Journal of Reproduction and Development, Vol. 65, No 4, 2019

—Original Article—

Comparative evaluation of the cost and efficiency of four types of sexing methods for the production of dairy female calves

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Abstract. This study was conducted to evaluate and compare the economic benefits of different embryo sexing methods, based on the cost per female dairy calf produced. Female calves were produced from four kinds of female embryos: (1) those collected from superstimulated donors at 7–8 days after artificial insemination (AI) with X-sorted semen; (2) those sex-determined by loop-mediated isothermal amplification assay of a biopsy sample of embryos collected from superstimulated donors after AI with conventional unsorted semen; (3) those obtained by *in vitro* embryo production (IVEP), using X-sorted semen and *in vitro*-matured oocytes collected from donors by ovum pick-up (OPU); and (4) those obtained by IVEP, using X-sorted semen and oocytes collected by OPU after dominant follicle ablation and follicle growth stimulation of the donors. The respective productivities of female calves per technical service and the total production cost per female calf of each sexing method were compared. The production cost per female calf (66,537 JPY), as calculated from the number of female calves per service (1.30), pregnancy rate of transfer (42.9%), rate of female calves obtained (92.9%), and total cost of the method (56,643 JPY plus embryo transfer fee), was less for IVEP with X-sorted semen and follicular growth-stimulated (FGS) oocytes than for the other groups ($P < 0.05$). The results demonstrate that embryo production with X-sorted semen and FGS oocytes provides a more efficient method for producing female calves than the other embryo sexing methods.

Key words: Bovine female embryo, Embryo transfer cost performance, *In vitro* embryo production, Ovum pick-up, X-sorted semen

(**J. Reprod. Dev. 65:** 345–352, 2019)

Multiple ovulation and embryo transfer (MOET) is a powerful technology used in genetic manipulation, most notably for the genetic improvement of Holstein cattle [1]. MOET is used with sexing equipment in dairy management for the systematic production of females. Diagnostic embryo sexing from a biopsy sample [2–4] and MOET with X-sorted semen [5, 6] has been previously used in the dairy farming industry.

Currently, breeders only use MOET to supply young sires for the artificial insemination (AI) industry, as this technique is too expensive for use by commercial dairy farmers as a conventional reproductive method to produce female cattle [7]. Although approximately 50,000 calves are produced from over 100,000 embryos obtained from almost Japanese Black cattle origin per year in Japan [8], these results do not account for even 1% of the total number of domestic dairy cattle produced. In other words, because 99% of calves are assumed to be obtained from AI, the remaining rare calves must have been produced using MOET techniques in Japan. Japanese dairy farmers are not sufficiently satisfied with the utility of MOET as the services required to obtain one female calf are too expensive and the technique does not always produce consistent results [9]. Therefore, although MOET provides dairy production with many benefits, its cost-effectiveness for female calf production per technical service is too low to be fully embraced by farmers in Japan.

Ovum pick-up (OPU) coupled with *in vitro* fertilization (IVF) has been regarded as a more efficient method than MOET for embryo production [10]. Embryos derived from OPU-collected oocytes, after dominant follicle ablation (DFA) treatment of the donor, developed into blastocysts at high rates *in vitro* [11]. The *in vitro* development

Received: February 28, 2019
Accepted: May 10, 2019
Published online in J-STAGE: June 10, 2019

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of blastocysts derived from *in vivo*-matured oocytes was generally higher than that of blastocysts derived from *in vitro*-matured (IVM) oocytes [12, 13]. Follicle stimulation by the follicle-stimulating hormone (FSH) before OPU is known to improve the recovery rate and developmental competence of bovine oocytes [14]. Furthermore, the developmental capabilities were increased in blastocysts derived from oocytes matured *in vivo* after a luteinizing hormone (LH) surge, compared with those derived from oocytes recovered during the preovulatory surge [15], as the meiotic resumption of bovine oocytes in the ovulatory follicles was initiated by the preovulatory LH surge [16]. Matoba *et al.* [17] established an efficient method for producing embryos from cows via *in vitro* embryo production (IVEP), using *in vivo*-matured oocytes collected by OPU from donors stimulated with FSH treatment after DFA. Akiyama *et al.* [18] demonstrated that female embryo production improved when using IVEP with X-sorted frozen sperm and follicular growth-stimulated (FGS) oocytes collected by OPU from superstimulated dairy Holstein cows after DFA. Consequently, IVEP using *in vivo*-matured oocytes with X-sorted semen could maximize the number of transferable female embryos in dairy cattle reproductive programs.

