REPRODUCTIVE INNOVATIONS FOR SWINE PRODUCTION: FUTURE IMPACTS OF GENDER PRE-SELECTION, EMBRYO TRANSFER AND CLONING

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ABSTRACT

It is estimated that in the past five years, flow cytometric sorting of gender pre-selected sperm using DNA as the marker has produced over 30,000 offspring. The majority of these offspring were cattle for two reasons: 1) The cattle industry has accepted the use of gender specific sperm for commercial reproduction and 2) Cattle have a distinct advantage over swine in requiring a significantly lower number of spermatozoa for fertilization. In the swine industry, using gender pre-selected sperm has not progressed at the same pace. Nevertheless, the ability to pre select gender of the offspring in the pig is one of the most sought after reproductive innovations because it would have a huge economic impact on pork production by reducing animal maintenance costs and supporting production goals. However, the current methods for producing gender pre-selected sperm and then delivery to the uterus require development to make them more productive, efficient and cost effective in swine production.

While porcine embryo transfer (ET) has been practiced for about 50 years in a research setting, it has been employed more recently to salvage a specific genotype from a disease scenario or for international transfer of valuable genetics. While ET is a practical application for modern genetic propagation, it has not received wide acceptance as a method of choice for reproduction because it requires skillful surgical embryo recovery and transfer. Further development of embryo recovery technique and non-surgical embryo transfer will lower the cost of ET and make the technology more user friendly in swine production.

While one considered the future of animal reproduction, cloning (-via embryo splitting-) and nuclear transfer (embryo from somatic cell DNA) are now the reality of today. When the lamb "Dolly", was born in 1996 as the first domestic animal cloned from an adult animal somatic cell, the fascination and fervor for the potential benefits resulting from the cloning process were launched. Since then, nuclear transfer has been successfully used to produce clones in many different species. Cloning technology will not replace traditional population genetic approaches to swine reproduction but will augment the potential to further genetic progress, increase production efficiency and improve protein quality for consumers throughout the world.

GENDER PRE-SELECTION

Physical Cell Sorting

The ability to pre-select gender of potential offspring has huge economic implications in the swine industry. Many attempts have been made to separate X and Y sperm in the past 70 years. Mechanical methods and physical differences between X and Y sperm have been relatively ineffective in obtaining a higher proportion of either sex in sperm sex ratio or offspring. Current research efforts are attempting to develop mass sorting techniques based on markers or chromosome specific proteins on the surface membranes of the X and Y sperm. Using 2D electrophoresis, over 1000 proteins have been isolated and characterized on the surface of sperm cells with no differences found between X or Y sperm (Hendriksen et al., 1996; Johnson and Clarke, 1988). This method of sorting sperm from a specific protein on the sperm surface has no published scientific evidence as a viable option to semen sorting at this time.

Sorting Living X and Y Sperm Based on DNA

With the improvement of staining methods and techniques and the understanding of the orientation of the sperm cell in flow cytometric sorting, relatively small differences in staining intensity between X and Y sperm can be detected and sorted (Johnson and Pinkel, 1986). Improved use of fluorochromes and utilization of vital stains to label the DNA of living sperm cells led to the sorting of X and Y bearing sperm and the Beltsville Sperm Sexing Technology (Johnson et al., 1987a). More recently, this technology has been improved with the use of the MoFlo high speed sorter after several modifications (Johnson et al, 1987b; Johnson and Clarke, 1988). There are now several other high speed sorters on the market today.

