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Fertility risk factors in transferring Japanese Black embryos into dairy heifers: An epidemiological study

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ABSTRACT

The aims of this study were 1) to summarize the current status of Japanese Black (JB) embryo transfer into Holstein heifers, which is carried out on a commercial basis in Japan, and 2) to reveal fertility risk factors, including those from the environment (year and season of transfer), recipient (age, number of transfers, clinical status of the ovaries) and embryo (quality, stage, state, genetic background). We used data from 4467 JB fresh or frozen embryo transfers into Holstein heifers conducted by Zen-noh Embryo Transfer Center during 2016–2018, and the differences in fertility risk due to factors related to the environment, recipient, and embryo were statistically evaluated. Differences in fertility risk due to each variable were observed, leading to significant differences in fertility with respect to year of transfer, embryo quality, embryo state, and embryo breed. These results suggest that the fertility of JB embryos might depend on differences in genetic background. There have been no previous reports of differences in embryo fertility due to the differences among JB's bloodline combinations. In the future, overall reproductive efficiency must be monitored, including the effects of different bloodline combinations on the success of embryo recovery and transfer.

1. Introduction

The Japanese Black (JB; known as "Wagyu") is the most common high-quality beef cattle breed in Japan, with a share of 90% (MAFF, 2016). There are three basic bloodlines (pedigrees) of JB, each of which has unique and specific characteristics. The characteristics of the Tajiri-line are its marbling quality (highest), smaller frame, and lower growth rates. The characteristics of the Kedaka-line are its larger frame and good growth, but its meat quality is inferior as compared with the other lines. The Fujiyoshi-line is medium framed with average growth rates and good meat quality (Facioli et al., 2020; Hirayama et al., 2019). It has also been reported that there are differences in reproductive characteristics, such as the response to superovulation, among the bloodlines (Hirayama et al., 2019). A desirable characteristic of JB beef is the marbled fat (intramuscular fat) distributed through the muscle fibers, which is extremely tender and desirable. These unique features of JB beef are not found in other beef cattle breeds, making JB beef an extremely valuable commodity (Gotoh, Takahashi, Nishimura, Kuchida, & Mannen, 2014).

According to the Japanese national statistics, the number of JB breeding cattle peaked at 684,000 in 2010, and decreased to a low of 580,000 in 2015 before slightly recovering to 629,000 in 2019. It is concerning, however, that the JB population has suffered due to a decrease in the number of breeding cattle (MAFF, 2020). As a means to complement the decrease in JB breeding cattle, the Japanese government has recommended JB embryo transfer (ET) into dairy cattle. The body size of JB calves at birth is smaller than that of dairy calves (Isogai, Shirai, & Ikeuchi, 1994); therefore, ET with JB embryos is unlikely to cause dystocia in dairy heifers. Moreover, JB calves are purchased as feeder livestock at higher prices than dairy calves, so a JB calf can represent precious income to dairy farms. Currently, in Japan, JB embryos are transferred into about 100,000 dairy cows per year, and about 42,000 JB calves are birthed (Oro, 2019). Japanese Black calves produced by ET make up about 8% of all JB calves produced annually,

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bolstering the population of JB cows (Oro, 2019). However, over the past 20 years, the fertility risk associated with ET in Japan has been stagnant at 52% for fresh embryos and 46% for frozen embryos (MAFF, 2015). To achieve more efficient production of JB cattle, further improvement of ET technology is required.

Reproductive epidemiology is an essential tool for assessing reproduction problems (Koketsu, Sasaki, Ichikawa, & Kaneko, 2010). However, there is very little statistical information detailing the current status of ET in Japan, so it is difficult to understand its distribution, and verify the causality of fertility risk factors on ET. There are several reproductive epidemiological reports in JB cattle (Irikura, Uematsu, Kitahara, Osawa, & Sasaki, 2018; Sasaki, Uematsu, Kitahara, & Osawa, 2016), but very few studies addressing ET using JB embryos. It is essential to conduct an epidemiological analysis in order to propose effective measures to improve ET of JB embryos. Since the bloodline is regarded as important in the production of JB cattle, embryos of various bloodlines have been used in the production of JB cattle by ET to dairy cattle according to the demand of the dairy farmers. It is known that the fertility of JB cattle is affected by the bloodline (Hirayama et al., 2019), therefore the differences bloodline may also affect the conception rate in ET using JB embryos. However, there are no studies investigated the effects of the bloodline of JB embryo on the conception rate in ET to dairy cattle. Clarifying the fertility of embryos among bloodline combinations will contribute to improving JB beef productivity in the future.