Reports of the costs of various reproductive techniques, such as MOET for dairy herds [1, 7, 19], IVEP for beef calf production [20], and X-sorted sperm for Holstein calf production [5, 6, 21], have been published in the literature. However, there are no economic reports that describe the utility of female embryos for dairy cattle management. Therefore, this study was conducted to clarify the embryo transfer (ET) techniques as well as sexing methods and their consequent economic benefits in dairy cattle management compared to other methods in terms of female calf production efficiency and the cost for female calf production per technical service.

Materials and Methods

Basic experimental conditions

The experiments for investigating female calf production were conducted in three prefectures in Japan using the MOET and IVEP systems and four kinds of sexing methods. All experimental procedures involving animals were reviewed and approved by the animal care committees of each prefectural livestock center and the Institutional Animal Experiment Committee and the Animal Ethics Committee of Nippon Veterinary and Life Science University (Approval No. S26K-1). Lactating Holstein cows, used as oocyte and embryo donors, were housed in a loose barn and milked twice daily at 0900 h and 1800 h. The cows were fed a total mixed ration (consisting of corn silage, hay, and concentrates) twice daily at 1000 h and 1600 h. The control diet was designed according to the Japanese Feeding Standard for Dairy Cattle [22] to meet the estimated requirements for total digestible nutrients, crude protein, and neutral detergent fiber. The recipient dairy farm cows were fed and managed similarly. Unless otherwise stated, all chemicals and reagents used for this study were obtained from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan).

Methods of female embryo production

Superstimulation of donors: Superstimulation of the lactating cows was conducted as described in an earlier report by Akiyama *et al.* [18]. Briefly, a progesterone-releasing device (CIDR; controlled internal drug release Livestock Improvement Association of Japan, Tokyo, Japan) was inserted into the dairy cows intravaginally (CIDR insertion = day 0). At day 5 after CIDR insertion, the ovaries of the animals were treated with DFA in order to aspirate all follicles with a diameter over 8 mm. Following this, 30 Armour units (AU) of FSH (Antrin R10; Kyoritsu Seiyaku, Tokyo, Japan) were administered intramuscularly, twice daily at 0900 h and 1600 h for 4 days, in decreasing doses (6, 6, 4, 4, 3, 3, 2, and 2 AU, respectively) from the evening of day 6 to the morning of day 10 to stimulate follicular growth. All cows were administered 0.15 mg of prostaglandin $F_2\alpha$ ($PGF_2\alpha$, D-cloprostenol, Dalmazin; Kyoritsu Seiyaku) intramuscularly on the evening of day 8. The CIDR device was removed on the morning of day 9 to induce estrus. A gonadotropin-releasing hormone (GnRH) analog (200 µg of fertirelin acetate, Spornen; Kyoritsu Seiyaku) was administered intramuscularly to induce the LH surge by the growing follicles at 0900 h on the morning of day 10 [17].

Collection of follicular oocytes by ovum pick-up: The oocytes were collected by OPU as described by Imai *et al*. [23]. The cumulus–oocyte complexes (COCs) were collected from the cows by OPU with the aid of an ultrasound scanner (SSD-900; Aloka, Tokyo, Japan) and a 7.5-MHz convex-array transducer (UST-9109P-7.5; Aloka) with a stainless steel needle. All visible follicles from the IVM oocyte group with a diameter over 2 mm were aspirated from the cows, using a 17-gauge × 500-mm disposable needle attached to a 1,500-mm polyvinyl chloride tube (COVA Needle; Misawa Medical Industry, Tokyo, Japan) connected to 120 mmHg of vacuum pressure (FV-4; Fujihira Industry, Tokyo, Japan), with an aspiration rate of approximately 24 ml/min. The follicular contents were gathered into a 50-ml conical tube containing approximately 5 ml of aspiration medium consisting of phosphate-buffered saline (PBS) supplemented with 1% newborn bovine serum (NBS; S0750-500; Biowest SAS, Nuaillé, France) and 10 IU/ml heparin (Novo-Heparin Injection 1000; Aventis Pharma, Tokyo, Japan) held at 37°C in a heat block (FV-5; Fujihira Industry). The FGS oocytes were collected, as described by Akiyama *et al.* [18], 25–26 h after administrating the cows with GnRH analog on day 11. All visible follicles with a diameter over 5 mm were aspirated using the OPU system, as described above.

