Animal Reproduction Science 205 (2019) 156–164

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

In vitro production of sex preselected cattle embryos using a monoclonal antibody raised against bull sperm epitopes

M.M.R. Chowdhury[a,e,1], Lianguang Xu[b,1], Rami Kong[b,d,1], Bun-Young Park[d], Ayman Mesalam[b,f], Myeong-Don Joo[b], Fahmida Afrin[a], Jong-In Jin[d], Hyun-Tae Lim[b,c], Il-Keun Kong[b,c,*]

[a] *Animal Genetic Resources Research Center, National Institute of Animal Science, RDA, Namwon 55717, Jeollabuk-do Province, Republic of Korea*
[b] *Department of Animal Science, Division of Applied Life Science (BK21 Plus), Republic of Korea*
[c] *Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Gyeongsangnam-do Province, Republic of Korea*
[d] *GAST, Gyeongsang National University, Jinju 52828, Gyeongnam Province, Republic of Korea*
[e] *Department of Physiology and Pharmacology, Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Bangladesh*
[f] *Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig, 44519, Egypt*

ARTICLE INFO

Keywords:
WholeMom
Sorted sperm
Genetic up-gradation
IVEP

ABSTRACT

Sex preselection has always generated great interest among livestock producers. Among the prevalent sperm sorting methods, there is much evidence that sex sorting has a negative effect on sperm quality with an altered pattern of sperm motility, ultimately reducing the period of cell viability. In this study, we have established a new approach for the preselected embryo production by using WholeMom®; a monoclonal antibody developed against bull sperm epitopes for simple and easy separation of X- and Y-sperm. There were no significant differences ($P > 0.05$) in the percentage of presumptive zygotes between the control and the X-sperm sorted group, but there was a difference in early cleaving embryos with there being 81.2 ± 1.4%, 78.3 ± 1.0%, and 66.7 ± 1.1% for the control, X-sperm sorted, and Y-sperm sorted groups, respectively. Similarly, the percentage of embryos that developed to the blastocyst stage (Day 7) were also greater ($P < 0.05$) in the control and X-sperm sorted group compared with the Y-sperm sorted group being 34.8 ± 1.0%, 32.1 ± 0.8%, and 23.7 ± 1.0% in the control, X-sperm sorted, and Y-sperm sorted groups, respectively. Furthermore, B-*SRY* F2 and B-*SRY* R2 gene expression data indicated there was a detection accuracy of 81.0% for the female embryos and 72.5% for the male embryos produced in vitro. In conclusion, in cattle in vitro derived embryo production using pre-selected sexed semen and subsequent embryo transfer can facilitate the mass production of individuals that are genetically superior.

1. Introduction

Currently, there is an increased worldwide demand for dairy and beef products which has resulted in greater attention on improving embryo production efficiency (Holden and Butler, 2018). The use of sex preselection along with use of other genomic, proteomic and

* Corresponding author at: Department of Animal Science, Division of Applied Life Science (BK21 Plus), Gyeongsang National University, Jinju 660-701, Gyeongnam Province, Republic of Korea.
E-mail address: ikong7900@gmail.com (I.-K. Kong).
[1] These three authors equally contributed to this work.

https://doi.org/10.1016/j.anireprosci.2018.11.006
Received 23 August 2018; Received in revised form 23 October 2018; Accepted 14 November 2018
Available online 16 November 2018

phenomics technologies, provide a platform for development of a promising breeding strategy to meet the increased demand for milk or meat (Chen et al., 2012) and also are promising as a means for sustainable production of elite dairy or beef cattle (Rath et al., 2012). Sex sorting technology would be the primary way of obtaining genetically elite cattle embryos with pre-selected sexes. For example, dairy cattle farmers prefer high yielding dairy cattle for milk production, while the elite bulls are selectively produced for their greater hereditary impact and economic importance (Chen et al., 2012). In the dairy industry, there is extra production of unwanted male calves. Male dairy calves increase the risk of dystocia as compared with heifer calves, and are a surplus by-product of breeding with conventional semen resulting in calves with relatively lesser economic value than heifer calves. Integrating sexed semen into the breeding programs can reduce the number of unwanted male dairy calves and reduce dystocia. Furthermore, provision of sexed semen could result in generation of herd replacements and additional heifers for herd expansion at a faster rate (Holden and Butler, 2018).

Although there are various sperm sorting techniques none can be used that have a 100% accuracy. Furthermore, sex preselection before the formation of a conceptus is an ethically justified practice (Eftekhaari et al., 2015) whereby there is sorting of sperm with X or Y chromosomes prior to fertilization of the oocyte to generate either a male or a female offspring. Sex preselection could be accomplished before insemination by sorting of the sperm with a X or Y chromosome based on the distinctive characteristics of these two cell types. The success of separation of sperm with a X from those with a Y chromosome increased because of the desire of a significant number of commercial and registered dairy breeders to use in vitro fertilization (IVF) technologies to obtain elite females using preselected sires with superior genomic values (Garner et al., 2013). Nevertheless, there are some disadvantages with this being an economically expensive process. Some of the challenges with use of this process include a reduced life span of sex-sorted sperm, slowness of the process of sperm sorting per h (15×10^6 sperm/h of each sex), lesser sperm numbers used per insemination dose (2×10^6), and relatively lesser in vivo fertility compared to when sperm are used that has not been sex sorted. Furthermore, there needs to be expertise available for use of other technologies including artificial insemination (AI) (Garner et al., 2013).

