Reproductive biotechnologies: current status in porcine reproduction

B.N. Day *

Animal Science Unit, University of Missouri, College of Agriculture, 159 Animal Sciences Center, Columbia, MO 65211, USA

Abstract

During the past decade, considerable attention has been directed toward the development of reproductive technologies for both research purposes and for more controlled swine reproduction. Artificial insemination is an example of a technology that has continued to be expanded from early use in European countries to the USA and Canada where it is now estimated that a majority of the sows bred are artificially inseminated. In addition, several significant technological advancements have been made in the genetic modification of swine and interest has been generated in the possible use of swine as donors of specific tissues and of organs for the improvement of human health. At the same time, the systems for production of swine for human food continue to undergo major changes including, in some countries, the consolidation of swine into large, integrated units. These swine operations are very receptive to the use of technologies to reduce labor costs as well as a basis for increased production efficiency. Therefore, the combined interest in swine reproductive technologies by both the medical field and the swine industry creates an increased effort for the development of new technologies as well as for the implementation of existing ones. One of the more rapid technological advancements this decade has been the progress in in vitro production (IVP) of swine embryos. Major advancements have been made on the development of procedures for production of large numbers of embryos from oocytes collected at slaughter houses which are then matured (IVM) and fertilized (IVF) in the laboratory. Success in IVP has stimulated increased research in other areas that can be enhanced by the availability of embryos without a requirement for surgical collection from gilts or sows. One example is the combined use of IVF, gender-sorted sperm cells, and embryo transfer to produce offspring of a predicted sex. In a related area, instrumentation for non-surgical embryo transfer has recently been...

* Tel.: +1-573-882-7555; fax: +1-573-884-7827.
E-mail address: dayb@missouri.edu (B.N. Day).
developed that results in significant improvement in this technology. Similar achievements have been gained in cryopreservation of embryos by vitrification. These developments will be reviewed with emphasis on the in vitro production of embryos from immature oocytes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Swine; Reproduction; Embryo culture; In vitro technology

1. Introduction

Considerable interest has developed during the past decade for the use of established as well as emerging reproductive technologies for both research purposes and for more controlled swine production. Several significant technological advancements have been made in the genetic modification of mammals and interest has been generated in the possible use of swine as donors of specific proteins and of organs for the improvement of human health. This interest by the scientific community has resulted in a major increase in the use of swine in biomedical research studies. At the same time, the systems for production of swine for human food continue to undergo major changes including, in some countries, the consolidation of swine units into large, integrated units very receptive to the use of technologies to reduce labor costs as well as a basis for increased production efficiency. Therefore, the combined interest in swine reproductive technologies by both the medical field and the swine industry creates an increased desire for the development of new technologies as well as for the implementation of existing ones.

One research area being given particular emphasis at the present time is studies on oocytes and early pre-attachment embryos. Major strides have been made during the past decade on the development of procedures for the in vitro production (IVP) of large numbers of embryos from oocytes collected at slaughter houses which are then matured (IVM) and fertilized (IVF) in the laboratory. Success in IVP has then stimulated increased research in other areas that can be enhanced by the availability of embryos without a requirement for surgical collection from gilts or sows. One example is the combined use of IVF, gender-sorted sperm cells, and embryo transfer to produce offspring of a predicted sex.

The following review will give emphasis to recent advancements in IVP and also the present status of related embryo biotechnologies. A major effort is underway to be able to provide large numbers of embryos for research being conducted in several areas including the manipulation of embryos to provide genetically modified swine to be used to improve human health. Similarly, numerous approaches are being taken for the micromanipulation of swine embryos for the purpose of more efficient pork production. Biotechnologies with the capability to produce larger numbers of high-quality embryos by IVP procedures would enhance progress in these related research studies. In addition, this resource would be a stimulus for increased research on factors controlling early embryonic development as directed toward the large economic loss due to the high rate of early embryonic mortality in livestock.
2. In vitro production of swine embryos

2.1. Historical

For many years, a scientific interest has existed in the capture of genetics present in the ovaries of superior sows in a manner similar to the use of artificial insemination in order to expand genetics of superior boars within the population. Swine are of particular interest in this regard since the ovaries contain large numbers (> 200,000) of primordial follicles (Grupen et al., 1995). During the early 1980s, media were developed to culture successfully pig embryos for a short period of time prior to transfer to recipient gilts. However, it wasn’t until the early 1990s that one- to two-cell embryos recovered from donor gilts could be cultured through the “four-cell block” to the blastocyst stage of development (Petters and Wells, 1993; Beckmann and Day, 1993). The presence of reduced NaCl concentration or the presence of organic osmolytes in the embryo culture medium was found to provide the capability to culture single-cell embryos through the four-cell stage. These established characteristics of pig oocytes have been later applied in the development of media for the in vitro maturation of oocytes.