In vitro embryo production using *in vitro*-matured oocytes: The collected COCs were matured, fertilized, and cultured *in vitro* as described in previous reports [17, 18]. In brief, the follicular contents in the conical tubes were rinsed with aspiration medium in an EmCon filter (Immuno System, Spring Valley, WI, USA) and then transferred to 90-mm Petri dishes. The recovered oocytes were classified as having either expanded or non-expanded cumulus cells. COCs with compact cumulus cells were washed in aspiration medium and then cultured in 35-mm Petri dishes (153066 TC Dish 35 × 10 Vents Nunclon; Nalge Nunc International, Rochester, NY, USA) in groups of 10–20 in 100-µl droplets of maturation medium consisting of 25 mM HEPES-buffered Tissue Culture Medium 199 (TCM-199; 12340-030; Life Technologies, Grand Island, NY, USA) supplemented with 5% NBS and 0.02 AU of FSH in paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan), for 24 h at 39.0°C in humidified air with 5% CO_2. Oocytes surrounded by excessive cumulus layers were trimmed with a blade in aspiration medium. To ensure the finalization of nuclear maturation, 10–20 oocytes covered by a few layers of expanded cumuli were cultured in 50-µl droplets

of maturation medium for approximately 3–5 h at 30 h after GnRH analog administration.

In vitro embryo production by *in vitro* fertilization: All frozen X-sorted semen of four Holstein sires, containing 2 million spermatozoa, were purchased from Genetics Hokkaido Association (Hokkaido, Japan). There was a statistical difference in the ability of IVEP among X-sorted semen [24]. One of X-sorted semen with a low IVEP ability was avoided and was not clustered between the groups as possible. The samples were thawed in a 37°C water bath for 14 sec, then layered onto a 45% and 60% Percoll density gradient (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 710 × *g* for 10 min at 37°C [24]. The sperm pellet was washed once with 5.5 ml of IVF100 medium (Research Institute for Functional Peptides, Yamagata, Japan) for 5 min at 500 × *g* and 37°C. The final sperm concentration was then adjusted to 3×10^6 sperm/ml in IVF medium. Oocytes were transferred to an 80-µl droplet of the sperm suspension in IVF medium and the system was covered with paraffin oil for 6 h at 39.0°C in humidified air with 5% CO_2 to allow fertilization to proceed.

In vitro embryo production by *in vitro* culture: The embryos obtained by IVF were first denuded by gentle pipetting with a fine glass pipette and then cultured in 125-µl droplets of Charles Rosenkrans medium (CR1) [25], consisting of amino acids supplemented with 5% NBS and 0.25 mg/ml of linoleic acid albumin (L-8384; Sigma-Aldrich), for 9 days at 39.0°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The female embryos that were transferable to recipients were classified according to the quality codes recommended by the International Embryo Transfer Society [26]. Those that had developed into the expanded blastocyst stages of code 1 (morphologically excellent and good embryos) to code 2 (poor embryos) on days 7–9 post IVF were used in this study.

In vivo female embryo production by multiple ovulation and embryo transfer: A total of four straws containing frozen–thawed X-sorted semen, with two straws each attached to an AI gun (Mo-1; Misawa Medical Industry), were inseminated into each deep-uterus horn of superstimulated donors on the morning of day 11. In the biopsy MOET group, conventional unsorted semen was inseminated into the uterus body of a superstimulated donor on the morning of day 11. Embryos were collected from the MOET groups by flushing the uterine horn of the donors with 500–700 ml of PBS supplemented with 1% NBS at 7–8 days after AI.

In vivo-collected embryos derived from conventional unsorted semen were biopsied using a microblade (Bio-cut blade 730; Feather Safety Razor, Tokyo, Japan) attached to a micromanipulator (Narishige, Tokyo, Japan). The biopsied embryos were cultured for 3–5 h in TCM-199 supplemented with 0.1 mM beta-mercaptoethanol (M7522; Sigma-Aldrich) + 5% NBS at 39.0°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 until the sexing diagnosis was completed. The sex of the small pieces of biopsied embryos was analyzed using a loop-mediated isothermal amplification (LAMP) kit (Eiken Chemical, Tokyo, Japan). The embryo sex was judged as female when the male–female common reaction was positive and the male-specific reaction was negative in the LAMP assays [27]. Female embryos that had developed into the expanded blastocyst stages of code 1 to code 2 were used for this study [26].

