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Calves Derived from in Vivo Frozen-Thawed Embryos Collected from Canada Holstein Friesian Cows with High Genetic Background

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Abstract : Embryo Transfer (ET) is one of the assisted reproductive technologies and a useful tool for improving herds. The purpose of this study is to produce the calves using frozen embryos which were produced in the top one percent Holstein in Canada by ET. One hundred seventeen recipients were used for surrogate mothers and seventy cows were diagnosed to be pregnant. Fifty seven calves were born successfully and thirteen out of them failed to produce viable calves (abortion: 4, stillbirth: 9). Their gestational length, birth body weight and sex ratio for all the viable calves (n = 57) were 278.1 ± 3.6 days (range: from 271 to 286 days), 44.0 ± 3.0 kg (range: from 37 to 49 kg) and 57.9 vs. 42.1 % (male 33 and female 24), respectively. Microsatellite analysis confirmed that they were derived from frozen embryos. In conclusion, this study demonstrated that viable calves derived from frozen-thawed embryos from Canada were born by ET.

Key words **:** frozen embryos, embryo transfer, calves, microsatellite.

Introduction

Assisted reproductive technologies (ARTs), such as in vitro or in vivo produced embryos, embryo culture, and cryopreservation, have made great advances in bovine species (6). Artificial insemination (AI) has been used all over the world (4). Currently, millions of cow are bred by AI, and more than a half million embryos are transferred annually in worldwide, and are produced by Multiple Ovulation and Embryo Transfer (MOET). Besides, the effective programs to control estrus induction and synchronization, superovulation, embryo collection, cryopreservation, and management of recipients have been well developed and applied in local farms for improving dairy herd performances (1,5,14).

MOET which consists of donor selection, superovulation, AI, embryo collection, and embryo transfer (or cryopreservation) is a powerful tool to speed up genetic improvement within short periods in cattle (12). Recently, owners of local farms have generally considered MOET as a great approach for improving herd performances in South Korea. As results, in 2007, 3,886 embryos from 726 donor cows were collected and 1,981 embryos were transferred to recipient cows in Korea (*personal communicated with Korean Embryo Transfer Society*). However, resources for donor cows with high genetic background are practically limited. A way for overcoming this issue is to import in vivo frozen embryos, which are produced in dam with the top level of genetic background. Accordingly, in this study, we carried out to produce the calves from in vivo frozen-thawed embryo collected in the top one percent Holstein Friesian cows in Canada for improving herd performances in domestic dairy farms.

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Materials and Methods

Source of embryos

In this study, in vivo frozen embryos were imported from Canada Holstein Friesian cows through the project, "Production of young elite sires by transfer of embryos imported from Canada with high genetic backgrounds" by the Ministry of Food, Agriculture, Forestry and Fisheries, Republic of Korea. A total of 117 embryos were brought to embryo research center at Seoul Dairy Cooperative. The morphological evaluation of embryos including assessment of the developmental stage and quality of the embryos were done by following the guidelines from the International Embryo Transfer Society (17). All the embryos, which were frozen with 1.5M ethylene glycol for direct transfer, were loaded into 0.5 mL straw and imported by the Ministry of Food, Agriculture, Forestry and Fisheries.

Recipient preparation

One hundred sixty dairy heifers (age range: from 12 to 19 months) were initially chosen as candidates for surrogates. All of them were subjected to investigation to confirm they are free from five infectious diseases (BLV, FMD, Brucellosis, Tuberculosis and Johne's disease). Ninety seven of them, which

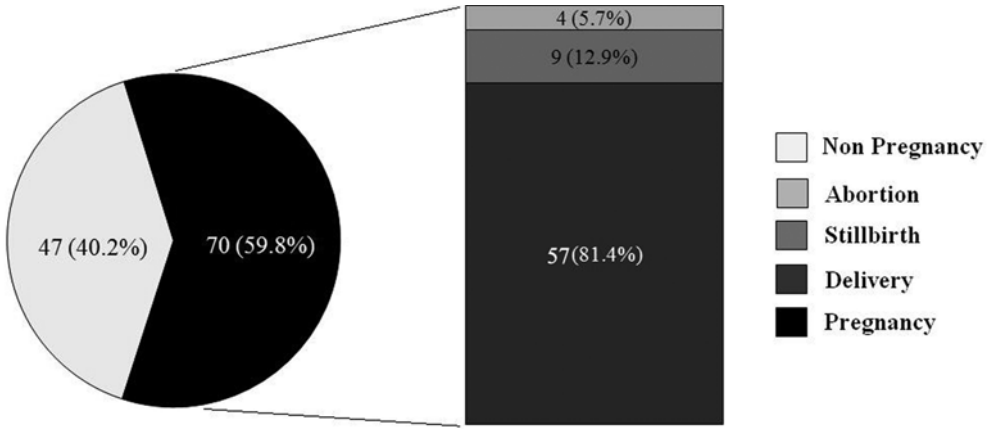

Fig 1. Summary after transfer of in vivo frozen-thawed embryos collected in Canada Holstein cows. One hundred seventeen embryos were transferred into uterine horn of recipients and seventy recipients were diagnosed to be pregnant. Among them, fifty seven recipients gave birth to the viable offspring.