The effectiveness of this system has been validated by flow-cytometric re-analysis of sorted sperm cells (Johnson et al., 1987a), fluorescence in situ hybridization (FISH) procedures (Kawarasaki et al., 1998) counting the microsatellite DNA probe on the Y sperm, and by PCR (Welch et al., 1995). All methods have verified with good accuracy that the Beltsville Sperm Sexing Technology is consistent and repeatable for altering the sex ratio of offspring in livestock. Besides laboratory validation, gender pre-selected sperm cells were used in combination with in vitro fertilization of in vivo matured oocytes. Cleavage rates after IVF were 56% of the embryos. The viable 2-4 cell embryos were surgically transferred to asynchronous gilts (n=4) with two pregnancies resulting (Rath et al., 1997). The litters from these two pregnancies were 6 and 4 pigs and all piglets were females. Further studies conducted at Beltsville produced offspring in 9 litters. The control litters gave a sex ratio of 52% male and 48% female offspring while the 6 litters with sexed IVF embryos gave 97% females pigs (Rath et al., 1999). Another study showing the effectiveness of the orientating nozzle of the high speed sorter used semen selected for both the X and Y in the IVF program. Five litters were born with 97% females from the X sorted sperm and three litters from the Y sorted sperm where 100% were males (Abeydeera et al., 1998). Researchers found that sperm must be used rather quickly after cell sorting and polyspermy is common, particularly in swine. Pigs have recently been produced using intracytoplasmic sperm injection (ICSI) from cytometrically sorted boar semen (Probst and Rath, 2003). The ICSI technique greatly

extends the use of sorted porcine sperm cells as it only takes one sperm per oocyte. While the use of these assisted reproductive technologies enables the use of gender pre-selected sperm in swine production, these approaches are impractical for everyday use.

Insemination of Flow Cytometric Sorted Semen

Surgical intratubal inseminations are effective for producing offspring from gender pre-selected sperm (Johnson, 1991), and pregnancies have also resulted from deep intra-uterine horn inseminations with gender pre-selected semen (Rath et al., 2003: Vasquez et al., 2003). The use of traditional artificial insemination in pigs using gender pre-selected sperm is not practical at this time because of the large number of sperm cells needed for insemination and the inability of techniques to sort the large number of sperm cells required.

New Technology in Gender Pre-Selection

Monsanto® recently announced a new machine designed to sort cattle sperm cells. It significantly speeds up cell sorting under lower pressure and reads by laser from multiple angles causing fewer traumas to the sperm. A sorter of this speed and detection technology has not been developed for swine.

Current research efforts are attempting to develop mass sorting techniques based on markers of chromosome specific loci on the X and Y chromosomes.

EMBRYO TRANSFER

The swine industry has become increasingly more aware of embryo transfer (ET) as a means of reproduction to reduce the risk of disease as new genetics are introduced for herd replacement and genetic progress (Holtz et al., 1987). The first documented surgical embryo transfer in swine appeared in 1951 (Kvasnicki, 1951). It was not until the late ‘60s that the first pregnancy resulting from non-surgical embryo transfer in a pig (Polge and Day, 1968) was reported.

Surgical Embryo Transfer

The widespread acceptance and use of ET in the swine industry is so far limited because surgical methods are required to recover and transfer embryos. These procedures make it difficult to coordinate sterile or semi-clean surgical locations and arrange transportation of embryos. Embryos are perishable and easily lost if the plane is delayed or papers are not in order. The factors that affect the success rate of surgical ET in the pig are different than cattle because of the high fecundity rate in pigs. Surgical ET and even non-surgical ET in swine are impacted by several factors such as; 1) selection and stimulation of the donor sow, 2) recovery of embryos, 3) embryo handling, including embryo assessment, transportation, medias, and storage, 4) selection and synchronization of the recipient sow and 5) transfer of recovered and washed embryos. A number of documented results for surgical embryo transfer are listed in the Table 1 below (Brüssow et al., 2000)

Table 1. Results of embryo transfer following surgical embryo transfer.*

No of transfers (n)	Pregnancy Rate (%)	Litter size (mean)	References
27	70	5.7	Dziuk et al., 1964
77	73	6.2	Schlieper, 1983
46	68	6.7	Kruff, 1985
206	53	7.0	Holtz, 1988
39	80	8.1	Cameron et al., 1989
112	63	7.7	Brüssow, 1990

* Table modified from Brüssow et al., 2000.

