THE EFFECT OF GROWTH HORMONE ON PIG EMBRYO DEVELOPMENT IN VITRO AND AN EVALUATION OF SPERM-MEDIATED GENE TRANSFER IN THE PIG

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(ABSTRACT)

The objective of part one of this study was to determine if the presence of porcine growth hormone (pGH) during oocycte in vitro maturation (IVM) affected subsequent embryo development. Pig cumulus-oocyte complexes (COC) (n=987) were aspirated from slaughterhouse derived ovaries and cultured in BSA-free NCSU 23 medium containing porcine follicular fluid (10% v/v), cysteine (0.1 mg/ml) and hormonal supplements (eCG and hCG, 10 IU/ml each), 10 ng/ml EGF, and with or without pGH (100 ng/ml) for 22 h. The COC were then cultured in the same medium with or without 100 ng/ml pGH, but without hormonal supplements for an additional 22 h. After the completion of maturation culture, cumulus cells were removed and oocytes were co-incubated with frozen-thawed spermatozoa for 8 h. Putative embryos were transferred to NCSU 23 containing 0.4% BSA and cultured for 144 h. Embryo development was assessed on d 6 of culture. The treatment groups were as follows: treatment 1 = control group cultured in IVM medium alone; treatment 2 = 100 ng/ml pGH present of the first 22 h of maturation culture and absent for the second 22 h of maturation culture; treatment 3 = 100 ng/ml pGH absent for the first 22 h of maturation culture, but present for the second 22 h of maturation culture; and treatment 4 = 100 ng/ml pGH present throughout the entire IVM period. Embryos were visually scored for developmental stage at 144 h following fertilization. Each oocyte in the study received a developmental score, based on a scale of 1 = uncleaved, 2 = 2-cell embryo, 3 = 4- to 8-cell embryo, 4 = 9- to 16-cell embryo, 5 = morula, and 6 = blastocyst. The addition of pGH did not affect porcine embryo development as compared to the control (1.57 ± .08, 1.67 ± .08, 1.47 ± .08, and 1.60 ± .08, respectively; $P > .10$). Replicates within the study differed significantly from each other ($P < .01$) primarily because the development in replicate 6 was greater than for all others. There was a significant treatment by replicate interaction ($P < .05$); pGH added during the first 22 h of IVM and pGH added during the second 22 h of IVM in replicate 6 resulted in higher development scores than for controls and continuous pGH addition. However, in replicate 2, continuous pGH resulted in the greatest development. These results suggest that pGH may exert a stimulatory effect on embryo development when present in the IVM media; however, further studies using pGH in IVM culture are necessary.

The objectives of the second part of the study were to examine aspects of intracytoplasmic sperm injection (ICSI) using membrane-disrupted spermatozoa, in vitro

fertilization (IVF), and sperm-mediated gene transfer in the pig. Porcine oocytes were shipped overnight in maturation media at 39°C in a portable incubator. After 22 h of maturation culture, oocytes were washed in maturation medium without gonadotropins and cultured for an additional 22 h. Cumulus cells were removed and oocytes were divided into four treatment groups: treatment 1 = ICSI using membrane-damaged spermatozoa coincubated with linear green fluorescent protein (GFP) DNA; treatment 2 = ICSI using membrane damaged spermatozoa; treatment 3 = IVF with frozen-thawed spermatozoa coincubated with linear GFP DNA prior to IVF; treatment 4 = IVF with frozen-thawed spermatozoa with no DNA coincubation. Embryos were scored for developmental stage at 144 h following fertilization. Each oocyte in the study received a developmental score, based on a scale of 1 = uncleaved, 2 = 2-cell embryo, 3 = 4-cell embryo, 4 = 5- to 8-cell embryo, 5 = 9- to 16-cell embryo, 6 = morula, and 7 = blastocyst. Although no overall difference in development score was observed following the four different treatments, a treatment difference among cleaved oocytes was observed when comparing only the two ICSI treatments ($P < .05$); development scores were greater in the ICSI treatment in which sperm were not coincubated with linear GFP DNA prior to injection than when the coincubation was performed ($3.76 \pm .21$ vs. $3.13 \pm .17$, respectively). No differences in development score were observed in the two IVF treatments. The percentage of embryos expressing the GFP transgene on d 6 of culture following fertilization was 7.3% in the ICSI+GFP group and 0% in all other treatment groups. Thus, sperm-mediated gene transfer using ICSI in the pig has been demonstrated, although success rates were low.

Keywords: Embryo development, Growth hormone, Intracytoplasmic sperm injection, In vitro fertilization, In vitro maturation, Pig oocytes, Trangenesis

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CHAPTER I
INTRODUCTION

In recent years, genome modification of domestic species has been the focus of intense research efforts. The creation of transgenic domestic animals is important in the production of pharmaceutical products as well as in the process of xenotransplantation of organs and tissues in which the donor organs are from animals that have been genetically modified to preclude immune rejection by the recipient. The production of recombinant proteins in non-human organisms has resulted in the worldwide marketing approval of more than a dozen therapeutics to treat genetic or disease initiated deficiencies.

A major problem in the production of transgenic livestock is the limited supply of early embryos at the proper stages of development. Additionally, it is a very expensive and labor-intensive process to obtain zygotes by superovulation and surgical collection, and this often results in an asynchronous population of embryos. An alternative is to use in vitro generated zygotes for gene transfer experiments. Although piglets have been born from in vitro derived embryos, the porcine in vitro system continues to present numerous problems including poor cytoplasmic maturation, poor male pronuclear formation, polyspermy, and reduced embryo development.

In an effort to improve the porcine embryo in vitro production (IVP) system, development of a superior culture media has been a research focus in recent years. Various factors have been shown to improve the nuclear or cytoplasmic maturation of pig oocytes as assessed by maturation of oocytes to metaphase II, fertilization, male pronuclear formation, cleavage, or blastocyst development. They include follicular fluid, oviductal fluid, steroids, other types of serum from animals, and gonadotropins. Along with this supplementation, various growth factors have been added to culture medias and their effects on oocyte maturation have been examined. The role of growth hormone (GH) supplementation in the maturation media has recently been studied in the both the bovine and porcine in vitro embryo production systems.

Matured porcine oocytes can be penetrated in vitro by spermatozoa; however, problems with in vitro fertilization (IVF) including poor pronuclear formation and a high incidence of polyspermy continue to plague the porcine IVP system. Pronuclear microinjection is currently the most widely used technique in the production of transgenic pigs. This technique involves the injection of a transgene directly into either the male or female pronucleus shortly after fertilization and a large number of zygotes at this developmental stage are required for this technique. Additionally, the efficiency of the technique is low, so the development of a new, more efficient technique in transgenic pig production is desirable.

Recently, a sperm-mediated gene transfer method, in which sperm cells are used as vectors to introduce foreign DNA into oocytes, has been combined with intracytoplasmic sperm injection (ICSI) to produce transgenic mouse embryos and offspring following embryo transfers. This technique could be applied to the porcine system with the hopes of increasing efficiency of transgenic pig production as their importance is growing in both the pharmaceutical and organ transplantation fields.

CHAPTER II

REVIEW OF LITERATURE

In vitro maturation

Developmental arrests and losses of viability are often observed when embryos are cultured in vitro. There have been many studies done in an attempt to determine what conditions are needed during the in vitro maturation (IVM), fertilization and culture processes to maximize embryo production. The maturational (cytoplasmic and nuclear) and developmental (fertilization, pronuclei formation and cleavage) competencies of oocytes are influenced by both the presence of follicular fluid and the size of the follicle from which the cumulus-oocyte complex (COC) is harvested (Vatzias and Hagen, 1999). In pigs, as in many other species, immature oocytes removed from ovarian follicles can resume meiosis and complete maturation in culture.

The pig has an advantage over other domestic species in that it produces large numbers of ovulations. In pigs, numerous follicles survive the recruitment and selection phases of folliculogenesis to become dominant follicles. A size limitation does exist in terms of which follicles can be considered competent and contain a healthy oocyte that can become a viable embryo. Motlik et al. (1984) measured small antral follicles of cycling gilts and found that follicles smaller than 0.7 mm were incapable of resuming meiosis. The same study also showed that larger follicles, > 3 mm, contained some oocytes that were unable to become viable embryos in culture.

Porcine oocytes initiate meiosis during the fetal stage of development and are arrested at the diplotene stage (germinal vesicle stage) of prophase immediately after birth; oocytes resume meiosis at the time of the gonadotropin surge prior to ovulation (Illera et al., 1998). A mature, antral follicle is composed of several layers of thecal cells separated from the inner granulosal cells by a vascularized basement membrane (Racowsky, 1991). The oocyte is housed in the antrum, a fluid filled cavity that is enclosed by granulosal cells. The oocyte is surrounded by layers of cumulus cells and attached to the granulosa cells by a stalk of cumulus cells. The COC is made up of an oocyte enclosed by a glycoprotein coat, the zona pellucida, and is surrounded by tight, compact layers of cumulus cells (Racowsky, 1991). These cells have finger-like projections that extend through the zona pellucida and into the pervitelline space of the oocyte. Some of these projections interact with the surface of the oolemma.

Before the gonadotropin surge occurs, many gap junctions are present at these points of contact (Racowsky, 1991). The luteinizing hormone (LH) surge causes a disruption of the follicular gap junctional network, which interrupts the transfer of cyclic adenosine 5'-monophosphate (cAMP) into the oocyte, from its cellular source, the granulosal compartment. This reduced amount of cAMP present in the oocyte is then reduced to a level below the minimum threshold required for maintenance of meiotic arrest. Cyclic AMP has been shown to inhibit the resumption of meiosis in pig oocytes in vitro (Rice and McGaughey, 1981). In addition to the action of cAMP on the maintenance of meiotic arrest in the pig, other factors seem to be involved (Racowsky, 1985). The component could act as an arrester, which then undergoes a decrease in intra-oocyte concentration after the LH surge, or as a maturation stimulator, which may bypass the elevated levels of cAMP to induce germinal vesicle breakdown.

Following such follicular gonadotropin stimulation, the oocyte becomes irreversibly committed to resume meiosis. The process of nuclear maturation begins with the recession of the nuclear membrane of the oocyte, the germinal vesicle. This process is known as germinal vesicle breakdown and is followed by changes in the chromatin configuration of the oocyte. The chromatin of the oocyte condenses at diakenesis, in which the chromosome pairs are set free into the cytoplasm and become arranged on the equatorial plate of the spindle. This primary oocyte then undergoes two meiotic divisions to transform into the secondary oocyte. In the first division, two daughter cells are formed, but one contains most of the cytoplasm. The smaller cell is the first polar body, which in addition to chromosomes, contains mitochondria, ribosomes and cortical granules (Gordon, 1994). The egg is now referred to as a secondary oocyte. The second meiotic division then begins and proceeds up to metaphase II, but is not completed unless sperm penetration occurs or the oocyte is otherwise activated. It is the completion of this second division that entails the extrusion of the second polar body into the PVS, leaving the oocyte with the haploid number of chromosomes.

This process is known as oocyte maturation. Edwards (1965) described nuclear maturation in the pig to proceed to the metaphase II stage in 43 to 46 h in vitro. Pig oocytes require almost twice the amount of time in vitro to transform the prophase nucleus, the germinal vesicle, into condensing chromatin as do other large animals including cattle, sheep and goats (Edwards, 1965). A similar situation exists in vivo, as the time between the LH surge and germinal vesicle breakdown is 20 to 24 h (Edwards, 1965).

In addition to the nuclear maturation described above, cytoplasmic maturation is an integral part in the maturation of an oocyte. In cytoplasmic maturation, an oocyte undergoes changes that allow it to transform from a developmentally incompetent cell into one with the capacity to direct and support the events of fertilization and embryonic development (Izadyar et al., 1998). The cumulus cells are thought to be particularly important in the inductive phase of cytoplasmic maturation. During the process of cytoplasmic maturation, cell organelles such as cortical granules, mitochondria, golgi apparatus and endoplasmic reticulum undergo reorganization within the oocyte; the oocyte also acquires the ability to undergo the cortical reaction, in which cortical granules are exocytosed upon the activation of the oocyte at fertilization to act as a block to polyspermy (Ducibella et al., 1990; Ducibella et al., 1993; Yoshida et al. 1993). The ability of the oocyte to decondense the chromatin of spermatozoa is also a component of cytoplasmic maturation (Motlik and Fulka, 1974).

Pig oocytes are often cultured in maturation media supplemented with gonadotropins due to their beneficial effects on cumulus cell expansion and nuclear maturation. Follicle-stimulating hormone (FSH) can induce expansion of COCs in vitro (Singh et al., 1993a; Singh et al., 1993b). Mattioli et al. (1991) reported that FSH and LH both act to accelerate and facilitate the meiotic progression of the oocyte. Further, this study demonstrated that LH alone selectively improved the cytoplasmic maturation of the oocyte, which is required for the formation of the male pronucleus. Within the ovary, FSH acts to increase aromatase activity, increase progesterone synthesis, stimulate cAMP binding, and induce the appearance of LH receptors on the granulosa cells in preparation for the LH surge (Dorrington et al., 1983; Hsueh et al., 1984; Richards et al., 1976). This

LH surge then triggers resumption of meiosis of the oocyte, rupture of the follicle resulting in ovulation and luteinization of the follicular cells to form the corpus luteum.

In addition to the supplementation of maturation media with gonadotropins, the effects of porcine follicular fluid (pFF) have also been examined. Naito et al. (1988) found that pFF stimulated male pronuclear formation when present in maturation media. The addition of pFF also acted to increase the nuclear maturation of oocytes (Yoshida et al., 1992a). The beneficial effects of pFF on oocyte maturation, fertilization, and developmental capacity were also shown by Vatzias and Hagen (1999). Yoshida et al. (1992b) suggested that the substance(s) present in pFF that acts to improve male pronuclear formation, nuclear maturation, normal fertilization and development is an acidic substance with a molecular weight of 10 to 200 kDa.

