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Porcine *In Vitro* Fertilization: Advances in Medium, Components and Equipment

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Abstract

There have been a number of modifications to *in vitro* fertilization (IVF) systems to allow efficient production of viable porcine embryos. Although *in vitro* production of pig embryos has been studied for over 20 years, the overall blastocyst production rate remains low. This is mainly due to two physiological events: polyspermic oocyte penetration and low rate of male pronucleus formation. Both of these pathological conditions appear to be inherent problems in the porcine *in vitro* production system and many of the mechanisms involved are still unknown. Many scientists have investigated the medium and techniques used during the various stages of *in vitro* production, but improvements to the equipment used during IVF remained unchanged until recently. This chapter discusses the preparation of spermatozoa, co-incubation times, changes in media composition, and development of modified equipment to improve the conditions used during IVF of porcine oocytes.

Key words: porcine, *in vitro* fertilization, embryo, polyspermy.

Invited Review

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Introduction

The *in vitro* production of porcine embryos has been a challenge for researchers for many years (Nagai *et al.*, 1984; Cheng *et al.*, 1986; Hunter, 1990, Yoshida *et al.*, 1990; Prather *et al.*, 1991; Rath, 1992; Zheng & Sirard, 1992; Coy *et al.*, 1993[a,b,c]; Niwa, 1993; Yoshida *et al.*, 1993[a,b]; Dubuc & Sirard 1995, 1996; Nagai, 1996; Xu *et al.*, 1996[a,b]; Abeydeera & Day, 1997[b]; Funahashi & Day, 1997; Kikuchi *et al.*, 1999; Suzuki *et al.*, 2000). Although immature oocytes can be recovered from ovarian follicles of prepubertal gilts and sows and matured under proper conditions, penetration by spermatozoa has resulted in low rates of pronuclear formation and a high incidence of polyspermy. These two issues still remain significant problems in the development of an efficient porcine IVF system (Abeydeera & Day, 1997[a]; Wang *et al.*, 1998[a]; Kouba *et al.*, 2000). The zygotes produced from a polyspermic insemination do not develop normally and, therefore, negatively affect the overall *in vitro* production of pig embryos. Additionally, the overall percentage of *in vitro*-produced pig blastocysts is only approximately 30 percent using the best existing systems (Abeydeera & Day, 1997[a]). Any improvements in the swine IVP system would revolutionize not only the reproductive management of swine but increase the use of pigs for biotechnological and biomedical applications.

In addition to the obvious agricultural applications of swine breeding, disease prevention and genetic improvement, the use of *in vitro*-produced swine embryos for biotechnology is becoming ever more important to biomedical research. At present, *in vitro* production of pig embryos would be of particular interest to researchers that create animals containing exogenous DNA, "transgenic animals" (Kubisch *et al.*, 1995). These transgenic animals are important tools for research involving pharmaceuticals, biomedicine and biotechnology (Huguet & Esponda, 2000; Wheeler & Walters, 2001). Additionally, there is research that is dedicated to developing different techniques to create transgenic animals. There is a variety of methods currently used to introduce the DNA into embryos. However, most of these methods rely on the ability to handle, manipulate or produce embryos/embryonic cells *in vitro*. Improvements in IVP will have a profound impact on these technologies.

The equipment and methodologies used for *in vitro* fertilization (IVF) research is a field of study that is constantly being investigated and re-invented. Emerging technology involving microfluidic environments for embryo culture and *in vitro* production of embryos could revolutionize the swine industry and embryo biotechnology (Glasgow *et al.*, 1998; Davis, 2000; Raty, 2000; Zeringue, 2000; Clark *et al.*, 2001; Raty, 2001; Raty *et al.*, 2001; Walters *et al.*, 2001; Beebe *et al.*, 2002; Clark *et al.*, 2002). The reported microfluidic devices (microchannel) provide a culture environment that mimics the oviduct and uterine environment and allows study of static as well as dynamic culture systems (Raty, 2001; Râty *et al.*, 2001). Additionally, this technology would allow for the integration of the different steps of the *in vitro* production process (*in vitro* maturation/fertilization/culture) within the same equipment with appropriate exchange of the media for each stage of the process.