Conventional cryopreservation and thawing of female embryos

Most female embryos with a quality code of 1–1.5 were frozen in basal solution (TCM-199 supplemented with 20% NBS) containing 10% glycol and 0.1 M sucrose (S-1888; Sigma-Aldrich). The embryos were loaded individually into 0.25-ml straws (Plastic Cassou AI mini straw; IMV Technologies, L'Aigle, France) and seeded at –7°C into the methanol chamber of a programmable freezer set at –7°C (ET-1; Fujihira Industry). The straws were cooled at –0.3°C/min to –30°C and then plunged into liquid nitrogen for storage until further use. The frozen straws loaded with female embryos were thawed by warming for 10 sec in room temperature air and 10 sec in a 39°C water bath with gentle shaking [20].

Female calf production by embryo transfer into recipients

The estrous cycles of some recipients were synchronized through the administration of a CIDR device for 7–14 days and the injection of 0.225 mg of $PGF_2\alpha$ at 1 day before the removal of the CIDR device. Recipients in the estrus cycle were observed for over 30 min at least twice daily in the morning and evening. A straw loaded with a fresh female embryo of code 1–2 or a frozen–thawed female embryo of code 1–1.5 produced by the four different kinds of sexing methods was randomly attached to ET guns (Cassou ET syringe; IMV Technologies) to transfer the embryo into the uterine horn ipsilateral to the corpus luteum of each synchronized recipient on days 7–8 after estrus. Pregnancy was confirmed by the observation of a fetus with a detectable heartbeat in the intraluminal uterine fluid and of an embryonic membrane by ultrasonography on day 35 after embryo transfer (HS101V; Honda Electronics, Toyohashi, Japan). The sex accuracy and gestation period of the calves were assessed at birth.

Calculation of the cost for the four types of sexing methods

To determine whether the sexing methods were economically beneficial for the dairy farmers, the total expenses of female calf production for the four kinds of sexing methods were compared. To estimate the cost of a technical service, the expenses accrued for each sexing method were multiplied. The establishment of a Japanese AI center was necessary to provide the consumables, drugs, materials, and equipment for AI, superovulation, oocyte/embryo collection, embryo manipulation, embryo freezing, and ET. For a private specialized MOET clinic to be economically viable, it must be able to collect embryos at least 200 times annually and perform approximately 1,000 ETs to recipients per year [19]. The depreciation and amortization costs of MOET-related equipment, including a clean bench, a warming plate, a stereo microscope, a programmable freezer, an incubator, a straw labeler, an embryo transporter machine, and a gas sterilizer, were divided individually by their annual usage frequency (200 times) and the service life of the purchased facilities and common equipment. These expenses were considered the cost of the facilities and equipment for MOET. The depreciation and amortization costs of the special equipment required for sexing, including an inverted microscope with micromanipulators, a turbidity measuring instrument for biopsied specimens, a centrifuge machine for IVF, an ultrasound apparatus, a probe, an aspirator, and an incubator for OPU, were also calculated as described above, where the expenses accrued for each sexing method were multiplied. The fee for the consumables for each sexing method per service was estimated from the amount and

Table 1. Outline of the sexing methods

Female embryo production method	MOET with X-sorted semen	MOET and biopsy sexing	IVEP with X-sorted semen and IVM oocytes	IVEP with X-sorted semen and FGS oocytes
Dominant follicle ablation	○	○	-	○
Follicle growth treatment	○	○	-	○
Ovum pick-up	-	-	○	○
Artificial insemination	4 semen	○	-	-
In vitro maturation	-	-	○	○
In vitro fertilization	-	-	○	2 times
In vitro culture	-	-	○	○
Embryo collection from uterus	○	○	-	-
Embryo freezing	○	○	○	○
Embryo transfer	○	○	○	○
Female calf production	○	○	○	○

The symbols in the table indicate whether the samples were treated (○) or not treated (-) in the sexing method. MOET: multiple ovulation and embryo transfer; IVEP: *in vitro* embryo production; IVM oocytes: *in vitro*-matured oocytes; FGS oocytes: follicular growth-stimulated oocytes.

price of the drugs and expendable material supplies. The technical cost of ET per recipient was inferred as the average of the fees in the three cooperating prefectures.

The following were excluded from the cost calculations: the cost of the feed and depreciation of the donor and recipient cows; the building costs of land charges and AI office equipment; commonly used AI equipment, including an electronic balance, a liquid nitrogen refrigerator, an autoclave, and a biological microscope; and lighting and fuel expenses, as well as the cost of consumables and drugs for hygiene and sterilization; the cost of estrus synchronization of the recipients and related technical fees; the technical costs of embryo collection, manipulation (e.g., morphological evaluation), and freezing; personnel expenses and labor costs; and the profits of the engineers. Under the criteria described above, the actual expenses for producing a female calf without profit and loss were calculated for the respective sexing methods.