Studies have also been performed to look at the effect of a coculture maturation system with follicular shell pieces. Beneficial effects on maturation were observed when everted follicles were present in the culture media, indicating that the effects of follicles may be mediated by soluble factors (Niwa, 1993). Mattioli et al. (1988) found that when pig oocytes were cultured with everted follicles, increased male pronuclear formation was observed in penetrated oocytes. When oocytes were matured in the presence of follicular shell pieces, improved cytoplasmic maturation and subsequent development to the blastocyst stage were observed (Abeydeera et al., 1998a). It has been shown that the maturity of the follicular shells is important as follicular cells with different maturity secrete a different quantity and/or quality of factors that stimulate cytoplasmic maturation of ooctyes (Ding and Foxcroft, 1994a).

In Vitro Fertilization

A major problem encountered with the in vitro fertilization (IVF) of porcine oocytes is the high incidence of polyspermy (up to 91% polyspermy using standard IVF procedures). Polyspermy, which results in embryonic death, could be due to inadequate maturation and/or fertilization conditions (Niwa, 1993). In vivo, there are two systems that act to prevent polyspermy in pigs. The first is a method by which the female reproductive tract decreases the number of spermatozoa that actually reach the oocyte in the isthmus of the oviduct (Hunter, 1991). In addition to the role of "sperm selection" by the female, the cortical granule reaction also acts to prevent polyspermy. The cortical granule contents are exocytosed into the PVS upon penetration of the spermatozoa into the oocyte. The cortical granules release hydrolytic enzymes into the PVS and this results in the formation of a new coat, uniformly distributed over the oocyte; this enveloping of the oocyte is followed by the modification of zona proteins and inactivation of sperm-binding receptors (Dandekar and Talbot, 1992).

Kim et al. (1996) found that incubating oocytes in a medium containing 10% or 30% oviductal fluid prior to fertilization increased the incidence of monospermy without decreasing sperm penetration. This suggests that a factor(s) from the oviductal fluid is important for the maintenance of monospermic fertilization. Additionally, a 2.5 h sperm-oviduct culture prior to fertilization reduced the rate of polyspermy 40 to 50% (Nagai and Moor, 1990). Co-culture of oocytes with oviductal epithelial cells also resulted in a higher percentage of monspermic oocytes (Kano et al., 1994).

There are many factors that are important to the success of the IVF procedure. Sperm-oocyte incubation time, sperm concentration, and IVF media are all integral

components. Abeydeera and Day (1997a) evaluated sperm-oocyte incubation times ranging from 3 h to 12 h. They found that 6 h was the optimal incubation time as judged by maximal penetration at 6 h (81%) with a moderate amount of polyspermy (39%). As incubation time increased, there was no further increase in penetration rate; however, the number of polyspermic oocytes increased with incubation time. Rath (1992) reported a high correlation between the incidence of polyspermy and the number of spermatozoa present at fertilization. Abeydeera and Day (1997a) support this finding; when sperm concentration was increased, the mean number of sperm (MNS) per oocyte increased also.

A variety of media have been evaluated for their use in IVF. In vitro fertilization is usually carried out in one of the following medias: tissue culture medium 199 (Yoshida et al., 1990; Funahashi et al., 1994; Nagai and Moor, 1990), Brackett and Oliphant (BO) medium (Wang et al., 1995; Kikuchi et al., 1993), Krebs-Ringer bicarbonate medium (Naito et al., 1988), or a modified Tris buffered medium (mTBM) (Abeydeera and Day 1997b). The mTBM contains a higher calcium concentration than the other medias and no bicarbonate ions (Abeydeera and Day 1997a). Fraser (1995) reported that mammalian spermatozoa require extracellular calcium for capacitation and maximal acrosomal exocytosis. Further, it has been shown by Harrison et al. (1993) using flow cytometry that bicarbonate stimulated a large influx of calcium into boar spermatozoa which, in the absence of external calcium, resulted in a bicarbonate mediated increase in cell death. In addition to calcium, bovine serum albumin (BSA) and caffeine are important modulators of sperm penetration (Abeydeera and Day 1997b). Abeydeera and Day (1997b) reported that the MNS per oocyte increased significantly with the addition of BSA to the IVF medium. Caffeine has also been shown to promote the capacitation of boar spermatozoa and thus fertilization (Wang et al., 1991). Thus, there are many factors that are pertinent to the successful IVF of porcine oocytes.

In Vitro Culture of Embryos

The final step in the in vitro production (IVP) of embryos is in vitro culture (IVC). Many factors determine the success of IVC, but the most important is providing adequate nutrients for the developing embryo. Supplementation of culture medium with oviductal fluid enhances the in vitro development of one-cell and two-cell pig embryos (Archibong et al., 1989). Since oviductal fluid is not readily available for routine embryo culture, early stage embryos (one to eight cells) are most often cultured in media using BSA as a protein supplement (Petters and Wells, 1993). Pig embryos can develop from the one-cell stage using glucose or glutamine as the sole energy source (Petters et al., 1990). NCSU-23, a culture medium containing taurine and hypotaurine, has been shown to support high levels of embryonic development (Petters and Reed, 1991, Reed et al., 1992). Taurine may act to serve as an osmoprotectant (Petters and Wells, 1993).

The effects of osmolarity on developing pig embryos have been evaluated. Beckman and Day (1993) showed that increased sodium concentration and not necessarily osmolarity had a negative effect on development in vitro. Embryo development (84% morula or blastocyst compared to 3% morula or blastocyst) was greater in media with a higher NaCl concentration than in media with a lower NaCl concentration. In summary, many advances have been made in the development of

culture media, but no standard media has been accepted as the sole medium for maturation, fertilization or embryo culture.

Growth Factors in the Porcine System

Even though LH and FSH are essential for follicular development, they can be termed "coarse tuners" of the system, while other factors produced locally within the follicle can operate as "fine tuners" (Dorrington et al., 1987). Peptide growth factors have been implicated as autocrine/paracrine regulators of ovarian function and the role of growth factors in oocyte maturation is gaining increasing attention. Epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) are among the most widely studied regulators of ovarian function. Singh et al. (1995) reported that mRNA for EGF and its receptor were present in the pig oocyte, cumulus and granulosa cells. Insulin-like growth factor-I is also present in significant quantities in the oviductal fluid of the pig (Wiseman et al., 1992).

Epidermal growth factor and IGF-I have both been evaluated for their effects on the IVM and IVF of porcine oocytes. Further, their effects on early embryonic development have also been examined. Epidermal growth factor is thought to regulate resumption of meiosis through the disruption of communications between the oocyte and surrounding cumulus cells (Knecht and Catt, 1983; Dekel and Sherizly, 1985). Reed et al. (1993) found that the addition of EGF to the oocyte maturation media lacking follicular fluid significantly stimulated the resumption of pig oocyte nuclear maturation in vitro. Epidermal growth factor also enhanced the cytoplasmic maturation of oocytes through interactions with gonadotropins (Ding and Foxcroft, 1994b).

The effect of EGF on porcine oocytes is likely to be mediated, in part, by cumulus cells since denuded oocytes show no significant increase in germinal vesicle breakdown when treated with EGF (Coskun and Lin, 1994). Treatment of porcine oocytes with FSH increased the number of EGF binding sites on granulosa cells (Feng et al., 1987, Fujinaga et al., 1992). The number of EGF binding sites was decreased when oocytes were treated with LH or human chorionic gonadotropin (hCG) (Feng et al., 1987). The mechanism(s) of the interaction between EGF and gonadotropins is most likely not due to a change in steroid production in follicular tissues, since EGF has no significant effect on the production of estrogen or progesterone in vitro (Ding and Foxcroft, 1994b).

Abeydeera et al. (1998b), demonstrated that EGF present at a concentration of 10 ng/ml in the maturation media enhanced post-cleavage development of porcine embryos to the blastocyst stage; however, when the concentration of EGF was increased to 40 ng/ml, increased blastocyst formation was not observed. Additionally, blastocyst stage embryos obtained from oocytes matured in media containing 10 ng/ml EGF had greater numbers of cells per blastocyst than contol embryos (Grupen et al., 1997). There appears to be no additional benefit in adding EGF to the embryo cutlture media (Abeydeera et al., 1998b). The above demonstrates that EGF can enhance both the nuclear maturation and cytoplasmic maturation of oocytes and early embryo development when added to the maturation media. Transforming growth factor-alpha (TGF-α), which shares the same receptor with EGF on porcine COCs, also enhanced the maturation of oocytes while transforming growth factor beta (TGF-β) inhibited the IVM of porcine oocytes (Coskun and Lin, 1994).

Insulin-like growth factor-I has been shown to be beneficial to porcine embryo development when it is present in either the maturation or culture media (Xia et al., 1994). The investigators demonstrated that cleavage rate and embryo development beyond the 8-cell stage were improved when oocytes were matured in the presence of IGF-I; the treatment of putative zygotes following IVF with IGF-I also increased total oocyte cleavage and embryo development to the 8- to 16-cell stage. In contrast, studies performed by Reed et al. (1993) and Grupen et al. (1997) found that addition of IGF-I to the maturation media had no beneficial effect on the maturation of oocytes or subsequent embryo development. Singh and Armstrong (1997) found that IGF-I enabled porcine cumulus cells to expand in response to FSH; they observed full cumulus expansion in a chemically defined maturation medium containing IGF-I

The IGF-I receptor is present on pig granulosa cells (Adashi, 1992) and there is IGF-I induced stimulation of granulosa cell proliferation and an overall increase in synthetic activity, both of which are likely to enhance embryo development (Xia et al., 1994). The mechanism(s) by which IGF-I acts to improve embryo development remains unclear. The increased development could be due to autocrine stimulation of granulosa and/or cumulus cells by IGF-I, or, IGF-I may act directly via a paracrine interaction with IGF-I or insulin receptors on preimplantation embryos (Xia et al., 1994).

Growth Factors in the Bovine System

More work has been done in the bovine system than the porcine system with IVM, IVF, and embryo culture. Studies have investigated the effects of a large number of growth factors including EGF, IGF-I, TGFα and growth hormone (GH). Epidermal growth factor was shown to stimulate cumulus expansion and increase oocyte fertilization when present in maturation media (Kobayashi et al., 1994). While no significant enhancement in cumulus expansion, oxidative metabolism, nuclear maturation or cleavage was observed with the addition of IGF-I alone to the maturation media, the addition of IGF-I in combination with EGF increased cumulus expansion, oxidative metabolism and nuclear maturation, suggesting that the effects of the two growth factors are additive (Rieger et al., 1998). Transforming growth factor-alpha acted to increase cumulus expansion, nuclear maturation and fertilization rate in the bovine system when compared to a control medium (Kobayashi et al., 1994).

Growth Hormone

The presence of physiological levels of GH in the ovary has been determined, indicating a potential relevance of GH in reproductive biology (Apa et al., 1994). Izadyar et al. (1997a) showed the presence of growth hormone receptor (GHR) mRNA in granulosal cells, cumulus cells and in the oocyte. While IGF-I has been shown to mediate several GH functions, including skeletal growth (Nicola, 1994), this is not the case in the oocyte maturational process (Izadyar et al., 1997b), as the presence of an IGF-I antibody in culture with GH did not affect the stimulatory effects of GH on oocyte maturation. The stimulatory effect of GH on oocyte maturation and cumulus expansion was shown to be mediated by a cAMP signal transduction pathway.

Growth hormone present at concentrations of 100 ng/ml and 1000 ng/ml acted to accelerate nuclear maturation, induce cumulus expansion and increase cleavage rate and blastocyst formation in the bovine (Izadyar et al., 1996). The addition of GH also

accelerated cytoplasmic maturation of bovine oocytes (Izadyar et al., 1998). A study by Hagen and Graboski (1990) found evidence that the presence of GH enabled porcine oocytes to respond to an activation stimulus at an earlier point during the maturation process, indicating accelerated maturation.

Intracytoplasmic Sperm Injection (ICSI)

During normal fertilization, the sperm nucleus is incorporated into an oocyte through membrane fusion between the two gametes. Intracytoplasmic injection (ICSI) is a micromanipulation technique currently used in the clinical setting to alleviate male factor infertility. "Male factor" is a term used to describe an individual with one or more semen abnormalities that may impair their fertilizing capability. The ICSI procedure overcomes many barriers of fertilization and involves the injection of a single spermatozoon directly into the ooplasm of a mature, metaphase II oocyte.

The earliest recorded attempt of sperm injection occurred in 1914 when G.I. Kite microinjected starfish spermatozoa into starfish oocytes but with inconclusive results (Lillie, 1914). Hiramoto (1962) microinjected sea urchin spermatozoa directly into sea urchin oocytes to examine oocyte activation and post fertilization events. In this study, although no activation or sperm decondensation occurred, when the injected eggs were subsequently inseminated with spermatozoa using conventional IVF techniques, the injected oocytes underwent activation and sperm decondensation with 40-50% exhibiting polyspermic cleavage. This result suggested that the injected sperm may take part in the mitotic process when the egg was activated from the outside as only 10% of the control eggs exhibited polyspermy. Hiramoto concluded that the substance in the spermatozoon that activates the sea urchin egg during fertilization may not be released unless the spermatozoon reacts directly with the egg surface from the outside.

Uehara and Yanagamachi (1976) microinjected isolated nuclei from hamster and human spermatozoa into hamster oocytes. When hamster sperm nuclei were injected into oocytes, 69% of oocytes injected demonstrated formation of both male and female pronuclei. In the same study, similar results were obtained when frozen-thawed or freeze-dried nuclei of human spermatozoa were injected into hamster oocytes as 72% of the microinjected hamster eggs contained decondensing sperm heads or male pronuclei. This study indicated that unlike sea urchin oocytes, hamster eggs could in fact be activated as a result of the injection, perhaps by the mechanical stimulation of the egg plasma membrane or the egg cytoplasm by the micropipette. Furthermore, the investigators concluded that sperm nuclei are stable organelles and the egg cytoplasmic factors controlling the transformation of sperm nuclei into male pronuclei are not strictly species specific since human sperm nuclei developed into apparently normal pronuclei in hamster ooctyes.

