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Increase of Production Ratio of Pre-selected Superior Dairy Female Offspring by Combination of OPU derived Oocytes and X-bearing Semen

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ABSTRACT

This study was designed to evaluate the possibility of increase through dairy female offspring's ratio by transfer of pre-selected transferrable blastocyst that was produced by pre-selected X-bearing semen with OPU derived oocytes. Elite dairy female cow is demanded strongly compared with male, the so called, farmer wants to produce only an elite female dairy offspring as a candidate female dairy cow for producing milk. In our study, we selected 2 elite dairy bull semen from National Agricultural Cooperative Federation to pre-select X-bearing semen and 5 elite dairy female cows as donor for collecting of OPU derived oocytes. OPU derived embryo production system was carried out an aspiration of immature oocytes from 5 donor cows 2 times per week, total 200 times for 2 to 7 months by an ultrasonographic guided follicular aspiration system and then produced *in vitro*-produced blastocysts by *in vitro* maturation, fertilization and culture. Dairy donor semen selected H-319, 320 bull in National Agricultural Cooperative federation was sorted X-bearing semen by flow-cytometer and frozen for using IVF with OPU derived oocytes. Donor cows were selected 5 elite dairy cows from Gyeongju Dairy Cow Community and then disease tests such as 4 kinds of disease before selecting was checked. Oocyte proportion of grade 1 to 3 from total collected oocytes was significantly lower in donor A and B than those in donor C, D and E (82.16 and 70.03% vs. 90.0, 91.78 and 93.57%), respectively ($p<0.05$). However, number of oocytes per session in donor A, C and E was significantly higher than those in donor B and D (7.77 ± 3.26, 5.85 ± 2.10 and 7.03 ± 2.14 vs. 4.68 ± 2.61 and 5.21 ± 1.97 oocytes), but donor A was significantly higher than donor C ($p<0.05$). Development to blastocyst in donor B, C and E was significantly higher than those in donor A and D (31.0, 25.0 and 25.0% vs. 14.3 and 4.5%), but donor A was not different in donor C and E ($p<0.05$). Nine out of 10 blastocysts (90.0%) derived from OPU blastocysts were confirmed male embryos that was induced with Y-bearing semen to confirm sex ratio only. Total 96 blastocysts derived from female bearing semen were transferred into synchronized recipients and then confirmed 42 recipients (43.8%) pregnancy rate, 36 offspring (37.5%) and 91.7% female sex ratio (33 female vs. 3 male offspring). Taken together all data, elite dairy female offspring could be produced effectively by *in vitro* production system between pre-selected x-bearing semen and OPU derived oocytes that would be influential breeder in the breeding of dairy farm to increase effectively elite dairy offspring ratio as well as net income in the dairy farmer.

(Key words : pre-selected female offspring, x-bearing semen selection, dairy cow, OPU derived embryo, embryo transfer)

INTRODUCTION

Embryo transfer offered a means by which their numbers could be multiplied rapidly. For several years, the most common use of embryo transfer in animal production programs was the proliferation rapidly, the so-called desirable phenotypes was occurred. Through the MOET (multiple ovulation and embryo transfer) programs intensity of selection could be increased,

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generation intervals, therefore, reduced and ultimately genetic gains would be improved. The establishment of nucleus herds and "Juvenile MOET" in heifer offspring was shown to result in genetic gains that approached twice those achieved with traditional progeny test schemes. Embryo transfer is now commonly used to produce artificial insemination (AI) sires from proven cows and bulls (Teepker and Keller, 1989). In addition, new genomic techniques are being used increasingly to select embryo donors, especially for selection of dairy bull dams for super-stimulation, where a genomic analysis would be essential (Seidel, 2010). Results supported the theory, but physiology was a limiting factor in practice; super-ovulatory results made it difficult to produce the desired number of female offspring for genetic testing.