Numerous factors such as health issues including sex-linked disease, has been stimulated farmers to select traditional or up-to-date techniques in selecting the gender of the offspring. Several of these approaches are the use of the following methods: swim-up or washing of spermatozoa, percoll gradient sperm separation, use of glass wool column filter, albumin separation, microsort using FISH (Fluorescence in situ hybridization), free electrophoresis, pH adjustment, pre-implantation genetic diagnosis (PGD) and flowcytometry (Eftekhaari et al., 2015). Of these attempts, the only method proven to be commercially viable is flow cytometry or fluorescence-activated cell sorting (Garner et al., 2013). Factors that should be taken into consideration during sorting process are: accuracy of sexing sperm, fertility when sexed sperm is used for breeding, genetic gain from use of sex-sorted sperm, and the economic value of sex-sorted sperm (Garner et al., 2013). During sorting, sorting pressure, speed, electrical deviation, and laser radiation, synergistically all lead to membrane alteration and pre-capacitation like changes in sperm that have been sex sorted, resulting in a reduced fertility when these sperm are used (Garner et al., 2013). Even with these limitations, production of sex-sorted semen usually followed by cryopreservation is being used commercially for cattle production. Development of technologies that would allow for the sorting rate to be more accurate without affecting sperm viability and fertility is still an evolving area of research.

To overcome the existing limitations, the present studies were designed for sperm sorting based on the distinctive physiological differences between sperm with a X or Y chromosome and we hypothesized that these differences will be significant enough to enable successful separation of sperm with a X from those with a Y chromosome. WholeMom is a monoclonal antibody used to separate the sperm with a Y- from those with a X chromosome. WholeMom antibody binds with the plasma membrane of the heads of sperm with the Y chromosome. After treatment with WholeMom antibody for 20 to 25 min, most of Y-sperm heads are attached together by head-to-head agglutination and these sperm precipitated in the bottom. WholeMom antibodies do not bind to the sperm with the X chromosome. Thus, sperm with the X chromosome are freely motile with the capacity for transport to the site of oocyte fertilization in the female reproductive tract. We hypothesized that use of the WholeMom monoclonal antibodies would allow for the accurate sex selection of embryos without negatively affecting embryo development to the blastocyst stage.

2. Materials and methods

2.1. Reagents

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

2.2. Animal ethics

All of the methods and experimental procedures were conducted according to the approved (Approval ID: GAR-110502- X0017) guidelines and regulations by the institutional animal care and use committee (IACUC) of Gyeongsang National University, Republic of Korea.

2.3. Cumulus-oocyte complex (COC) recovery

Ovaries from Korean native cows (Hanwoo) were collected after slaughter at the local slaughterhouse, placed in physiological saline (0.9% NaCl) at approximately 37.5 °C, and transported to the laboratory within 4 h of slaughter. After washing the ovaries with fresh Dulbecco's phosphate buffered saline (D-PBS), COCs were retrieved as described (Chowdhury et al., 2017). COCs were recovered from follicles (2–8 mm diameter) using an 18-G needle attached to a vacuum pump. Aspirated follicular fluid was expelled into dishes containing TL-HEPES medium (6.662 g/L sodium chloride, 0.238 g/L potassium chloride, 0.168 g/L sodium bicarbonate,

0.040 g/L sodium biphosphate, 0.85 g/L sodium lactate, 0.101 g/L magnesium chloride, 0.101 g/L calcium chloride, 2.383 g/L HEPES, 1 μL/mL phenol red, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin), and the oocytes were retrieved using a stereo-microscope as per (Chowdhury et al., 2018). Good-quality oocytes with more than three layers of compact cumulus cells and homogenous cytoplasm were selected and washed three times in TL-HEPES medium.

2.4. In vitro maturation (IVM) and fertilization (IVF)

Oocytes were cultured in IVM medium as described by (Mesalam et al., 2017). In brief, COCs were washed three times in IVM medium (tissue culture media-199 [TCM-199]) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 μg/mL estradiol-17β, 10 μg/mL follicle-stimulating hormone, 0.6 mM cysteine, and 0.2 mM sodium pyruvate and transferred to NUNC 4-well plates (45–55 oocytes per well) containing 700 μL of IVM media. *In vitro* matured COCs were fertilized with frozen-thawed sex-sorted sperm from a Hanwoo Bull (KPN-684) and KPN-917.

2.5. Preparation of sex-sorted spermatozoa using Whole Mom

At first, one vial of WholeMom monoclonal antibody (20 μL) was thawed for 2–3 minutes. KPN-684 and KPN-917 bull semen straw was thawed at 38.5 °C for 1 min, and then the sperm were treated (One vial per one sperm straw) with WholeMom (Lot # NUCOD15-01, http://www.nurisci.com/) and placed vertically for 25 min in a water bath with stoppage of vibration (without any disturb which may cause mixing X-sperm and Y-sperm together) at 38.5 °C. WholeMom protein binds specifically the plasma membrane of sperm head. After 25 min we observed, the Y-sperm agglutinated head to head as shown in (Fig. 2) and settle down in the bottom of vial and the pellet was collected and mixed with 100 μL heparin and termed as Y-sperm as the WholeMom protein can bind only with Y-sperm and kept at 38.5 °C in a humidified atmosphere of 5% CO_2 for 15 min. The Whole Mom-treated rest of the two-thirds volume of semen was washed in 10 mL Dulbecco's phosphate buffered saline (D-PBS) and then centrifuged at 317 × g for 5 min. The supernatant was discarded and the sperm pellet was carefully diluted in 200 μL heparin (20 μg/mL) and termed as X-sperm as the WholeMom protein cannot not bind with X-sperm and then was kept at 38.5 °C in a humidified atmosphere of 5% CO_2 for 15 min. After maturation of oocytes, the IVM medium was removed and the COCs were denuded by adding 500 μL of IVF media, which is Tyrode's lactate solution (TL-Fert) supplemented with 6 μg/mL bovine serum albumin (BSA), 22 μg/mL sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin sulphate. In case of Y-sperm, the matured oocytes were inseminated with 100 μL capacitated motile spermatozoa, whereas for the X-sperm, the matured oocytes was inseminated with 200 μL capacitated motile sperm and kept at 38.5 °C in a humidified atmosphere of 5% CO_2 for 18–20 h (Figs. 1, Fig. 2). An in vivo study was conducted as a field trial in which artificial insemination was performed using WholeMom as depicted in Supplementary Fig. 3.

