

# Cryopreservation of Porcine Gametes: A Chilly Future in the Swine Industry

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## Abstract

There are many reasons why cryopreservation of gametes are important: 1) maintenance of genetic diversity in domestic and wild species populations (Wildt 1992; Wildt 1997; Critser and Russell 2000), 2) facilitating the distribution of “genetically superior” domestic species lines, 3) treatment of human infertility (Kuczynski et al. 2001; Ranganathan et al. 2002; Tash et al. 2003; Agarwal et al. 2004; Nalesnik et al. 2004), and 4) genetic banking of genetically modified animal models of human health and disease (Critser and Russell 2000; Knight and Abbott 2002). Although cryopreservation of gametes has been routine in many other industries such as the dairy industry, the swine industry is still in its infancy. Birth of live offspring has been reported from cryopreserved sperm and embryos, but success is still extremely low. From an industry perspective the low success rate has too much of an economic impact therefore the integration of the technology has been slow. However, the improvements in the technologies are slowly improving pregnancy rates, farrowing rates and litter size. Integration of cryopreservation into the swine industry is coming and will have a huge impact on movement of genetic material internationally and domestically.

## Introduction

The swine industry has continued to change with the demands of the public and has become a worldwide industry. With increased distance between grand-parent herds and commercial herds the need to utilize reproductive technologies has increased. Most of the interest has been with cryopreservation of porcine gametes largely as an easy way to move germplasm from one farm to another farm. In addition with the outbreak of Foot and Mouth disease in the UK in 2001, cryopreservation of porcine gametes is seen as a method for disease elimination if proper

washing techniques are utilized. However, the utilization of these technologies is currently limited by the on-farm success of these techniques.

Embryo cryopreservation in many domestic animals is routine, however in the pig there has been limited success (Dobrinsky 2001c). Pig embryos have an extreme sensitivity to hypothermic exposure which impedes the ability to use conventional slow cooling protocols. However, development of vitrification methods using an open pulled straw (Vajta et al. 1997) has increased the survivalability of porcine embryos but still has limitations for the swine industry. The biggest limitation of embryo cryopreservation for the swine industry is the reduced farrowing rates and litter size, and lack of nonsurgical embryo collection and transfer procedures

Similar to embryo cryopreservation, porcine sperm cryopreservation success is limited again due to the extreme sensitivity of pig sperm to hypothermic exposure. Despite the potential for a huge impact on the industry the use of frozen-thawed semen is >1% of the AI being performed (Wagner and Thibier 2000) because of the reduced economics compared to either fresh or liquid-cooled semen. Currently, the use of frozen-thawed boar sperm during insemination results in a reduction in farrowing rates and litter size by 50% and three piglets per litter, respectively (Johnson 1985).

### **Cryopreservation of porcine embryos**

Although porcine embryos have been successfully frozen and produced live offspring (Dobrinsky 2001), the impact of cryopreserved embryos on the swine industry is limited. This limitation is due to the difficulty collecting in vivo derived pig embryos, their hypothermic sensitivity (i.e. the cryopreservation procedure), and the lack of a commercially viable non-surgical embryo transfer procedure (Martinez et al. 2004). Pig embryos have an extreme sensitivity to cooling so have limited the success of cryopreservation to vitrification versus slowing cooling. Peri-hatching porcine embryos have the greatest survival rate (Dobrinsky 2001; Dobrinsky 2001a) but are not currently used in the industry due to guidelines set forth by the International Embryo Transfer Society which restricts the cryopreservation to zona intact embryos for international and domestic shipping (Stringfellow 1998). Risk of disease transmission increases as the zona-free embryos becomes exposed to the natural surroundings.

However, currently most of the embryo cryopreservation work requires some manipulation of the embryo prior to cryopreservation which also severely limits its impact in the swine industry. Currently most of the success with porcine embryo cryopreservation involves damage to the zona pellucida independent of embryonic stage. Either the zona is completely removed as with the peri-hatching blastocyst or a small incision is made in the zona to delipate (remove the lipid) the embryos. The reason that much of the work requires manipulation of the embryo and damaging of the zona prior to the cryopreservation protocol is the lipid content of the embryo. Pig embryos have a large amount of lipid compared to other species, it was found that removal of the lipid increased the survival of cryopreserved porcine embryos (Nagashima et al. 1995). Typically the intracellular lipid content of porcine embryos is composed of triacylglycerols (Sturmeay and Leese 2003). Removal of the lipid from the embryos requires centrifugation and micromanipulation which compromises the zona pellucida thus increasing the risk of disease exposure and transmission. However, currently there is work being done to remove the lipid without compromising the zona, either by polarizing of the lipid (centrifugation without micromanipulation) or chemical delipation. Polarization of lipid in the pig embryos is a technique to minimize damage to the zona prior to cryopreservation. Polarization of the lipid

involves centrifugation of the embryos at a relatively high speed to cause the lipid to collect at the bottom portion of the embryo. Initially Cameron et al., (2000) reported the birth of the first vitrified zona intact pig embryos, however, the pregnancy rate and embryo survival was extremely low. Beebe et al., (2005) modified the freezing protocol by changing the base medium and decreasing the plunging temperature to  $-204^{\circ}\text{C}$  from  $-196^{\circ}\text{C}$  which resulted in an increase in pregnancy rate and embryo survival. In a large on-farm trial, Beebe et al., (2005) reported that using this improved cryopreservation protocol; the litter size with vitrified embryos was 8.2 total born and 7.7 born alive.