Commercial applications and use of surgical embryo transfer have some of their own trade secrets and report slightly higher efficiencies of reproductive success. Still, for ET to gain wide spread appeal in the swine industry and to move genetic material, the goal needs to reduce the need to use surgery for collection and transfer of the embryos.

Non-surgical Embryo Transfer

Although, it was demonstrated in the late 60's that embryos from the pig could be transferred non-surgically, greater efforts toward this goal were further demonstrated in the 90's. The later technique showed that deposition of embryos into the body of the uterus or in the caudal end of the horn could be done with no anesthesia and produce farrowing rates up to 40% with 5-7 piglets born (Hazeleger and Kemp, 1994; Galvin et al., 1994; Hazeleger et al., 2000). These successes still leave room for improvement in reproductive performance before invoking the confidence of the pork producer or genetic company to use this as a technique to transfer genetic material. Data from surgical ET would suggest that the uterine body might not be the best location for deposition of embryos and that a site much further up toward the cranial end of the uterine horns might improve both farrowing rate and litter size (Stein-Stefani et al., 1987; Wallenhorst and Holtz, 1999). This data showed a pregnancy rate of 12% in the body of the uterus, 81% for the caudal end of the horn and 88% for the middle of the uterine horn. Survival rate of these embryos at day 28-34 was only 3% at the uterine body, 29% at the caudal end of the uterine horn and 41% at the middle of the horn. It is not known whether placement of the embryos affects survival with nonsurgical ET.

This data suggests that a nonsurgical ET method in swine with embryo deposition further up into the uterine horn would likely be a benefit to reproductive success. In one procedure, a modified flexible catheter (43 cm in length) is inserted through a traditional artificial insemination Spirette. The Spirette is inserted into the cervix of a non-sedated sow. The inner catheter is guided up into one of the uterine horns and 24-31 embryos are deposited. The average insertion of the catheter takes about 2.5 minutes. A study conducted with this method of nonsurgical ET reported 70.8% farrowing rate with an average litter size of 6.9 pigs on 17 females (Martinez et al., 2004). Table 2 shows some results of non-surgical transfer.

Table 2. **Results of embryo transfer following non-surgical embryo application.***

No. of Transfers (n)	Pregnancy rates (%)	Litter size (mean)	References
58	9	5.2	Reichenbach et al., 1993
21	33	6.7	Hazeleger and Kemp, 1994
46	22	4.3	Galvin et al., 1994
16	31	6.2	Li et al., 1996
25	64	3.1	Yonemura et al., 1996
27	59	10.9	Hazeleger and Kemp, 1999
24	70.8	6.9	Martinez et al., 2004
19	53	6.9	Dyck et al., 2005

* Table modified from Brüssow et al., 2000.

This nonsurgical method is relatively simple to use but requires on-farm training to become proficient at the insertion of the inner catheter. This method provides the beginning of a simple and practical method to perform non-surgical embryo transfer.
Additionally, new technologies such as cyropreservation of porcine embryos can add practicalities of storage and shipment of embryos (Dobrinsky 1997; Dobrinsky et al., 2000).

CLONING

Brief History of Cloning

1984 – Danish scientist, made a genetic copy of a lamb from early sheep embryo cells (Willadsen, 1986). This technique, eventually called "twinning", led many other scientists to follow with production of "twin" cattle, pigs, goats, rabbits, and rhesus monkeys.
1993 - Creation of calves by transferring the nuclei from cultured embryonic cells (Simms and First, 1994)
1995 – Differentiated embryo cells to clone two sheep. (Campbell et al., 1996)
1996 – Dolly, the first mammal to be cloned from adult cells. (Wilmut et al., 1997)
2000 – First pigs are cloned. (Betthauser et al., 2000, and Polejaeva et al., 2000)

Principles of Cloning

The foundation for cloning is an embryology program with controlled testing of media, oocyte maturation and blastocyst development rates, IVF success, and equipment for manipulation of oocytes and donor cells. When excellent blastocyst formation is accomplished with oocytes extracted from ovaries collected from females slaughtered in an abattoir, then the lab is ready to try its hand at producing clones. Therefore, the first and most important component of a successful cloning program is an outstanding in vitro embryo production lab.