The aims of this study were 1) to summarize the current status of ET of JB embryos into Holstein heifers, which is carried out on a commercial basis in Japan, and 2) to reveal the differences in fertility risk due to factors such as the environment (year and season of transfer), recipient (age, number of transfers, clinical status of the ovaries) and embryo (quality, stage, state, genetic background).

2. Material and methods

This study was carried out in accordance with the guidelines for the care and use of laboratory animals of Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan.

2.1. Study area and animals

Data used in this study were collected from the Naitai Plateau public ranch in the Tokachi region in Hokkaido, Japan. The Tokachi region is well known for dairy production, and has a subpolar climate with a mild summer and very cold winter. All recipient animals (0.9–2.5 year old Holstein-Friesian heifers) were fed via an equal self-sufficient feeding and management system. All recipients were confirmed negative for bovine viral diarrhea-mucosal disease (BVD-MD) and had also been vaccinated twice a year with five mixed (infectious bovine rhinotracheitis virus; IBRV, bovine viral diarrhea virus; BVDV, bovine parainfluenza virus; BPIV, bovine respiratory syncytial virus; BRSV, bovine adenovirus; BAdV) inactivation vaccine. In this study, data from 4467 embryo transfers of JB fresh or frozen embryos conducted between 1 April 2016 and 30 October 2018 by Zen-noh Embryo Transfer Center were analyzed.

2.2. Procedures

Recipients were prepared by natural estrus or estrous synchronization using hormone drug treatment prior to transfer. Synchronized treatments were performed using a single intramuscular injection of PGF2α (0.15 mg; Dalmazine; D-cloprostenol, Kyoritsuseiyaku, Tokyo, Japan), or insertion of an intravaginal progesterone-releasing device (OVAPRON-V; Kyoritsuseiyaku, Tokyo, Japan) for 8 days with intramuscular injection estradiol (2 mg; OVAHORMON, estradiol benzoate; ASKA Animal Health, Tokyo, Japan) at progesterone device insertion. The recipients received PGF2α intramuscular injection two days prior to OVAPRON removal. After induction of estrus, heifers were observed for estrus expression twice per day; estrus was detected by standing heat and rectal palpation. Additionally, a recipient's corpus luteum (CL) was examined by transrectal ultrasonography (HS-101 V; FHK, Tokyo, Japan, with 5 MHz linear probe) one day prior to ET. The presence (or absence), size (diameter including cavity), and location (left or right-side ovary) of the CL were confirmed. Then embryos were transferred (nonsurgically) into the deep uterus horn ipsilateral to the CL 7–8 days after detection of estrus using a disposable ET catheter (YT gun; YAMANETECH, Nagano, Japan). Pregnancy was diagnosed 53 days after ET via ultrasonography.

All embryos in this study were collected at day 7 (day 0 = estrus) via non-surgical recovery from super-ovulated JB after artificial insemination (AI) with frozen JB semen (sex-unsorted). Recovered embryos were evaluated according to the International Embryo Technology Society (IETS) classification. Among them, the quality code 1 (IETS code 1) and the quality code 2 (IETS code 2) embryos were classified into 'excellent' or 'good' or 'fair' categories. Fresh embryos were encapsulated in a straw and immediately non-surgically transferred to recipients. Some of the embryos classified as 'excellent' were cryopreserved within 3 h of recovery, as described by Aoyagi et al. (1996), and non-surgically transferred directly after thawing into heifer recipients at a later date.

2.3. Statistical analyses

Outcomes were summarized using summary statistics, and statistically analyzed using a multiple logistic regression model. The comparison of categorical variables was evaluated using chi-square and Cochran–Armitage trend tests using data from contingency table analyses among groups. The dependent variable in the logistic regression model was the fertility status. The independent variables were year of transfer (2016, 2017, or 2018), season of transfer (Spring, Summer, Autumn, or Winter), age of recipients, number of transfers, CL side (Left or Right), CL diameter ($\leq$ 20 mm or $>$ 20 mm), embryo quality (Excellent, Good, or Fair), embryo stage (Morula, Early blastocyst, Blastocyst, or Expanded blastocyst), embryo state (Fresh or Frozen-thawed), and embryo breed. Embryo breed was categorized into nine-categories, combining sire and donor of each of three-major JB bloodlines. In the analyses, we categorized the age of the recipients into four groups based on quartile points ($\leq$ 25%, 25–49.9%, 50–74.9%, and $\geq$ 75%), and CL diameter into two groups based on the median ($<$ 50% and $>$ 50%). In the categorization of embryo breed, variables present in less than 5% of the total number of recipients were combined and categorized as 'others'. Interaction effects between two categorical independent variables were included in the model, but insignificant interactions were removed from the final model ($P \geq 0.05$). 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).