The technique of ultrasound-guided transvaginal follicular aspiration for ovum pick-up (OPU) is a non-invasive procedure for recovering oocytes from antral follicles in live animals. An ultrasonic-guided aspiration of bovine follicular oocytes was first proposed in Denmark (Callesen *et al.*, 1987) and a real OPU was first established in cattle by a Dutch team (Pieterse *et al.*, 1991). Together with *in vitro* fertilization of oocytes, OPU has been taken as a most flexible and repeatable technique to produce embryos from any given live donor. Unlike MOET, OPU does not interfere with the normal reproduction and production cycles of the donor. It has been shown to be a feasible and practical alternative to the conventional MOET program (Bousquet *et al.*, 1999; Kruip *et al.*, 1991), and it is being more used for commercial applications in the world (Faber *et al.*, 2003; Pontes *et al.*, 2010).

Both OPU and *in vitro* fertilization (IVF) could be seen as mature technologies in the current world. The total number of transferable *in-vitro*-produced (IVP) bovine embryos worldwide was 453,471 in 2011 (Stroud, 2011), which were included OPU embryos and abattoir embryos. In addition, with the complementing of bovine genome sequencing and key genes for traits of economic interest becoming available in the recent years, OPU/IVP has proven invaluable in rapidly multiplying rare genes and provides the more advanced technologies such as cloning (Yang *et al.*, 2005, 2008) and transgenic. The original OPU procedure includes no hormone stimulation. It routinely performs twice a week, which allows the maximum recovery of oocytes of suitable quality for embryo production in a given time interval compared to once-a-week OPU, because no dominant follicle develops when all visible follicles are aspirated in the OPU process.

Sex-sorting of sperm cells by flow cytometry is an established method that has been commercially used in cattle (Seidel, 2007; Garner and Seidel, 2008; Rath *et al.*, 2013). This technology is an important tool for the dairy and beef industry, leading to greater supply of replacement heifers and the consequent hastening on genetic gain (De Vries *et al.*, 2008; Chebel *et al.*, 2010). The separation of sperm bearing X and Y chromosomes is possible due to the differences on the DNA content of these cells (X bearing sperm has about 4% more genetic material than Y bearing sperm) identified by flow cytometry (Johnson, 2000). The possibility to choose the sex of the offspring in a herd in species of economic interest is a much desired goal in animal production. The benefits of early identification of sex in the acceleration of the genetic progress, when associated with AI and/or ET techniques, were reported by some authors (Taylor *et al.*, 1985). One of the first studies to evaluate the feasibility of using sex-sorted sperm in super-ovulation programs was conducted by Sartori *et al.* (2004). The advantage of this study was the use of equal amounts of sperm by treatment (sex-sorted sperm or non sex-sorted). And so the sex-sorted sperm in OPU programs is one of the alternative ways to increase the genetic improvement as well as female offspring production scheme in diary industry, especially in Republic of Korea.

The objective of the present study was to evaluate whether the ratio of female offspring in dairy cattle can be increased by OPU derived embryos with sex-sorted female sperm.

MATERIALS AND METHODS

Unless indicated, all reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Care and use of animals was conducted in accordance with guidelines prescribed by Gyeongsang National University (approval no. GNU-130902-A0059).

1. Donor Information for OPU Derived Oocyte Aspiration

Five Holstein dairy cows approximately 2 to 7 years old, which belonged to the Institute of Agriculture and Life Science, Gyeongsang National University, were used. These cows were selected based on a general clinical examination and normal ovarian cyclicity, as determined by transrectal ultrasonography and also milk production ability, of which was 12,000 to 15,000 kg per year and also checked up 4 kinds of disease such as

foot-and-mouth disease, Johne's disease, tuberculosis and brucellosis. The donor cows were housed in a single group and were fed grass silage *ad libitum*.