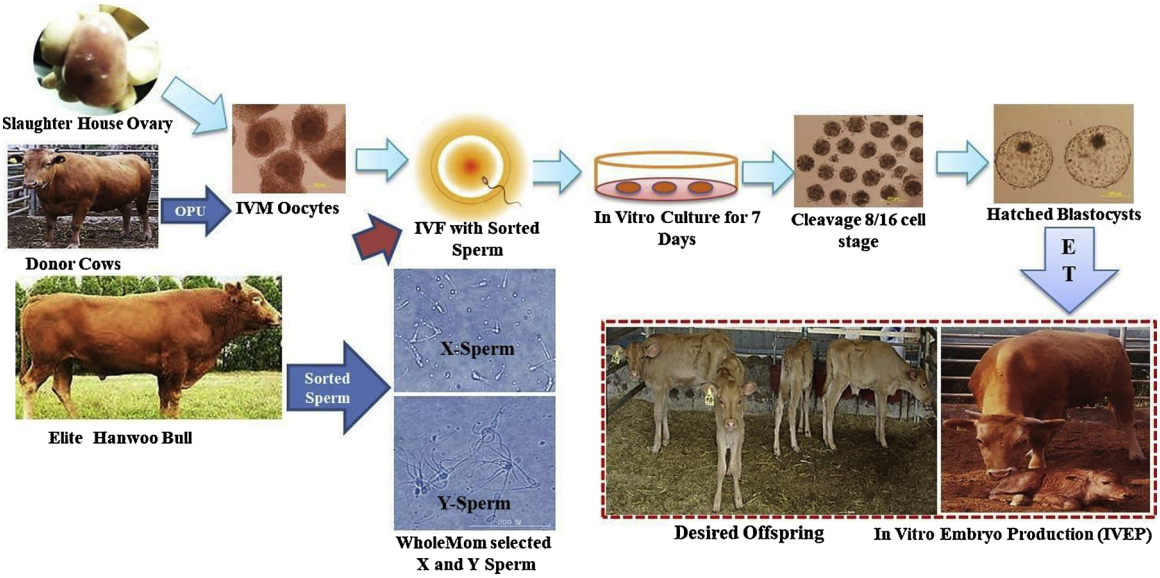

Fig. 1. Experimental protocol for the IVF with WholeMom antibody-selected X-sperm or Y-sperm to obtain preselected embryo. Oocytes were cultured in IVM medium for 22 h and then in vitro matured COC's were fertilized with frozen-thawed WholeMom treated sex-sorted sperm from Hanwoo Bull (KPN-684 and KPN-917). After fertilization, cumulus cells were removed and the denuded presumptive zygotes were transferred into culture in media IVC1 (Day 1 to Day 3) and IVC2 (Day 4 to Day 7) [Day 0 = day of IVF]. At Day 9 to Day 11, the hatched blastocysts were transferred to the selected heifer for implantation in to the uterus.