Chemical delipitation of the pig embryos is a new technique that is being developed to keep embryos zona intact. Lipolysis of triacylglycerols is regulated by many hormones but there are also several chemicals that are capable of lipolysis. Forskolin is a chemical that has lipolytic activity that have been used to chemically delipitate pig embryos prior to cryopreservation. Men et al., (2006) reported the use of Forskolin for chemical delipitation of pig embryos prior to cryopreservation with increased survival. They stated that when pig blastocysts were treated with Forskolin and an apoptosis inhibitor, approximately 50% of the embryos survived vitrification compared to 23% survival for controls (Men et al. 2006). However, they did not report any embryo transfer survival or live offspring. Although survival rate increased with the treatment, for an industry impact it must result in acceptable pregnancy and farrowing rates, and litter size. With litter sizes of 8.2 total born and 7.7 born alive, further implementation of embryo cryopreservation into the swine industry is not too far away.

### **Cryopreservation of Boar Sperm**

Preservation of boar sperm was developed in the 1970s (Pursel and Johnson 1975), however the method used was different than that for other species. Specifically in 1975, Pursel and Johnson developed a “pellet” method that was successful in freezing boar sperm. First, samples were cooled to  $5^{\circ}\text{C}$  at a rate of  $0.22^{\circ}\text{C}/\text{min}$ . At this temperature, cooled media containing extender and glycerol was added. After the addition of glycerol, aliquots of the samples were placed directly on a block of dry ice ( $-79^{\circ}\text{C}$ ) and then plunged to liquid nitrogen ( $\text{LN}_2$ ;  $-196^{\circ}\text{C}$ ). This pellet method was relatively effective in terms of post-thaw motility but the major drawback was the inability to individually label the pellets and the difficulty involved with shipment of the samples. In recent years, other methods have been developed such as “maxi” (5 ml) and “mini” (0.25 or 0.5 cc) straws, which allow individual identification and ability to ship germplasm domestically and internationally (Bwanga et al. 1990; Bwanga 1991). There has been limited progress in boar sperm cryopreservation in the past several years due to the fact that most of the work uses an empirical approach instead of a fundamental cryobiology approach. Fundamental cryobiology approach investigates and takes into account the biophysical characteristics of the sperm when developing cryopreservation protocols.

Successful sperm cryopreservation requires maintaining the post-thaw structural and functional integrity. Maintaining functional integrity is critical, the compartments (*i.e.* acrosome, flagella, midpiece) of sperm will be affected by cryopreservation differently and need to be fully protected so that frozen-thawed sperm can undergo normal fertilization under *in vivo* conditions. While motility may be protected at a high level, acrosome integrity may be severely damaged under a similar physical alteration such as osmotic stress (Gilmore et al. 1998; Agca et al. 2002; Guthrie et al. 2002; Walters et al. 2005).

The semipermeable nature of the plasma membrane that surrounds sperm cells causes volume changes when exposed to anisotonic solutions. The degree of volume response to specific anisotonic solutions is unique for each cell type. Therefore, knowledge of the volume response to anisotonic conditions relies on a fundamental understanding of biophysical characteristics of the cells of interest. Several projects have begun to gain the understanding of the physical and biophysical characteristics of boar spermatozoa, which is critical to the development and optimization of cryopreservation protocols. With the knowledge of fundamental cryobiological properties associated with osmotic changes such as: 1) permeability to water ( $L_p$ ) and cryoprotectants ( $P_s$ ); 2) activation energies ( $E_a$ ); and 3) osmotic tolerance limits (OTL) (Gilmore et al. 1998), we can begin to mathematically model cryopreservation protocols to determine the optimal addition and removal of cryoprotective agents (CPA), as well as cooling and warming rates.

There is a potential for osmotic injury to the cell with equilibration of high concentrations of permeating CPA which causes the cell to shrink and swell in response to the influx and efflux of water and CPA. Gilmore et al., (2005) reported that spermatozoa from boars have reduced osmotic tolerance relative to sperm from other mammalian species. In order to maintain 90% motility, the cell volume excursions must be maintained between 99% and 101% of the initial isosmotic volume, which is much narrower than the osmotic tolerance of human sperm (75% and 110% of their isosmotic volume) (Gao et al., 1995; Gilmore et al., 1998). Further studies have reported that boar sperm OTL can be extended with the addition of extender components such as cholesterol (Walters et al., 2006 unpublished data). It is believed that the extender components extend OTL by altering membrane permeability characteristics as well as the potentially the temperature dependences of these characteristics (Walters et al., 2006, unpublished data).