were totally free from the enlisted diseases, were employed for embryo transfer. In the majority of cases, recipients that had been observed in natural standing estrous were used, however CIDR programs for estrous induction (8) were applied if necessary. However, when some of the recipients were failed to be pregnant, they were re-employed to ET program.

Embryo transfer

The straw with frozen embryos were thawed at 35°C water for at least 15 seconds before embryo transfer and then it put into the embryo transfer gun. After standing heat was detected (Day 0), thawed embryos with stage codes 4, 5, and 6 (Third manual of IETS 1998, (17)) were transcervically transferred into the middle uterine tube (horn) of surrogates on Day 6.5, 7.0, and 7.5, respectively. Each stage code means as followings: 4 = compact morula, embryos that had undergone compaction, 5 = early blastocysts, embryos with blastocoels comprising less than 50% of the embryonic mass, and 6 = mid-blastocysts, embryo with blastocoels comprising more than 50% of the embryonic mass and on thinning of the zona pellucida. Before embryo transfer, the existence of corpus luteum was palpated by rectal examination and the embryos were ipsilaterally transferred.

Pregnancy Diagnosis

Initial pregnancy diagnosis was done by rectal palpation on Day 50-55 after the embryo transfer. Thereafter, only surrogates which were diagnosed to be pregnant on Day 90 were completely considered and recorded as pregnant.

Genotyping

Parentage analysis was performed on all the viable calves to confirm their genetic identity with the donor embryos used for embryo transfer. Genomic DNA from blood of calves was extracted according to instructions of the G-spin™ Genomic DNA Extraction Kit (Intron, Seoul, Republic of Korea). The isolated genomic DNA samples were dissolved in 50 μl TE and used for microsatellite assay with bovine specific markers [BM1824, BM2113, CSSM36, ETH10, HEL1, INRA23, SPS115, TGLA122, TGLA126, and TGLA227] labelled with one of the fluorescent dyes, FAM, NED or HEX. The PCR amplification was carried out for one cycle with denaturing at 95°C for 15 min, and subsequently for 35 cycles with denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. Ten micro liters of PCR products were fractionated on a 2.0% agarose gel and stained with ethidium bromide. Length variations were assayed by PCR amplification with fluorescent-labeled locus-specific primers and polyacrylamide gel electrophoresis (PAGE) on an automated DNA sequencer (ABI 373: Applied Biosystems, Foster City, CA). Proprietary software (GeneScan and Genotyper; Applied Biosystems) was used to estimate PCR product size in nucleotides.

Results

Seventy (59.8%) out of 117 surrogates were diagnosed to be pregnant. And number of abortion and stillbirth were four (5.7%) and nine (12.9%), respectively (Fig 1). Pregnancy rate in view of embryo stage code (4, 5 and 6) and quality code (1 and 2) were (62.5, 59.0, and 58.3%) and (58.9 and 80.0%), respectively (Figs 2 and 3). And pregnancy rate in view of recipient age (12-13, 14-15, 16-17 and 18-19 months) and number of transfer trials (1st and 2nd) were (48.1, 61.4, 60.7 and 100%) and (55.7 and 72.4%), respectively (Figs 4 and 5). Gestational length, birth body weight and sex ratio for all the viable calves (n = 57) were 278.1 ± 3.6 days (range: from 271 to 286 days), 44.0 ± 3.0 kg (range: from 37 to 49 kg) and 57.9 vs. 42.1% (male 33 and female 24), respectively. Parentage analysis on all the viable calves demonstrated the offspring were derived from frozen-thawed embryos (Table 1, all data not shown).

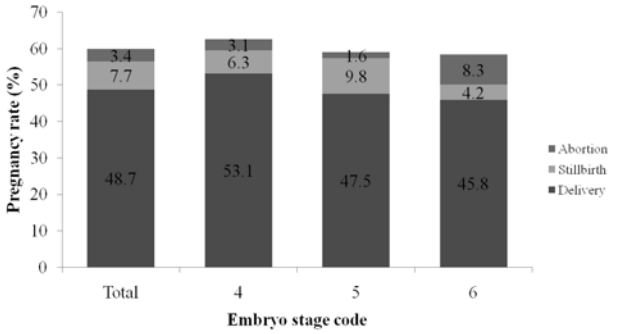

Fig 2. Pregnancy rate after transfer of in vivo frozen-thawed embryos collected in Canada Holstein cows according to the embryo stage code. Embryo stage code (4, 5, and 6) as followed from International Embryo Transfer Society Manual. Each stage code means as followings: 4 = compact morula, embryos that had undergone compaction, 5 = early blastocysts, embryos with blastocoels comprising less than 50% of the embryonic mass, and 6 = mid-blastocysts, embryo with blastocoels comprising more than 50% of the embryonic mass and on thinning of the zona pellucida. Recipient numbers for each stage code (4, 5, and 6) were 32, 61, and 24, respectively.

