PRODUCTION OF PIGLETS PRESELECTED FOR SEX FOLLOWING IN VITRO FERTILIZATION WITH X AND Y CHROMOSOME-BEARING SPERMATOZOA SORTED BY FLOW CYTOMETRY

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ABSTRACT

In vivo-matured porcine oocytes were fertilized in vitro with X and Y chromosome-bearing spermatozoa, and sorted for sex on the basis of DNA content by flow cytometry. Developmental competence of the sexed embryos was determined through established pregnancies after embryo transfer. Spermatozoa were stained with Hoechst 33342 and sorted using a flow cytometry cell sorter. Purity of sorting was 83% for Y spermatozoa and 92% for X spermatozoa. A total of 387 mature cumulus-oocyte-complexes (COC) was collected from 18 superovulated prepuberal gilts shortly before ovulation. In vitro fertilization with sorted spermatozoa was performed in 4 replicates. After 18 h of sperm-oocyte co-culture at 39°C, the zygotes were placed into culture medium (NCSU-23) for another 24 h. The average cleavage rate was 56.2%. Ninety-two embryos produced from X-sorted sperm cells were transferred surgically into the uterus of 2 recipients. Two gilts farrowed and delivered 6 and 4 healthy female piglets, respectively. Additionally, 2 gilts were inseminated intratubally via surgical laparotomy with either X or Y sorted spermatozoa (2x10⁵) per oviduct. The 2 sows farrowed producing 15 piglets. Thirteen of the 15 piglets were of the predicted gender (85%).

INTRODUCTION

Economical and ecological perspectives with respect to livestock industry demand optimal herd management. Presently, maximum utilization of facilities has been achieved and sex related diseases can be avoided altogether or successfully treated, and genetic progress is being made with both male and female animals. In past years, various attempts were made to sort sperm populations based on their sex determining characteristics. Through the use of flow cytometry, sperm sorting (Beltsville Sperm Sexing Technology; BSST) gender preselection in mammals can now be realized (4,5,7). Offspring have been produced after surgical insemination in rabbits (7) and swine (4), after in vitro fertilization (IVF; 1,2) or deep uterine insemination in the bovine

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Earlier attempts to use IVF in the pig with sorted X and Y sperm cells (11) met with limited success. The objective of this study was to test an improved IVF protocol in conjunction with spermatozoa sorted by flow cytometry where the sorting process was appropriately synchronized to the IVF protocol. Embryo development in vitro, surgical insemination, and embryo transfer with the subsequent birth of piglets were chosen as the mean to measure the effectiveness of these procedures.

MATERIALS AND METHODS

Semen Collection and Flow Cytometry

Semen was collected from 2 boars of proven fertility. These boars were the result of a gilt and boar mating in which both animals had been produced from spermatozoa sorted for sex by the BSST system (4). The sperm rich fraction was diluted with pre-warmed Beltsville TS (BTS; 1:2, v/v; 6) immediately after collection and was evaluated for motility at x 400 magnification with a phase-contrast microscope. Sperm concentrations were determined using an improved Neubauer counting chamber (Clay-Adams, NY). Aliquots of 15x10⁶ spermatozoa were diluted in 1ml BTS (0.1% BSA). Hoechst 33342 fluorochrome (Calbiochem, La Jolla, CA) was added to achieve a final concentration of 6.25 µM, and samples were incubated for 20 min at 32°C in 1.5-ml Eppendorf tubes (4,7). Sperm cells were sorted according to the procedure described by Johnson et al. (7), using a modified EPICS V/750 series flow cytometer cell sorter (Coulter Corporation, Miami, FL; 8) at 150 mW ultraviolet (UV) light (351 and 364 nm; 5-watt 90-5 Innova Argon-ion laser, Coherent Inc., Palo Alto CA). The sorting flow rate was adjusted to 2,300 sperm/sec, for a sorting rate of 70 to 110 sperm/sec. The PBS supplemented with 0.1% BSA (Sigma, St. Louis, MO) and 2.5% EDTA (Sigma) was used as sheath-fluid, which was prefiltered through a 0.2-µm filter (Naigene, NY). Presiliconized microcentrifuge tubes (0.6 ml, Intermountain Scientific, Kaysville, UT) used for collection were coated with BSA by filling, soaking and decanting a 1% BSA solution (7). The sort collection media was TEST extender containing 2% hen egg yolk rather than the standard 20% (4). A total of 60 µl was placed in each sort collection tube prior to sorting. Approximately 3.5x10⁶ to 4x10⁶ sperm cells were sorted into each tube after 1.5 h. The sorted semen samples were centrifuged at 800 x g for 5 min, and the remaining sperm pellet was resuspended with 200 µl fertilization medium (TALP).