Hamster oocyte activation following cytoplasmic injection was further examined by Uehara and Yanagamachi (1977a). Hamster oocyte activation was mechanically induced with glass pipets greater than 5 μm in diameter in the presence of calcium ions. The investigators stated that the disruption in the oolemma followed by formation of a new membrane may change the permeability of the oocyte membrane, resulting in an increased ion influx and/or efflux and increased intracellular levels of free calcium ions, resulting in subsequent oocyte activation.

Uehara and Yanagamachi (1977b) also investigated the decondensation and pronuclear formation ability of immature and mature sperm nuclei after injection into hamster eggs. The investigators microinjected the isolated nuclei from testicular, caput and cauda epididymal hamster spermatozoa into hamster oocytes. While all three types of nuclei decondensed in the egg cytoplasm, only sperm form the testes (68%) and cauda epididymides (61%) demonstrated pronuclear formation. Only 3% of oocytes injected with caput epididymal spermatozoa contained a male pronucleus. Since testicular sperm nuclei are the least stable, most immature sperm, the researchers suggested that the hamster sperm from the caput epididymis may contain a "factor" that prevents pronuclear formation, and that this factor may be absent or inactive in testicular spermatozoa and is added to or activated in caput nuclei and then removed or inactivated in spermatozoa in the cauda epididymis as the sperm maturational process takes place.

In addition to the early work done with ICSI in the hamster, fertilization following sperm injection has been investigated in many other species. Thadani (1980) looked at heterospecific sperm-egg interactions in the rat, mouse, and deer mouse using ICSI. In this study, when rat eggs were injected with mouse sperm, the eggs underwent normal initial cleavages and produced hybrid zygote nuclei that cleaved. Markert (1983) examined the fertilization of mouse oocytes following injection of mouse and deer mouse spermatozoa; normal fertilization events were observed in both cases. These results are similar to those shown in the hamster, exhibiting the ability of the sperm from one species to respond normally and form a pronucleus when injected into the oocytes of another species; this study also extended previous work by showing that mouse eggs injected with mouse sperm could develop to the blastocyst stage in vitro.

Hosoi et al. (1988) examined whether an intact rabbit spermatozoon injected into the cytoplasm of an ovulated oocyte could develop into the male pronucleus. Pronuclear formation was observed in 45% of surviving oocytes following injection and after transfer of twelve 2- to 4-cell embryos to two recipients does, two normal offspring were born. Keefer (1989) further explored the use of the rabbit and the ICSI procedure. In this study, both isolated sperm nuclei and whole spermatozoa were found to undergo pronuclear formation following injection into rabbit oocytes. However the method of sperm preparation used prior to injection was found to have an effect on pronuclear formation in activated ova. When capacitated spermatozoa were injected, significantly more pronuclear formation was observed than when non-capacitated spermatozoa were injected. This study further demonstrated that salt extraction of spermatozoa detrimentally affected the rate of oocyte activation.

A later study by Hosoi and Iritani (1993) using the rabbit model examined the use of activation factors in conjunction with the ICSI procedure. In this study, some oocytes were treated with 10 μM of the Ca^{++} ionophore, A23187, following sperm injection. The proportion of eggs that developed to the 2- to 4-cell stage increased from 20% in the untreated group to 50% in the group treated with the ionophore, indicating that in the rabbit, subsequent Ca^{++} ionophore activation acts to improve cleavage.

Kimura and Yanagamachi (1995) reported that sperm injection using a piezo-driven micropipette was far less traumatic to mouse oocytes than the conventional method using a mechanically driven pipette when performing ICSI. This piezo-electric effect is used to produce a stabbing, punctate movement to puncture the oolemma with minimal distortion of the oocyte. The incidence of normal fertilization was about ten

times higher in piezo-operated eggs than in eggs injected using the conventional method. Furthermore, the developmental ability of those oocytes injected with the piezo-driven pipette was much greater than those injected with the conventional method, 68% vs. 33% blastocyst development. One-hundred and six embryos that were obtained form ICSI were transferred into foster mothers at the 2-to 4-cell stage and 30 young pups were born and grew into normal adults.

Lacham-Kaplan and Trounson (1995) reported a 30% increase in mouse pronuclear formation when spermatozoa were treated with calcium ionophore prior to ICSI to induce the acrosome reaction. Pronuclear oocytes developed to blastocysts in vitro and to term when transferred to recipient mothers at similar rates to zygotes formed after insemination in vitro. The authors postulated that acrosome-intact spermatozoa remain resistant to sperm nuclear envelope breakdown and sperm head decondensation within the cytoplasm. This study also reported no increase in fertilization rates after activation of oocytes with 8% ethanol before or after the ICSI procedure and no evidence of parthenogenetic activation of oocytes by the sperm solution used in ICSI.

Studies using the mouse model demonstrated that spermatozoa motility and plasma membrane integrity are not essential for fertilization and the production of live offspring. In a study by Wakayama et al. (1998a), epididymal mouse spermatozoa were frozen in various media: CZB (Chatot et al., 1989), phosphate buffered saline (PBS), or isotonic saline, with or without cryoprotectant. In all groups live-dead staining confirmed that all spermatozoa were non-motile immediately after thawing except for a few (1-3%). Almost all oocytes injected with sperm heads (nuclei) from spermatozoa frozen with or without cryoprotectant fertilized normally and developed to the 2-cell stage (89-100%). While no differences in fertilization or cleavage were found to exist between the groups, development to the blastocyst stage was greatest when spermatozoa frozen in CZB medium were used for ICSI (80-94%). Normal fertile offspring were obtained in all treatments. In a different study by Wakayama and Yanagamachi (1998b), mouse caudae epididymal spermatozoa were freeze-dried and kept in vacuum-sealed ampules. The sperm were then rehydrated and injected into mouse oocytes. The majority of fertilized eggs developed into mourlae/blastocysts in vitro and normal offspring when transferred to surrogates. These studies demonstrate that non-viable spermatozoa can retain their genetic and reproductive potential and support normal development when injected directly into oocytes.

Kasai et al. (1999) reported that sperm immobilization and Triton X-100 treatment of human and mouse spermatozoa prior to injection into oocytes rendered the ICSI technique more effective. The researchers found that membrane-intact spermatozoa were the least effective in activating oocytes; most efficient oocyte activation was obtained when oocytes were injected with Triton X-100 treated sperm. The detergent-treated spermatozoa also decondensed and formed pronuclei much faster than those of immobilized or intact spermatozoa. The fast oocyte activation by the detergent-treated spermatozoa may be explained by the quick contact of the perinuclear material with ooplasm after injection. In contrast to this study, Ahmadi and Ng (1997) reported no difference in fertilization rates following ICSI of membrane damaged mouse sperm that had been treated with Triton X-100 into mouse oocytes. Further, rates of development to the blastocyst stage, inner cell mass (ICM) number, and total embryo cell number were not different among embryos that had been injected with intact and detergent-treated

sperm. Finally, this study showed no differences in implantation rates and live fetus rates between the two groups after transfer to recipient females. These data, in contrast to the data presented by Kasai et al. (1999), show that destruction of the sperm plasma membrane with detergent prior to ICSI does not affect fertilization and further development in the mouse.

In addition to using conventional laboratory animals as models for ICSI, the technique has been attempted in a number of species of farm animals. Catt and Rhodes (1995) demonstrated that ovine, bovine, and porcine oocytes could undergo normal fertilization, as evidenced by pronuclear formation, and limited development without exogenous activation. Goto et al. (1990) reported the birth of live calves after utilization of the ICSI technique. This procedure used capacitated epididymal spermatozoa that were killed by freezing at -20ºC. Following sperm injection, oocytes were treated with the calcium ionophore, A23187, to induce activation. In a later report (Goto, 1993), developmental rates were compared when bovine oocytes were injected with intact, non-viable sperm and sperm heads derived from sonication treatment to separate the heads from tails. Development of oocytes injected with whole sperm was significantly greater (10.7% morula/blastocyst) compared to oocytes injected with sperm heads alone (2.4% morula/blastocyst). The authors postulate that the decreased development may be due to nuclear damage that occurred during the sonication process.

A study by Motoishi et al. (1996), using the bovine model evaluated the safety of the ICSI procedure using bovine zygotes. Oocytes were matured and inseminated in vitro. After in vitro fertilization, sham-ICSI treatments were performed on some oocytes to determine if the ICSI technique itself is detrimental to embryonic development and quality. Intracytoplasmic injection of either medium alone or medium containing polyvinylpyrrolidone (PVP) into bovine oocytes had no harmful effects on the rate of normal fertilization, blastocyst development, or the number of cells present per blastocyst.

The ICSI technique was recently evaluated in the pig (Lee et al., 1998). The investigators determined that the normal fertilization processes, as determined by pronuclear formation, pronuclear opposition, and syngamy occur following intracytoplasmic sperm injection of round spermatids into in vitro matured pig oocytes. This study also showed that the microtubule dynamics and chromatin configuration of porcine oocytes fertilized by ICSI were similar to those observed during conventional fertilization. Kim et al (1998) demonstrated that porcine oocytes injected with either a spermatozoon or isolated sperm head were capable of development to the blastocyst stage in vitro at efficient rates (45% and 31%, respectively).

Kolbe et al. (1999) further investigated the ICSI technique in the pig using fresh ejaculated or frozen-thawed epididymal spermatozoa injected into either in vivo or in vitro matured oocytes. The best result was obtained when fresh in vitro capacitated spermatozoa were injected into in vivo matured oocytes. The proportion of fragmented oocytes resulting from injection was also significantly lower in in vivo matured oocytes compared to in vitro matured oocytes. Finally, this study also examined the effect of calcium ionophore treatment of oocytes following sperm injection. It was found that ionophore treatment was ineffective at 100 μM and at 50 μM a significant reduction in cleavage rate was observed. No favorable effect of the calcium ionophore was observed.

Recently, Martin (2000) reported the birth of three piglets following ICSI and subsequent transfer of developing embryos to a recipient.

Intracytoplasmic sperm injection has also been investigated in the horse. Dell' Aquilla et al. (1997) compared conventional IVF to ICSI by fertilization rates of aspirated oocytes. The percentage of normally fertilized oocytes showing two pronuclei or cleavage was significantly higher with ICSI than IVF (29.8% vs. 17.1%). Guignot et al (1998) injected in vitro matured equine oocytes and subjected them to activation with 10 μM calcium ionophore following ICSI. Sixty-eight percent of injected oocytes fertilized normally and 46% of the oocytes exhibited cleavage. Foals have been born after ICSI of in vitro matured oocytes (Grondahl et al., 1997).

Rhesus monkey oocytes injected with sperm obtained form fertile males resulted in a 76.6 ± 14.9% fertilization rate (Hewitson et al., 1998). These injected oocytes were cultured in vitro and developed to the blastocyst stage with a morphologically normal appearance. The transfer of fourteen 3- to 8-cell rhesus monkey embryos to 9 recipient females resulted in 6 (66%) pregnancies. Four live births and one still birth were observed as a result.

To determine the ability of human oocytes to undergo and survive the ICSI procedure, normal sperm were injected into in vitro matured human oocytes. Thirteen hours following injection, oocytes were fixed and examined (Lanzendorf et al, 1988). Six of 20 oocytes demonstrated normal male and female pronuclei. In a report by Ng et al. (1991), 38 human oocytes were injected with sonicated sperm nuclei, resulting in 4 pronuclear embryos and no pregnancies after embryo transfer. In 1992, Palermo and coworkers (Palermo et al., 1992) reported the first human pregnancies following ICSI and subsequent embryo transfers, two singleton and one twin pregnancy. The birth of two healthy boys from the singleton pregnancies and a healthy boy and girl from the twin pregnancies was also reported in the same study.

Oocyte activation following ICSI

The ability of the spermatozoa to induce oocyte activation following injection is a crucial requirement for successful fertilization. Oocyte activation is a cell signalling event that results in events including the cortical granule reaction, resumption of meiosis as evidenced by second polar body extrusion, and later events such as pronuclear formation and DNA synthesis (Cuthbertson et al., 1981; Sun et al., 1992; Vincent et al., 1992). Two models have been proposed to explain the mechanisms by which the spermatozoon induces the activation of the mammalian oocyte: the receptor model and the sperm factor model.

In the receptor model, it is the spermatozoa interacting with the egg plasma membrane that results in egg activation. In this model, an oocyte surface receptor is coupled to a G-protein (Miyazaki et al., 1990) or a tyrosine-kinase-mediated (Ben-Yosef et al., 1998) signalling pathway and when activated by a spermatozoa, leads to the release of calcium from intracellular stores. These sperm induced calcium oscillations usually last several hours after the initial calcium increase and the frequency shows considerable variation between species (Jones, et al., 1998). The sperm factor model, on the other hand, suggests that egg activation is initiated only after sperm-egg fusion, by introducing a sperm factor into the egg cytoplasm (Stice and Robl, 1990; Swan et al., 1990). When homogenized rabbit sperm was microinjected into rabbit oocytes, activation rates were

higher than in oocytes injected with medium alone; activated oocytes exhibited cortical granule exocytosis, pronuclear formation and cleavage (Stice and Robl, 1990). Additionally, sperm extracts do not appear to be species specific as rabbit sperm fractions activated both rabbit and mouse oocytes following injection into the cytoplasm (Stice and Robl, 1990). Finally, the ICSI procedure itself bypasses sperm-egg binding and fusion, and its success also supports the soluble sperm factor model.