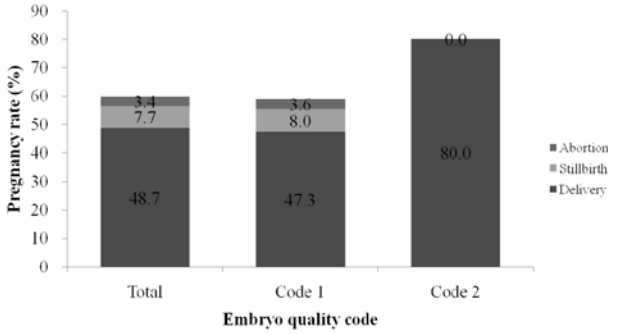

Fig 3. Pregnancy rate after transfer of in vivo frozen-thawed embryos collected in Canada Holstein cows according to the embryo quality code. Embryo quality code (1 and 2) as followed from International Embryo Transfer Society Manual. Recipient numbers for each code 1 and 2 were 112 and 5, respectively.

Discussion

This study demonstrated that the frozen-thawed embryos of Holstein from Canada with high genetic background were successfully implanted into surrogate mothers and gave birth to their calves. A total of 117 recipients were used and seventy out of them were diagnosed to be pregnant. This efficiency on pregnancy rate (59.8%, Fig 1) showed higher compared to the previous studies (3,13,15-16). The reason for higher efficiency in this study is ascribed to procure improved technical skills in bovine assisted reproduction technologies being practiced for over than 10 years such as embryo culture, embryo transfer, and cryopreservation (9-11).

Firstly, as we analyzed pregnancy efficiency in terms of code for embryo development and quality, it was not different from the previous studies (16). The gestation periods and

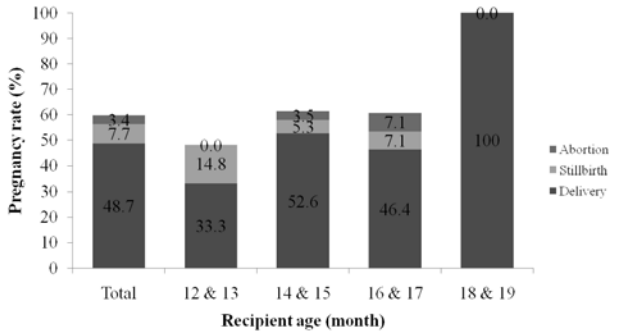

Fig 4. Pregnancy rate after transfer of in vivo frozen-thawed embryos collected in Canada Holstein cows according to the recipient age. Recipient numbers for each age (12&13, 14&15, 16&17, and 18&19) were 22, 57, 28, and 5, respectively.

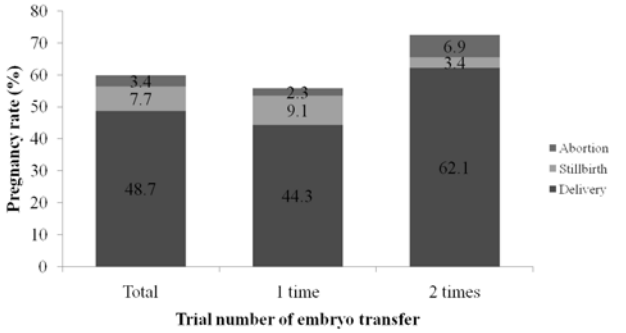

Fig 5. Pregnancy rate after transfer of in vivo frozen- thawed embryos collected in Canada Holstein cows according to the trial number of transfer. Recipient numbers for each trial number, 1time and 2 times were 88 and 29, respectively.

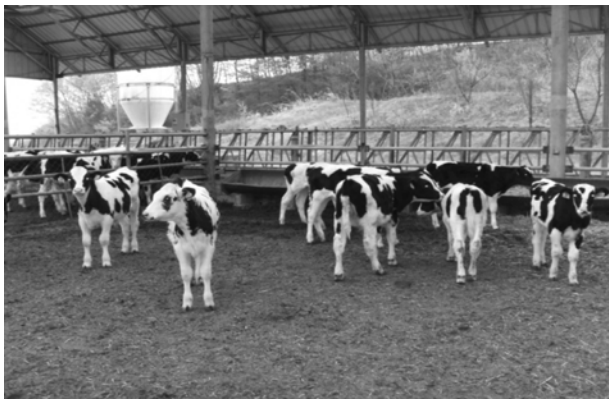

Fig 6. Calves produced from transfer of in vivo frozen-thawed embryos collected from Canada Holstein cows.

body weight for the calves were 278.1 ± 3.6 days and 44.0 ± 3.0 kg, respectively. Those values were within normal range (2).