Shortly prior to the development of technology to culture in vivo fertilized one- to two-cell embryos to the blastocyst stage, in vitro fertilization of in vivo matured oocytes was achieved (Chen et al., 1986). Therefore, it became possible to produce live offspring from the in vitro fertilization of in vivo matured oocytes. The missing link for the in vitro production of blastocysts from immature oocytes recovered from ovaries was the ability to mature oocytes in vitro (IVM). Focus has been directed toward research in this area during the past decade.

2.2. In vitro maturation of oocytes

In general, oocytes surrounded by a compact cumulus cell mass when collected from control follicles of slaughtered peripuberal gilts have been used for in vitro maturation. Although oocytes collected from sexually-mature sheep and cattle have generally been found to be superior to those from prepuberal animals, this comparison has not been reported for pigs using currently recommended maturation media.

Early studies on IVM clarified the importance to developmental competence of both nuclear and cytoplasmic maturation. Nuclear maturation can be more clearly defined by the development of a metaphase II spindle from the germinal vesicle; whereas, indirect measures must be employed to measure cytoplasmic maturation. In earlier studies, it was noted that a high incidence of nuclear maturation could be attained in media which did not support cytoplasmic maturation (see Niwa, 1993; Funahashi and Day, 1996). However, in a series of studies involving the addition of cysteine (Yoshida et al., 1993), follicular fluid (Funahashi and Day, 1993a), cysteamine (Grupen et al., 1995) and beta-mercaptoethanol (Funahashi and Day, 1997; Abeydeera et al., 1998a), this problem has been minimized (Table 1). Other approaches including reduction of duration of hormone exposure during maturation to 20 h rather than 40 (Funahashi and Day, 1993b)
Table 1
Recent improvement in blastocyst development*

<table>
<thead>
<tr>
<th>Maturation medium(^b)</th>
<th>Modification(^b)</th>
<th>% Blastocysts</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCSU 37 + 10% pFF</td>
<td>control</td>
<td>9</td>
<td>Funahashi et al. (1997b)</td>
</tr>
<tr>
<td>NCSU 23 + 10% pFF</td>
<td>cAMP for 20 h</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>NCSU 23 + 10% pFF</td>
<td>control</td>
<td>18</td>
<td>Abeydeera et al. (1998c)</td>
</tr>
<tr>
<td>TCM 199 + 25% pFF</td>
<td>Follicular shell pieces</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>TCM 23 + 10% pFF</td>
<td>control</td>
<td>12</td>
<td>Grupen et al. (1995)</td>
</tr>
<tr>
<td>NCSU + 10% pFF</td>
<td>500 μM cysteamine</td>
<td>12</td>
<td>Abeydeera et al. (1998a)</td>
</tr>
<tr>
<td>NCSU 23 + 10% pFF</td>
<td>12.5 μM BME</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>NCSU 23 + 10% pFF</td>
<td>25 μM BME</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>NCSU 23 + 10% pFF</td>
<td>1 ng/ml EGF</td>
<td>33</td>
<td>Abeydeera et al. (1998b)</td>
</tr>
<tr>
<td>TCM-199 + 0.1% PVA</td>
<td>control</td>
<td>22</td>
<td>Abeydeera et al. (1999)</td>
</tr>
<tr>
<td>TCM-199 + 0.1% PVA</td>
<td>10 ng/ml EGF</td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Day et al., 1999 (modified).
\(^b\)mWM: modified Whitten’s medium; NCSU: North Carolina State University; pFF: pig follicular fluid; PVA: polyvinylalcohol.
\(^c\)BME: β-mercaptoethanol; EGF: epidermal growth factor.

and use of reduced media concentrations of NaCL (Funahashi et al., 1994) have also been found to enhance pronuclear development.

### 2.3. Protein free medium

Although high rates of pronuclear formation had been accomplished by the addition of porcine follicular fluid, follicular shell pieces, and conditioned media (Day and Funahashi, 1996), a preferred medium for maturation would be one which did not require co-culture with somatic cells or their secretions. Abeydeera et al. (1999) have developed such a medium following a series of studies involving the substitution of polyvinylalcohol (PVA) for previously used biological materials added to NCSU23, Whitten’s, Waymouth, and TC199. Results not different from NCSU23 plus porcine follicular fluid were obtained when oocytes were matured in TC199 without follicular fluid but with added PVA.