Experimental groups

MOET with X-sorted semen: Two straws of frozen–thawed X-sorted semen were inseminated into each deep-uterus horn of superstimulated donors on the morning of day 11 after treatment. A total of four straws were used for each superstimulated donor. Female embryos were collected from the uterine horn of the donors by flushing with PBS (containing 1% NBS) at 7–8 days after estrus (17–19 days after treatment). Female embryos that had developed into compacted morula and onto the expanded blastocyst stages of code 1–2 were used for ET in this study (Table 1).

MOET and biopsy sexing: Conventional unsorted semen was inseminated into the uterus body of a superstimulated donor on the morning of day 11 after treatment. Code 1–2 embryos collected from superstimulated donors at 7–8 days after estrus were biopsied using a microblade. The sex of the biopsied embryos was predicted by analyzing small pieces of the embryos by LAMP assay. The female biopsied embryos with a quality code of 1–2 were used for ET in this study.

IVEP with X-sorted semen and IVM oocytes: COCs were collected from all visible follicles of over 2 mm in diameter by OPU. The recovered COCs were cultured in maturation medium for 24 h and then fertilized with X-sorted semen. The resultant female embryos were cultured *in vitro* for 9 days. Blastocysts classified as code 1–1.5 were used for the subsequent ET examination.

IVEP with X-sorted semen and FGS oocytes: Oocytes stimulated with FSH with a diameter over 5 mm were collected from follicles 25–26 h after administrating cows with GnRH analog on the morning of day 11 after treatment. The recovered oocytes were classified as having expanded or non-expanded cumulus cells at 30 h after the GnRH analog administration on day 11. Two types of IVF with X-sorted semen were performed for this group; the first, using oocytes with expanded cumulus cells at 30 h after GnRH analog administration on day 11, and the second, using oocytes with non-expanded cumulus cells at 24 h after maturation. The resultant female embryos were cultured *in vitro* for 9 days. Blastocysts classified as code 1–1.5 were used for subsequent ET examination.

The cost of four types of sexing methods, consisting of two types of MOET and two types of IVEP using OPU-collected oocytes with X-sorted semen, were compared, where the cost was denoted as the number of female calves produced per technical service and the total technical cost per female calf. The efficiency of female calf production per technical service was calculated using the number of transferable female embryos produced, the pregnancy rate after ET to recipients, the number of full-term developments, and the birth rate of female calves. As the production competency of transferable female embryos is restricted by the number of follicles present in the donors in OPU [23], and the number of corpora lutea assessed indicates the ovulation numbers of superovulated donors in MOET [19], we counted the follicle numbers in OPU and the corpora luteum numbers before collection. The total technical cost per female calf was calculated on the basis of the total cost of technical services, the production rate of female calves per technical offer, and the cost of ET per recipient. On the basis of those results and the aforementioned costs, the cost performance of the respective sexing methods was evaluated.

Table 2. Efficiency of female embryo production using the different sexing methods

Female embryo production method	No. of donors used	No. of responses of donors * (M ± SEM)	No. of collected embryos or oocytes ** (M ± SEM)	No. of transferable female embryos (M ± SEM)
MOET with X-sorted semen	35	9.8 ± 0.8 [a]	6.4 ± 0.8 [a, c]	1.6 ± 0.4 [a]
MOET and biopsy sexing	34	11.6 ± 1.0 [a]	9.3 ± 1.3 [a]	1.4 ± 0.3 [a]
IVEP with X-sorted semen and IVM oocytes	36	19.5 ± 1.6 [b]	12.5 ± 1.5 [d]	1.3 ± 0.3 [a]
IVEP with X-sorted semen and FGS oocytes	23	27.3 ± 3.1 [b]	17.7 ± 2.3 [b]	4.2 ± 0.9 [b]

M ± SEM: mean ± standard error of the mean; MOET: multiple ovulation and embryo transfer; IVEP: *in vitro* embryo production; IVM oocytes: *in vitro*-matured oocytes; FGS oocytes: follicular growth-stimulated oocytes. * No. of corpora lutea in MOET and follicles in the ovum pick-up of donors. ** Embryo numbers collected from donors in MOET, and oocytes numbers after ovum pick-up. [ab, cd] $P < 0.05$.