Recently, a hypothesis combining the two major models was proposed (Tesarik, 1998). In this "trigger and oscillator" hypothesis, the role of the trigger or initiation of calcium oscillations, normally realized by sperm-oocyte cell surface interactions in normal fertilization, is substituted for in ICSI by an artificial calcium influx generated by the ICSI procedure itself. In this hypothesis, the sperm cytosolic factors play the role of the oscillator.

Mammalian Transgenesis

Several methods have been used successfully to introduce exogenous DNA into a developing embryo. Currently, the most widely used method is pronuclear injection. Pronuclear injection involves the injection of the transgene into either the male or female pronucleus of a zygote shortly after fertilization. Due to timing difficulties, pronuclear injection and subsequent DNA integration may occur after replication of the genome has occurred, resulting in the production of mosaics (Page et al., 1995). Mosaicism appears to be a particular problem in pigs since more than half of transgenic founders transmit the transgene to less than half of their progeny (Nottle et al, 1997). The efficiency of pronuclear injection is low. Between 0.3% and 4.0% of injected embryos result in the production of a transgenic pig (Pursel and Rexroad, 1993).

Other methods that have been employed in the creation of transgenic animals include retroviral infection of blastomeres and gene targeting in embryonic stem cells. Retroviral infection limits the size of the transgene that can be inserted to about 7 kilobases (Rulicke, 1996). The production of embryonic stem cells is a very rigorous process. In the mouse, the production of embryonic stem cells and introduction of these cells into a developing embryo to produce a chimera can take 6 to 12 months before germline transmission is evident (Nottle et al., 1997). This process would take considerably longer and be much more costly in the pig due to the longer generation time involved. Recently, transgenic rhesus monkeys possessing the GFP gene were produced by the injection of a retroviral vector containing the GFP gene into the perivitelline space of oocytes followed by ICSI (Chan et al., 2001).

Sperm-Mediated Gene Transfer

Sperm cells have also been used as vectors to introduce foreign DNA into ooctyes, resulting in the creation of transgenic animals. Transgenesis has been reported in mice (Lavitrano et al., 1989), cattle (Schellander et al., 1995), and pigs (Lavitrano et al., 1997) by incubating sperm with DNA prior to routine IVF or artificial insemination. Considerable controversy surrounds this approach however, since the results were highly variable and other laboratories have failed to produce similar results (Perry et al., 1999).

Transgenesis by ICSI

Perry et al. (1999) recently reported the production of transgenic mice using ICSI. They demonstrated efficient production of transgenic embryos as well as live offspring by coinjection of membrane-disrupted sperm and exogenous DNA into the cytoplasm of unfertilized oocytes. Important findings included that the sperm and DNA must be incubated together prior to microinjection for transgenesis to occur; and that membrane-disrupted spermatozoa were more efficient in producing transgenesis than fresh (membrane-intact) spermatozoa (77% vs. 26%, respectively. These findings indicate that a critical step for efficient SMGT is the association of the exogenous DNA and sperm submembrane structures prior to the events that occur during fertilization.

In a subsequent study (Chan et al., 2000), the investigators used ICSI-mediated transgenesis with tagged plasmid bound spermatozoa. In this study, rhodamine-tagged DNA encoding for green fluorescent protein (GFP) was incubated with sperm and used for in vitro fertilization and ICSI. The rhodamine signal could be traced using dynamic imaging and was found to be lost at the egg surface during IVF, but remains as a marker on injected sperm in the oocyte cytoplasm following ICSI. The GFP transgene was expressed in preimplantation embryos produced by ICSI, but not IVF, as early as the 4-cell stage with the number of expressing cells and the percentage of expressing embryos increasing during embryogenesis to the blastocyst stage. The GFP fluorescence was found to be undetectable by direct GFP imaging, and for detection of GFP expression, immunocytochemistry was used. Following seven embryo transfers, none of the offspring born were positive for the transgene.

Shim et al. (2000) injected isolated porcine sperm heads with membranes that had been damaged by treatment with either 1.0 M NaOH or 0.02% Triton X-100 and then incubated with the GFP transgene. In some treatments, oocytes were electroactivated just prior to injection. Of the injected oocytes, 20 to 26% of embryos reached the 4-cell stage or greater, and the percentage of those embryos expressing the GFP construct ranged from 11.8 to 58.8% as evidenced by fluorescence of the embryos.

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CHAPTER III

EFFECTS OF PORCINE GROWTH HORMONE PRESENT DURING IN VITRO MATURATION ON SUBSEQUENT EMBRYO DEVELOPMENT

ABSTRACT

The objective of this study was to determine if the presence of porcine growth hormone (pGH) during porcine oocyte in vitro maturation (IVM) affected subsequent embryo development. Pig cumulus-oocyte complexes (COC) (n=987) were aspirated from slaughterhouse derived ovaries and cultured in BSA-free NCSU-23 medium containing porcine follicular fluid (10% v/v), cysteine (0.1 mg/ml) and hormonal supplements (eCG and hCG 10 IU/ml each), 10 ng/ml EGF, and with or without pGH (100 ng/ml) for 22 h. The COC were then cultured in the same medium with or without 100 ng/ml pGH, but without hormonal supplements for an additional 22 h. After the completion of maturation culture, cumulus cells were removed and oocytes were co-incubated with frozen-thawed spermatozoa for 8 h. Putative embryos were transferred to NCSU 23 containing 0.4% BSA and cultured for 144 h. Embryo development was assessed on d 6 of culture and each oocyte in the study received a developmental score based on a scale of 1 = uncleaved, 2 = 2-cell, 3 = 4- to 8-cell, 4 = 9- to 16-cell, 5 = morula, and 6 = blastocyst. The treatment groups were as follows: treatment 1 = control group cultured in IVM medium alone; treatment 2 = 100 ng/ml pGH present of the first 22 h of maturation culture and absent for the second 22 h of maturation culture; treatment 3 = 100 ng/ml pGH absent for the first 22 h of maturation culture, but present for the second 22 h of maturation culture; and treatment 4 = 100 ng/ml pGH present throughout the entire IVM period. The addition of pGH used in the four treatments did not affect porcine embryo development as compared to the control (1.57 ± .08, 1.67 ± .08, 1.47 ± .08, and 1.60 ± .08, respectively; $P > .10$). Replicates within the study differed significantly from each other ($P < .01$) primarily because the development in replicate 6 was greater than in all others. There was a significant treatment by replicate interaction ($P < .05$); pGH added during the first 22 h of IVM and pGH added during the second 22 h of IVM in replicate 6 resulted in higher development scores than for controls and continuous pGH addition. However, in replicate 2, continuous pGH resulted in the greatest development. These results suggest that pGH may exert a stimulatory effect on embryo development when present in the IVM media; however, further studies using pGH in IVM culture are necessary.

Keywords: Embryo development, Growth hormone, In vitro maturation, Pig oocytes

INTRODUCTION

In pigs, as in many other species, immature oocytes obtained from follicles can resume meiosis and complete maturation in culture. Nuclear maturation proceeds to the metaphase II stage in 43 to 46 h in vitro (Edwards, 1965). In addition to the nuclear maturation, the oocyte undergoes cytoplasmic maturation, which includes changes that allow it to transform from a developmentally incompetent cell into one with the capacity to direct and support the events of fertilization and embryonic development. Although porcine oocytes matured in vitro can be penetrated by spermatozoa in vitro, high incidences of polyspermy, low incidences of male pronuclear formation, and low subsequent embryo development are common.

In vitro maturation (IVM) culture with gonadotropins has been used to improve oocyte maturation. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) act to accelerate and facilitate the meiotic maturation of the oocyte, while LH selectively improves cytoplasmic maturation (Mattioli et al., 1991). In addition to investigating the role of gonadotropins for improving oocyte maturation, the role of growth factors and their effects on the in vitro maturation, fertilization, and subsequent embryo development has been evaluated in porcine systems.

Epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) have both been evaluated for their effects on the IVM and IVF of porcine oocytes. Further, their effects on early embryonic development have also been examined. Epidermal growth factor is thought to regulate resumption of meiosis through the disruption of communications between the oocyte and surrounding cumulus cells (Knecht and Catt, 1983; Dekel and Sherizly, 1985). Reed et al. (1993) found that the addition of EGF to the oocyte maturation media lacking follicular fluid significantly stimulated the resumption of pig oocyte nuclear maturation in vitro. Epidermal growth factor also enhanced the cytoplasmic maturation of oocytes through interactions with gonadotropins (Ding and Foxcroft, 1994b). Transforming growth factor-alpha (TGF-α), which shares the same receptor with EGF on porcine COCs, enhanced the maturation of oocytes while transforming growth factor beta (TGF-β) inhibited the IVM of porcine oocytes when present in the maturation media (Coskun and Lin, 1994).

Recently, the presence of physiological levels of growth hormone (GH) in the ovary has been determined, suggesting a potential role in reproductive biology (Apa et al., 1994). In the pig, porcine growth hormone (pGH) treatment increased the number of small and medium follicles on the surface of the ovary of non-superovulated gilts (Nottle et al, 1997). Izadyar et al. (1997a) showed the presence of growth hormone receptor (GHR) mRNA in granulosa cells, cumulus cells, and in the oocyte itself. While IGF-I acts to mediate several GH functions, including skeletal growth (Nicola, 1994), this was not the case in the oocyte maturational process (Izadyar et al., 1997b). The presence of IGF-I antibody in maturation culture with GH did not affect the stimulatory action of GH on oocyte maturation; GH exerted the stimulatory effects on oocyte maturation through a cyclic adenosine 5'-monophosphate (cAMP) signal transduction pathway.

Studies have been conducted to analyze the effect of bovine growth hormone (bGH) on bovine oocyte in vitro maturation. The presence of bGH in the maturation media at concentrations of 100 ng/ml and 1000 ng/ml acted to accelerate the rate of nuclear maturation, induce cumulus expansion, and increase cleavage rate and blastocyst

formation (Izadyar et al., 1996). The addition of bGH also improved the cytoplasmic maturation of bovine oocytes (Izadyar et al., 1998). Hagen and Graboski (1990) concluded that the presence of pGH during in vitro maturation enabled porcine oocytes to respond to an activation stimulus at an earlier point during the maturation process, indicating accelerated maturation

The present study was conducted to determine the effect of the presence of pGH during different stages of IVM on subsequent embryo development in the pig.

MATERIALS AND METHODS

Culture Media

The media used for COC collection and washing was modified TL-HEPES-PVA medium (Funahashi et al., 1997). The maturation medium was BSA-free North Carolina State University 23 (NCSU-23) medium (Petters and Wells, 1993) supplemented with 0.1 mg/ml cysteine, 10% (v/v) porcine follicular fluid, 10 IU/ml eCG, 10 IU/ml hCG, and 10 ng/ml EGF. The basic medium for IVF was modified Tris-buffered medium (mTBM; Abeydeera and Day, 1997b) supplemented with 0.2% BSA and 5 mM caffeine. The long term embryo culture medium was NCSU-23 supplemented with 0.4% BSA. The maturation, fertilization, and culture media were equilibrated at 39ºC in an atmosphere of 5% CO_2 in air for a minimum of 12 h before use. Porcine follicular fluid was collected from follicles 3 to 6 mm in diameter, centrifuged at 1900 X g for 10 min, and kept at -20ºC until use.

Recovery and Culture of Oocytes

Ovaries were collected from prepubertal gilts and sows at a local slaughterhouse in Lynchburg, VA and rinsed twice in 0.9% NaCl. Ovaries were placed in Dulbecco's phosphate buffered saline (DPBS) containing 100 µg/ml penicillin G sodium and 100 µg/ml streptomycin sulphate maintained at 25 to 30ºC in an insulated cooler and transported to the laboratory. Time from ovary collection until follicular aspiration ranged from two to five hours. Oocytes were aspirated from medium size follicles (3 to 6 mm in diameter) on the surface of the ovaries with an 18-gauge needle fixed to a 10 ml disposable syringe, washed 3 times with modified TL-HEPES-P\VA medium, and then collected into a 35 X 10 mm^2 polystyrene sterile culture dish (Becton and Dickinson Labware, Franklin Lakes, NJ) of fresh modified TL-HEPES-PVA medium. Oocytes surrounded by a complex cumulus mass and with an evenly granulated cytoplasm were selected and washed three times in maturation medium containing no hormonal supplements. Then, groups of 40 to 50 randomly allocated oocytes were transferred into each well of a Nunc 4-well multidish (Roskilde, Denmark) containing 500 µl of maturation medium containing hormonal supplements, with or without pGH, that had been previously covered with warm paraffin oil and equilibrated in a CO_2 incubator. After 22 h of maturation culture, the oocytes were washed three times in hormone-free maturation medium and transferred into 500 µl of the same medium for an additional 22 h of culture.

In Vitro Fertilization

Three hours before the completion of IVM culture, an aliquot of a commercially prepared frozen semen straw (Swine Genetics, International, Ltd., Cambridge, IA) prepared from a single ejaculate was placed into DPBS containing 0.1% BSA, 75 µg/ml potassium penicillin and 50 µg/ml streptomycin sulphate (pH 7.2), pre-warmed to 39ºC and spun for 5 min at 36.3 X *g* for 5 min in a 15 ml polypropylene centrifuge tube (Fisher Scientific, Pittsburg, PA). The supernatant was collected into a pre-warmed 15 ml centrifuge tube and washed two times by centrifugation at 553 X *g* for 5 min. At the end of the washing procedure, the sperm pellet was resuspended in 100 µl of mTBM to give a final concentration of 1 to 3 X 10^7 sperm/ml. Sperm concentration was determined using a hemocytometer. Spermatozoa were preincubated at 39ºC for 2.5 h before addition to the oocytes.