All organisms are influenced by the interaction of genes with their environment. This is sometimes referred to as epigenetic effects. The impact of the environmental influences may

cause clones to differ phenotypically; however, they will still have the same genetic information.

Factors which Impact Cloning:

1. Source and quality of the oocytes. Seasonal variation in the quality of oocytes can be significant.
2. Culture media, laboratory cleanliness and technique.
3. Timing of the different processes is critical to success.
4. Recipient management- sows versus gilts, time of the year, natural timing of estrus versus hormonally synchronized timing. Whether the sows are from maternal lines or paternal lines will have a significant impact on results.
5. Different cell culture lines can have differing results in cloning. (Forsberg et al., 2002)

Success rates for reconstructed embryos leading to live births remain relatively low. Most losses occur in early development (first trimester), however, cloned animals also die in late pregnancy or soon after birth, often due to respiratory and physiological dysfunction. Increased abnormal placental development, increased fetal losses, large offspring syndrome in cattle and sheep, and a generally higher incidence of abnormalities have all been observed. However, research suggests that both cloned animals and their offspring are safe for milk and meat production and consumption. Several countries such as Denmark, Japan and Germany have passed legislation that allows the introduction of cloned offspring into the food chain. It is anticipated that the US will soon release a study that will also provide evidence that food from offspring of clones is safe for the food chain. A recent survey conducted by KRC research and released November 4, 2005 reported that two thirds of US consumers would either buy or consider buying meat and milk made from clones.

How to Clone From Adult Cells

Somatic cell chromatin transfer is the process of making a genetic copy of a desired animal that will carry the genetic material from the source animal. This process differentiates itself from blastomere separation or blastocyst division, which produces clones of the embryo (a genetic combination of both parents). The chromatin transfer technique has the advantage of allowing for the selection and multiplication of the adult traits that one desires. The following steps outline somatic cell chromatin transfer, a technology licensed from Hematech® by Minitube of America.

Oocyte Aspiration

Ovaries are typically purchased from packing plants and brought back to the embryology lab according to strict bio-security measures. Oocytes surrounded by the cumulus cells or cumulus-oocyte complexes (COCs) are aspirated from properly sized follicles on the ovaries to obtain the ideal stage of a pre-ovulatory follicle. The oocytes are placed in maturation media and mature in vitro. Each oocyte goes through meiosis to yield a metaphase II oocyte and a polar body that passes out of the oocyte to a location under the zona pellucida.

In-vivo Derived Oocytes

Oocytes may be collected from a given population of sows, synchronized for ovulation, to select the proper timing to flush the in-vivo matured oocytes. Oocytes from a known source and status of the sow will usually give an advantage in cloning success rates and may be preferred by customers in the cloning of their own animals.

Enucleation

Mature oocytes of normal morphology are selected for enucleation (DNA removal). Their chromosomes are stained to be visible in florescent light under inverted microscopes fitted with hydraulically-controlled micromanipulators. These micromanipulators allow the technician to hold and manipulate the oocyte while locating the polar body and removing the chromosomes. Identifying the polar body location helps the technician to identify the location of the chromosomes lying in the cytoplasm of the oocyte. With smooth precision, the technician inserts a glass needle under the zona pellucida of the oocyte and removes a karyoplast containing the polar body and chromosomes from the oocyte. Remaining is an enucleated egg that is a cytoplast devoid of chromatin material.

Chromatin Transfer

The next step is to isolate the cultured adult somatic cells and place one of these cells under the zona pellucida of the cytoplast. It is important to insert the cell through the opening made when enucleating the egg in order to prevent any further damage to the zona pellucida. Once the donor cell is placed under the zona pellucida the donor cell is electrically fused with the enucleated oocyte.