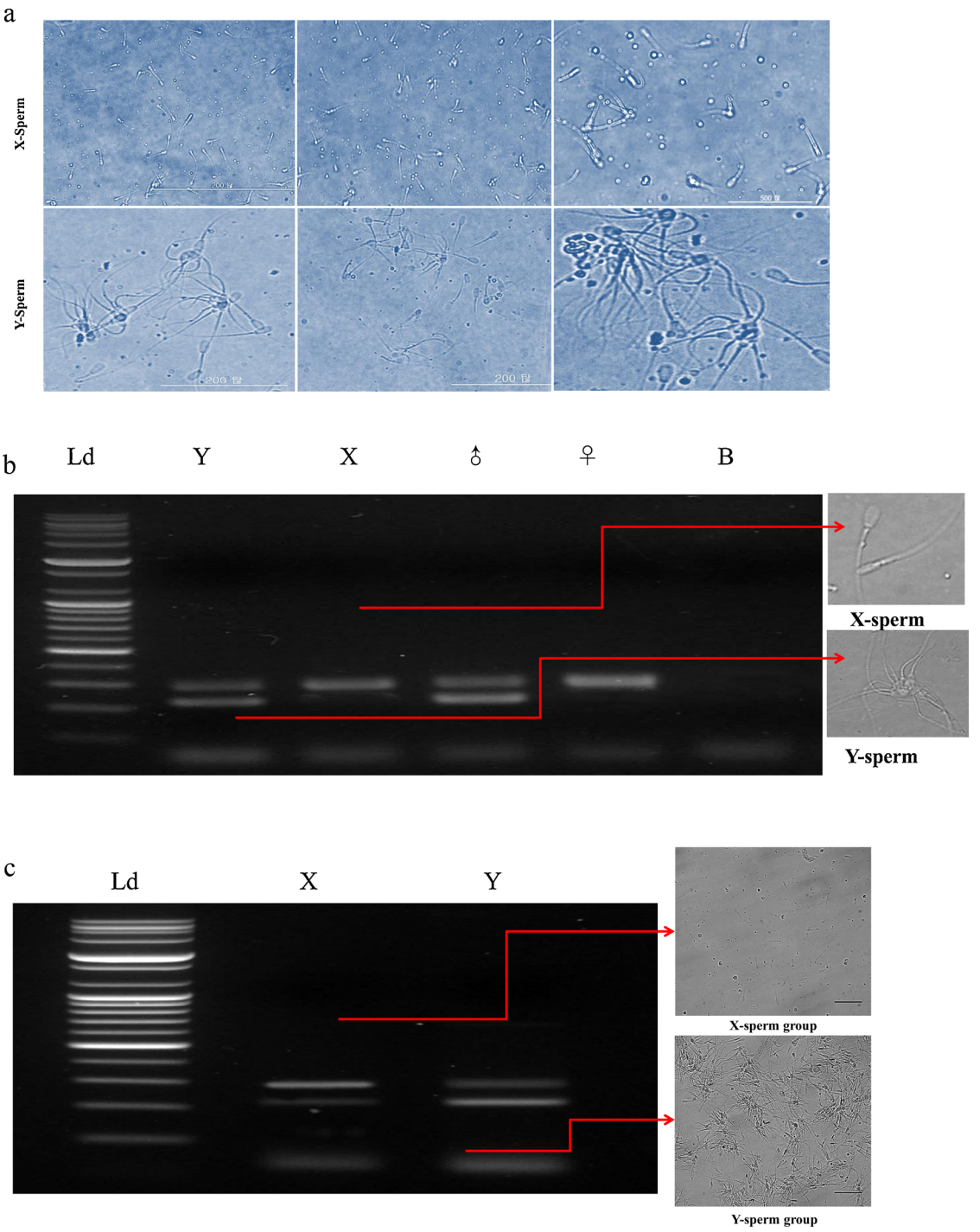

Fig. 2. (a) Representive pictures of X- and Y-sperm after 25 min of treatment with WholeMom anti-body. When the sperm were treated with WholeMom protein for 25 min at 37.5 ℃, there was agglutination of Y-sperm by binding head to head. Agglutinated Y-sperm had inhibited movement or swimming capacity for fertilization of oocytes whereas X-sperm had typical motility for fertilization of oocytes as the WholeMom antibodies can bind only to the sperm with a Y chromosome. (b) Representative gel electrophoresis data of WholeMom antibody-selected "Pure" sperm (KPN-917) using polymerase chain reaction (PCR) to check either X-sperm or Y-sperm at DNA level. WholeMom positive sperm represents Y-specific band, but WholeMom negative sperm is the X-sperm enriched due to treatment with surface antibodies that supposed to agglutinate the Y-sperm and subsequently has no Y-specific band. Samples were processed by amelogenin (*aMEL*), which shows a 270 base pair (bp) band for X sperm and two bands (270 bp and 214 bp) for Y sperm. From left to right: Ladder (Ld-100 bp); Y = Y sperm; X = X sperm; male control (♂); female control (♀); B = blank. (c) Representative gel electrophoresis data of WholeMom antibody-selected X-sperm group and Y-sperm group (KPN-917) using polymerase chain reaction (PCR) to check either X-sperm or Y-sperm at DNA level. WholeMom positive sperm represents Y-specific band, but WholeMom negative sperm is the X-sperm enriched due to treatment with surface antibodies that supposed to agglutinate the Y-sperm and subsequently has no Y-specific band. Samples were processed by amelogenin (*aMEL*), which shows a 270 base pair (bp) band for X sperm and two bands (270 bp and 214 bp) for Y sperm. From left to right: Ladder (Ld-100 bp); X = X-sperm group; Y = Y-sperm group.

2.6. In vitro culture

After fertilization, cumulus cells were removed by repeat pipetting in TL-HEPES medium. The denuded presumptive zygotes were transferred into 50 µL droplets of modified CR1-aa culture medium supplemented with 44 µg/mL sodium pyruvate ($C_3H_3NaO_3$), 14.6 µg/mL glutamine, 10 IU/mL penicillin, 0.1 mg/mL streptomycin, 3 mg/mL BSA, and 310 µg/mL glutathione for 3 days (IVC1 media) (Mesalam et al., 2017a; Khan et al., 2018). From Day 4–7 of embryonic development (Day 0 = day of IVF), presumed zygotes were cultured in media of the same composition, except that BSA was replaced with 10% (v/v) FBS (IVC2 media). Day 7 blastocysts were washed three times in TL-HEPES, transferred to fixative (4% [v/v] paraformaldehyde prepared in 1 x phosphate buffered saline [PBS]), and stored at 4 °C for further study. For gene expression analysis, blastocysts (n = 1) were transferred to a 1.5-mL Eppendorf tube, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.7. Evaluation of embryos

Embryos with a blastocoel were evaluated on Day 7 and those with an inner cell mass were classified as blastocysts using criteria that were previously described (Bermejo-Alvarez et al., 2010). Percentages of embryos that had undergone cleavages and that developed to the blastocyst stage were determined relative to the number of oocytes placed with the X- and Y-sperm.

2.8. Analysis of embryonic sex by PCR

Genomic DNA was isolated from the embryos by *in vitro* culture of bovine oocytes, and target loci were PCR amplified from 50-100 ng of genomic DNA with Ex Taq DNA polymerase (Clontech®TakaRa Bio Inc. Japan). The *SRY* gene sequences of all of the 6 major domesticated species of the Bovidae family (Prashant et al., 2008) were downloaded from GenBank and aligned using ClustalW software (http://align.genome.jp/) to locate the conserved HMG box region. The PCR primers were B-*SRY*-F_2 (5´- GAA CGA AGA CGA AAG GTG GC-3´) and B-*SRY*-R_2 (5´-CTG TGA CCG GCT TAA TTG GC -3´) and amelogenin (aMEL) (F: CAG CCA AAC CTC CCT CTG C; R: CCC GCT TGG TCT TGT CTG TTG C) with GenBank accession no: NM-001014984 (*AMEL-X*), NM-174240 (*AMEL-Y*) (Trigal et al., 2012). PCR was performed in 0.2-mL thin-walled PCR tubes in a total volume of 20 µL. Each tube contained 2 µL of 10 X Ex Taq buffer (Clontech®TakaRa Bio Inc. Japan), 1.6 µL of deoxynucleotide mix (dNTP, 10 mM, stock solution, Clontech), 3 µL of $MgCl_2$ (25 mM, Clontech®), 0.1 µL of forward and reverse primers (10 µM each), 0.1 µL of Ex Taq polymerase (5 units/µL) and 50–100 ng of genomic DNA. The volume was made up to 20 µL with RNase and DNase-free water, and PCR rounds were performed in a Bio-Rad thermocycler with one cycle of 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 sec, annealing temperatures of 58 °C for 30 sec, and 72 °C for 30 sec, and a final extension step of 72 °C for 10 min. Products were visualized on a SYBR® Safe stained 2% agarose gel. The gel was visualized under ultraviolet illumination, where single fragments of 332 base pairs (bp) were assigned as male (Extended data Fig. 3). Every PCR reaction was carried out with two controls: male (testis tissue) genomic DNA and a negative control with deionized water.