During the cryopreservation procedure, loss of motility is hypothesized to be associated with one or more cellular injuries. Cellular injury resulting from concentrated solutions during the cryopreservation procedure is associated with either 1) an osmotic effect, or 2) a solution effect. Solution effects are a collective characterization of cellular injury as a result of concentration of solutes as a result of ice formation (Mazur et al., 2000). It has been suggested that solution effects are exacerbated by slow cooling rates due to the fact that the exposure time to the highly concentrated solution is increased. On the other hand, the osmotic effect, results in cellular injury due to the shrinkage and swelling of the cell in response to changes in the extracellular osmolality. Understanding of the osmotic effects on boar sperm from different genetic backgrounds, coupled with membrane permeability parameters, one can engineer CPA addition and removal procedures specifically tailored to each strain's sensitivity, and begin to development of breed-specific cryopreservation protocols.

As stated before most of the work has used an empirical approach to develop cryopreservation protocols for the boar. Currently, methods are being developed to freeze boar sperm by alterations of the freezing medium composition such as the addition of the antioxidants (Funahashi and Sano 2005), various forms of packaging the semen for cryopreservation (Bwanga 1991), and storage prior to cryopreservation (Guthrie and Welch 2005). There has been an effort to investigate the effects of reactive oxygen species on cryopreservation of boar sperm by the addition of antioxidants to the extender prior to freezing. In addition there is a large boar to boar variation as well as the intra-boar (ejaculate variation) in the ability of the sperm to undergo cryopreservation.

Recently there has been a desire to develop a simple and effective test for determining "good" versus "bad" freezers for a way for the industry to decide which boars to keep in the

herd. Thurston et al., (2002) used amplified restricted fragment length polymorphism technology to find 16 different molecular markers linked to freezability that potentially could be used for identifying inter-boar variation. In addition, Thurston et al., (2002) reported that the inter-boar variation may be genetically predetermined as they investigated differences between three breeds of pigs (Landrace, Large White, Duroc). As of now, there is no good method to determine if boars are “good” or “bad” freezers during selection.

In the swine industry, producers will limit the use of frozen-thawed semen if they have to thaw 10-15 0.5cc straws to achieve the desired AI dose. However, if the producer can thaw one flatpack (containing 5ml of sperm) and dilute to achieve an AI dose in combination with good farrowing rates and litter sizes, frozen-thawed boar sperm will have a huge impact on the industry. However, currently the dose of frozen thawed sperm is  $5-6 \times 10^9$  which is twice the “normal” AI dose, as a large percentage of the sperm are lost during the freeze-thaw procedure. Furthermore, the lost of sperm is not limited to the cryopreservation procedure, frozen thawed sperm have a limited life span in the female tract. With this limited life span of frozen-thawed sperm, the need for more accurate heat detection and proper AI technique increases. There are alternative methods to improve the fertility of frozen thawed sperm such as timed AI, and deep uterine insemination (DUI). One of the advantages that DUI offers is the use of a low dose insemination with the frozen-thawed sperm. But a disadvantage of DUI is timing of insemination relative to the ovarian status of the female. This timing between ovulation and insemination may account for some of the differences between farms using frozen-thawed semen. Bolarin et al., (Bolarin et al. 2006) found that when using DUI with frozen thawed sperm that peri-ovulatory (some ovulation had occurred) ovarian status of the females increased pregnancy, farrowing rates and litter size compared to either pre-ovulatory or presence of corpus hemorrhagica. In this study, Bolarin et al., (2006) compared two farms with different management styles and found there was a difference between the two farms in terms of success with DUI in combination frozen thawed sperm. One of the big differences between the two farms was the ovarian status of the females used for this trial as a larger percentage of the females were peri-ovulatory at one farm versus the other farm. The different management styles between the farms probably accounts for the differences seen in the results with DUI in combination with frozen-thawed sperm. In farm A (farm with the largest peri-ovulatory group) boar exposure was minimal as there was no “habituation” of the boars with the females, however, in farm B there was continuous boar exposure (Bolarin et al. 2006). Suggesting that management practices in particular boar exposure and heat detection is critical for DUI in combination with frozen thawed sperm.

## **Conclusions**

The cryopreservation of porcine gametes has made huge improvements in the last several years however; the potential impact in the swine industry has been limited. There are still many factors that have to be addressed before cryopreservation of porcine gametes will be beneficial to the swine industry but steps are being taken to make this a reality. In addition, there are several reproductive technologies such as nonsurgical embryo collection and transfer procedures that have to be optimized as they will be critical for the future of cryopreservation of porcine gametes. The integration of cryopreservation in the swine industry has a bright and chilly future.

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