### 2.4. Epidermal growth factor (EGF)

A further improvement in available medium for embryo maturation has been obtained by the addition of EGF to either a medium containing follicular fluid (Abeydeera et al., 1998b) or a protein-free medium (Abeydeera et al., 1999). By the addition of EGF, approximately one-third of oocytes subjected to in vitro maturation form blastocysts following IVF. Intracellular GSH concentration as well as blastocyst cell numbers are increased by the addition of 10 ng/ml of EGF to these maturation media.
2.5. Stage of germinal vesicle at gonadotropin stimulation

Only recently has attention been directed toward the nuclear morphology of oocytes collected from ovaries of slaughtered animals (Funahashi et al., 1997a,b; Guthrie and Garrett, 1999; Hirao et al., 1995). The germinal vesicle stage of oocytes selected for in vitro maturation varies considerably when classified according to morphological characteristics developed by Motlik and Fulka (1976). In contrast, nuclear morphology of the GV stage is closely synchronized in gilts at the approximate time of endogenous luteinizing hormone release to induce ovulation and oocyte maturation (Funahashi et al., 1996). Improved embryonic development has been obtained by modification of culture condition to synchronize the germinal vesicle stage of oocytes between follicular aspiration and germinal vesicle breakdown (GVB). Exposure of oocyte–cumulus complexes (OCC) to dibutyryl cyclic adenosine 3', 5'-monophosphate (dbcAMP), for initial 20 h of maturation culture, synchronized germinal stage and improved embryonic development. Further, simply holding oocytes in culture for 12 h prior to exposure to gonadotropins improved blastocyst formation following IVF. Further research in this area appears to be indicated in order to more clearly establish the most desirable germinal vesicle stage at time of treatment with gonadotropins in order to achieve maximal developmental competence.

2.6. In vitro fertilization (IVF)

Along with the development of highly successful methods for the penetration of in vivo or in vitro matured oocytes, there has also occurred a high frequency of polyspermy. Although many of the previous investigations of IVF in pigs have used freshly ejaculated sperm, frozen–thawed sperm techniques have now been developed which provide the major advantage of continued use of the same semen collection over repeated experiments (Wang et al., 1991; Abeydeera and Day, 1997). However, the availability of frozen–thawed sperm has still not led to the development of IVF procedures which yield acceptable rates of monospermy. Reduced polyspermy has been noted following additions to fertilization medium of porcine oviductal cells and secretions (Nagai and Moor, 1990; Kano et al., 1994; Kim et al., 1996), porcine follicular fluid (Funahashi and Day, 1993a) and semi-purified porcine oviduct specific glycoprotein (Kouba et al., 1999). Detailed comparative examination of cortical granule release following IVF of in vivo matured, oviductal and IVM oocytes has not revealed significant differences in degree or time of cortical granule exocytosis following IVF (Wang et al., 1997, 1998), which could be interpreted to be a potential cause of polyspermy.

In summary, polyspermy remains the major obstacle to successful production of large numbers of IVP embryos with high level of developmental competence.

2.7. Embryo culture

Several media are available for the successful culture of in vivo embryos to the blastocyst stage of development (approximately 75% success). These include modified
Whittens medium (MWM) (Beckmann and Day, 1993), North Carolina State University 23 (NCSU23) medium (Petters and Wells, 1993), Iowa State University (ISU) medium (Youngs et al., 1993) and Beltsville embryo culture medium 3 (BECM-3) (Dobrinsky et al., 1996). Recently, a comparison was made of the rate of development to blastocysts of IVM/IVF derived embryos in the above media. A wide variation of 5% to 30% blastocyst development was found with the NCSU23 medium, the most successful and MWM the least. Further preliminary testing of the NCSU23 medium indicated that culture for the initial 72 h in NCSU23 medium without glucose was also beneficial (Abeydeera, unpublished).