2. OPU Derived Oocyte Aspiration from Superior Dairy Cow

In all donor cows, OPU was performed twice weekly (every 3 or 4 d) starting on d 3 or 4 of the estrous cycle of the first follicular wave of follicular development, as previously described (Garcia and Salaheddine, 1998). Five Holstein cyclic cows were used for 14 to 57 sessions of OPU at intervals of 3 to 4 d (day of estrus = D 0). The donor cows were first restrained in a chute, and were given caudal epidural anesthesia (3 to 4 ml of 2% Lidocaine). The number of follicles in both ovaries was counted with ultrasonography. Thereafter, OPU was performed with an ultrasound device (Scanner 100S; Esaote-Pie Medical, Maastricht, The Netherlands) with a 7.5-MHz convex transducer connected to a vacuum suction pump (negative suction pressure, 85 to 90 mm Hg). All ovarian follicles 2- to 8-mm in diameter were retrieved. The follicular content of each cow was aspirated individually into TL-HEPES medium (114 mM sodium chloride, 3.2 mM potassium chloride, 2 mM sodium bicarbonate, 0.34 mM sodium biphosphate, 10 mM sodium lactate, 0.5 mM magnesium chloride, 2.0 mM calcium chloride, 10 mM HEPES, 1 μl/ml phenol red, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin). Cumulus-oocyte complexes (COCs) were examined under stereomicroscope and classified into four grades, based on number of cumulus cell layers and the homogeneity of the cytoplasm (Dey *et al.*, 2012) separately for each donor. The COCs were washed three times in TL-HEPES medium.

3. Pre-selection of X-bearing Semen by Flow Cytometer System

Flow sorting and cryopreservation were performed as described by Schenk *et al.* (1999), and Suh and Schenk (2003). Briefly, aliquots of spermatozoa from H- 319 and H-320 candidate dairy bulls (Korean National Agriculture Cooperative Federation: NACF, http://rd.dcic.co.kr) were diluted in modified Tyrode's albumin-lactate-pyruvate (TALP) buffer (Schenk *et al.*, 1999), stained with 125 mM Hoechst 33342 at 200′ 10^6 spermatozoa/ml for 45 min at 34℃, and then fur ther diluted to 100× 10^6 spermatozoa/ml with TALP containing 4% (w/v) egg yolk and 0.002% (w/v) food coloring dye (FD&C #40, Warner Jenkinson, St. Louis, MO, USA). Stained spermatozoa were sorted for the X-chromosome-bearing population by the MoFlo^{TM} XDP system, equipped with a 70 μm internal diameter nozzle and laser source of 150 mW. Sorted spermatozoa were collected in a 50 ml centrifuge tube containing TRIS catch fluid supplemented with 20% (w/v) egg yolk (Schenk *et al.*, 1999). The unsorted control (i.e., the sample was not passed through the sorter) was prepared from the same ejaculate as the sorted sperm. Sperm samples in plastic tube racks were cooled to 5℃ in a cold room for 90 min. Thereafter, samples were diluted with an equal volume of 12% (v/v) glycerol containing Tris extender (SortEnsure^{TM}, XY Inc., Navasota, TX, USA) and centrifuged at 850× g for 20 min using a swinging-bucket centrifuge precooled to 5℃. After the supernatant was aspirated, the resulting sperm pellet diluted and the final sperm concentration was adjusted to 20× 10^6 spermatozoa/ml. Sorted and unsorted samples were packaged in to 0.5-ml straws containing 2× 10^6 and 20× 10^6 total spermatozoa, respectively, and frozen in static liquid nitrogen vapor by a routine procedure on racks (Schenk*et al.*, 1999).

4. *In Vitro* Maturation (IVM), *In Vitro* Fertilization (IVF) and *In Vitro* Culture (IVC)

The collected COCs that were selected only grade 1 to 3 were washed twice in maturation medium (TCM-199) supplemented with 10% (v/v) FBS, 1 μg/ml of estradiol-17β, 10 μg/ml of FSH, 0.6 mM of cystein and 0.2 mM of sodium pyruvate, 50 μg/ml gentamycin sulphate and transferred in 700 μl of IVM medium at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 23～24 h, and cultured as described previously (Dey*et al.*, 2012). *In vitro*-matured COCs were fertilized with frozen-thawed sperm previously used in our laboratory (Dey *et al.*, 2012). To confirm sex ratio of IVP blastocyst, Y-bearing semen was only used to produce and confirm sex ratio of IVP blastocyst. To produce OPU derived IVP blastocyst, X-bearing semen was used to produce and embryo transfer of OPU derived IVP blastocyst. Semen was thawed (36℃ for 1 min), and sperm were then washed and pelleted in Dulbecco's PBS (D-PBS) by centrifugation at 750 × g at room temperature for 5 min. The pellet was diluted with 500 μl of heparin (20 μg/ml) in IVF medium [Tyrodes lactate solution supplemented with 6 mg/ml of BSA (A-6003), 22 μg/ml of sodium pyruvate, 100 IU/ml of penicillin, and 0.1 mg/ml of streptomycin] and incubated at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 15 min. Capacitated sperm were diluted in IVF medium (1×10^6 sperm/ml). Matured oocytes were transfer red in to IVF medium (700 μl) containing sperm and cultured at 38.5℃ for