The process of *in vitro* production (IVP) of porcine embryos has been studied for over 30 years and technological advancements are continually occurring (Coy *et al.*, 1999; Day, 2000). Tremendous efforts have been made to improve the IVP process in pigs through changes in culture medium, number of spermatozoa used during insemination and quantity of medium used to perform the various steps (Coy *et al.*, 1999; Day, 2000). However, little had changed in the equipment used to mature, fertilize or culture the oocytes and embryos (Beebe *et al.*, 2002). The technology used to produce IVP pig embryos

not only involves study of the various biological aspects of the procedure but also the design and engineering of the equipment used to successfully create live piglets from embryos derived at the lab bench. Recently, research has concentrated on the modifying the methodology and equipment used for *in vitro* fertilization in order to reduce the exposure of oocytes to an excessive number of sperm cells (Funahashi & Nagai, 2000). The development of microscale environments (microchannels) has opened exciting new avenues for manipulation of the IVP system to improve the efficiency and overall production of porcine embryos (Clark *et al.*, 2001; Walters *et al.*, 2001; Beebe *et al.*, 2002; Clark *et al.*, 2002). These devices have provided new information that will lead to significant reduction of polyspermy on porcine IVF systems (Clark, Beebe & Wheeler, unpublished data).

In Vitro Fertilization of Porcine Oocytes

In Vitro Fertilization

The porcine ovary is populated with a large number of oocytes arrested in meiosis within the follicular cells (Telfer, 1998). The oocytes in the Graafian follicles resume meiotic divisions just prior to ovulation and are ovulated in response to a surge of luteinizing hormone (LH) from the anterior pituitary gland. This process is mimicked in the *in vitro* system by aspiration of oocytes within the follicle. Whether in the *in vivo* or *in vitro* culture system, oocytes are under the influence of hormonal stimulation to resume meiosis. The oocytes then undergo meiotic maturation, which is described as progression from the dictyate stage (oocyte) to metaphase II (egg) of meiosis (Wassarman & Albertini, 1994; Telfer, 1998). The process of meiotic maturation will not proceed until parthenogenic activation (Wang *et al.*, 1998[b]) or fertilization occurs. Parthenogenic activation is performed *in vitro* whereas fertilization can occur under both *in vivo* and *in vitro* conditions. Only oocytes that have undergone maturation, extruded the first polar body and are arrested in meiosis with chromosomes aligned on the metaphase II spindle have the ability to become fertilized and emit the second polar body (Wassarman & Albertini, 1994).

In the IVF system, there are a large number of sperm cells present near the oocytes. Depending on the IVF system, the oocytes may have a large amount of cumulus cells surrounding them or they may have none (Ka *et al.*, 1997). In contrast, *in vivo*-produced oocytes will have few if any cumulus cells on them and there are very few spermatozoa in the immediate vicinity of the oocytes in the oviduct. The oviduct acts as a reservoir for sperm and then serves as a "gate" which allows sperm cells to enter the ampullary-isthmus junction near the time that the oocytes are arrested at metaphase II and ready to be fertilized (Hunter, 1981; Hunter, 1984; Topfer-Peterson *et al.*, 2000). The mechanisms by which sperm cells are maintained in the reservoir may be binding of the spermatozoa to the oviductal epithelium, physical obstruction to forward progression by thick, viscous secretions, and decreased motility of the sperm tails (Raychoudhury & Suarez, 1991; Mburu *et al.*, 1996)