In Vitro Fertilization

Eighteen prepuberal gilts, 165 d of age and averaging 76 kg, were superovulated with 1500 IU PMSG (Brumegon, Hydrochemie, Oberschleißheim, Germany), followed by 750 IU hCG (Ovogest, Hydrochemie, Oberschleißheim, Germany) 72 h later. The gilts were slaughtered 38 h after hCG treatment, and their reproductive tracts were transported in a prewarmed thermos container (38°C) to the laboratory within 5 min of slaughter. The ovaries were placed into Dulbecco’s PBS (Sigma), supplemented with penicillin (Sigma 0.06 g/l) and streptomycin (Sigma 0.05 g/l). Mature follicles were punctured with an 18-gauge needle, connected to a 1-ml syringe and prefilled with
0.1 ml flushing medium. Aspirates were individually placed into Petri dishes and thereafter were collected into a 35-mm Petri dish containing 2 ml flushing medium. Cumulus-oocyte complexes (COC) were evaluated for cumulus expansion and morphological integrity of the cytoplasm. Only COC with maximally expanded cumulus cell layers and evenly granulated cytoplasm were used for the experiments. Subsequently, the COC were placed into 1 ml of fertilization medium, and 4x10^5 sorted sperm cells/ml were added to the culture dish. Sorted X spermatozoa were used for IVF, and were co-cultured with the COC for ~18 h. Thereafter, zygotes were washed 5 times and then transferred into the culture medium North Carolina State University (NCSU 23; 9) for another 24 h. Developing embryos (2 to 8 cell stages) were transferred to 0.25 ml modified French straws (12) for transfer to the surgery room.

Transfer of IVF Embryos

Two cyclic crossbred gilts with 3 known previous estrous cycles were synchronized with the oocyte donors at Day 17 to 18 of the cycle with a single injection of 750 IU hCG, making the recipients and donors asynchronous by minus 24 h, and were checked daily for estrus. For embryo transfer, the gilts were anesthetized with a combination of ketamine (Ketaset), tiletamine/zolazepam (Telazol; Fort Dodge, IA), butorphanol tartrate (Torbugesic, Fort Dodge, IA), xylazine (Rompun) and atropine (Phoenix Inc., St. Joseph, MO). The gilts were prepared for surgery in dorsal recumbency, and the ovaries and oviducts were exposed by midventral laparotomy. North Carolina State University culture medium (NCSU 23; 9) without taurine or hypotaurine and supplemented with 5.96 g/L HEPES was used as the transfer medium. A straw (12) containing embryos in transfer medium was inserted into the oviduct of the recipient gilts, and 46 embryos were pushed into the respective ampulla using a mandrin. The reproductive tract was then returned to the abdominal cavity.

Surgical Insemination

Cyclic crossbred gilts (n=2) were prepared for surgical insemination on the day of expected ovulation as described above. After surgical exposure of the reproductive tract, 100 μl TEST-yolk extender containing ~200,000 spermatozoa were placed into each oviduct by inserting a blunt 20-gauge needle through the utero-tubal junction into the isthmus of the oviduct as previously described (4). The first surgical insemination was performed with Y chromosome-bearing spermatozoa and the second surgical insemination with X-bearing spermatozoa.

RESULTS

Sperm Sorting

The average motility of the neat semen after collection and before dilution with BTS was 82 ± 3%. After sorting, sperm motility dropped slightly to 76 ± 6% and remained at this level (63 ± 10%) at least for 4 h. Even after 24 h of co-culture with the oocytes in fertilization medium 10 ± 8% of the sperm cells showed active movement. The purity of
the respective sorted population was 89% for X chromosome-bearing spermatozoa (predicted sex, Table 1) and 95% for the Y-bearing sperm cells (Table 1).

In Vitro Fertilization

A total of 387 COC (21.5 per donor) was aspirated from 424 follicles (recovery rate 91.2%). Of these, 379 COC were mature and met the required quality (maximal cumulus expansion and evenly granulated cytoplasm) for use in IVF. Average cleavage rate was 56.2% (n=213). Forty-eight hours after the start of IVF, 33 embryos were at the 2-cell stage, 74 at the 4-cell stage, and 106 at the 8-cell stage. From these, 92 cleaved embryos were transferred into 2 recipients, (46 embryos per recipient). Both gilts became pregnant and delivered litters of 6 and 4, respectively. Table 1 shows the distribution of the sex ratio and average birth weight per litter.