18～20 h. After IVF, cumulus cells were removed by pipetting and the denuded presumptive zygotes were placed in 700 μl of CR1-aamedium (Rosenkrans *et al.*, 1993) supplemented with 44 μg/ml of sodium pyruvate, 14.6 μg/ml of glutamine, 10 μl/ml of penicillin/streptomycin, 3 mg/ml of BSA, and 310 μg/ml of glutathione for 3 d (IVC-I). Presumptive zygotes were then cultured until Day 8 of embryonic development (Day 0 = day of IVF) in a medium of the same composition (IVC-I), except that the BSA was replaced with 10% (v/v) FBS (IVC-II). Cleavage rate was recorded on Day 3 after IVF (72 hpi). On Day 8, the stage of development and embryo quality were evaluated for each donor.

5. PCR Confirmation of Sexed Male Embryos

Samples of the IVF embryos were digested with 20 μl of K-buffer containing 0.1 μg/μl proteinase K (Invitrogen-Life Technologies, Carlsbad, USA) at 56℃ for 45 min and then held at 95 to 100℃ for 10 min to inactivate proteinase K. A multiplex amplification of bovine specific autosome and Y-chromosome fragments was performed by one round of PCR (Tominaga and Hamada, 2004). A bovine-specific primer pair, β-actin RNA was designed to amplify a fragment of 21 bp from a bovine 1.715 satellite DNA as follows: forward: 5'-GAAGATGACCCAGGTCAGTGG-3'; reverse: 5'-GTACATGGCAGGGGTGTTGA-3'. The Y-specific RNA, ZRSR2Y (20 bp, GenBank accession number: GQ426330.1) was amplified using male-specific primers (forward: 5'-GTCAGTTGCAACCTGGAACC-3'; reverse: 5'-GCCATATTCCATTGGGTCAC-3'). One microliter of embryo lysate was used for PCR amplification in a total volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP (DNTP100A), 0.4 M oligonucleotide primers, and 0.5 unit of REDTaq DNA polymerase (Invitrogen-Life Technologies, Carlsbad, USA). The PCR was initiated with an initial cycle of 95℃ for 2 min followed by 30 cycles of 95℃ for 30 s, primer annealing at 64℃ for 30 s, primer extension at 72℃ for 30 s, and a final hold at 72℃ for 10 min. Ten microliters of PCR products were analyzed on a 2% agarose gel.

6. Embryo Transfer of OPU Derived Pre-selected Blastocyst

Presumptive female dairy blastocysts were transferred into recipients to examine their developmental potential *in vivo*. Recipient cattle breeds consisted of Holstein cattle on several dairy farms. Recipients were chosen according to criteria that included: age, health status, breeding history, size and weight, as well as the farm's nutritional management. Recipients were synchronized by a regimen of GnRH injection (Day 0), prostaglandin $F_2\alpha$ (Lutalyse, Upjohn Co., Kalamazoo, MI; 25 mg/injection, i.m.) injection (Day 7), GnRH injection (Day 9), detected estrus induction (Day 10) and then embryo transfer (Day 17) (Jin *et al.*, 2014). On D 7 following estrus, recipients were selected by palpation per rectum to verify the presence and the size of the CL. Blastocysts (1/straw) were loaded into 0.25-ml French straws containing TCM-199 media. Straws were maintained at 39℃ in a portable incubator for transportation to the farms. A single embryo was deposited nonsurgically into the uterine horn ipsilateral to the ovary with the CL. Pregnancy was determined by palpation per rectum on Day 70 after transfer and induced delivery at full term.