3. Results

The fertility risks by category for each environmental, recipient, and embryo factor are shown in Table 1. Significant differences were observed in the embryo quality, embryo state, and embryo breed variables. In addition, a significant trend was observed in the year of transfer and embryo quality variables (Table 1). The relationships between fertility risk and ten confounding variables were analyzed using a multiple logistic regression model (Table 2). Bloodline information is depicted anonymously as capital letters of the alphabet. Significant differences were observed in year of transfer, embryo quality, embryo state, and embryo breed variables.

4. Discussion

In this study of 4467 embryo transfers, we found no significant differences in environmental factors (season of transfer) or recipient factors (age, number of transfers, clinical status of the ovaries) between

Table 1
Difference in fertility status in various affecting factors (Three bloodlines are shown anonymously) (Total 4467 transfers of Japanese Black embryos to Japanese Holstein-Friesian heifers, Tokachi region, Hokkaido, Japan, 2016–2018).

Variable	Category	*N*	Fertility risk		*P*	*P*-trend
			n	%		
Year of transfer	2016	1173	810	69.1	0.0837	0.0264
	2017	1847	1307	70.8		
	2018	1447	1056	73.0		
Season of transfer	Spring (March–May)	1140	829	72.7	0.0896	–
	Summer (June–August)	1232	893	72.5		
	Autumn (September–November)	1233	847	68.7		
	Winter (December–February)	862	604	70.1		
Age of recipients (year)	≤1.11	1117	803	71.9	0.6778	0.7294
	1.11 <– ≤ 1.22	1118	793	70.9		
	1.22 <–≤ 1.33	1116	778	69.7		
	1.33 <	1116	799	71.6		
Number of transfers	First time	3574	2551	71.4	0.5609	0.4091
	Secondtime	702	487	69.4		
	Third time or more	191	135	70.7		
CL side	Left	1866	1319	70.7	0.6658	–
	Right	2601	1854	71.3		
CL diameter	≤ 20mm	2660	1877	70.6	0.4027	–
	> 20mm	1807	1296	71.7		
Embryo quality	Excellent	3514	2572	73.2	< 0.0001	< 0.0001
	Good	621	395	63.6		
	Fair	332	206	62.0		
Embryo stage	Morula	963	696	72.3	0.2433	0.6642
	Early blastocyst	2217	1554	70.1		
	Blastocyst	1108	803	72.5		
	Expanded blastocyst	179	120	67.0		
Embryo state	Fresh embryo	3876	2814	72.6	< 0.0001	–
	Frozen–thawed embryo	591	359	60.7		
Embryo breed[1)]	*A* × *A*	323	218	67.5	0.0147	–
	A × *B*	340	262	77.1		
	A × *C*	1006	719	71.5		
	B × *A*	1049	706	67.3		
	B × *B*	3	3	100.0		
	B × *C*	345	256	74.2		
	C × *A*	1210	870	71.9		
	C × *B*	112	80	71.4		
	C × *C*	79	59	74.7		

N: number of recipients.
n: number of pregnant recipients.
P: $\chi 2$.
P-trend: cochran–armitage trend test.
[1)]Sire x dam bloodline combination.

successful and failed fertility groups. On the other hand, the year of transfer, embryo quality, embryo state, and embryo breed were identified as risk factors. The results of this study have great clinical significance for further improving the efficiency of JB calf production by ET into dairy Heifers.