Surgical Insemination

Because neither of the recipients had ovulated at the time of insemination prominent mature follicles were present in both animals. After insemination, both gilts became pregnant and delivered a total of 15 piglets. Table 1 shows the distribution of the sex ratio and the average piglet weight per litter.

Table 1. Farrowing results from gilts receiving embryos purchased from spermatozoa sorted for the X chromosome and from gilts surgically inseminated with spermatozoa sorted for either X or Y chromosome

<table>
<thead>
<tr>
<th>Fertilization method</th>
<th>Sperm cells sorted for</th>
<th>Litter size n</th>
<th>Average birth weight (kg)</th>
<th>Male offspring n</th>
<th>Female offspring n</th>
<th>Predicted sex (%)b</th>
</tr>
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<tbody>
<tr>
<td>IVF 1a</td>
<td>X chromosome</td>
<td>6</td>
<td>2.1</td>
<td>0</td>
<td>0</td>
<td>100</td>
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<tr>
<td>IVF 2b</td>
<td>X chromosome</td>
<td>4</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
<td>100</td>
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<tr>
<td>First surgical</td>
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<td>insemination</td>
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<td>Second surgical</td>
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a 45 embryos transferred per recipient gilt.
b Based on re-analysis of an aliquot of sorted X or Y chromosome-bearing spermatozoa for DNA content (4,5).
DISCUSSION

Many attempts have been made during the past 50 to 60 yr to skew the natural sex ratio from the standard 50:50 to some proportion in favor of males or females depending on the desired sex. Only the sorting method based on flow cytometry (Beltsville Sperm Sexing Technology) has a proven record of accurately identifying X and Y chromosome-bearing spermatozoa, thus making it possible to shift the sex ratio to the desired gender (1,2,4,5). The method is dependent on distinguishing the DNA content of individual sperm cells and sorting the 2 populations into separate tubes (4) at a limited rate of 3 to 4 x 10^5/h as individual sperm cells are sorted one by one. Either IVF or surgical insemination are efficient means for delivering sorted spermatozoa to the fertilization site. Surgical insemination has been shown to produce rabbits (7) and pigs (4) of the desired sex, while IVF has produced calves of the desired sex at rates up to 90% (1,2). In our earlier study (12), we produced sexed pig embryos utilizing gamete intra-fallopian transfer (GIFT).

Here we report on the birth of 2 litters of pigs produced by IVF using spermatozoa sorted for sex. It is the first report of the birth of piglets following IVF with spermatozoa preselected for sex based on the difference in X and Y chromosome-bearing sperm DNA. In our previous study (11), we showed that IVF with sorted spermatozoa was possible. However, in that study the number of embryos produced was limited and thus there was an insufficient number for embryo transfer. In the present study we improved the procedures for both IVF and sperm processing after sorting, which led to higher cleavage rates that were comparable to those of IVF with unsorted spermatozoa (13). In another earlier experiment (13) we utilized TCM 199. The TALP medium used in our present experiments seemed to be better suited for use with the TEST yolk (2%) extender that is necessary as a collection medium for sorted spermatozoa (4). Although all sorted sperm samples were centrifuged after sorting, small amounts of egg yolk are transferred with the spermatozoa to the fertilization medium. This minimal amount of egg yolk, however, did not appear to interfere with fertilization.

In our present study, cleavage rates after IVF, pregnancy rates, and litter size were nearly comparable to those of IVF with unsorted spermatozoa (10,15) or of the GIFT procedure (12). This indicates that neither sperm treatment with Hoechst 33342 fluorochrome nor the sorting procedure affected the fertilizing capacity of the sorted spermatozoa, and that the sperm-oocyte interaction was sufficiently synchronized to result in pregnancies. All offspring were healthy and the average weight was normal. No differences were found between litters derived from IVF and surgical insemination, but fewer sperm cells were needed per oocyte in the IVF system than in the surgical insemination procedure. Compared with other IVF experiments (10) using unsorted spermatozoa, the fertilization and pregnancy rates in this study were similar. Additional experiments are underway to confirm the present results.

An infection with a Pseudomonas subspecies was found in the ejaculates of 1 boar. This infection likely prevented pregnancies following 4 other transfers that were performed. The infection, however, did not affect IVF and in vitro development up to the
4-cell stage, as is indicated by the high cleavage rates (56.2%). Commercial application of IVF with sorted X or Y chromosome-bearing spermatozoa to produce piglets of a preselected sex will require improved efficiency of sexed embryo production as well as improvement in embryo transfer technology. Nonsurgical transfer, currently in the developmental stage (3), offers the potential for expanded use of gender selection in animal production.

REFERENCES