7. Confirmation of Paternity Identification

Total 20 offspring and recipients were collected coat hairs from each farm to analysis of paternity identification, and X-bearing bull semen also. Genomic DNA extraction from sample was used E-prep kit (Prepgene CO., LTD, U.S.A). Total 11 MS markers were used to analysis of the allele as shown in Table 3 (Lim *et al.*, 2010). The multiplex-PCR was performed in a final volume 15 μl containing 2 μl genomic DNA (50 ng/μl), 8.25 μl primer mix, 0.4 μl Hot start Taq DNA polymerase (Bioneer, Korea), 1.5 μl of 10× buffer, 1.2 μl of 10 mM dNTP, 1.1 μl of 20 mM $MgCl_2$, 0.25μl DMSO. The multiplex-PCR was carried out in touch down. The touchdown PCR was performed with an initial denaturation step at 94℃ for 15 min followed by 9 cycles, which involved a denaturation step at 94℃ for 1 min, annealing at 58℃ for 1 min, extension at 72℃ for 1 min in the initial cycle and at decreasing temperatures by 1℃ /9 cycle until a temperature of 56℃ was reached in the subsequent cycles (denaturation at 94℃ for 1 min; extension at 72℃ for 1 min). After the touchdown program, 16 cycles at 94℃ for 1 min, 56℃ for 1 min, 72℃ for 1 min followed by a final extension was at 65℃ for 30 minutes and then it was stored at 8℃. Electrophoresis was performed on an ABI-3130*xl* (Applied Bioststems, USA) and each PCR product was resolved by the size and color. GeneMapper version 3.7 (Applied Biosystems, USA), was used for genotyping and Microsoft Excel (Microsoft, USA) was used for data processing.

8. Statistical Analysis

Results that are no. of oocytes per session are presented as mean ± S.D.; differences of $p \leq 0.05$ were considered significant. The blastocyst development and pregnant data were tested by one-way ANOVA followed by Duncan's multiple range test to detect differences among groups. All data were analysed using the SAS program, version 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

1. Efficiency of IVP Blastocyst Production by OPU Derived Oocytes with Pre-selected Female Sperm

OPU derived oocytes and embryos production efficiency was shown in Table 1. OPU derived oocytes collection was carried out total 200 sessions with 5 donor cows for 2 to 7 months as 57, 57, 41, 14 and 31 of donor A, B, C, D and E. The proportion of Grade 1 to 3 oocytes to induce IVF were greater in donor C, D and E than those in donor A and B (90.0, 91.78 and 93.57% vs. 82.16 and 70.03) ($p<0.05$). Numbers of collected oocytes per session were significantly greater in donor A, C and E than those in donor B and D (7.77, 5.85 and 7.03 vs. 4.68 and 5.21 oocytes), but donor A was significantly higher than that in donor C (7.77 vs. 5.85) ($p<0.05$). Developmental competence to blastocyst was greater in donor B, C and E than those in donor A and D (31.0, 25.0, 25.0% vs. 14.3, 4.5%) ($p<0.05$), but donor A was not different in donor C and E (14.3 vs. 25.0 and 25.0%).

2. Confirmation of Pre-selected Male Blastocyst by PCR Analysis

Pre-selected Y-bearing semen was used to confirm pre-selected sex blastocyst, because X-bearing semen used for producing of female blastocyst to embryo transfer. So total 10 blastocysts derived from Y-bearing semen were analysis by PCR to confirm the male blastocyst (Fig. 1). Nine over ten blastocysts were expressed male specific marker gene and so 90% was male blastocyst produced by *in vitro* fertilization of Y-bearing sperm with OPU derived oocytes.

3. Production of Female Dairy Offspring

To produce dairy female offspring, total 96 pre-selected blastocysts were transferred into recipients and 42 out of 96 recipients (43.8%) were diagnosis as pregnancy at Day 70 and then 36 out of 96 recipients were delivered (37.5%) as shown in Table 2. Thirty three female offspring was produced from total 36 offspring that was 91.7% female ratio (Fig. 2).