Polyspermy and Pronuclear Formation

There have been many experiments conducted to enhance porcine *in vitro* fertilization systems (Nagai *et al.*, 1984; Cheng *et al.*, 1986; Hunter, 1990; Coy *et al.*, 1993[a,b]; Wang *et al.*, 1994; Rath *et al.*, 1995; Abeydeera & Day, 1997[a,b]; Wang *et al.*, 1997; Abeydeera *et al.*, 1998[a,b], 1999; Coy *et al.*, 1999; Iwasaki *et al.*, 1999; Kikuchi *et al.*, 1999). The single most important barrier to the successful production of IVF pig embryos is the high incidence of polyspermy (Nagai *et al.*, 1984; Nagai & Moor, 1990; Hunter, 1991; Rath, 1992; Nagai,

1994; Dubuc & Sirard, 1995; Kim *et al.*, 1996; Abeydeera & Day, 1997[a]; Wang *et al.*, 1998[a]; Kouba *et al.*, 2000). Polyspermy is defined as the "entry of more than one spermatozoa into the cytoplasm of oocytes" (Hunter, 1991). There have been numerous studies on the mechanisms of polyspermy in the pig and ways in which to prevent its occurrence, e.g., decreasing the sperm concentration in both ejaculated and epididymal semen (Zheng & Sirard, 1992; Coy *et al.*, 1993[c]; Wang *et al.*, 1994; Dubuc & Sirard, 1995; Xu *et al.*, 1996[a,b]; Rath & Niemann, 1997). Coy *et al.* (1993[c]) fertilized oocytes with 3 different concentrations of sperm cells to determine their effects on penetration rates and polyspermy. These two issues have proven to be rather extensive stumbling blocks to the success of IVF in swine. One theory why polyspermy is a problem in porcine IVF systems is "the abnormally high number of competent spermatozoa reaching the oocyte surface approximately simultaneously" (Coy *et al.*, 1993[c]). The oocyte is bombarded with an excessive population of capacitated sperm cells that are all in its immediate vicinity. This is not the case in the *in vivo* situation where there are very few spermatozoa present, near the egg. Polyspermic fertilization is considered to be a pathological condition in mammalian species, which results in developmental anomalies and failure of the zygote to produce a viable offspring. According to Hunter (1991), "penetration of the vitellus by large numbers of spermatozoa may lead to the appearance of chromatin aggregates formed from sperm heads that have failed to de-condense into accessory pronuclei".

It is the zona pellucida of the oocyte that actually blocks polyspermy, but this is not an instantaneous process. The blockage of polyspermy is not the only function of the zona pellucida at fertilization, but it is a critical step that would lead to the development of pig embryos. At fertilization, the zona pellucida acts as a selective "screen" to spermatozoa and then as protective capsule to the developing embryo (Brown & Cheng, 1986). The primary block to polyspermy is the cortical granule (CG) reaction, which results in a biochemical change in the composition that induces hardening of the zona pellucida (Hatanaka *et al.*, 1992). These granules are "small, spherical membrane-bound organelles located in the cortical region of the unfertilized oocyte" (Wassarman & Albertini, 1994) that release their contents after fusion with oolemma at the time of fertilization into the perivitelline space (Dandekar & Talbot, 1992). The contents released into the perivitelline space are composed of proteinases and ovoperoxidase that alter the properties of the zona pellucida in a manner such that polyspermy is blocked. These reactions only occur after the sperm cell has penetrated the zona pellucida and exocytosis of the CGs has spread rapidly along the entire surface of the egg. One study (Cran & Cheng, 1986) reported that the cortical reaction of the pig egg could be induced experimentally with the addition of calcium without fetal calf serum to the medium.

One of the other fundamental developmental anomalies that occur during *in vitro* fertilization of porcine oocytes is either a low rate of or asynchronous pronuclear formation (Iritani *et al.*, 1978; Naito *et al.*, 1988; Hunter, 1990). According to the literature, asynchronous pronuclear formation is characterized by "a delay in male pronuclear development following the normal formation of the female pronucleus after fertilization" (Grupen *et al.*, 1995). The formation of the male pronucleus is dependent on proper cytoplasmic maturation of the oocyte and synthesis of male pronucleus growth factor(s), which provide the oocytes the ability to de-condense sperm heads (Nagai *et al.*, 1984; Grupen *et al.*, 1995; McLay & Clarke, 1997). Synthesis of other substances, such as glutathione, has also been reported to increase the ability of oocytes to form male pronuclei (Yoshida, 1993; Yoshida *et al.*, 1993[a]; Funahashi *et al.*, 1994; Grupen *et al.*, 1995). The compounds present in the maturation medium can dramatically affect the capacity of the

