71	0.5880	0.824	0.408	1.662
(1st test record)	3.57≤ to <4.39	147	0.2678	0.707	0.382	1.306
	4.39≤	73	Ref			
Confounding variable						
Days in milk at transfer	<97	70	0.7383	0.890	0.449	1.763
	97≤ to <125	75	0.6983	1.146	0.576	2.279
	125≤ to <157	73	0.3378	1.401	0.703	2.793
	157≤	73	Ref			
Parity of recipients	Primiparous cow	96	0.2810	1.340	0.787	2.282
	Multiparous cow	195	Ref			
Season of transfer	Spring (March–May)	72	0.7588	0.901	0.463	1.753
	Summer (June–August)	56	0.2660	0.668	0.328	1.361
	Autumn (September–November)	80	0.6187	0.846	0.439	1.633
	Winter (December–February)	83	Ref			
Embryo state	Fresh embryo	119	0.0192	1.851	1.105	3.099
	Frozen−thawed embryo	172	Ref			
Embryo type	*in vivo*	268	0.4065	1.462	0.597	3.581
	in vitro	23	Ref			

N: number of recipients, *P*: probability of the reference category in the variable, OR: odds ratio, 95% CL: 95% confidence limit.

Table 3. Multiple logistic regression analysis determined the association between fertility status and the second milk protein

Variable	Category	*N*	*P*	OR	95% CL	
Independent variable						
Milk protein (%)	<2.79	69	0.2978	1.442	0.724	2.874
(2nd test record)	2.79≤ to <3.07	142	0.0637	1.737	0.969	3.115
	3.07≤	80	Ref			
Confounding variable						
Days in milk at transfer	<97	70	0.8250	0.925	0.463	1.847
	97≤ to <125	75	0.4486	1.311	0.651	2.641
	125≤ to <157	73	0.2757	1.472	0.734	2.950
	157≤	73	Ref			
Parity of recipients	Primiparous cow	96	0.3846	1.268	0.742	2.165
	Multiparous cow	195	Ref			
Season of transfer	Spring (March–May)	72	0.9336	0.972	0.497	1.900
	Summer (June–August)	56	0.2630	0.665	0.325	1.359
	Autumn (September–November)	80	0.4591	0.777	0.399	1.515
	Winter (December–February)	83	Ref			
Embryo state	Fresh embryo	119	0.0100	1.951	1.173	3.243
	Frozen−thawed embryo	172	Ref			
Embryo type	*in vivo*	268	0.3280	1.561	0.639	3.813
	in vitro	23	Ref			

N: number of recipients, *P*: probability of the reference category in the variable, OR: odds ratio, 95% CL: 95% confidence limit.

week of lactation [30]. As the DHI test is administered once a month and the DIM at the time of the first test can vary by less than 30 days between cows, the records of cows that passed the transition period [13] of 3 weeks before and after parturition, which occurs in NEB, were included in the first test records. The median DIM in the first test was 21. Therefore, the first DHI record was defined as an index of early lactation, which may not accurately reflect fluctuations in FAT levels during the transition period. Van Hoeck *et al*. [37] have reported decreased oocyte developmental capacity at elevated NEFA concentrations and its effects on the quality and viability of early embryos. Fertility assessment in this study was based on fertility risk in transfer using externally derived normal embryos, which may have bypassed the adverse effects of elevated maternal NEFA levels on reproduction. In this study, FAT levels were categorized and compared based on quartiles. Future analyses need to be conducted after the reevaluation of the reference FAT value, as Kristula *et al.* [22] indicated abnormal FAT levels at ≥4.5% for the first postpartum test.

Table 4. Multiple logistic regression analysis determined the association between fertility status and the second milk urea nitrogen

Variable	Category	*N*	*P*	OR	95% CL	
Independent variable						
Milk urea nitrogen (mg/dL) (2nd test record)	<9.20	69	0.0274	0.450	0.221	0.915
	9.20≤ to <13.60	147	0.2654	0.707	0.384	1.302
	13.60≤	75	Ref			
Confounding variable						
Days in milk at transfer	<97	70	0.8462	0.934	0.468	1.863
	97≤ to <125	75	0.5491	1.238	0.616	2.488
	125≤ to <157	73	0.2523	1.504	0.748	3.028
	157≤	73	Ref			
Parity of recipients	Primiparous cow	96	0.3726	1.273	0.749	2.165
	Multiparous cow	195	Ref			
Season of transfer	Spring (March–May)	72	0.8526	0.938	0.480	1.834
	Summer (June–August)	56	0.3121	0.692	0.339	1.413
	Autumn (September–November)	80	0.8719	0.946	0.481	1.861
	Winter (December–February)	83	Ref			
Embryo state	Fresh embryo	119	0.0093	1.967	1.182	3.276
	Frozen−thawed embryo	172	Ref			
Embryo type	*in vivo*	268	0.4268	1.439	0.586	3.534
	in vitro	23	Ref			

N: number of recipients, *P*: probability of the reference category in the variable, OR: odds ratio, 95% CL: 95% confidence limit.