2.9. Statistical analyses

Statistical analyses were performed using SPSS version 18.0 for Windows (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA). For inter-group comparisons, data of 12 replicates of each treatment were analyzed using one-way analysis of variance (ANOVA) with Group (Control, X-, or Y-sperm as the independent variable) followed by multiple pairwise comparisons (Duncan's test). The blastocyst that developed from the groups where there was sorting for the sperm with a X- or Y-chromosome, after confirmation that there was B-*SRY* F2/ B-*SRY* R2 gene expression, were further analyzed using a chi-square test to assess the accuracy of male and female embryo characterization based on DNA. In all cases, 95% confidence levels were considered significant at $P < 0.05$.

3. Results

3.1. Cleavage and embryo developmental rates with use of semen sorted to contain sperm with a X or Y chromosome

The effects of use of sex-sorted sperm (KPN-684 bull) on cleavage at day 3 and blastocyst developmental rates at day 7 were

Table 1
Blastocyst developmental competence of sorted and control bull sperm (KPN-684).

Groups	Total Presumed zygote	Cleavage (mean % ± SE)	Day-7 Blastocyst (mean % ± SE)
Control	648	525 (81.16 ± 1.39)[a]	234 (34.78 ± 1.03)[a]
X-sperm	600	468 (78.33 ± 1.04)[a]	186 (32.06 ± 0.75)[a]
Y-sperm	425	278 (66.69 ± 1.09)[b]	95 (23.73 ± 1.04)[b]

Note: Data obtained from the 12 replicates and were analyzed by using One Way ANOVA among the Group (Control, X-sperm and Y-sperm as independent variable). Different superscript letter a, b in the same column indicate significant difference (P < 0.05).

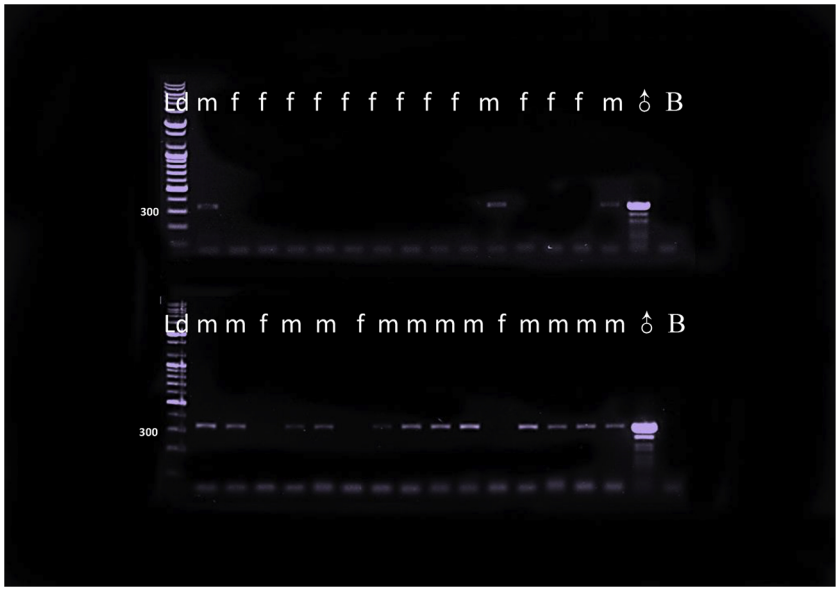

Fig. 3. Representative gel electrophoresis data of confirmed embryos produced by WholeMom antibody-selected sperm (KPN-684) using polymerase chain reaction (PCR) to check the embryo accuracy at DNA level. WholeMom positive sperm shows Y-specific band, but WholeMom negative sperm has no Y-specific band. Samples were processed by B-*SRY* F2 and B-*SRY* R2 gene (bovine Y- specific gene), which shows a 332 base pair (bp) band for males. From left to right: Ladder (Ld-100 bp); m = male; f = female; male control (♂); B = blank.

Table 2
Percentages of blastocyst (X- and Y-sperm) confirmed after B-*SRY* F2 and B-*SRY* R2 gene expression using KPN-684 bull semen.

Groups	Total blastocyst analysis	Blastocyst confirmed (%)
X-sperm	58	47 (81.03)
Y-sperm	51	37 (72.54)

Note: Embryos obtained from the treatment with the X- and Y-sperm afterwards confirmed through B-*SRY* F2 and B-*SRY* R2 gene expression and were analyzed by using chi-square test to check the embryo accuracy at DNA level in between the Group (X- sperm and Y-sperm).

assessed on embryo development. There were no differences ($P > 0.05$) between the control (81.2%) and X-sorted sperm (78.3%) groups when the percentage of embryos cleaving during early developmental stages was assessed. For the Y-sorted sperm group, as compared with the other two groups (control and Y-sorted), there was a lesser cleavage percentage of embryos with percentages being 81.2 ± 1.4%, 78.3 ± 1.0%, and 66.7 ± 1.1% in the control, X-, and Y-sperm sorted groups, respectively. There were similar group differences for percentage of embryos developing to the blastocyst (Day 7) stage. The percentage of embryos that developed into blastocysts (Day 7) was greater ($P < 0.05$) in the control, and X-sperm sorted compared with the Y-sperm sorted group, being 34.8 ± 1.0%, 32.1 ± 0.8%, and 23.7 ± 1.0%, respectively (Table 1).