cumulus-oocyte-complexes to achieve cytoplasmic and nuclear maturation and therefore improve *in vitro* fertilization and development of *in vitro*-produced embryos (Rath *et al.* 1995; Funahashi & Day, 1997; Abeydeera *et al.*, 1998[a,b]; Iwasaki *et al.*, 1999)

Another reason for asynchronous pronuclear formation is incomplete sperm capacitation under *in vitro* conditions (Yoshida *et al.*, 1990). Capacitation is defined as "the physiological (functional) changes that render the spermatozoa competent to fertilize' (Yanagimachi, 1994). This process is known to be complete by the time the spermatozoa reach the isthmus and await ovulation of the ova. The precise mechanisms involved in capacitation of spermatozoa are still unknown. According to Yanagimachi (1994), there has been evidence reported that supports changes in the plasma membrane and increased activity of adenylate cyclase and cAMP availability. This is a difficult area of research due to the fact that not all sperm cells will live and, therefore, cannot undergo capacitation.

This paper will review many of the modifications made to improve *in vitro* fertilization of porcine oocytes, particularly in reference to polyspermy. There are many aspects from the sperm/boar side of the IVF system that can add variability to the results obtained from this technology. Individual boar variability with regard to the ejaculated semen is a concern but advancements have been made to minimize these effects. The use of cryopreserved ejaculated and epididymal sperm cells, can also reduce the variability of porcine IVF and may enhance the evolution of more efficient porcine IVF systems.

Preparation of Spermatozoa for IVF

Concentration of Sperm

There have been numerous studies investigating the mechanisms of polyspermy in the pig including possible methods to prevent its occurrence (Nagai *et al.*, 1984; Zheng & Sirard, 1992; Coy *et al.*, 1993[c]; Wang *et al.*, 1994; Dubuc & Sirard, 1995; Xu *et al.*, 1996[a,b]; Rath & Niemann, 1997). The overriding theme of the studies was decreasing the sperm concentration in both ejaculated and epididymal semen. As one would expect, the majority of these experiments reported an increase in the penetration rates and polyspermic penetration of oocytes as the concentration of sperm increased.

Coy *et al.* (1993[c]) fertilized oocytes with 3 different concentrations (3×10^5, 6×10^5 and 12×10^5 cells/mL) of sperm cells to determine their effects on penetration rates and polyspermy. This study reported that 6×10^5 sperm/mL was the optimal concentration for co-culture of oocytes for 4 h in the medium TCM-199. This concentration of sperm achieved comparable fertilization rates to the highest concentration as well as similar monospermic penetration rates at the lowest concentration. Finding the optimal concentration of sperm cells that result in the highest fertilization rates of oocytes with the lowest number of polyspermic inseminations still remains the goal of IVF systems in the pig.

Fresh or Stored Ejaculated or Epididymal Sperm

There have been reports of successful *in vitro* fertilization of pig oocytes by fresh or frozen-thawed ejaculated or epididymal sperm (Iritani *et al.*, 1978; Nagai *et al.*, 1984; Nagai *et al.*, 1988; Wang *et al.*, 1994). The use of frozen sperm would allow the testing of various *in vitro* conditions using the same sperm with certain fertilization characteristics (Wang *et al.*, 1991). Additionally, there would be no need to schedule collection of fresh ejaculated sperm for each set of experiments. The advantage of ejaculated over epididymal semen is that it can be obtained repeatedly from the same

boar. Epididymal sperm, however, can only be collected from boars that have been euthanized or recently deceased. However, techniques to freeze such sperm have been developed, which alleviate some of the pitfalls of using epididymal semen (Ikeda *et al.*, 2002).