Many factors, such as breed, nutrition, and other management factors are involved in a successful embryo transfer (Hasler, 2014; Mapletoft, Steward, & Adams, 2002; Peixoto, Bergmann, Suyama, Carvalho, & Penna, 2007; Takahashi, Sawada, Kawate, Inaba, & Tamada, 2013). Gonella-Diaza, Holguín, Montaña, and Valbuena (2013) concluded that CL diameter was an important factor affecting pregnancy rates *in vitro* embryos (fertility: 5495 recipients/17,521 ETs, 31.4%). In our study showing no effects of CL size, it should be noted that the fertility risk was much higher because we used in vivo fertilized embryos (fertility: 3173 recipients/4467 ETs, 71.0%). Differences in the type of embryos transferred may be possible reasons for the conflicting results in fertility differences between studies. Although we found no effect of recipient age on fertility risk, it should be noted that these study data were obtained from a single well-managed farm for heifers only. Fertility of the recipients is significantly affected by environmental factors such as year, season of transfer (Hasler, 2014; Peixoto et al., 2007), and heat stress associated with increased temperature-humidity index (Ferraz et al., 2016). In this study, year-round self-supplied feed management and the characteristic climate in the Tokachi region may have affected fertility risk in recipients. Further consideration of these factors, which may fluctuate from year to year, will be needed.

Furthermore, embryo stage was not identified as a significant factor in our results; Vieira et al. (2014) supported this result, but Ferraz et al. (2016) reported the opposite finding. Embryo quality is well known to be a significant factor in pregnancy rate (Hasler, 2004). In addition, Hasler (2004) reported that pregnancy rates resulting from transfer of frozen-thawed embryos are approximately 10 percent lower than those for fresh embryos of similar quality. Our results also clearly showed that embryo quality and state (fresh or frozen-thawed) have great impact on fertility risk, as was also seen in previous studies.

This is the first study to show that the embryo breed affects the conception rate in the ET of JB embryo. The reason why embryo breed appeared to be a risk factor may be due to differences in reproductive characteristics among JB's bloodlines. Hirayama et al. (2019) showed that differences in genetic background among bloodlines are involved in the response to superovulation in JB cattle. Tatsumi et al. (2018) reported that lipid droplets, which are necessary nutrients for early embryo development in mammalian embryos, play an important role in mouse embryo survival. Sturmey, Reis, Leese, and McEvoy (2009) determined that oocytes and early embryos utilize endogenous lipids as an energy substrate. Moreover, Takahashi et al. (2013) reported that supplementation with rumen bypass polyunsaturated fatty acids improve the likelihood of production of viable embryos in JB. These results suggest that lipid metabolism in embryos affects embryo development and quality. It is fairly simple to gain lipids in JB by genetic

Table 2
Association between fertility status and ten confounding variables as determined by Multiple logistic regression analysis (Three bloodlines are shown anonymously) (Total 4467 transfers of Japanese Black embryos to Japanese Holstein-Friesian heifers, Tokachi region, Hokkaido, Japan, 2016–2018).

	Variable	Category	*N*	EV	SE	*P*	OR	95% CI	
Independent variable	Embryo breed[1)]	*A* × *A*	323	Ref					
		A × *B*	340	0.505	0.179	0.005	1.657	1.166	2.355
		A × *C*	1006	0.224	0.142	0.113	1.251	0.948	1.651
		B × *A*	1049	0.005	0.139	0.971	1.005	0.766	1.319
		B × *C*	345	0.317	0.174	0.068	1.373	0.976	1.932
		C × *A*	1210	0.158	0.138	0.252	1.171	0.894	1.534
		Others (*B* × *B*, *C* × *B*, *C* × *C*)	194	0.238	0.205	0.245	1.269	0.849	1.895
Confounding variable	Year of transfer	2016	1173	Ref					
		2017	1847	0.110	0.085	0.198	1.116	0.944	1.320
		2018	1447	0.308	0.095	0.001	1.360	1.130	1.638
	Season of transfer	Spring (March–May)	1140	Ref					
		Summer (June–August)	1232	0.011	0.094	0.911	1.011	0.840	1.216
		Autumn (September–November)	1233	−0.155	0.093	0.096	0.857	0.714	1.028
		Winter (December–February)	862	−0.131	0.103	0.202	0.877	0.717	1.073
	Age of recipients (year)	≤ 1.11	1117	Ref					
		1.11 <–≤ 1.22	1118	−0.021	0.097	0.833	0.980	0.810	1.185
		1.22 <–≤ 1.33	1116	−0.092	0.098	0.347	0.912	0.753	1.105
		1.33 <	1116	−0.047	0.117	0.689	0.954	0.760	1.199
	Number of transfers	First time	3574	Ref					
		Second time	702	−0.137	0.107	0.201	0.872	0.707	1.076
		Third time or more	191	−0.121	0.186	0.514	0.886	0.615	1.275
	CL side	Left	1866	Ref					
		Right	2601	0.032	0.068	0.642	1.032	0.903	1.179
	CL diameter	≤ 20mm	2660	Ref					
		> 20mm	1807	0.065	0.069	0.346	1.067	0.932	1.221
	Embryo quality	Excellent	3514	Ref					
		Good	621	−0.581	0.097	< 0.0001	0.559	0.462	0.677
		Fair	332	−0.695	0.125	< 0.0001	0.499	0.391	0.638
	Embryostage	Morula	963	Ref					
		Early blastocyst	2217	−0.155	0.089	0.081	0.856	0.720	1.019
		Blastocyst	1108	−0.041	0.106	0.699	0.960	0.780	1.181
		Expanded blastocyst	179	−0.244	0.181	0.176	0.783	0.550	1.116
	Embryo state	Freshembryo	3876	Ref					
		Frozen–thawed embryo	591	−0.681	0.097	< 0.0001	0.506	0.419	0.612