To assess the variations of semen quality among bulls for the preselected embryo production using sex-sorted sperm, there was use of semen from another bull (Bull KPN-917) that was treatment with WholeMom. The data indicate a lesser embryo developmental potential using semen for the KPN-917 bull compared with that from the KPN-684 bull. There, however, was no difference between the control and X-sorted sperm groups in percentage cleavage rate and development to the blastocyst stage. The Y-sorted sperm group had a lesser blastocyst development as compared with the other two (control and Y-sperm) groups (Supplementary Table 1 and Supplementary Table 1). Furthermore, the sexes of calves were 76% female and 24% male embryos in field studies using WholeMom treated sperm for artificial insemination (Supplementary Table 3).

3.2. B-SRY F2 and B-SRY R2 transcripts of blastocysts derived from use of semen sorted to contain sperm with X or Y chromosomes

To confirm the sexing efficiency, DNA was assessed using gel electrophoresis after PCR assessment for the B-*SRY* F2 and B-*SRY* R2 genes (bovine Y-specific genes) in blastocysts derived using sexed sorted sperm. Blastocysts derived with use of WholeMom positive sperm had a Y-specific band and blastocysts derived using WholeMom negative sperm did not have a Y-specific band. When KPN-684 semen was used there was an 81.0% accuracy in producing female and 72.5% for producing male embryos when there was assessment of B-*SRY* F2 and B-*SRY* R2 gene expression after use of 58 sperm that were characterized to be sperm with an X- and 51 as sperm with a Y chromosome (Fig. 3 and Table 2). When the KPN-917 semen was used, there was a lesser accuracy of embryo sexing

with the use of semen from the KPN-684 bull. There was a 72% accuracy for the female embryos and 62% for the male embryos with the assessment for B-*SRY* F2 and B-*SRY* R2 gene expression from the 43 and 34 blastocysts characterized to have a X or Y chromosome, respectively (Supplementary Fig. 1. Fig. 3, Table 2 and Supplementary Table 2).

4. Discussion

The prevalent sex sorting techniques, even though being used commercially need to be further refined for mass scale use of sexed semen. Semen sorting techniques that are being used commercially have 90% accuracy (Holden and Butler, 2018), but the fertility when using sex sorted sperm is less than that when semen is used that is not sex-sorted (Palma et al., 2008). This insufficiency when using sex-sorted semen decreases the efficacy of its use in the dairy industry. The lesser fertility with use of sex-sorted semen will reduce the financial benefits from the sorted semen usage on farms. The assessment of why there is lesser fertility rates with use of sex sorted spermatozoa is confounded because of using smaller doses of spermatozoa per insemination than normally occurs (Garner et al., 2013). When the same concentration of spermatozoa/dose was used, pregnancy rates with sexed spermatozoa were 60% to 80% compared to when there was use of non-sorted spermatozoa (Garner et al., 2013). The use of bulls with the greatest fertility when using sex-sorted sperm results in greater fertility when using sex-sorted semen as compared to when bulls with relatively lesser fertility are used (Frijters et al., 2009). In the present study, therefore, semen from different bulls was assessed before starting of the experiment and it was observed that the semen from bull KPN-684 provided the most desirable results compared to use of semen from other bulls. Semen from bull KPN-684 bull, therefore, was used for further studies (Supplementary Table 1 and Table 2). This may be due to the variation of semen quality among bulls resulting in a different in vitro embryo developmental competence and efficiency with use of the two bull's semen, however, embryo sorting efficiency was similar for the two bulls. With use of the sex sorting techniques that were utilized in the present study, the embryo cleavage percentage ($\geq$ 8–16 cell stage) with use of X-sorted sperm and control sperm was greater ($P > 0.5$) compared with use of the sperm with the Y- chromosome (Table 1). The reason for this result is because the WholeMom monoclonal antibody binds specifically to the head of the spermatozoa with the Y chromosome. Consequently head to head Y-sperm agglutination occurs with inhibition of movement or swimming capacity for fertilization of oocytes resulting in a lesser embryo developmental potential. Sperm with the X chromosome, however, have typical motility patterns that result in a greater probability of contributing to the oocyte fertilization process.

Fresh semen is used when conducting commercial sorting techniques, however, the present study was performed with cryopreserved sperm. Usually with cryopreserved sperm there are 40% to 50% dead and 50% to 60% live sperm (Palma et al., 2008). The percentage of embryos developing to the blastocyst stage with use of sperm with the X chromosome was greater ($P > 0.5$) compared with use of the sperm with the Y chromosome. There was no significant difference in embryos developing to the blastocyst stage with use of sex-sorted and unsorted cryopreserved sperm (control; Table 1). Results from previous studies indicate the efficiency of in vitro embryo production when using sex-sorted sperm has been improved and the rates of embryo developing to the blastocyst stage has been reported to be as great as 45% (Underwood et al., 2010), while in the majority of studies, the mean rate was 25% (Puglisi et al., 2006; Bermejo-Álvarez et al., 2008). The primary cause for these lesser fertilization rates with use of the sex-sorted spermatozoa may be due to the procedures associated with sorting, handling of sperm, bull selection, instrumentation, mode of insemination, and management as well as other factors (Garner and Seidel, 2008; Garner et al., 2013). Results of the present study are consistent with those of previous studies where there was the production of embryos using IVF with sex-sorted sperm with the efficiency of production being less than with use of sperm that was not sex-sorted (control). In the previous studies, sperm sorted that contained the X chromosome was used for IVF (Schenk et al., 2009), insemination (Schenk et al., 2009) and embryo transfer programs (Hayakawa et al., 2009) and there was a reduced fertility with use of the sperm sorted to have the X chromosome as compared with sperm where sex sorting had not occurred. The evidence suggests that this is due to damage induced during the sorting process, as the cells are exposed to a number of potential hazards, including dilution, centrifugation, incubation, exposure to DNA stains, high pressure, laser light, and electrical charges (Frijters et al., 2009).