4. Confirmation of Paternity Identification

Paternity identification was confirmed by total 11 MS mar-

Table 1. OPU related embryo production efficiency depend on donor cows

Donors	Sessions	No. of oocytes (%)				No. (%) of oocytes G1-3 per session	No. of total oocytes	No. of oocytes per session (Mean ± S.D.)	No. (%) of cleaved embryos*	No. (%) of blastocyst*
		G1	G2	G3	G4					
A	57	137 (31.0)	95 (21.4)	132 (29.8)	79 (17.8)	364 (82.16)^{b,c}	443	7.77 ± 3.26^{a}	85 (23.4)^{b}	52 (14.3)^{b,c}
B	57	48 (18.0)	49 (18.4)	90 (33.7)	80 (29.9)	187 (70.03)^{c}	267	4.68 ± 2.61^{c}	87 (46.5)^{a}	58 (31.0)^{a}
C	41	135 (56.3)	46 (19.2)	35 (14.5)	24 (10.0)	216 (90.0)^{a,b}	240	5.85 ± 2.10^{b,c}	105 (48.6)^{a}	54 (25.0)^{a,b}
D	14	21 (28.8)	17 (23.3)	29 (39.7)	6 (8.2)	67 (91.78)^{a,b}	73	5.21 ± 1.97^{c}	16 (23.9)^{b}	3 (4.5)^{c}
E	31	128 (58.7)	41 (18.8)	35 (16.1)	14 (6.4)	204 (93.57)^{a}	218	7.03 ± 2.14^{a,b}	82 (40.2)^{a}	51 (25.0)^{a,b}
Total	200	469 (37.8)	248 (20.0)	321 (25.8)	203 (16.4)	1,038 (83.64)	1,241	6.21 ± 2.88	375 (36.1)	218 (21.0)

* Data of cleavage and blastocyst development was calculated from total no. of grade 1 to 3 oocytes. Grade 1 to 3 oocytes were only used for IVF, not grade 4.

$^{a\sim c}$ Values with different superscripts in same column were significantly different ($p<0.05$).

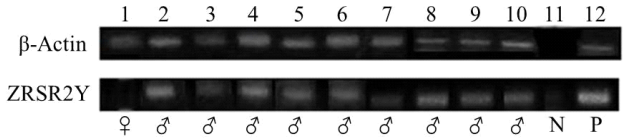


Fig. 1. Representative gel of PCR sexing of bovine blastocysts fertilized *in vitro* by sorted Y-bearing sperm. Amplification of bovine specific autosome and Y-chromosome RNA fragments was performed by a multiplex PCR as described in the Materials and Methods. The ZRSR2Y of Y-linked gene was expressed in 1～10 lines. ACTIN was used as a control gene. Line 11 was analyzed as negative control. Line 12 of male testis cDNA was analyzed as positive control.

Table 2. Female offspring production by pre-selected OPU derived female blastocyst

Items	No. and (%) of data
No. of blastocysts transferred	96
No. of pregnancy	42 (43.8)
No. of offspring	36 (37.5)
Sex ratio (female vs. male)	33 (91.7)* vs. 3(8.3)

* Value with superscripts in same line was denoted significantly different (p<0.05).

Fig. 2. Photograph of OPU derived presumed female blastocyst and female offspring by embryo transfer of pre-selected OPU derived female blastocyst.