The preparation of the sperm for fertilization generally includes a pre-incubation period to induce capacitation of the sperm cells. In a recent study (Ikeda *et al.*, 2002), it was shown that frozen epididymal sperm cells do not need a pre-incubation period prior to addition to oocytes *in vitro*. All of the factors that induce capacitation of sperm cells are still unknown, but it is speculated that the freezing procedure may initiate the capacitation process as well as the binding ability of sperm cells to the oocytes via the acrosome reaction. "Results indicate that, under the *in vitro* conditions studied, boar spermatozoa undergoes capacitation and a true acrosome reaction during co-incubation with oocytes even when not washed or pre-incubated." (Martinez *et al.*, 1996).

The addition of caffeine to the fertilization medium has been used extensively to induce capacitation of sperm used in IVF systems (Yoshida, 1987; Nagai *et al.*, 1984; Mattioli *et al.*, 1989; Wang *et al.*, 1991; Funahashi & Day, 1993). Wang *et al.* (1991) showed that the penetration rates of oocytes by frozen-thawed spermatozoa were increased proportionally to the concentration of caffeine in the medium. The addition of heparin to the medium, however, combated the beneficial effects of the caffeine. Recently, adenosine has been examined for its ability to improve penetration characteristics of frozen-thawed spermatozoa (Funahashi & Nagai, 2001). Both caffeine and adenosine have increased penetration rates compared to control oocytes, but only the adenosine maintained the monospermic penetration rate seen with the control oocytes as compared to the caffeine-treated system.

Co-incubation Time Periods

There have been several experiments (Nagai *et al.*, 1984; Coy *et al.*, 1993[b], Choi *et al.*, 1995) performed to determine the proper conditions and concentration of sperm cells to be used for in assisted reproductive technologies. The length of co-incubation of fresh, ejaculated sperm has been determined to be between 4 to 6 h, which was the interval where both adequate penetration and reduced incidence of polyspermy using a high concentration of sperm (2×10^6 sperm/mL) occurred (Coy *et al.*, 1993[b]). Nagai *et al.* (1984) previously had investigated co-incubation parameters for use of epididymal spermatozoa in IVF. This study found that pre-incubation in a defined medium at concentrations of $4\text{-}16 \times 10^8$ cells/mL achieved 71-75% penetration rates, but penetration rates were reduced to 11% at lower concentrations (0.8×10^8 cells/mL). In spite of these many studies, the fact still remains that regardless of the sperm concentration used, the length of time that oocytes and sperm cells are allowed to interact generally increases the incidence of sperm penetration and, therefore, polyspermy.

Medium and Components

Fertilization Medium

IVF of porcine oocytes has been accomplished in a variety of different medium formulations (Abeydeera, 2002). Some of the more common formulations that have been used for IVF in swine include Tissue Culture Medium 199 (TCM-199), modified Tris-buffered medium (mTBM), modified Whitten's (mWM) and modified Tyrode's medium

(mTALP). These different media have been analyzed for penetration rates and the incidence of polyspermy. Researchers have compared two different IVF media, mTBM and mTALP for their ability to enhance penetration rates and limit polyspermy (Martinez-Madrid *et al.*, 2001; Kidson *et al.*, 2001). Both these studies showed that mTBM had higher penetration rates and lower incidence of polyspermy than oocytes fertilized in mTALP. However, these results were dependent on sperm concentration at IVF. From these studies, it seems that proper selection of an appropriate IVF medium may also be a critical factor in minimizing polyspermy.

Supplements

There have been a number of experiments performed to determine which medium will produce viable embryos with a decreased incidence of polyspermy and acceptable penetration rates (Choi *et al.*, 1995). According to the literature, there are various molecular factors in the female reproductive tract that regulate the incidence of polyspermy *in vivo* (Nagai & Moor, 1990; Hunter, 1991). Although the mechanisms by which these factors affect sperm penetration are unknown, it is known that the fluid in the follicles and oviduct assist in the fertilization process. Funahashi and Day (1993) investigated the effect of follicular fluid on sperm penetration and found that with the addition of 10% follicular fluid increased the percentage of monospermic penetration.