In this study, no significant association was observed between PRO levels and fertility risk in ET. However, PRO is a milk constituent strongly associated with energy balance [9], and PRO levels in the early lactation phase have been shown to exhibit a positive correlation with later reproductive performance [25]. In this study, PRO levels were derived from the second test results of the DHI, and many of the embryos were transferred several months after the second test (DIM at transfer: lower quartile 97, median 125, and upper quartile 157). The concentration of PRO during the breeding period exhibits a stronger association with conception success than during the early lactation period [26]. An NEB during early lactation is unlikely to affect PRO concentrations considerably during mid- and late lactation [12]. A likely reason for the lack of a significant association between PRO concentrations and fertility risk in ET in this study was the time lag between the 2nd test and ET, which may have resulted in improvement in PRO concentrations in the early lactation until the breeding period. In the future, the effects of PRO concentrations on fertility risk in ET need to be comprehensively investigated during the breeding period.

The MUN levels at the peak of lactation were significantly associated with a marked trend toward fertility risk in ET, with the lowest fertility risk in the category with MUN <9.2 mg/dL. MUN levels are an indicator used to evaluate the nitrogen nutritional status of dairy cows [2] and reflect milk nitrogen excretion [20]. In general, high MUN levels correlate negatively with fertility [14, 32], are toxic to early embryos [27], and affect oocyte cleavage and blastocyst formation [14]; however, their adverse effects on early embryos (day 0 to day 6) may have been circumvented by the transfer of externally derived normal embryos (*in vivo* embryos recovered on day 7 or *in vitro* embryos). In addition, MUN levels correlate positively with lactation volume [10] and increase in response to physiological changes during early to mid-lactation [7]. Therefore, low MUN levels during early lactation indicate a severe deficiency in dry matter intake [4]. During peak lactation, when MUN levels are physiologically elevated, low MUN levels can be attributed to inadequate protein supply and an imbalance between the levels of proteins and fermentable carbohydrates in the rumen [33], which may have exerted the strongest effect on subsequent fertility risk in ET. Furthermore, as observed with the impact of PRO levels on fertility risk in ET, we cannot exclude the possibility that the time lag between the 2nd test and ET may have affected fertility in ET in response to variations in MUN levels. In the future, we need to reconsider the relationship between MUN and fertility risk in ET during the breeding period.

Among the confounding variables, we observed a significant association between the dependent variable and embryo state. Regarding the effects of the state of the embryo on fertility risk in ET, the conception rate in ET with frozen embryos is reported to be approximately 10% lower than that with fresh embryos of similar quality [17], consistent with the results of this study.

The following limitations must be considered while interpreting the data obtained in this study. First, the data were mostly gathered from DHI subscribers; therefore, the sample population may not fully reflect all farms in the study area. Second, sampling for herd testing was performed once every 30 days, and the tests performed may have resulted in up to a 30-day difference in DIM between individuals. Moreover, cows with inadequate CL formation and a less than 20 mm CL diameter were excluded in this study. Therefore, including recipient cows that were not eligible for ET should be reconsidered in future studies. Finally, this analysis identified MUN as the only variable significantly linked to ET fertility, with no significant associations observed for FAT or PRO. However, previous studies have indicated that FAT and PRO levels during early lactation could influence subsequent fertility [22, 23, 25]. The inconsistency in our findings may be unique to ET, as opposed to artificial insemination. In the future, more detailed analyses must be

conducted using large datasets that include additional confounding variables such as feed protein amount and quality, feeding system, and stall type. In addition, continued research is required in the future, such as exploring specific MUN values (e.g., setting cutoff values) that can determine whether ET is possible.

In summary, this study investigated the relationship between fertility risk as the dependent variable and three milk compositions as the independent variable to test the hypothesis that early lactation milk composition in recipient cows can predict fertility risk in subsequent ET. The results identified MUN levels at peak lactation as a key determinant of fertility risk in ET, while FAT and PRO levels had no significant impact. MUN levels below 9.2 mg/dL suggest reduced dry matter intake, insufficient protein supply, and an imbalance between protein and fermentable carbohydrates, potentially reducing fertility risk. Monitoring MUN levels can help predict fertility risk in ET, assist in selecting appropriate recipient cows, and offer a cost-effective approach using production records.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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