Then, we investigated whether the age of recipients or trial number of ET would influence the ET results. While this study showed lower abortion rates compared to a previous report in Korea (7), some calves did not survive in the middle of delivery with higher percentage (7.7%). Particularly, as

Table 1. Representative microsatellite analysis on offspring derived from in vivo frozen-thawed embryos collected from Canada Holstein cows

	DAM	SIRE	1th calf	DAM	SIRE	2nd calf	DAM	SIRE	3rd calf
BM1824	180/188	178/182	180/182	182/188	180/188	180/188	180/188	188/188	188/188
BM2113	125/127	125/135	125/125	125/135	127/135	125/127	135/135	135/139	135/135
CSSM36	169/179	173/179	179/179	-	-	-	-	-	-
ETH10	209/219	217/225	219/225	223/225	219/223	218/225	217/219	219/219	217/219
HEL1	104/112	104/112	112/112	-	-	-	-	-	-
INRA23	202/210	214/214	210/214	214/214	210/214	214/214	206/214	206/210	206/210
SPS115	248/254	248/254	248/248	248/248	248/256	248/256	248/248	248/248	248/248
TGLA122	143/183	143/183	183/183	163/183	149/183	163/183	163/183	149/163	149/163
TGLA126	117/121	117/123	117/117	117/121	115/117	115/117	117/117	117/119	117/123
TGLA227	93/97	87/89	89/93	83/89	87/103	87/89	89/97	81/97	81/97

shown in Fig 4, stillbirth ratio in recipients with low age (12 to 13 months) was the highest. We can infer the reason that because recipients with younger age i.e. < 13 months don't have completely matured reproductive tract or body condition (i.e. body weight) to support full term pregnancy, they were not compatible to be surrogate mothers for embryo transfer. Moreover, when recipients were re-employed as surrogate mothers for embryo transfer, there was no different pregnancy efficiency. In summary, whereas embryonic parameters (stage code, 4~6 and quality code, 1~2) do not have crucial influence on pregnancy and delivery rate, because the recipient parameters had effect on the efficiency for producing the calves, we recommend more than 13-month old heifer to be a surrogate since it has sufficiently developed reproductive organ to capacitate complete pregnancy.

Microsatellite analysis showed the genomic evidence that all viable calves were derived from frozen-thawed embryos. These calves can be the first generation with genetic identification in Republic of Korea. As the calves are to be grown up, pregnant, and delivered second generation offspring, we can not only make exact and objective pedigree based on this DNA analysis, but also evaluate that these cattle have outstanding features.

In conclusion, the viable calves by ET were born from frozen-thawed embryos, which were collected from the top one percent Holstein in Canada, and their parentage was confirmed by microsatellite analysis. The calves are growing well without any abnormalities so far. They can be resources not only for sires and dams, but also for improving herd in dairy farms at Republic of Korea.

Acknowledgements

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캐나다산 고능력 젖소에서 생산된 동결-융해 배아 유래의 송아지 생산

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요 약 : 소에서 유전적 군 (herd)의 향상을 위한 유용한 도구는 보조 생식술 중 배아 이식 (embryo transfer)으로 알려져 왔다. 본 연구의 목적은 캐나다 유전 능력 상위 1% 이내의 젖소에서 생산된 냉동 배아를 수입하여, 국내 수란우군을 이용한 배아이식을 적용한 후, 송아지를 생산하는 것이다. 117번의 배아 이식이 이루어졌고, 그 중 70 마리에서 임신이 확인 되었으며, 총 57 마리의 송아지가 성공적으로 태어났다. 그들의 임신기간, 체중, 성별은 각각278.1 ± 3.6 일 (범위: 271 에서 286일), 44.0 ± 3.0 kg (범위: 37 에서 49 kg) and 57.9 vs. 42.1% (수컷: 33, 암컷: 24) 이었다. 미세위성체검사를 통하여, 태어난 송아지는 모두 수입된 배아 유래임이 증명되었다. 결론적으로, 본 연구를 통하여 캐나다에서 수입된 배아로부터 살아있는 송아지를 성공적으로 얻을 수 있었으며, 이 송아지들은 앞으로 국내 낙농산업에 있어 종모우와 종빈우가 되는 좋은 유전자원이 될 것이다.

주요어 : 동결배아, 배아이식, 송아지, 미세위성체.