Caffeine is also typically added to the fertilization medium. The mechanism by which caffeine effects fertilization is that it increases intracellular cyclic AMP (cAMP) in the sperm resulting in increased motility. Caffeine is a phosphodiesterase inhibitor, the enzyme that breaks down cAMP, and the net result of its addition is accumulation of cAMP in sperm. Recently, Funahashi *et al.* (2000) examined the effect of caffeine and showed pig oocytes had greater penetration (98%) then controls when caffeine was added to the medium. They also examined the effects of fertilization promoting peptide (FPP) and adenosine supplementation to fertilization medium. These two molecules were not as effective as caffeine at promoting oocyte penetration with 75% penetration for FPP and 71% penetration for adenosine. However, there were a greater percentage of polyspermic oocytes with caffeine (87%) as compared to FPP (25%) or adenosine (21%).

Among the other modifications to IVF procedure has been the addition of hyaluronan (0.5 mg/mL) (Suzuki *et al.*, 2000), glutathione (GSH) (Boquest *et al.*, 1999) and oviduct-specific secretory glycoprotein (pOSP; Buhi *et al.*, 2000) to the fertilization medium. The addition of the hyaluronan had a beneficial effect on monospermic penetration during the IVF process. This study also showed that the improvement in monospermic penetration was enhanced by the addition of seminal plasma. However, a later study (Suzuki *et al.*, 2002) observed a detrimental effect when seminal plasma (10%) alone was added to the fertilization medium. The addition of GSH resulted in higher blastocyst development and the mechanism, although unknown is thought not to be increased GSH in the zygotes but a protective effect from reactive oxygen-species generated by dead or dying sperm in the fertilization drops (Boquest *et al.*, 1999). Finally, a porcine oviduct-specific secretory glycoprotein (pOSP), which is estrogen-dependent, was added to the medium and oocytes were cultured in this solution for 4 hours before and during sperm incubation in the IVF drops. The result of exposure to pOSP was a decrease in polyspermy (29%) compared to the controls (61%) with no decrease in sperm penetration (Kouba *et al.*, 2000). All of these studies indicate that there are specific modifications to the sperm incubation medium that can decrease the incidence of polyspermy during fertilization *in vitro*.

Co-culture of Sperm Cells with Other Cells

It has been shown that oviductal cells have a dramatic effect on penetration of the oocyte by sperm cells under *in vitro* conditions. Nagai and Moor (1990) were among the first researchers to attempt to recreate the environmental conditions of the oviduct during fertilization by co-culturing sperm cells with oviductal cells prior to IVF. This study suggested that there are products of the epithelial cells of the oviduct that affect the sperm penetration characteristics and, therefore, affect polyspermic penetration of the oocytes.

There have been experiments performed that support the theory that oviductal epithelial cell co-culture with oocytes (instead of sperm) was beneficial in decreasing polyspermy while maintaining penetration rates (Kano *et al.*, 1994). Additionally, Dubuc and Sirard (1995) co-cultured spermatozoa with oviductal cells prior to *in vitro* fertilization and found that it was successful in reducing the incidence of polyspermy and increasing the proportion of cells with 2 pronuclei without having detrimental effects on penetration rates. If steroids, such as estradiol and progesterone, were added to the system, different effects were observed on monospermic penetration. The pre-treatment of isthmus cells with estradiol decreased polyspermic penetration when compared to pre-treatment of ampulla cells with estradiol or either cells type treated with progesterone (Dubuc & Sirard, 1996).

Park and Sirard (1996) tested various pre-incubation times (0, 1, 2, 3, and 4 h) for frozen-thawed sperm cells with oviductal vesicles and epithelial cell monolayers. There were no significant advantages observed in the various time periods except when pre-incubating sperm for 1 or 2 h with oviductal vesicles and epithelial cell monolayers. These two time periods maintained penetration rates without adversely (*i.e.*, increasing) affecting polyspermy rates during IVF.