Table 3. Results of female calf production obtained using the different sexing methods

Female embryo production method	Embryo	No. of embryos transferred	No. of pregnancies (%)	No. of abortion (%)	No. of female calves (%) *	Birth weight (kg ± SEM)	Gestation period (day ± SEM)
MOET with X-sorted semen	Fresh	8	3 (37.5)	0	14 (93.3)	40.8 ± 0.9	278.6 ± 0.9
	Frozen	27	12 (44.4)				
MOET and biopsy sexing	Fresh	13	6 (46.2)	1	15 (88.2)	44.1 ± 1.4	280.7 ± 0.9
	Frozen	32	12 (37.5)				
IVEP with X-sorted semen and IVM oocytes	Fresh	7	4 (57.1)	2	15 (93.8)	43.7 ± 1.6	280.4 ± 1.9
	Frozen	30	14 (46.7)				
IVEP with X-sorted semen and FGS oocytes	Fresh	6	3 (50.0)	4	13 (92.9)	44.1 ± 1.4	278.9 ± 1.4
	Frozen	36	15 (41.7)				

MOET: multiple ovulation and embryo transfer; IVEP: *in vitro* embryo production; IVM oocytes: *in vitro*-matured oocytes; FGS oocytes: follicular growth-stimulated oocytes. * A total of 5 male calves were delivered.

Statistical analysis

All data were assessed using the Kruskal-Wallis test. When a significant difference was detected, the Mann-Whitney U test was used for multiple comparison testing with Holm's adjustment. Statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R foundation for Statistical Computing, Vienna, Austria), that is, a modified version of the R commander, designed to add statistical functions that are frequently used in biostatistics [28]. Differences with a probability value of less than 0.05 were considered statistically significant.

Results

Table 2 presents the female embryo production results. For the MOET methods, of the total number of embryos collected, the number of transferable female embryos was 1.6 ± 0.4 of 6.4 ± 0.8 for the X-sorted semen group and 1.4 ± 0.3 of 9.3 ± 1.3 for the biopsy sexing group. Although the recovery of OPU-collected oocytes from the FGS group was not higher than that from the non-stimulated group ($P > 0.05$), the number of transferable female embryos per treatment (4.2 ± 0.9) in the FGS group was significantly higher ($P < 0.001$) than that of the other groups. Among all the groups, the highest number of female embryos was obtained from IVEP with the FGS oocytes.

Table 3 presents the results of female calf production after the transfer of the female embryos into recipients, according to the various sexing methods. The accuracy rate was 91.9% for a total of 57 females and 5 males derived from embryos identified as female. No significant difference ($P > 0.05$) was found in the percentages of pregnancies, frequencies of abortion, rates of female calf production, or birth weight among the groups. The average gestation period of the Holstein calves was 279.2 ± 0.6 days. No large offspring syndrome or abnormality was found among the groups.

Table 4 presents the technical costs of the various sexing methods. The costs of the facilities and equipment are shown as the amortization cost (6,780 JPY) of the facilities, common equipment, and special equipment for the respective sexing methods.

Table 5 presents the productivity of the four kinds of sexing methods, compared by the total cost per female calf and number of female calves produced per technical service. The fee for ET to a recipient was calculated as 7,140 JPY. More than one female calf was produced by IVEP with X-sorted semen and FGS oocytes. The cost performance of this method per female calf produced was also less (66,537 JPY) than that of the other groups.

Discussion

The results of this study indicated that by using IVEP with X-sorted semen and oocytes collected by OPU from superstimulated dairy cows after DFA, the number of transferable female embryos obtained and female calves produced per service was higher than the other sexing methods. Accidents and difficult deliveries depending on the prolongation of the gestation period, increased birth weight in calves, and cases of large offspring syndrome have been reported

Table 4. Technical costs of the sexing methods

Female embryo production method	Cost of facilities and equipment *	Total fee for frozen semen	Consumption fee **	Cost of the method ***
MOET with X-sorted semen	6,780	26,132	35,644	68,556
MOET and biopsy sexing	9,214	4,355	37,752	51,321
IVEP with X-sorted semen and IVM oocytes	7,897	6,533	12,556	26,986
IVEP with X-sorted semen and FGS oocytes	7,897	13,066	35,680	56,643

MOET: multiple ovulation and embryo transfer; IVEP: *in vitro* embryo production; IVM oocytes: *in vitro*-matured oocytes; FGS oocytes: follicular growth-stimulated oocytes. * Shown as the depreciation and amortization costs divided by 200 times of the annual use and service life of facilities, common equipment, and special equipment. ** The price (JPY) of drug and expendable supplies of materials. *** The cost of the technical service was estimated from the cost of facilities and equipment, total fee for frozen semen, and consumption fee.