The reasons are not clear why sex-sorted sperm have a lesser fertilizing capacity in vivo than the unsorted sperm and also have a lesser survivability after cryopreservation (Seidel and Schenk, 2008; Seidel, 2014). Changes in viability/motility and capacitation/acrosome reaction of sex-sorted sperm could be the reason for the reduced initial fertilization rates in in vitro and in vivo studies. There are several reports on the effects of the sorting procedure on sperm, including altered motility patterns (Suh et al., 2005), a reduced life span (Hollinshead et al., 2003), premature induction of the acrosome reaction (Mocé et al., 2006) and increases in the proportion of capacitated sperm (Maxwell et al., 2004). Data from the present study indicate there was greater development of presumptive zygotes and also improved fertilization rates using the protocol for the present study compared to the use of sperm where there was not sex-sorting and this may be due to the advanced capacitation state of sex-sorted sperm paralleling that of the control sperm. This would result in the sex sorted sperm progressing more rapidly to an acrosome reacted state than the sperm that were not sex-sorted (Mocé et al., 2006).

Furthermore, an attractive feature of the IVF technique used in the present study is using a sperm concentration of 1.7×10^6 (confirmed by computer-assisted sperm analysis-CASA), although the commonly used insemination dose in cattle with sperm that have not been sex sorted is usually 10×10^6 or more for cryopreserved sperm (Garner and Seidel, 2008). This aspect of the present study also indicates there is a greater sorting efficiency with the techniques used with a minimum of stressors on sperm cells. The sperm numbers needed to obtain 95% of the maximal conception rate ranged from 1×10^6 to 11×10^6 (Garner et al., 2013). Results of a previous study indicated the minimum number of sperm per insemination dose that is important to achieve satisfactory conception rates in cattle as assessed by non-return to estrus. About 50% non-return rate could be achieved with as few as 0.38×10^6 live sperm post-thaw per insemination (Garner and Seidel, 2008). Previous studies, however, were compromised regarding fertility assessments to a greater extent than what occurred in the present study when the embryo sexing was performed with preselected sex-sorted sperm. In the present

study, fertility was not compromised with use of sex sorted semen compared to use of sperm in which sex sorting had not occurred. Additionally, the in vitro data are consistent with the in vivo field study for heifer production where using the WholeMom treated sperm for artificial insemination confirmed that sex sorting resulted in 76% female and 24% male embryos (Supplementary Table 3).

In the present study, determination of blastocysts with a X from those with a Y chromosome was confirmed by using polymerase chain reaction (PCR) amplification by using primers for the Y-linked *SRY* gene The approach in using Y-chromosome-specific primer sequences is the most popular among the techniques of sex determination as compared with in situ hybridization, Southern/Dot blotting and others (Miller, 1991). In the present study, there was confirmation of the accuracy of sexing embryos at the DNA level. This accuracy was assessed by using gel electrophoresis, where for WholeMom positive sperm there is a Y-specific band, but for WholeMom negative sperm there is not a Y-specific band. The X/Y ratio was subsequently calculated. Several observations suggest that both the X- and Y-genes are acquired and amplified in the male germline. The X genes undergo recombination during meiosis (Bachtrog, 2014). The Y-chromosome-specific gene, SRY, is one of the key genes involved in sex determination in mammals. The *SRY* gene encodes a testis-specific transcription factor that has an important role in sexual differentiation and development in males and is located on the distal region of the Y-chromosome (Shahid et al., 2004; Rajender et al., 2006). When a developing sperm cell undergoes crossover during meiosis, the *SRY* gene remains a part of the Y chromosome. If the SRY sequence is transferred to the X chromosome, the resulting Y chromosome will not have a SRY gene and can no longer initiate male gonad development. The X chromosome that results after this crossover contains a *SRY* gene, thus, having the capacity to initiate male gonad development. The Y chromosome-specific gene, SRY, is the principal regulator of male sex determination and sperm production through ampliconic gene families and is thought to modulate the sex-determination pathway (Bellott et al., 2014).

Further studies are being conducted to assess data from in vitro experiments using mice and also for commercially applying field data using Ovum pick-up (OPU) of oocytes after estrous synchronization in heifers. In vitro production of pre-selected embryos using OPU oocytes with subsequent embryo transfer to recipients can occur with use of a combination of technologies that has been increasingly commercialized in the cattle-breeding industry (Mesalam et al., 2017). Even though these sperm sorting techniques have some limitations, it is expected that utilization of these approaches will overcome the constraints of the prevalent sorting techniques such as exposure of sperm to the DNA-binding dye, Hoechst 33342 (Garner et al., 2013). Furthermore, there will not be need for use of expensive equipment and associated maintenance costs. There is also need for skilled personnel for operation and supervision of machine operation and the amount of time to conduct the process is also a problem. Another problem is that about 50% of the sperm samples cannot be sex-sorted leading to a large amount of semen being wasted with only about 30% of sperm being sex-sorted (Garner et al., 2013). Another problem is that efficiency of sexing sperm is greatest with fresh sperm and there is lesser in vivo fertility, including a reduced life span of sex-sorted sperm (Garner and Seidel, 2008). The technique used in the present study could be applied to the dairy industry and commercial breeders to produce desired offspring by using sex-sorted semen. Sex-sorted semen is expected to be widely used in the future because of continued improvements in fertility when using sex-sorted semen, increased sex-sorting capacity, possible pre-selection of dams, and the rate of genetic progress is expected to increase by as much as 15% and milk production by as much as 90% (Holden and Butler, 2018).

5. Conclusion

Results of the present study indicate there were no differences in cleavage and blastocyst developmental rates with use of sperm with the X chromosome and sperm where there was no sorting of sperm with X or Y chromosomes (control). Furthermore, using semen from the KPN-684 bull, the blastocyst accuracy percentages with use of sperm sorted to have X or Y chromosomes was 81.0% female and 72.5% male embryos based on B-*SRY* F2 and B-*SRY* R2 gene expression. It is emphasized that these data were obtained using cryopreserved sperm and that there may be more desirable results with use of fresh semen. The time taken to conduct techniques used in the present study is less and there is less stress on sperm cells and thus less detrimental effects to the sperm. This is the case because there is no use of the dye and radiation that is used with most conventional sex sorting techniques. Furthermore, there is a lesser cost for the sorted sperm production compared with the conventional techniques being used at present, thus, there may be uptake of the techniques used in the present study in the dairy industry and by commercial breeders to increase the population of dairy or beef cattle with elite genetics for productivity.