After completion of culture for IVM, cumulus cells were removed with 0.1% hyaluronidase in NCSU-23 medium and washed three times with mTBM. After washing, 25 to 35 oocytes were placed in 50 µl drops of the same medium that had been covered with warm paraffin oil in a 35 X 10 mm^2 polystyrene culture dish. Oocytes were preincubated in mTBM 30 min prior to addition of spermatozoa. The concentrated sperm solution was diluted appropriately with mTBM to give a final concentration of 2 X 10^6 sperm/ml. Fifty microliters of this sperm suspension were introduced into 50 µl of the mTBM that contained the oocytes for a final sperm concentration of 1 X 10^6 sperm/ml. Oocytes were co-incubated with spermatozoa for 8 h at 39ºC in an atmosphere of 5% CO_2 in air.

Following sperm-oocyte coincubation, putative zygotes were washed three times in NCSU-23 medium supplemented with 0.4% BSA, transferred (40 to 50 zygotes) to a Nunc 4-well multidish containing 500 µl of the same medium covered with paraffin oil and incubated at 39ºC in 5% CO_2 in air. At 48 h and 144 h after IVF, cleavage rate and embryo development, respectively, was evaluated under a stereomicroscope. Embryos were scored for developmental stage at 144 h following fertilization. Each oocyte in the study received a developmental score, based on a scale of 1 = uncleaved, 2 = 2-cell embryo, 3 = 4- to 8-cell embryo, 4 = 9- to 16-cell embryo, 5 = morula, and 6 = blastocyst.

Treatment Groups

The treatment groups were as follows: treatment 1 = control group cultured in IVM medium alone (-pGH/-pGH); treatment 2 = 100 ng/ml pGH (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA) present for the first 22 h of maturation culture and absent for the second 22 h of maturation culture (+pGH/-pGH); treatment 3 = 100 ng/ml pGH absent for the first 22 h of maturation culture, but present for the second 22 h of maturation culture (-pGH/+pGH); treatment 4 = 100 ng/ml pGH present throughout the entire IVM period (+pGH/+pGH). Porcine growth hormone was rehydrated according to manufacturer's guidelines.

Statistical Analysis

Data analysis was conducted using general linear model (GLM) procedures in the Statistical Analysis System (SAS®, 1985). Two different models were used to analyze embryo development data. The first model examined all oocytes in the study. The model for the dependent variable development score included treatment, replicate and the

interaction between treatment and replicate. The second model analyzed only cleaved oocytes. Chi-square analysis was used to determine percentages of embryos reaching the different developmental stages for each treatment.

RESULTS

The addition of 100 ng/ml pGH to the maturation media in the three treatments did not have an effect (Table 1) on subsequent embryo development as compared to the control group ($P > .10$, Table 2). Replicates within the study differed significantly from each other ($P < .01$, Table 3) primarily because the development in replicate 6 was greater than in all others. There was a significant treatment by replicate interaction ($P < .05$, Table 4); pGH added during the first 22 h of IVM and pGH added during the second 22 h of IVM in replicate 6 resulted in higher development scores than for controls and continuous pGH addition. However, in replicate 2, continuous pGH resulted in the greatest development.

In a separate analysis (Table 5) using only cleaved oocytes, the three GH treatments had no effect on embryo development ($P > .10$, Table 6), there was no difference among replicates ($P > .10$, Table 7), and no treatment by replicate interactions was present ($P > .10$, Table 8). However, overall development scores were higher when unfertilized ova were removed.

Table 1. Analysis of variance for development score of total oocytes.

Source	df	Mean Squares	P value
Treatment	3	1.58	
Replicate	5	13.45	**
Replicate*Treatment	15	2.83	*
Error	963	1.48	

*P < .05
**P < .01

Table 2. Least-square means (±SE) for development score for oocytes subjected to different pGH treatments.

Treatment	n	$\bar{X}$ ± SE
-GH/-GH	249	1.57 ± .08[a]
+GH/-GH	236	1.67 ± .08[a]
-GH/+GH	248	1.47 ± .08[a]
+GH/+GH	254	1.60 ± .08[a]

+GH: 100 ng/ml pGH present
-GH: no pGH present

Table 3. Least-square means (±SE) for development score of oocytes cultured for 6 d by replicate.

Replicate	n	$\bar{X}$ ± SE
1	183	1.38 ± .09[a]
2	181	1.62 ± .09[a]
3	88	1.53 ± .13[a]
4	153	1.39 ± .10[a]
5	169	1.47 ± .09[a]
6	213	2.07 ± .08[b]

[a,b] Values in the same column with different superscripts are different (P < .01).

Table 4. Least-square means (±SE) for development of oocytes for treatment by replicate interaction.

	Treatment			
Replicate	-GH/-GH	+GH/-GH	+GH/-GH	+GH/+GH
1	1.43 ± .18	1.24 ± .18	1.31 ± .18	1.53 ± .18
2	1.62 ± .18	1.38 ±.18	1.36 ± .18	2.11 ± .18[a]
3	1.70 ± .25	1.76 ± .27	1.32 ± .26	1.36 ± .26
4	1.43 ± .20	1.46 ± .23	1.19 ± .19	1.49 ± .18
5	1.41 ± .18	1.65 ± .19	1.61 ± .18	1.22 ± .19
6	1.83 ± .17	2.55 ± .16[b]	2.04 ± .17[c]	1.89 ± .16

[a] Value is significantly different than all values less than 1.62, P < .05.
[b] Value is significantly different from all values less than 2.11, P < .05.
[c] Value is significantly different from all values less than 1.45, P < .05.

Table 5. Analysis of variance for development score of cleaved oocytes.

Source	df	Mean Squares	P value
Treatment	3	.27	
Replicate	5	1.99	
Replicate*Treatment	15	1.66	
Error	199	1.32	

*P < .05
**P < .01

Table 6. Least-square means (±SE) for development score of cleaved oocytes subjected to different GH treatments.

Treatment	n	$\bar{X}$ ± SE
-GH/-GH	56	3.54 ± .16[a]
+GH/-GH	61	3.54 ± .17[a]
-GH/+GH	44	3.72 ± .21[a]
+GH/+GH	62	3.52 ± .18[a]

+GH: 100 ng/ml GH present
-GH: no GH present

Table 7. Least-square means (±SE) for development score of cleaved oocytes cultured for 6 d by replicate.

Replicate	N	$\bar{X}$ ± SE
1	27	3.54 ± .23[a]
2	39	3.86 ± .20[a]
3	18	3.58 ± .29[a]
4	26	3.32 ± .25[a]
5	33	3.35 ± .20[a]
6	80	3.84 ± .13[a]

Table 8. Least-square means (±SE) for development score of cleaved oocytes for treatment by replicate interaction.

	Treatment			
Replicate	-GH/-GH	+GH/-GH	-GH/+GH	-GH/-GH
1	3.50 ± .41	3.20 ± .51	3.80 ± .51	3.67 ± .38
2	4.50 ± .41	3.43 ± .43	3.67 ± .47	3.83 ± .27
3	3.67 ± .47	3.67 ± .47	3.33 ± .66	3.67 ± .66
4	3.00 ± .41	2.86 ± .43	3.67 ± .66	3.75 ± .41
5	3.00 ± .38	3.89 ± .38	4.00 ± .38	2.50 ± .47
6	3.59 ± .28	4.22 ± .22	3.83 ± .27	3.72 ± .27

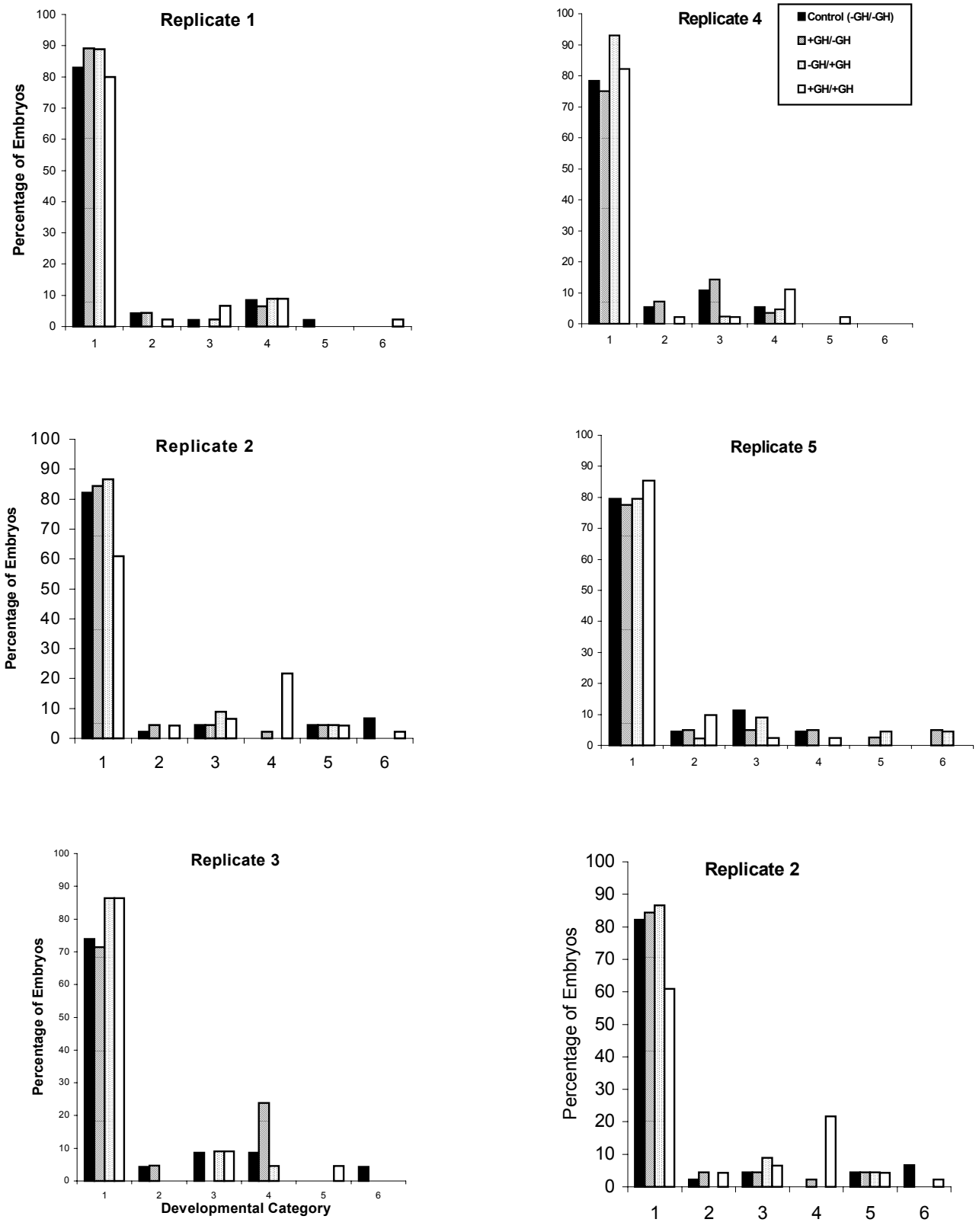

Figure 1. Treament by Development for each replicate. +GH: + 100 ng/ml pGH

DISCUSSION

In this study, the pGH treatments present in the maturation media did not act to improve embryo development. These data are in contrast to results obtained by Izadyar et al. (1996) using bGH in the bovine in vitro production system. They demonstrated that the proportion of embryos developing to the blastocyst stage was greater in the bGH supplemented group compared to the control. Izaydar et al. (1996) also reported a higher percentage of oocytes reaching metaphase II after 17 h culture when oocytes were matured in the presence of bGH and a correlation between the dose of bGH used and the percentage of oocytes at metaphase II at 17 h. The IVM media was supplemented with 0, 10, 100 or 1000 ng/ml bGH, and greater amounts of bGH present resulted in a larger proportion of oocytes reaching metaphase II. At all times bovine oocytes were examined during in vitro maturation culture (4 to 22 h after the onset of culture), the presence of 100 ng/ml bGH significantly enhanced germinal vesicle breakdown and the progression to the metaphase II stage. However, the number of oocytes reaching metaphase II beyond 22 h of maturation culture was not affected. Growth hormone at 100 and 1000 ng/ml induced significant cumulus expansion and cleavage, demonstrating that, in the bovine system the presence of bGH promoted nuclear maturation and developmental capacity of oocytes.

Growth hormone acted to increase the rate of nuclear maturation of bovine oocytes, rather than increasing the total number of oocytes reaching metaphase II stage after completion of the maturation culture. Culture of porcine oocytes (Hagen and Graboski, 1990) and rat oocytes (Apa et al., 1994) in the presence of GH also acted to accelerate the process of nuclear maturation. Porcine oocytes were able to respond to an activation stimulus at an earlier point during the maturation process when levels of supplemental pGH were present during culture (Hagen and Graboski, 1990). Apa et al. (1994) showed that the action of GH on rat oocytes, follicle enclosed and cumulus enclosed, was both time and dose dependent. Supplemental levels of GH had no effect on denuded oocytes, suggesting that the action of GH was exerted through cumulus cells.

Izaydar et al. (1996) concluded that in the bovine system, it is likely that the increased cleavage rate and blastocyst formation was due to the effect of bGH on the first polar body extrusion. It has been demonstrated that bovine oocytes that have an extruded polar body at either 16 or 20 h after the onset of maturation exhibit a higher rate of cleavage and blastocyst formation than oocytes that do not have an extruded polar body at this time (Van der Westerlaken et al., 1994).

More recently, bovine COCs matured in the presence of 100 ng/ml bGH had higher number of fertilized oocytes than those matured without bGH supplementation (80.2% vs. 67.6%, respectively) (Izadyar et al., 1998). Similarly, metaphase II oocytes matured in the presence of 100 ng/ml bGH exhibited a higher fertilization rate than metaphase II oocytes that were matured in the absence of bGH, indicating that bGH may act to improve cytoplasmic maturation of oocytes in addition to accelerating nuclear maturation. Some aspects of cytoplasmic maturation are initiated as a result of germinal vesicle breakdown and mixing of the GV contents with the ooplasm, especially processes related to fertilization and male pronuclear formation (Borsuk, 1991).