Since well-known morphological differences are known to exist between in vivo matured embryos exposed to the oviductal environment and in vitro embryos (Wang et al., 1998), additional research may reveal that reduced development of IVM/IVF embryos may not be due only to inadequate cytoplasmic maturation, but instead this is in combination with polyspermy and inappropriate embryo culture medium, particularly during initial cleavage stages. For example, embryo development to the morula/blastocyst stage of early one-cell hamster embryos collected before pronuclear development (3 h post-egg activation) was stimulated by an initial 6 h culture in the standard hamster culture medium treated to reduce intracellular free calcium levels (Lane and Bavister, 1998). Further, it has also been shown that one- to two-cell in vivo embryos recovered from one oviduct, and placed in culture for comparison with embryos recovered from the opposite oviduct 4 days later, are delayed approximately one cell cleavage from the embryos retained in vivo (Machaty et al., 1998). The inner cell mass was particularly delayed in development in the in vitro embryos.

2.8. Developmental competence

2.8.1. Blastocyst

With the improvements in cytoplasmic maturation, as measured by incidence and timing of male pronuclear development, and the decreased frequency of polyspermy during IVF, major increases have been obtained in the percentage of oocytes subjected to IVM that develop to the blastocyst stage of development (Table 1). At present, IVM and IVF procedures are available which will give repeated results of approximately 35% of subjected immature oocytes that form blastocysts. However, it is now known that fertilized oocytes with more than two male pronuclei form blastocysts at the same frequency as normally fertilized oocytes (Han et al., 1999a). There are no reported births of polyploid offspring in pigs and polyploidy may, therefore, be a cause of embryo/fetal mortality in recipient gilts receiving morphologically normal IVP embryos. The question remains open as to the fate of extra sperm in polypronuclear oocytes. It has been shown that blastocysts with both normal and abnormal ploidy develop from polypronuclear oocytes (Han et al., 1999b).

Since major uncertainties exist in the quality of morphologically normal IVP cleavage stage embryos and blastocysts and because of the lack of a non-invasive measure to assess blastocyst quality of pig embryos, it becomes increasingly important to extend the assessment of modifications in IVP procedures to include not only development to early embryonic stages but also to full term.
Table 2
Recent summary of embryo transfer results

<table>
<thead>
<tr>
<th>Oocyte source</th>
<th>Source of spermatozoa</th>
<th>Embryonic stage</th>
<th>Pigs/recipient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVM</td>
<td>Frozen-thawed</td>
<td>2–4-cell</td>
<td>7/1</td>
<td>Abeydeera et al. (1998b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-cell to morula</td>
<td>11/2</td>
<td></td>
</tr>
<tr>
<td>IVM</td>
<td>Frozen–thawed</td>
<td>2–4-cell</td>
<td>18/5</td>
<td>Abeydeera et al. (1998d)</td>
</tr>
<tr>
<td>IVM</td>
<td>X-sperm</td>
<td>8-cell to morula</td>
<td>24/5</td>
<td>Abeydeera et al. (1998c)</td>
</tr>
<tr>
<td></td>
<td>Y-sperm</td>
<td>8-cell to morula</td>
<td>9/3</td>
<td></td>
</tr>
<tr>
<td>IVM</td>
<td>Frozen–thawed</td>
<td>≥ 1-cell</td>
<td>17/3</td>
<td>Kikuchi et al. (1999)</td>
</tr>
<tr>
<td>IVM</td>
<td>Frozen–thawed</td>
<td>8-cell to morula</td>
<td>82/12</td>
<td>Abeydeera et al. (unpublished data)</td>
</tr>
</tbody>
</table>

*Day et al., 1999 (modified).

2.8.2. Pregnancy rate and litter size

Only during the past 4–5 years has IVP techniques been improved to the extent that expectations for full term development were sufficient to justify more than a very few embryo transfers to recipient gilts (Tables 1 and 2). With improvements in IVM/IVF, gradual increases in number of embryo transfers, pregnancy rate and litter size have occurred to the point whereby consideration can now be given to the use of IVP embryos in related studies in biotechnology (see review by Abeydeera et al., 1998c; Day et al., 1999). For example, it has recently been shown that adequate reproduction was obtained in recipient gilts receiving IVP embryos matured in a protein-free medium containing EGF, fertilized with frozen–thawed sperm and cultured to the eight-cell to morula stages of development. Of 25 recipient gilts, 22 (88%) were diagnosed by ultrasonography to be pregnant at 25–30 days post-estrus. Following slaughter on days 26–45 (n = 6) or at farrowing (n = 12), 18 of 25 gilts were pregnant with an average of 7.2 and 6.8 young, respectively, which represented 23% of the 30 IVM/IVF embryos transferred to each of these 18 recipients (Abeydeera, unpublished).