Funding information

This work is supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the Agri-Bio Industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (grant: # 315017-5, # 117029-3, the Agenda Program of Rural Development Administration, Republic of Korea (grant #, supported this research PJ01029304; Evaluation of genetic and reproductive properties on the Korean brindle cattle, Chikso) and the 2019 Post-Doctoral Fellowship Program of National Institute of Animal Science, Rural Development Administration, Republic of Korea.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The author wants to give special thanks to all the lab fellows and co-workers as well as the farmers for their help and support for this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2018.11.006.

References

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., Gutierrez-Adan, A., 2010. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proceedings of the National Academy of Sciences.

Bermejo-Álvarez, P., Rizos, D., Rath, D., Lonergan, P., Gutiérrez-Adán, A., 2008. Can Bovine *In Vitro*-Matured Oocytes Selectively Process X- or Y-Sorted Sperm Differentially? Biology of Reproduction 79, 594–597.

Chowdhury, M.M.R., Choi, B.H., Khan, I., Lee, K.L., Mesalam, A., Song, S.H., Xu, L., Joo, M.D., Afrin, F., Kong, I.K., 2017. Supplementation of lycopene in maturation media improves bovine embryo quality in vitro. Theriogenology 103, 173–184.

Chowdhury, M.M.R., Mesalam, A., Khan, I., Joo, M.D., Lee, K.L., Xu, L., Afrin, F., Kong, I.K., 2018. Improved developmental competence in embryos treated with lycopene during in vitro culture system. Molecular Reproduction and Development 85, 46–61.

Garner, D.L., Seidel, G.E., 2008. History of commercializing sexed semen for cattle. Theriogenology 69, 886–895.

Hayakawa, H., Hirai, T., Takimoto, A., Ideta, A., Aoyagi, Y., 2009. Superovulation and embryo transfer in Holstein cattle using sexed sperm. Theriogenology 71, 68–73.

Hollinshead, F.K., Gillan, L., O'Brien, J.K., Evans, G., Maxwell, W.M.C., 2003. *In vitro* and *in vivo* assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. Reproduction, Fertility and Development 15, 351–359.

Maxwell, W.M.C., Evans, G., Hollinshead, F.K., Bathgate, R., de Graaf, S.P., Eriksson, B.M., Gillan, L., Morton, K.M., O'Brien, J.K., 2004. Integration of sperm sexing technology into the ART toolbox. Animal Reproduction Science 82-83, 79–95.

Mesalam, A., Khan, I., Lee, K.L., Song, S.H., Chowdhury, M.M.R., Uddin, Z., Park, K.H., Kong, I.K., 2017. 2-Methoxystypandrone improves *in vitro*-produced bovine embryo quality through inhibition of IKBKB. Theriogenology 99, 10–20.

Miller, J.R., 1991. Isolation of Y Chromosome-specific Sequences and their Use in Embryo Sexing. Reproduction in Domestic Animals 26, 58–65.

Mocé, E., Graham, J.K., Schenk, J.L., 2006. Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. Theriogenology 66, 929–936.

Palma, G.A., Olivier, N.S., Neumuller, C., Sinowatz, F., 2008. Effects of sex-sorted spermatozoa on the efficiency of *in vitro* fertilization and ultrastructure of *in vitro* produced bovine blastocysts. Anat Histol Embryol 37, 67–73 give full name of journal name such as other one.

Prashant, Gour, D.S., Dubey, P.P., Jain, A., Gupta, S.C., Joshi, B.K., Kumar, D., 2008. Sex determination in 6 bovid species by duplex PCR. J Appl Genet 49, 379–381.

Puglisi, R., Vanni, R., Galli, A., Balduzzi, D., Parati, K., Bongioni, G., Crotti, G., Duchi, R., Galli, C., Lazzari, G., Aleandri, R., 2006. *In vitro* fertilisation with frozen–thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. Reproduction (Cambridge, England) 132, 519–526.

Rajender, S., Rajani, V., Gupta, N.J., Chakravarty, B., Singh, L., Thangaraj, K., 2006. SRY-negative 46,XX male with normal genitals, complete masculinization and infertility. MHR: Basic science of reproductive medicine 12, 341–346.

Rath, D., Barcikowski, S., Degraaf, S., Garrels, W., Grossfeld, R., Klein, S., Knabe, W., Knorr, C., Kues, W.A., Meyer, H., Michl, J., Moench-Tegeder, G., Rehbock, C., Taylor, U., Washausen, S., 2012. Sex selection of sperm in farm animals: status report and developmental prospects. Reproduction (Cambridge, England).

Schenk, J.L., Cran, D.G., Everett, R.W., Seidel, G.E., 2009. Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. Theriogenology 71, 717–728.

Shahid, M., Dhillion, V.S., Jain, N., Hedau, S., Diwakar, S., Sachdeva, P., Batra, S., Das, B.C., Husain, S.A., 2004. Two new novel point mutations localized upstream and downstream of the HMG box region of the *SRY* gene in three Indian 46,XY females with sex reversal and gonadal tumour formation. MHR: Basic science of reproductive medicine 10, 521–526.

Suh, T.K., Schenk, J.L., Seidel, G.E., 2005. High pressure flow cytometric sorting damages sperm. Theriogenology 64, 1035–1048.

Underwood, S.L., Bathgate, R., Pereira, D.C., Castro, A., Thomson, P.C., Maxwell, W.M.C., Evans, G., 2010. Embryo production after *in vitro* fertilization with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. Theriogenology 73, 97–102.