While with the bovine model, the presence of bGH during maturation accelerated nuclear maturation and increased cytoplasmic maturation and subsequent embryo

development (Izaydar et al., 1996; Izaydar et al., 1998), in the present study using the porcine model, no differences were observed with pGH addition to the maturation media. Hagen and Graboski (1990) examined the effects of pGH on porcine oocyte maturation and the oocytes' ability to respond to a subsequent activation stimulus. Abattoir sourced COCs were matured in medium containing 0, .25, .5, 1, 10 μg/ml pGH. At selected time points during culture (24, 30, 40, 42 h after the onset of maturation) COCs were removed and denuded oocytes were placed in activation media and electroporated. After 24 h, the percentage of oocytes that reached metaphase II was higher when pGH was present in the maturation media, but at 42 h, the percentage of activated oocytes was similar among treatments. The dosage of pGH did not affect the activation rate. No further studies have been reported with pGH in the porcine culture system.

In the present study, however, no differences were observed on development between treatments. The significant replicate effect may be due to the oocyte source. Ovaries were obtained from a small-scale local slaughterhouse, and there was extreme variability in the age and body weight (BW) of the animals slaughtered there. In addition to the visible variation among animals, there are many other variables that can affect oocyte quality, and variations in oocyte quality may have been responsible for masking the beneficial effects of GH treatment on embryo development reported elsewhere.

Factors that can affect oocyte quality include breed, maturity of the animals, environmental stressors including temperature and season, and nutritionally induced changes in follicle development. For example, Chinese Meishan pigs provide a better follicular environment than Large White Hybrid pigs. Xu et al (1998) showed that sperm penetration rates and male pronucleus formation were both higher in oocytes that were matured in Meishan-conditioned media than those matured in Large White hybrid-conditioned media, which consisted of everted preovulatory follicles from either Meishan or Large White Animals. It remains unclear what factors that are secreted by the follicles into the media are responsible for the increased maturation of oocytes.

A factor that may also account for oocyte variability is the maturity of the animals from which the oocytes are harvested. Nottle et al. (1997) reported that more ova were recovered and fertilized from sexually mature gilts compared with those recovered from prepubertal animals. Embryos from prepubertal gilts are also less competent in their ability to develop in vitro. Hajdu et al. (1994) reported developmental superiority of embryos collected from sexually mature animals, as both microinjected and noninjected embryos collected from prepubertal gilts had decreased developmental capacity.

Environmental stressors such as extreme temperatures can affect embryo viability. Exposure of pigs to high ambient temperatures can affect reproduction in both males and females. Reduced reproductive efficiency due to heat stress has been observed as decreased conception rates, reduced amounts of embryonic tissue present at a certain time in pregnancy, as well as reduced litter sizes. Wetterman and Bazer (1985) reported that these consequences to heat exposure may be a result of alterations in growth of the follicle, which subsequently results in decreased oocyte quality. A study in Holstein cows showed that following ultrasound-guided follicle aspiration, there was a decrease in oocyte quality as well as developmental capacity during months of high temperature and humidity (Rocha et al., 1998).

Maternal nutrition has an impact on pregnancy outcome in the pig (Ashworth et al., 1999). Gilts consuming 2.8 x maintenance rations during the estrous cycle preceding

mating had not only a higher embryo survival rate, but also the blastocysts were larger and had enhanced metabolic and secretory activity in vitro compared to controls (Ashworth et al., 1999). This suggests that the oocyte quality and subsequent development likely were influenced by nutritionally induced changes in follicle development.

Additionally, Zak et al. (1997) compared oocyte quality from sows that had feed restricted by 50% on d 22 to 28 of lactation (restricted) with sows that were fed to appetite from d 22 to 28 (refed). More oocytes from the refed group matured to metaphase II than those from the restricted group following in vitro maturation. Those oocytes that were matured in the presence of follicular fluid obtained from refed sows, had a greater rate of nuclear maturation than those oocytes incubated with follicular fluid from restricted sow, supporting the idea that nutrition affects follicular secretions and oocyte quality.

In conclusion, although no significant effects on embryo development were found with the GH treatments, the overall cleavage and blastocyst development for all of the groups including the control was lower than what is presented in the literature (Abeydeera et al., 1997a, Abeydeera et al., 1998a, Abeydeera et al., 1998b). An evaluation of cumulus cell expansion following maturation culture in the presence or absence of GH could be done to determine if there is any effect on the maturation process. It is possible that the in vitro embryo production system is too inefficient and any effects earlier on (i.e. in the maturation or fertilization steps) were missed. Further evaluation and optimization of the porcine IVM, IVF and IVC systems in our lab may be needed. Extreme variability in oocyte quality could have accounted for the differences between replicates. As the role and importance of GH in reproductive biology is becoming more clear, further studies using pGH with in vitro maturation culture will determine whether GH can act to improve oocyte maturation and/or subsequent embryo development in the porcine in vitro production system.

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CHAPTER IV

AN EVALUATION OF SPERM-MEDIATED GENE TRANSFRER IN THE PIG

ABSTRACT

The objectives of this study were to examine aspects of intracytoplasmic sperm injection (ICSI) using membrane-disrupted spermatozoa, in vitro fertilization (IVF), and sperm-mediated gene transfer in the pig. Porcine oocytes were shipped overnight in maturation media at 39°C in a portable incubator. After 22 h of maturation culture, oocytes were washed in maturation medium without gonadotropins and cultured for an additional 22 h. Cumulus cells were removed and oocytes were divided into four treatment groups: treatment 1 = ICSI using membrane-damaged spermatozoa coincubated with linear green fluorescent protein (GFP) DNA; treatment 2 = ICSI using membrane damaged spermatozoa; treatment 3 = IVF with frozen-thawed spermatozoa coincubated with linear GFP DNA prior to IVF; treatment 4 = IVF with frozen-thawed spermatozoa with no DNA. Although no overall difference in development score was observed following the four different treatments, a treatment difference among cleaved oocytes was observed when comparing only the two ICSI treatments ($P < .05$); development scores were greater in the ICSI treatment in which sperm were not coincubated with linear GFP DNA prior to injection than when the coincubation was performed. No differences in development score were observed in the two IVF treatments. The percentage of embryos expressing the GFP transgene on d 6 of culture following fertilization was 7.3% in the ICSI+GFP group and 0% in all other treatment groups. Thus, sperm-mediated gene transfer using ICSI in the pig has been demonstrated, although success rates were low.

Keywords: Intracytoplasmic sperm injection, In vitro fertilization, Trangenesis

INTRODUCTION

During normal fertilization, the sperm nucleus is incorporated into the oocyte through membrane fusion between the two gametes and paternal genetic material is introduced into the egg. Intracytoplasmic sperm injection (ICSI) is a micromanipulation technique currently used in the clinical setting to alleviate male factor infertility, in which the male has one or more sperm abnormalities that may impair their fertilizing capabilities. The ICSI procedure overcomes many barriers of fertilization and involves the injection of a single spermatozoon directly into the ooplasm of a matured, metaphase II oocyte.

The direct injection of spermatozoa into the egg cytoplasm is not a recent innovation. It was performed in sea urchins in 1962 (Hiramoto et al., 1962). The hamster has been the model most extensively used for ICSI studies, because the oocytes are easy to inject without excessive damage and the cytoplasm of hamster eggs allows for pronuclear formation with sperm from many different species (Catt and Rhodes, 1995). The first report of ICSI in domestic animals was in 1995. Catt and Rhodes (1995) demonstrated that ovine, bovine, and porcine oocytes could undergo pronuclear formation and at least limited development without exogenous activation following ICSI. However, porcine oocytes were found to be temperature sensitive during the ICSI procedure and during early stages of development.

The ICSI technique was further evaluated in the pig by Lee et al. (1998). In vitro matured porcine oocytes underwent normal fertilization processes, as determined by pronuclear opposition and syngamy following ICSI of round spermatids. Microtubule dynamics and the chromatin configuration of the oocytes fertilized by ICSI were similar to those observed during conventional fertilization. Successful fertilization and subsequent development to blastocysts followed ICSI of either a spermatozoon or isolated sperm head injection into in vitro matured oocytes (Kim et al., 1998). At 7 d following injection of spermatozoa, 38% of oocytes developed to blastocysts, while 22% of oocytes developed to blastocysts following injection of isolated sperm heads.

Kolbe et al. (1999) investigated the ICSI technique in the pig using fresh ejaculated or frozen-thawed epididymal spermatozoa injected into either in vivo or in vitro matured oocytes. The best cleavage (14%) was obtained when fresh in vitro capacitated spermatozoa were injected into in vivo matured oocytes. The proportion of fragmented oocytes was significantly lower with in vivo matured oocytes compared to in vitro matured oocytes. No beneficial effect on cleavage rate was observed with addition of either 50 μM or 100 μM calcium ionophore.

Perry et al. (1999) reported the production of transgenic mice by ICSI, demonstrating efficient production of transgenic embryos as well as live offspring by coinjection of membrane-disrupted sperm and exogenous DNA into the cytoplasm of unfertilized oocytes. Important findings included that the sperm and DNA must be incubated together prior to microinjection for transgenesis to occur; and that membrane-disrupted spermatozoa were more efficient in producing transgenesis in mice than fresh (membrane-intact) spermatozoa (77% vs. 26%, respectively). These findings indicate that a critical step for efficient sperm mediated gene transfer (SMGT) is the association of the exogenous DNA and sperm submembrane structures prior to the events that occur during fertilization.

In a subsequent study, Chan et al. (2000a) examined ICSI-mediated transgenesis in the rhesus monkey using tagged, plasmid-bound, membrane intact spermatozoa. Rhodamine-tagged DNA encoding GFP was incubated with sperm and used for IVF and ICSI. The rhodamine signal could be traced using dynamic imaging and was lost at the egg surface during IVF, but remained as a marker on injected sperm in the oocyte cytoplasm following ICSI. The GFP transgene was expressed in preimplantation embryos produced by ICSI, but not IVF, as early as the 4-cell stage with the number of expressing cells and the percentage of expressing embryos increasing during embryogenesis to the blastocyst stage. In this study, the GFP fluorescence was undetectable by direct GFP imaging, and for detection of GFP expression, immunocytochemistry was used. Following seven embryo transfers, none of the offspring born were positive for the transgene.

Shim et al. (2000) injected isolated porcine sperm heads with membranes that had been damaged by treatment with either 1.0M NaOH or 0.02% Triton X-100 and then incubated with the GFP transgene. In some treatments, oocytes were electroactivated just prior to injection. Of the injected oocytes, 20 to 26% of embryos reached the 4-cell stage or beyond, and the percentage of embryos expressing the GFP construct ranged from 11.8 to 58.8% as evidenced by fluorescence of the embryos.

The present study was conducted to examine ICSI-mediated transgenesis in the pig using in vitro matured oocytes.

MATERIALS AND METHODS

Oocyte Maturation

Porcine oocytes were purchased (Bomed, Inc., Madison WI) and shipped overnight in 3 ml maturation medium at 39ºC in a temperature-controlled portable incubator (Minitube of America, Madison, WI). Maturation medium consisted of tcm199 with earle's salts, supplemented with 5 µg/ml insulin, 10 ng/ml EGF, 0.6 mM cysteine, 0.2 mM sodium pyruvate, 25 µg/ml gentamicin, 10% pFF, and 5µg/ml FSH. After 22 h of maturation culture, oocytes were washed in maturation medium without gonadotropins and 50 to 60 oocytes were cultured in each well of a Nunc 4-well multidish (Roskilde, Denmark) containing 500 µl of the same medium covered by 500 µl of parafin oil.

In Vitro Fertilization

Three hours before the completion of IVM culture, an aliquot of a commercially prepared frozen semen (ejaculated) straw (Swine Genetics, International, Ltd., Cambridge, IA) prepared from a single ejaculate was placed into Dulbecco's phosphate buffered saline (DPBS) containing 0.1% BSA, 75 µg/ml potassium penicillin and 50 µg/ml streptomycin sulphate (pH 7.2) pre-warmed to 39ºC and spun for 5 min at 36.3 X *g* for 5 min in a 15 ml polypropylene centrifuge tube (Fisher Scientific, Pittsburg, PA). The supernatant was collected into a pre-warmed 15 ml centrifuge tube and washed two times by centrifugation at 553 X *g* for 5 min. At the end of the washing procedure, the sperm pellet was resuspended in 100 µl of modified Tris Buffered Medium (mTBM; Abeydeera et al., 1997b) to give a final concentration of 1 to 3 X 10^7 sperm/ml. Sperm concentration was determined using a hemocytometer. Spermatozoa were preincubated for 2.5 h at 39ºC before addition to oocytes.

After completion of culture for IVM, cumulus cells were removed with 0.1% hyaluronidase in NCSU-23 medium and washed three times with mTBM. After washing, 25 to 35 oocytes were placed in 50 µl drops of the same medium that had been covered with warm paraffin oil in a 35 X 10 mm^2 polystyrene culture dish (Becton and Dickinsion Labware, Franklin Lakes, NJ). Oocytes were preincubated in mTBM 30 min prior to addition of spermatozoa. The concentrated sperm solution was diluted appropriately with mTBM to give a final concentration of 2 X 10^6 sperm/ml. Motility was evaluated and 50 µl of this sperm suspension were introduced into 50 µl of the mTBM that contained the oocytes for a final sperm concentration of 1 X 10^6 sperm/ml. Oocytes were co-incubated with spermatozoa for 8 h at 39ºC in an atmosphere of 5% CO_2 in air.