Fusion

To fuse the donor cell nucleus into the oocyte, the cell membrane must be in direct contact with the oocyte cytoplasm. Fusion is accomplished with an electrical pulse generated by a special device that causes the donor cell to fuse with the oocyte. A few hours after fusion, the reconstructed cloned embryos are activated to trigger a response like fertilization. If all works well, the somatic cell chromatin material now inside the cell is reprogrammed and acts as a fertilized embryo to begin development.

Embryo Transfer

In pigs, approximately 100+ reconstructed embryos are surgically transferred into the oviducts of a synchronized recipient. About 50% of the females become pregnant and of these, about 70-80% farrow. Therefore, roughly 30% of the sows surgically implanted will give birth to cloned piglets. Generally, cloned embryos will have gestation length a few days longer than normal (117-118 days).

Uses for Cloning

The application of cloning technology is an excellent way to replicate valuable animals for widespread dissemination of desirable traits. The greatest potential impact for the swine industry will be the replication of genetically superior boars for placement in boar studs for distribution of their genetically superior semen. This application of cloning simply multiplies "normal" top genetic animals to be used by the swine industry for the efficient and increased production of meat protein to feed the world. Anticipated goals for the swine industry include improved rates of weight gain, feed conversion and reduced product variation. Gene marker technologies may be used to identify animals with a particular disease resistance. These animals can be cloned and used for breeding to produce disease-resistant progeny resulting in protecting the swine industry from annual losses of billions of dollars.

Clones may also be used to reduce variation in experimental models, thereby reducing the number of experimental animals needed to realize statistical significance in each experiment. The far reaching impact of using cloned animals as experimental models accelerates the output of scientific information for use by the swine industry. Cloning will also be used to help maintain or expand populations that are nearing extinction. There are even suggestions to bring back populations of animals that are already extinct. This, however, would not be possible unless a preserved source of unbroken or uncorrupted DNA and a source of oocytes from a closely related species to allow embryonic development in the surrogate recipient mother are available.

Other applications of cloning will result from well-established methods to genetically modify cells before their use in cloning procedures. The ability to make changes in the genome of animals will enable strategies to directly add desirable traits and remove undesirable traits. Envisioned agricultural applications include safer, healthier and more economically priced food products with reduced environmental impact.

Health-care applications of genetically modified cloned animals include the production of therapeutic proteins in the milk or blood of animals (see GTC Biotherapeutics at www.transgenics.com and Hematech at www.hematech.com); the use of genetically modified animals for xenotransplantation; and the development genetically modified animal models for human diseases (Kolber-Simonds et al., 2004).

CONCLUSIONS

At the present time, gender pre-selection in the pig has only been accomplished using the Beltsville Sperm Sexing Technology. Currently, the slow sorting speed of the flow cytometers limits most of the use of gender pre-selected porcine semen to technologies such as deep intra-uterine inseminations with low sperm numbers, ICSI, and IVF fertilization of oocytes. As technologies advance in the equipment and in new sorting techniques for DNA staining, using gender pre-selected semen will become a reality in pork production.

Interest will increase in the movement of genetics between farms and countries because of the reduced risk of disease through technologies such as embryo transfer. Currently, most ET is done surgically. Development of nonsurgical ET techniques that will be adaptable for farms will increase and make the use of ET technology more widespread. New catheter developments have greatly improved the results of non-surgical ET; however the industry has a long way to go to perfect non-surgical flushing of embryos in the pig.

The success of research groups to successfully produce cloned pigs has resulted in the recognition of cloning and its role in agriculture. Several countries have now passed legislation to accept offspring of clones for use in their food chains. Successful clones are reliant on excellent embryology and embryo transfer programs where manipulation of the embryo does not decrease the survival rate and efficiency of producing offspring. To cloning animals found with genetic markers for disease resistance and extremes on production parameters such as feed efficiency, rate of gain will pay large economic dividends for the swine industry.

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