3. Embryo transfer

3.1. Surgical

Surgical techniques for embryo transfer have been available for several decades and are presently in use in several research programs.

3.2. Non-surgical embryo transfer

An interest in non-surgical embryo transfer has existed for many years (Polge and Day, 1968). A major obstacle in development of this technology has been the lack of effective instrumentation to transverse the torturous anatomy of the cervix in pigs. More
recently, increased research has been given to this technology and success has been achieved with improved designs in transfer instruments and also variations in synchronization of donors and recipients (see review by Hazeleger and Kemp, 1999). Further development of present non-surgical transfer techniques would provide major benefits toward the implementation of other swine biotechnologies including transfer of micro-manipulated IVP embryos.

It is interesting to speculate if the endoscopic technology described by Vazquez et al. (1999) for deep uterine AI may provide a much improved method for both non-surgical embryo collection and embryo transfer in pigs.

4. Cryopreservation of embryos

4.1. Freezing

Porcine embryos have been successfully frozen, thawed and produced offspring following transfer to recipient gilts (Nagashima et al., 1994; Dobrinsky, 1997). However, the successful birth of piglets from various approaches to cryopreservation has been inconsistent and has involved small numbers of litters.

4.2. Vitrification

Vitrification has become available as an alternative method for cryopreservation of animal embryos. Blastocysts, expanded blastocysts and hatched blastocysts have been shown to have high survival rates in culture following vitrification. More recently, significant improvements have been made in procedures which have resulted in several litters of offspring born from embryos transferred following vitrification (see Dobrinsky, 1999).

5. Gender preselection

5.1. Sorting of X and Y bearing spermatozoa

It is currently possible to separate X bearing spermatozoa by flow cytometry/cell sorting technology (see review by Johnson, 1997). The spermatozoa DNA are stained with a fluorescence dye which binds equal to the amount of DNA present. In turn, the slightly larger amount of DNA in X bearing sperm provides a basis for sorting the spermatozoa into two populations; i.e., X and Y pools. Offspring have been born as a result of using gender-selected sperm for surgical intratubal inseminations (Rath et al.,
6. Micromanipulation of germ cells

6.1. Cloning and transgenesis

The selection of pigs as a model for biomedical research on human cardiovascular disorders and the increased interest during the past 3 or 4 years on the modification of pig tissues and organs as transplants to humans have resulted in a dramatic increase in the number of laboratories involved in development of transgenic and cloned offspring (see Nottle et al., 1997; Piedrahita et al., 1997). Cloning and transgenesis technology is needed to have a sufficient resource for both desired modification of genotypes for biomedical studies including xenotransplantation and also for an availability of identical piglets. To date, a single cloned pig has been produced (Prather et al., 1989). Although advancements have been made in the processes utilized for nuclear transfer in pigs, such as the improved methods for activation of unfertilized oocytes with thimersol (Machaty et al., 1997), full development of additional cloned offspring has not been reported to date. However, chimeric embryos and a live offspring have been produced by transfer to blastocyst of genetically transferred primordial germ cells (Piedrahita et al., 1998).

7. Summary

Past and present research has established reproductive technologies which have become an integral part of the global swine industry. Artificial insemination is an example of a technology that has continued to be expanded from early use in European countries to the USA and Canada where it is now estimated that a majority of the 6–7 million sows bred are artificially inseminated.

Recommended surgical embryo transfer procedures are readily available and numerous research laboratories employ the technology.

Major research progress has been made in several emerging technologies and others are still struggling for significant solutions. One of the more rapid technological advancements this decade has been the progress in IVP of swine embryos. During this period, the technology has advanced from research efforts directed toward synchronized formation of male and female pronuclei in in vitro matured oocytes to the successful production of litters of live piglets following transfer of advanced cleavage stage IVP embryos to recipient gilts. IVP technology has been used in conjunction with gender-sorted sperm to demonstrate the high level of accuracy of this new technology. Instrumentation for non-surgical embryo transfer has recently been developed that results in significant improvement in this technology. Similar achievements have been
gained in cryopreservation of embryos by vitrification. Several laboratories await the birth of a cloned "Miss Piggy", hopefully, in the 20th century.

Acknowledgements

The author would like to acknowledge the very beneficial assistance from Dr. Hiroaki Funahashi and Dr. Lalantha Abeydeera in preparation of the manuscript and from long-standing editorial contributions by Betty Nichols.

References