Following sperm-oocyte coincubation, putative zygotes were washed three times in NCSU-23 medium supplemented with 0.4% BSA (Petters and Wells, 1993), transferred (40-50 zygotes) to a Nunc 4-well multidish containing 500 µl of the same medium covered with paraffin oil and incubated at 39ºC in 5% CO_2 in air. At 48 h and 144 h after IVF, cleavage rate and embryo development, respectively, were evaluated under a stereomicroscope.

There were two treatment groups for IVF. Control frozen-thawed spermatozoa not incubated with linear GFP; and frozen-thawed spermatozoa incubated with 300 ng/mL of linear GFP for 30 min prior to IVF. Both sperm samples were prepared together as above and separated 30 min prior to IVF for DNA incubation treatment before addition to oocytes.

Preparation of Sperm for ICSI: Membrane disruption treatment

The sperm rich fraction was collected from a boar by the gloved hand method. Three milliliters of this fraction was added to 12 ml of TALP-HEPES (Bavister, 1989). This mixture was incubated at 15ºC for 4 h. Motility was approximately 80% following incubation. Quick frozen pellets were made using a dry ice block. These pellets were immediately plunged into a liquid nitrogen bath, placed into Nunc cryovials (Roskilde, Denmark), and stored in liquid nitrogen until use. Following treatment, sperm viability was assessed by using a commercially available cell viability test kit (Live/Dead® sperm viability kit; Molecular Probes, Eugene, OR) which differentiates between plasma membrane-intact (live) and damaged (dead) cells according to the fluorescence staining pattern observed under an ultraviolet filtered microscope. All sperm cells were confirmed dead following this assessment.

DNA Preparation

The transgene construct CMV-EGFP was prepared according to Chauhan et al. (1999). Briefly, the inter-ribosome entry site of pIRES-EGFP (Clonetech Laboratories; Palo Alto, CA) was removed by *Bam*HI endonuclease digestion. The restriction products were separated and the vector-containing fragmnts were ligated, and ligation products were used to transform *E. coli* XL-Blu cells (Stratagene) with selection on ampicillin plates. The plasmid was isolated and purified using column absorption (Maxiprep; Qiagen, Inc.) and the linear DNA construct was obtained by overnight digestion of the plasmid with *NruI* and *XhoI*. The linear construct was separated from the plasmid by agarose gel electrophoresis and subsequently purifed using the QIAquick gel extraction kit (Qiagen, Inc.). The purified construct was reconstituted in 10 mM Tris and 0.25 mM EDTA at pH 7.4 at a concentration of 65 µg/ml, and stored at -20°C until use.

ICSI with membrane-disrupted spermatozoa

Following maturation culture, cumulus cells were removed with 0.1% hyaluronidase in NCSU-23 medium and washed 3 times in mTBM. All eggs were then combined in a 250 µl drop for randomization effect. Eighty oocytes were removed from the pool of oocytes and centrifuged for 10 min at 12,000 X *g* in 500 µl of HEPES-buffered Tyrode's medium (HbT; Prather et al., 1995).

A frozen pellet of the membrane-disrupted spermatozoa was thawed at room temperature and diluted with HbT to give a final concentration of 3 X 10^7 sperm/ml. Ten microliters of this sperm suspension was mixed with polyvinylpyrrolidone (PVP; average molecular weight 360,000; Sigma) solution to give a final concentration of about 10% (w:v) PVP.

A microdrop of this solution was then placed on the lid of a 100 X 10 mm^2 polystyrene culture dish (Becton and Dickinson Labware, Franklin Lakes, NJ). Manipulation was carried out in 10 µl droplets of HbT under parafin oil using Narishige manipulators and a Nikon inverted microscope equipped with Hoffman modulator optics. One droplet contained the sperm and the other droplet contained the oocytes. The oocytes were stabilized with a holding pipette with an outer diameter of about 200 µm and an inner diameter of about 50 µm. The sperm was injected using a micropipette with an outer diameter of 8 to 9 µm and an inner diameter of 6 to 7 µm. The polar body of the oocyte was placed at 6 or 12 o'clock and the point of injection was at 3 o'clock. The

oocyte was penetrated by the injecting micropipette and a small amount of cytoplasm was drawn into the micropipette to ensure penetration of the oocyte. Then, the cytoplasm, together with one sperm and a small amount of medium were injected into the oocyte. Immediately following ooplasmic injection, the injection pipette was withdrawn quickly and the oocyte released from the holding pipette to reduce the intracytoplasmic pressure. Oocytes were then washed 3 times in NCSU-23 medium containing 0.4% BSA, placed in 50 µl drops of the same medium, and cultured at 39º under 5% CO_2 in air. Embryos were examined for cleavage at 48 h following injection, and for development and fluorescence at 96 h and again at 144 h. Embryos were quickly examined under fluorescent light using a Nikon B-2E/C FITC fluorescence filter cube (#96107).

Electron Microscopy

Electron microscopy was performed on membrane-disrupted and frozen-thawed (membrane-intact) spermatozoa according to methods of Flesh et al. (1998). Briefly, sperm samples were fixed overnight at 4°C in Karnovsky fixative. Pellets were washed with 0.1 M sodium-cacodylate (pH 7.4), post-fixed with 1% osmium tetroxide in 0.1 M sodium-cacodylate (pH 7.4) for 1 h, washed with distilled water, and stained with 2% aqueous uranylacetate for 1 h. Samples were dehaydrated in graded series of acetone and embedded in Polybed 812 (Polysciences, Inc.; Warrington, PA). Ultrathin sections were cut on a Reichert microtome and stained for 2 min with Reynold's lead citrate.

Treatment Groups

The treatment groups were as follows: treatment 1 = ICSI with membrane-disrupted spermatozoa that were preincubated with linear GFP DNA (7 ng/µl) for 1 min prior to injection (ICSI+GFP); treatment 2 = ICSI with membrane-disrupted spermatozoa that were not preincubated with linear GFP DNA prior to injection (ICSI-GFP); treatment 3 = IVF with frozen-thawed spermatozoa that were preincubated with linear GFP DNA (300 ng/ml) prior to IVF (IVF+GFP); treatment 4 = IVF with frozen-thawed spermatozoa that were not preincubated with linear GFP DNA for 30 min prior to IVF (IVF-GFP).

Statistical Analysis

Data analysis was conducted using general linear model (GLM) procedures in the Statistical Analysis System (SAS®, 1985). Three different models were used to analyze embryo development data. The first model examined all oocytes in the study. The model for the dependent variable development score included treatment, replicate, and the interaction between treatment and replicate. The second model analyzed only cleaved oocytes. The third models analyzed only cleaved oocytes, but included either both ICSI treatments or both IVF treatments.

Embryos were scored for developmental stage at 144 h following fertilization. Each oocyte in the study received a developmental score, based on a scale of 1 = uncleaved, 2 = 2-cell embryo, 3 = 4-cell embryo, 4 = 5- to 8-cell embryo, 5 = 9 to 16-cell embryo, 6 = morula, and 7 = blastocyst.

RESULTS

This study incorporated the investigation of 929 oocytes. In comparing the different treatments, ICSI or IVF, with or without GFP, there were no significant treatment effects (Table 1) on overall embryo development. ($P > .10$, Table 2). Replicates differed from each other ($P < .01$, Table 3) primarily because the development in replicate 1 was greater than for all others. In a reduced model using only cleaved oocytes (Table 4), no significant treatment effect ($P > .10$, Table 5), replicate effect ($P > .10$, Table 6), or treatment by replicate interaction ($P > .10$) were present.

Data sets were reduced in a subsequent model comparing development score for only the two ICSI treatments (Table 7), there was a significant ($P < .05$, Table 8) treatment effect showing greater development for ICSI without GFP when compared with development with oocytes injected with sperm that had been coincubated with GFP DNA. No difference in development score was found for the two IVF treatments was observed (Table 9 and Table 10).

The percentage of embryos expressing the GFP transgene on d 6 of culture following fertilization treatment (Table 11) was 7.3% in the ICSI+GFP group and 0% in all other treatment groups. The percentages of transgenic embryos reaching various development stages are presented in Figure 1.

Table 1. Overall analysis of variance for development score of oocytes used in ICSI or IVF.

Source	df	Mean Squares	P value
Treatment	3	1.10	
Replicate	7	5.84	**
Replicate*Treatment	21	1.53	
Error	897	1.29	

* P < .05
** P < .01

Table 2. Least-square means (±SE) for overall development score for oocytes subjected to different fertilization treatments.

Treatment	n	$\bar{X}$ ± SE
ICSI+GFP	232	1.54 ± .08
ICSI-GFP	215	1.53 ± .08
IVF+GFP	241	1.39 ± .07
IVF-GFP	241	1.53 ± .07

ICSI+GFP: oocytes injected with membrane damaged sperm preincubated with GFP DNA prior to intracytoplasmic sperm injection.
ICSI-GFP: oocytes injected with non-incubated membrane damaged sperm.
IVF+GFP: oocytes fertilized in vitro with sperm that had been preincubated with GFP DNA.
IVF-GFP: oocytes fertilized in vitro with non-incubated sperm.

Table 3. Least-square means (±SE) for overall development score of oocytes cultured for 6 d by replicate.

Replicate	n	$\bar{X}$ ± SE
1	123	$2.00 \pm .10^{a}$
2	121	$1.31 \pm .11^{b}$
3	117	$1.44 \pm .11^{b}$
4	122	$1.57 \pm .10^{b}$
5	118	$1.47 \pm .10^{b}$
6	104	$1.29 \pm .11^{b}$
7	115	$1.42 \pm .11^{b}$
8	109	$1.47 \pm .11^{b}$

[a,b] Values in the same column with different superscripts are different (P < .01).

Table 4. Analysis of variance for development score of cleaved oocytes.[a]

Source	df	Mean Squares	P value
Treatment	3	2.82	
Replicate	7	1.62	
Replicate*Treatment	21	1.23	
Error	154	1.75	

[a]Fragmented and uncleaved ova were not included in the analysis.

Table 5. Least-square means (±SE) for development score of cleaved oocytes subjected to different fertilization treatments.

Treatment	n	$\bar{X}$ ± SE
ICSI+GFP	58	3.13 ± .18
ICSI-GFP	43	3.76 ± .23
IVF+GFP	36	3.50 ± .24
IVF-GFP	49	3.39 ± .25

ICSI+GFP: oocytes injected with membrane damaged sperm preincubated with GFP DNA prior to intracytoplasmic sperm injection.
ICSI-GFP: oocytes injected with non-incubated membrane damaged sperm.
IVF+GFP: oocytes fertilized in vitro with sperm that had been preincubated with GFP DNA.
IVF-GFP: oocytes fertilized in vitro with non-incubated sperm.

Table 6. Least-square means (±SE) for development score of cleaved oocytes cultured for 6 d by replicate.

Replicate	n	$\bar{X}$ ± SE
1	44	3.83 ± .21
2	15	3.46 ± .36
3	21	3.43 ± .29
5	29	3.45 ± .29
6	21	3.68 ± .31
7	14	2.85 ± .44
8	22	3.19 ± .29
9	20	3.68 ± .31

Table 7. Analysis of variance for development among ICSI[a] treatments of cleaved oocytes[b].

Source	df	Mean Squares	P value
Treatment	1	8.14	*
Replicate	7	1.48	
Replicate*Treatment	7	1.32	
Error	85	1.57	

* P < .05
** P < .01
[a]ICSI: intracytoplasmic sperm injection of oocytes using membrance-damaged sperm that was preincubated with GFP DNA or was not preincubated with the construct.
[b]Fragmented and uncleaved ova were not included in the analysis.

Table 8. Least-square means (±SE) for development score for cleaved oocytes subjected to the two ICSI treatments.

Treatment	n	$\bar{X}$ ± SE
ICSI+GFP	58	3.13 ± .17[a]
ICSI-GFP	43	3.76 ± .21[b]

[a,b] Values in the same column with different superscripts are different (P < .05).
ICSI+GFP: oocytes injected with membrane damaged sperm preincubated with GFP DNA prior to intracytoplasmic sperm injection.
ICSI-GFP: oocytes injected with non-incubated membrane damaged sperm.

Table 9. Analysis of variance for development among IVF treatments[a] of cleaved oocytes[b].

Source	df	Mean Squares	P value
Treatment	1	.17	
Replicate	7	.75	
Replicate*Treatment	7	1.45	
Error	69	1.97	

[b]Fragmented and uncleaved ova were not included in the analysis.

Table 10. Least-square means (±SE) for development score for cleaved oocytes subjected to the two IVF treatments.

Treatment	n	$\bar{X}$ ± SE
IVF+GFP	36	3.50 ± .25
IVF-GFP	49	3.39 ± .27

IVF+GFP: oocytes fertilized in vitro with sperm that had been preincubated with GFP DNA.
IVF-GFP: oocytes fertilized in vitro with non-incubated sperm.

Table 11. Embryos expressing the GFP transgene on d 6 of culture following fertilization treatment.

Treatment	N	cleaved (%)	dev. ≥ 4 cell (%)[*]	n (%) GFP+
ICSI+GFP	232	58 (25)	34 (14.7)	17 (7.3)
ICSI-GFP	215	43 (20)	36 (16.7)	0 (0)
IVF+GFP	241	36 (14.9)	27 (11.2)	0 (0)
IVF-GFP	241	49 (20.3)	38 (15.8)	0 (0)

ICSI+GFP: oocytes injected with membrane damaged sperm preincubated with GFP DNA prior to intracytoplasmic sperm injection.
ICSI-GFP: oocytes injected with non-incubated membrane damaged sperm.
IVF+GFP: oocytes fertilized in vitro with sperm that had been preincubated with GFP DNA.
IVF-GFP: oocytes fertilized in vitro with non-incubated sperm.
[*]number of embryos developing to 4-cell stage or beyond.