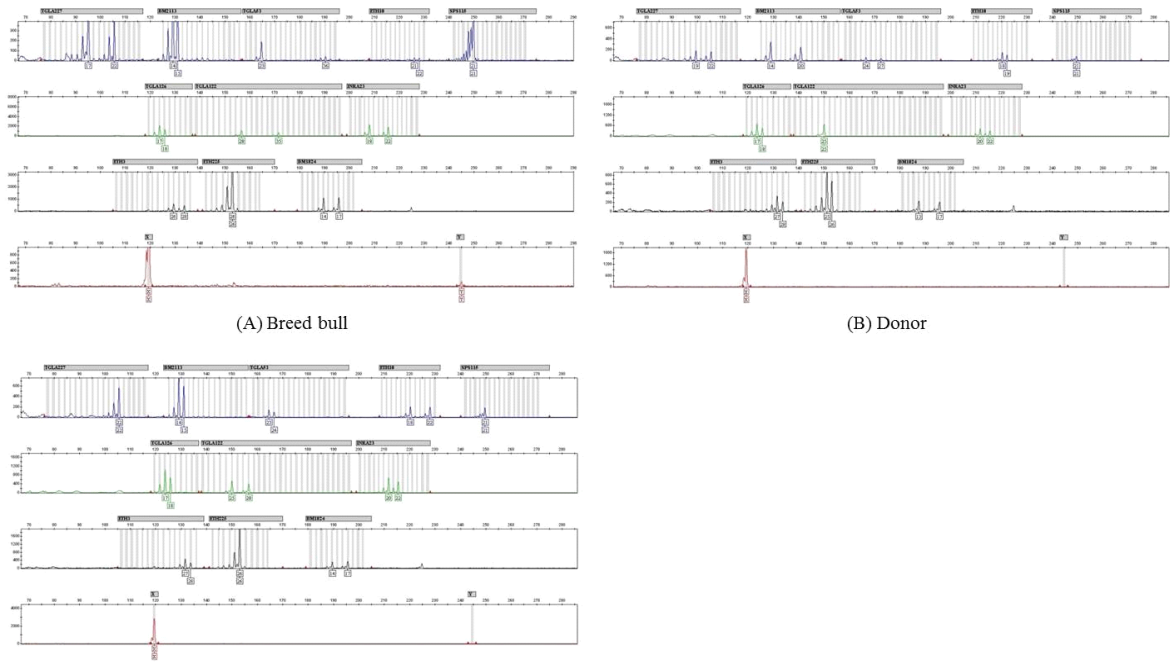
kers as shown in Table 3 and Fig. 3. Each 20 offspring and recipients were analysis and confirmed 100% perfectly identification between parent and offspring, even male offspring.

Table 3. Information of the 11 microsatellite markers used in this study

No.	Locus	Label	Size range (bp)
1	TGLA227	FAM	76～104
2	BM2113	FAM	123～143
3	TGLA53	FAM	154～188
4	ETH10	FAM	212～224
5	SPS115	FAM	246～260
6	BM1824	PET	178～192
7	TGLA126	VIC	116～122
8	TGLA122	VIC	137～181
9	INRA23	VIC	196～222
10	ETH3	NED	105～125
11	ETH225	MED	141～159

DISCUSSION

With the desire of controlling the sex of the offspring, for decade researchers have investigated different techniques to sexing sperm, but as mentioned by Seidel (2003), to date only one procedure has proven efficacious in any practical sense. That procedure utilizes flow cytometry to separate X- and Y-bearing spermatozoa based on the DNA content of individual sperm as described by Johnson (2000). This technique has been found to have excellent accuracy and results in sorted population of viable sexed sperm. The disadvantages of this technique are the slow speed of sorting, the decreased fertility of sexed sperm, especially in superovulated donor cows, the cost of the semen, and the availability of semen from specific bulls (Amann, 1999). For the embryo transfer industry, sex-sorted semen presently has its greatest use in IVP of bovine embryos. The possibility to choose the sex of the offspring in a herd in species of economic interest is a much desired goal in animal production. In order to obtain more oocytes from the donor, it is necessary to be more follicles on the bovine ovary, which is influenced by cattle breed, nutritional status and climate conditions. First, different cattle breeds present different follicle numbers on the ovaries in a follicular wave cycle. It was reported that *Bos indicus* breeds tend to have more follicular waves (Figueiredo *et al.*, 1997; Viana *et al.*, 2000) and a larger population of small follicles (<5 mm) compared to *B. taurus* breeds (Segerson *et*



(A) Breed bull (B) Donor

(C) Offspring

Fig. 3. Confirmation of paternity identification between donor, bull's semen and offspring by 11 MS markers.