[1)]Sire x dam bloodline combination.
N: number of recipients.
EV: estimated value.
SE: standard error.
P: probability of the reference category in the variable.
OR: odds ratio.
95%CI: 95% confidence intervals.

improvement moreover; lipid production and metabolism vary greatly depending on JB bloodlines (Facioli et al., 2020; Gotoh et al., 2014; Oka et al., 2002). Japanese Black also differs in their nutritional and metabolic capacities, and behavioral traits, depending on bloodlines (Uetake, Ishiwata, Kilgour, & Tanaka, 2012). Leroy et al. (2004) indicated that there was a significant correlation between the peripheral blood levels and the follicular fluids of lipid levels among dairy cows. Annes et al. (2018) indicated that the follicular microenvironment determines the oocyte developmental process. Taken together, it is possible that association exists between the lipid metabolism status of the donor and the lipid contained within the embryo. In fact, lipid metabolism in bovine embryos varies by breed between Jersey and Holstein-Friesian cows (Baldoceda et al., 2016). Therefore, lipid metabolism of JB embryos may also be affected by differences in bloodlines. Differences in lipid metabolism and reproductive characteristics caused by different genetic backgrounds among bloodlines may affect fertility risk in ET using JB embryos.

The differences in fertility of embryos among JB's bloodline combinations were clearly demonstrated. However, this study has some limitations. For example, the data used in this study only represent the ETs performed at a single facility. In addition, the effects of bloodline combination on conception rate of ET using only transferable embryos were analyzed. In other words, the effect of bloodline combination on embryo recovery performance, such as embryo fertilization rate and embryo quality, was not analyzed. Therefore, it is not possible to evaluate the impact of JB's bloodline combinations on overall reproductive performance, including the results of embryo recovery performance and transfer. This study is a limited research model aimed at determining the relationship between bloodline combinations in transferable embryos and their fertility outcomes. Future work should include a comparison of embryo recovery results with respect to bloodline combination. Moreover, more detailed information on the donors in addition to the recipients should be collected and analyzed, as there was little information available concerning the donors in this analysis. It is necessary to conduct multivariable statistical analysis by adding variables such as coefficient of inbreeding (Lazzari et al., 2011), and variables related to embryo productivity, such as donor age or parity (Ferraz et al., 2016; Hasler, 2014), to construct the best predictive models. In the future, if the impact of JB's bloodline combination on fertility can be fully understood, embryo production and transfer considering bloodline combination may improve the productivity of JB calves.

5. Conclusion

We performed a descriptive epidemiological analysis of ET of JB embryos into dairy heifers. We quantified the current status of ET in the Hokkaido region of Japan. Moreover, we identified the year of transfer, embryo quality, embryo state, and embryo breed as risk factors for

successful fertility. These results suggest that the fertility of JB embryos might depend on differences in genetic background.

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CRediT authorship contribution statement

Akira Goto: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Koh Hayama:** Conceptualization. **Manami Urakawa:** Conceptualization. **Yoshio Oono:** Conceptualization. **Ken Hazano:** Writing – review & editing. **Mitsunori Kayano:** Writing – review & editing. **Shingo Haneda:** Writing – review & editing. **Ken Nakada:** Data curation, Formal analysis, Writing – original draft. **Yrjö Tapio Gröhn:** Writing – review & editing. **Motozumi Matsui:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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