Table 5. Efficiency of female calf production per technical service

Female embryo production method	Price / technical service *	No. of transferable female embryos collected *	Production rate (%) of female calves **	Cost / female calf ***	No. of female calves / service ****
MOET with X-sorted semen	68,556	1.6	40.0	124,969	0.64 [a]
MOET and biopsy sexing	51,321	1.4	33.3	131,525	0.47 [a]
IVEP with X-sorted semen and IVM oocytes	26,986	1.3	40.5	68,885	0.53 [a]
IVEP with X-sorted semen and FGS oocytes	56,643	4.2	31.0	66,537	1.30 [b]

MOET: multiple ovulation and embryo transfer; IVEP: *in vitro* embryo production; IVM oocytes: *in vitro*-matured oocytes; FGS oocytes: follicular growth-stimulated oocytes. * The number of female embryos and the cost per sexing method were obtained from Tables 2 and 4. ** Female calf production rates are shown by the pregnancy rate after embryo transfer (ET) and the birth rates of female calves: No. of female calves produced / no. of embryos transferred (%). *** The multiplication cost (JPY) for a female calf as calculated using the technical fee, ET fee (7,140 JPY), and rate of female calf production: Price of technical service / no. of transferable female embryos collected / rate of female calves produced plus ET fee (7,140 JPY) / rate of female calves produced. **** Female calf production rates are shown by the pregnancy rate after ET and birth rates of female calves: No. of female calves produced / no. of embryos transferred (%). [ab] $P < 0.05$.

with recipients of transferred embryos produced from IVEP [20]. These issues were not observed among the groups in this study. Sexing of the female calves obtained from X-sorted semen was accomplished with a probability of over 90%. The high pregnancy rate (> 40%) and gestation range of calves (265–288 days) in the experimental groups were similar to those of normal parturition and those obtained by other studies [18, 29].

As an efficient method of embryo production, IVEP coupled with OPU is expected to be superior to MOET [10, 11]. However, although the cost of IVEP with OPU was lower, its embryo production rate was equivalent to that of the MOET method [10]. Akiyama *et al.* [18] reported efficient methods for the production of female embryos from Holstein dairy cows by IVEP with X-sorted sperm and oocytes collected by OPU from superstimulated donors. A similar result has been previously reported for IVM oocytes obtained from FGS donors, where micromorphological and immunomorphological functions contributed to a higher developmental competence than those of non-stimulated IVM oocytes [30]. Among all the methods examined in our study, the highest number of female embryos and calves produced per service was achieved in the IVEP with FGS oocyte group, indicating that follicle growth stimulation before OPU is important for obtaining oocytes with a high developmental ability.

The results showed that follicle growth stimulation was able to promote the number of follicles growing in the ovaries. However, after FGS treatment, only certain follicles underwent ovulation, as shown in Table 2. The remaining follicles degenerated without ovulation. In this study of IVEP with FGS oocytes, COCs of two kinds (i.e., oocytes surrounded by expanded cumulus layers and oocytes with non-expanded cumulus cells) were collected by OPU. It is likely that the follicles with expanded cumulus cells were able to attain ovulation, and underwent delayed regression. Female embryos obtained from pre-matured oocytes with the non-expanded cumulus cells not only developed into blastocysts in the *in vitro* culture, but also developed to term at the same rate as that of blastocysts derived from matured oocytes with expanded cumulus cells after transferring to recipients, as described by Akiyama *et al*. [18]. Given that IVF for embryo production with both matured COCs and immature COCs is difficult, twice the amount of processing is regarded as being worthwhile for the production of more female embryos.

A shorter life span and an absolute lower quantity of X-sorted semen have been suggested as reasons for the reduced fertility and pregnancy rates compared with those from conventional unsorted semen [31, 32]. The yield rate of transferable embryos in MOET after AI with X-sorted sperm is also lower than that achieved with conventional unsorted semen [33, 34]. However, it has been reported that deep-uterine insemination can compensate for the shortcoming of the X-sorted semen [35]. No significant difference was found in the numbers of transferable embryos, degenerated embryos, and unfertilized eggs collected by *in vivo* insemination with X-sorted semen deposited into the uterus horns compared with those of the conventional unsorted control semen [36]. Given that neither the sperm number per insemination dose nor the site of insemination is a factor determining the proportion of unfertilized eggs obtained when X-sorted semen is used in embryo production [30, 37], the cost

of female embryo production associated with MOET with X-sorted semen may be lower.