al., 1984). In the present study, we achieved average 6.21 ± 2.88 oocytes per session and 83.64% grade 1 to 3 oocytes of total collected oocytes. It is significantly lower compared with Korean native cow (Hanwoo), 8.2 ± 4.5 oocytes per session and 85.86% grade 1 to 3 oocytes (Jin *et al.*, 2014). Developmental competence to blastocyst using X-sorted sperm was average 21.0% in dairy cow that was significantly lower compared with 36.3% of unsorted Korean native sperm of cow (Jin *et al.*, 2014). Although dairy cows were different breed with Korean native cows, ability of X-sorted sperm has lower potential to cleavage and developmental competence rather than unsorted sex sperm. Embryonic development to the blastocyst stage was not delayed. *In vitro* fertilization is a feasible application for utilizing sorted sperm, based on current sexing technology (Lu *et al.*, 1999; Lu and Seidel, 2004; Wilson *et al.*, 2005). Previous studies with sexed IVF embryos (Cran *et al.*, 1993, 1995; Wilson *et al.*, 2006) indicated that embryos resulting from sexed sperm IVF had an inferior (12 to 27%), and sometimes delayed preimplantation development to blastocyst. In the present study, we demonstrated that there were both bull and sorting effects that resulted in differential embryo development in sexed IVF and that the bull effect had more influence than the sorting process. The bull effect for IVF is believed due to the difference of sperm capacitation for individual bulls during the process of fertilization (Parrish *et al.*, 1986). The 2 bulls used were selected for their genetic merits and availability, but not to their *in vitro* fertility. After sorting, sperm from bulls H-319, 320 maintained similar *in vitro* fertility to sperm that were not sorted; however, the fertility for other 2 bull was significantly decreased. These results indicated that the sorting process affected the *in vitro* fertility of sorted sperm in a bull specific manner, but it was not a significant factor for all bulls. The bull effect of the sorting process on the *in vitro* fertility may have reflected recent improvements in sperm sorting technology. Further, research is needed to optimize the use of sexed IVF by using different sperm concentration, as suggested by Lu and Seidel (2004).

Pre-selected Y-bearing semen was confirmed pre-selected sex blastocyst to apply for embryo transfer of X-bearing blastocyst. In this study, total 10 blastocysts produced by Y-bearing semen derived blastocysts, were confirmed 9 male blastocysts and so ZRSR2Y RNA primer was very well working to confirm male embryos. To apply embryo transfer, X-bearing semen was used to produce female embryos by IVP system. The real practical sense in using this biotechnology is closely related to the generation of offspring in satisfactory quantities and costs. There are few studies reported about the pregnancy per ET (P/ET) in their results are designed to find this answer, possibly due to

the difficulty in reaching large number of embryos in super-ovulation programs. Schenk *et al.* (2006) observed no difference in the pregnancy rate of embryos derived from sex-sorted or non sex-sorted sperm. However, in this study, the number of transferred embryos was small size of recipient numbers that were given only one female bearing blastocyst per recipient. An experiment performed by Baruselli *et al.* (2007), part of the embryos was transferred immediately after collection (fresh) at fixed time into synchronized recipients. Similar P/ET was observed at 30 and 60 days of gestation after transfer of embryos produced with sex sorted or non sex-sorted semen. After sexing by ultrasound, it was observed that sex-sorted semen resulted in 90.0% females and conventional semen resulted in 52.7% females. In the present study, we achieved 43.8% pregnancy rate, produced 37.5% offspring and successfully 91.7% female offspring in dairy cow, our findings therefore, could be applied to increase female offspring ratio in the dairy farm. In this study, 11 MS markers were used to confirm paternity identification between parents and offspring that were produced by female bearing blastocyst transfer. Total 20 offspring and recipients were confirmed 100% perfectly identification and so these 11 MS markers can be applied to analysis of paternity identification between parent and offspring.

Taken together our data, it can be overcome the obstacle that male offspring of dairy farm does not have value in Korea and so the most of dairy farmer intended very much to increase female offspring ratio by application of new biotechnology, just like embryo transfer of OPU derived blastocysts with X-bearing sperm. In this regards, this result can be applied in the dairy farm to increase female offspring ratio and then to improve net income in each farm as well as overcome the obstacle of male offspring production.

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