Equipment

Traditional Culture Equipment

During IVP in swine, oocytes, embryos and sperm are pipetted and washed through a variety of drops of different media. There may be greater than 20 different wash steps that occur in a typical IVP protocol. During the culture periods, gametes are placed into drops of specific medium for each stage of IVP and covered with oil in polystyrene dishes tested for embryo culture. For *in vitro* fertilization, matured oocytes are placed into a drop of fertilization medium covered with oil and sperm cells are added to this drop to achieve the appropriate predetermined concentration of the experiment. Oocytes and sperm cells are then co-incubated for a period of 4-6 h at 39°C in an humidified 5% CO_2 in air atmosphere.

Microfluidic Systems

The equipment and methodologies used for *in vitro* fertilization (IVF) research is also an area that is constantly being investigated and re-invented. Emerging technology involving microfluidic environments (microchannels) for embryo culture and *in vitro* production of embryos could revolutionize the swine industry (Clark *et al.*, 2001; Walters *et al.*, 2001; Beebe *et al.*, 2002; Clark *et al.*, 2002). The microfluidic devices provide a culture environment that mimics the oviduct and would allow studies involving static as well as dynamic culture systems. Additionally, microchannels would allow for the

integration of the different steps of the *in vitro* embryo production (*in vitro* maturation/ fertilization/culture) within the same equipment with appropriate exchange of the medium for each stage.

In vitro fertilization experiments in the microchannels resulted in a total of 113/236 (48%) and 90/210 (43%) embryos cleaved after fertilization in the 50 μL drops and microchannels, respectively. Additionally, 43/71 (61%) and 42/70 (60%) presumptive zygotes were fixed and stained with aceto-orcein and found to have male pronucleus formation. There was no significant difference ($p>0.05$) found between the two fertilization systems with regard to pronuclear formation, however, the incidence of polyspermy is decreased in the microchannels (Clark, Beebe & Wheeler, unpublished). These data suggest that the microchannel culture system supports IVF of porcine oocytes and may potentially be a method in which controlled delivery of sperm cells can be accomplished.

Recent data (Clark, Beebe & Wheeler, unpublished) involving the maturation and subsequent fertilization of oocytes within the microchannels revealed no significant difference ($p>0.05$) between the cleavage rates of those embryos and control *in vitro*-matured and fertilized oocytes. A total of 92/180 (51.1%) and 91/185 (49.2%) embryos cleaved after fertilization in the 50 μL drops and microchannels, respectively. These were the initial experiments involving medium changes without disturbing the oocytes during the processes of IVM and IVF and suggest that the microchannels can support *in vitro* oocyte maturation, IVF and subsequent cleavage of porcine embryos produced *in vitro*.

Other modifications to the equipment that have been used to effect the selection of sperm in IVF has been described as the climbing-over-a-wall (COW) method used by Funahashi and Nagai (2000). They used different concentrations of sperm (0.5×10^5, 1×10^5, 5×10^5, and 10×10^5 cells/mL) placed into the outer chamber filled with fertilization medium for insemination of denuded oocytes, which were placed in an inner well of the COW chamber. This experiment resulted in higher penetration rates at increased sperm concentration while still maintaining increased monospermic penetration compared to the controls. Additionally, there was a significantly higher incidence of monospermic penetration in the COW method as the concentration of sperm was decreased.

Concluding Remarks

Although there have numerous experiments have been performed to improve the efficiency of IVP of porcine embryos, it is evident that a substantial amount of progress still needs to be made in order to combat the two major inherent problems of the system: polyspermy and low male pronucleus formation. Novel research involving modifications to the methodology used during the process of IVF (Funahashi & Nagai, 2000; Clark *et al.*, 2002) shows exceptional potential in selecting sperm for penetration with a significant reduction in the polyspermic penetration rates. Further research needs to be performed to determine the factors and environment that more closely mimics that of the porcine oviduct, where fertilization *in vivo* actually occurs.

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