Although the pregnancy rate of the biopsied embryos was the same as that of the intact embryos, the pregnancy rates of both the frozen sexed embryos and the sexed embryos obtained from low-quality embryos were lower [3]. The biopsy-sexing of the low-quality embryos was inadequate for embryo manipulation since it could potentially reduce the viability of the embryos after ET [2, 4]. Moreover, the amplification of the male-specific DNA sequences of embryos is not financially optimal as approximately over 50% of the recovered embryos are of the undesired sex and are therefore wasted. As a result, sexing diagnosis using embryo biopsy is expected to diminish in industrial applications.

Most Japanese farmers are hesitant to use MOET for the production of offspring due to the negative factors associated with this technique, including high technical costs, low cost performance, and unsuitable productivity of female offspring [9]. Therefore, farmers are demanding that the ET industry produce multiple transferable female embryos, or at least more than one female calf per technical service [38]. If the difficulties associated with the MOET or IVEP techniques can be resolved, then dairy farmers will accept their utility as practical reproduction methods.

In this study, the proportion of transferable female embryos produced by IVEP with X-sorted semen and FGS oocytes was approximately three-fold that of other sexing methods. However, the cost performance for a private specialized MOET clinic must include all original expenses and the practical calculated expenses for technical services, such as the actual prices of materials and drugs, additional profits and losses, technical fees, and profits for the engineer performing the OPU, IVEP, and embryo manipulation. The pregnancy rate of female embryos obtained from X-sorted semen was lower than that of embryos obtained from unsorted semen [32]. Further analyses with model studies are needed in order to justify the use of embryo sexing throughout the dairy industry and allow dairy farmers to ascertain the difference between ideal and realistic outcomes.

The recovery rate of oocytes (recovered oocytes/observed follicles) was higher for those collected from superstimulated donors [14] and OPU operations by skilled handlers [12]. In humans, over 90% of matured oocytes are suitable for collection from the ovaries immediately before ovulation [39]. Because the recovery rate of oocytes from the FGS group was not significantly different from that of the other OPU groups in this study, increasing the practitioner's skills and improving the handling of the oocytes may increase productivity. Somfai *et al*. [30] reported that over one-third of bovine *in vivo*-matured oocytes did not reach a second metaphase stage under the same FGS-OPU protocol. As IVEP with immature oocytes decreases the rate of normal fertilization, the research results suggest that allowing an extended time for the *in vitro* maturation of the oocytes may increase the fertilization rate. Research has shown that the rates of IVF using spermatozoa with a shorter life span and/or damage were also improved when intracytoplasmic sperm injection was introduced into the IVEP industry [40, 41]. Recently, vitrification, an additional technique related to MOET, has been used as a method for preserving bovine embryos of low-grade quality [42, 43], although the pregnancy rate after cryopreservation depends on the quality of the embryos. This technical improvement could contribute to increases in female embryo and calf production by IVEP with X-sorted semen and FGS oocytes.

In conclusion, this study demonstrates that IVEP using X-sorted semen and oocytes collected by OPU from donor dairy cattle after DFA treatment and FSH superstimulation can greatly improve the efficiency of female embryo and calf production. This method provides superior results to conventional MOET as it reduces female calf production costs by half and reliably produces female calves. Since the results of the IVEP system are closely aligned to the expectations of dairy farmers, further studies will need to be conducted in order to increase female calf productivity via this method.

Acknowledgments

This study was supported by JSPS KAKENHI (grant number JP6450395), carrying on the research of the "Research and development projects for application in promoting new policy of agriculture, forestry and fisheries" (grant number 22016). The authors are grateful to Y Inaba, T Somfai, registered at the NARO Institute of Livestock and Grassland Science (Ibaraki, Japan), S Matoba, H Yoshioka, H Matsuda, Y Hashiyada, S Sugimura, and K Imai, registered at the National Livestock Breeding Center (NLBC, Nishigo, Japan), for their valuable technical advice related to IVEP with OPU. We also deeply appreciate the research staff of Y Sato in Niigata Prefecture, and M Kawai, Y Hori, K Nagai, and N Hayakawa in Ishikawa Prefecture of the respective research organizations. Frozen X-sorted semen for IVEP examination were purchased through special arrangement by H Hayakawa from Genetics Hokkaido.

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