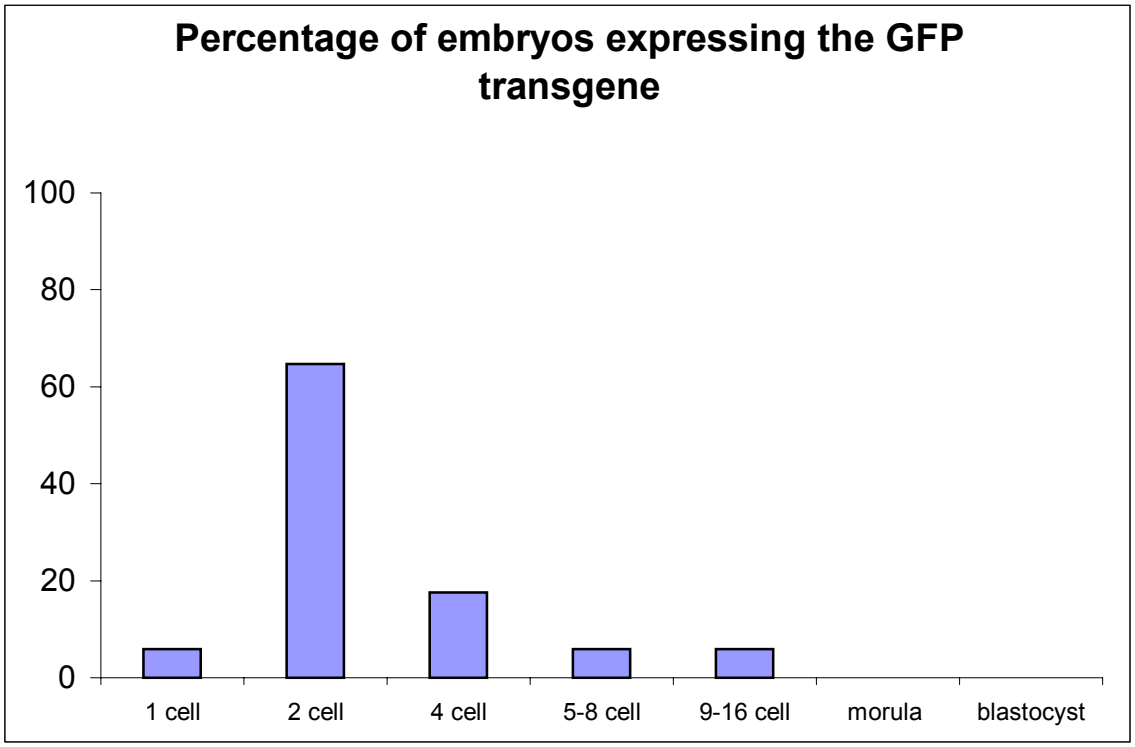

Figure 1. Percentage of embryos expressing the GFP transgene at different stages of development.

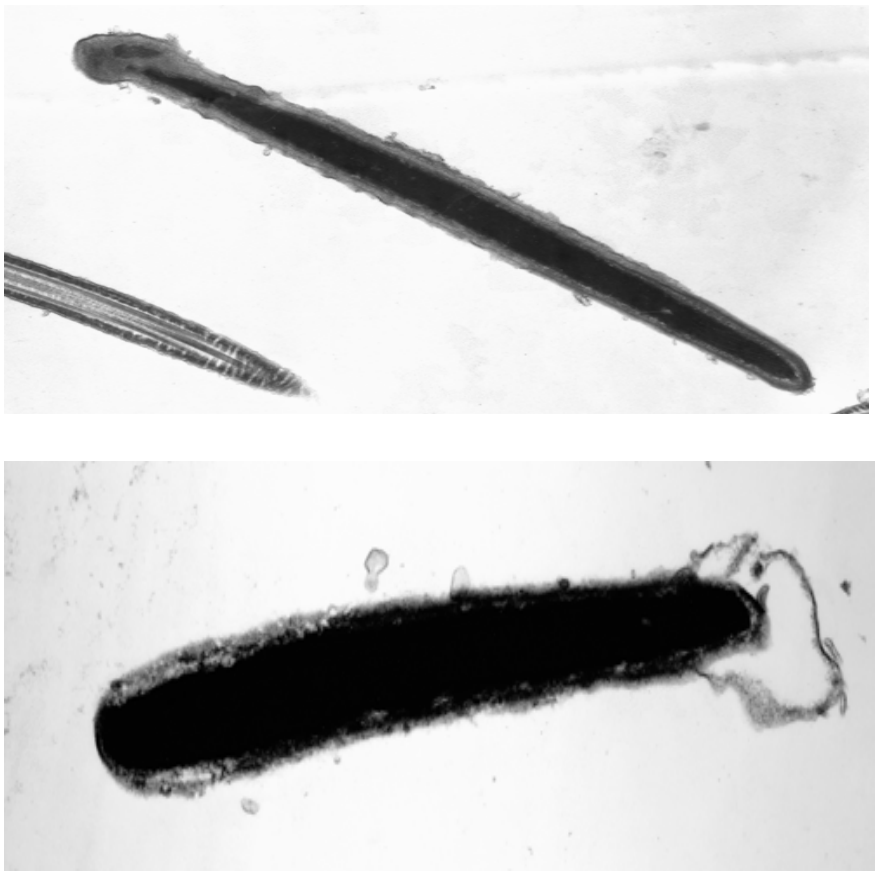

Figure 2. Electron micrographs of membrane-intact (top) and membrane-damaged porcine spermatozoa (bottom).

DISCUSSION

In the present study, aspects of ICSI, IVF, and sperm-mediated transgenesis were examined. It was demonstrated that porcine embryos expressing a GFP transgene can be produced by the injection of membrane-disrupted spermatozoa which were preincubated with linear plasmid DNA into matured, metaphase II oocytes. Embryo development in this study following injection of membrane-disrupted spermatozoa was consistent with previous reports that used similar methods in cattle (Goto et al., 1990), mice (Wakayama et al., 1998a; Wakayama et al., 1998b), and pigs (Shim et al., 2000). These studies demonstrated that motility and plasma membrane integrity are not necessary for fertilization and subsequent embryo development. In fact, Goto et al. (1990) reported the birth of two normal calves after injection of spermatozoa killed by freeze-thawing without cryoprotectants, and normal mice were born as well following injection of membrane damaged spermatozoa derived from either freeze thawing without cryoprotectant (Wakayama et al., 1998a) or freeze-drying (Wakayama et al., 1998b). These results further demonstrate that the production of live offspring is possible when the nuclei of non-viable sperm are injected directly into oocytes. Shim et al. (2000) reported embryo development to the blastocyst stage following ICSI of membrane disrupted spermatozoa into porcine oocytes.

Perry et al. (1999) recently reported the production of transgenic mouse embryos and live offspring by coinjection of membrane-disrupted sperm and exogenous DNA into the cytoplasm of unfertilized oocytes. They reported that the sperm and DNA needed to be preincubated with the spermatozoa for transgenesis to occur. Fresh spermatozoa were also coincubated with linear DNA and injected into metaphase II oocytes. Transgenesis rates were higher using membrane-damaged rather than fresh spermatozoa (77% vs. 26%, respectively), suggesting that a critical step in sperm-mediated gene transfer was a preinjection association between exogenous DNA and sperm submembrane structures. These structures could possibly include the basic proteins of the sperm perinuclear matrix (Longo et al., 1987).

In the present study, 7.3% of the ICSI+GFP embryos expressed the transgene; however, the majority of injected oocytes in both the GFP and control groups either did not cleave or became fragmented (75% of ICSI+GFP and 80% of ICSI-GFP, respectively). This may indicate that the freeze-thaw method used in the sperm preparation for membrane disruption was not optimal. Freezing sperm without cryoprotectants to damage the membrane can also result in unwanted damage of sperm genomic DNA. While the BSA used in the freezing media may have afforded the sperm some protection, since no cryoprotectant was used in the media, the sperm DNA likely suffered damage in the process (Wakayama et al., 1998a). This would support the theory that single-stranded breaks were present to allow for transgene integration (Perry et al., 1999).

Shim et al. (2000) reported the development of pig embryos to the blastocyst stage in treatments using ICSI with membrane-damaged spermatozoa and membrane damaged spermatozoa coincubated with linearized GFP DNA. To promote membrane disruption, sperm were treated with either 0.2% Triton X-100 detergent or 1.0 M NaOH. It is possible that these treatments are more optimal than the freeze-thaw method used in the present study and results in a lesser degree of genomic DNA damage.

In the present study, development to the morula/blastocyst stages was rarely observed in the two ICSI treatments (3.4% morula from the cleaved oocytes in the ICSI+GFP group and 7% morula/blastocyst from the cleaved oocytes in the ICSI-GFP group). The great degree of arrested development observed suggests possible injury to sperm genomic DNA during the freeze-thaw process which subsequently resulted in the disruption of the embryonic genome activity essential for normal preimplantation development (Wakayama et al., 1998a).

It is interesting to note the number (12/17, 70.6%) of embryos at the 1- or 2-cell stage that exhibited GFP transgene expression in this study (Figure 1). Embryonic genome expression in the pig does not begin until the 4-cell stage; when pig zygotes, 2-cell or early 4-cell embryos are placed in α-amanitin, a transcription inhibitor, development does not proceed to the 8-cell stage (Jarrell et al., 1991). Pig zygotes and 2-cell embryos rely on maternally derived mRNA to direct embryonic development and the timing of specific events in differentiation in the embryo are regulated by an 'intrinsic' time clock (Prather et al., 1993). It is possible that the transgene became integrated at the time of fertilization or shortly after, but that the embryos could not complete cytokinesis and remained arrested. Additionally, it is possible that the fluorescence observed in some or all of the embryos was due to transient expression. To determine which of the above is the case, PCR reactions would need to be done on the embryos expressing the transgene.

The embryos that contained the transgene but remained arrested at the 1- or 2-cell stage did not express GFP until d 4 following ICSI as determined by fluorescence imaging. All other embryos in the study that were GFP positive exhibited fluorescence at this time as well. It is possible that the arrested embryos' internal time clocks were able to activate expression of the GFP gene although prior checkpoints for cell division were likely not met perhaps due to the compromised DNA of the treated sperm used for fertilization and the embryos remained arrested. Clearly, future research into the initiation of transcription and the regulation of developmental events in the pig is needed.

The majority of embryos (11/17) examined for GFP expression were mosaic for transgene expression and had both GFP+ and GFP- blastomeres. In embryos in which all blastomeres expressed the transgene (6/17), integration likely occurred at the pronuclear stage, before DNA replication occurred (Chan et al., 1999). However, in the mosaic embryos, integration of the GFP transgene was likely delayed following the ICSI procedure. Perry et al. (1999) suggested that the exogenous DNA may be stabilized by sperm derived material and not subjected to degradation. They observed this delayed integration of the transgene was observed in the mouse only when the exogenous DNA was coinjected with sperm heads, and not when DNA was injected alone, and oocytes were subjected to subsequent parthenogenetic activation with the cytokinesis-blocking agent, cytochalasin B, even though efficient development of embryos was observed.

The kinetics of transgene integration appear different in the mouse using ICSI-mediated trangenesis and the bovine using pronuclear injection (Perry et al., 1999; Chan et al., 1999) as evidenced by the degree of mosaicsm observed. In the mouse model, 3 to 45% mosaicism was observed following ICSI with membrane disrupted spermatozoa incubated with linear GFP while in the bovine 62% was reported following pronuclear injection of linear GFP DNA. Perry (2000) implied that this difference could be due to either variations in the oocytes themselves due to the species, or more likely because that metaphase II eggs are used in ICSI-mediated transgenesis, while pronuclear zygotes are

used for pronuclear microinjection. In the present study, although metaphase II eggs were used to promote transgenesis, a very high degree of mosaicism, similar to that observed in the bovine pronuclear study was observed. This implies that since porcine oocytes are more similar to bovine oocytes than mouse oocytes, perhaps the difference among species plays a significant role in the amount of mosaicism obtained as well as stage of maturation the oocyte.

In the present study, when comparing cleaved oocytes in the two ICSI treatments, development scores for the ICSI+GFP group were significantly lower than in the ICSI-GFP group (3.13 ± .17 and 3.76 ± .21, respectively). Perry et al. (1999) observed a decrease in embryo development as the proportion of embryos that contained fluorescent blastomeres increased. The maturation mechanism of the GFP protein requires oxidation by O_2, and H_2O_2 is released with mature GPF chromophore. This may explain the deleterious effect of high level expression of the GFP protein as early embryonic development may be very sensitive to free radical generation (Tsien, 1998).

The ICSI technique has been evaluated in the pig using membrane intact spermatozoa, and results are promising. Lee et al. (1998) showed that normal fertilization processes as determined by pronuclear formation, pronuclear oppostion, and syngamy occur following injection of round spermatids into in vitro matured oocytes. Kim et al. (1998) showed that in vitro matured porcine oocytes injected with a spermatozoon or isolated sperm heads could develop in vitro to the blastocyst stage. Martin (2000) reported that live piglets could be born following the injection of live sperm into in vivo matured oocytes. These data demonstrate that ICSI is a viable option and alternative to IVF. However, it will be necessary to find a method to damage the outer membranes of the sperm while keeping the genomic DNA relatively unharmed in order for it to support full development.

There was no difference in embryo development scores for the two IVF treatment groups in the present study, and none of the IVF derived embryos that were fertilized with spermatozoa that were preincubated with linear GFP DNA prior to fertilization expressed the protein as evidenced by fluorescence. This does not support results in which transgenesis was reported in mice (Lavitrano et al., 1989), cattle (Schellander et al., 1995) and pigs (Lavitrano et al., 1997) with this procedure. In the above experiments, sperm was incubated with DNA prior to routine IVF or artificial insemination. Considerable controversy has surrounded this approach, however, as the results were highly variable and other laboratories have failed to produce similar results (Lavitrano et al., 1989; Brinster et al, 1989; Maione et al., 1989). It should be noted that the overall cleavage and blastocyst development in both of the IVF groups was significantly lower than values presented in the literature (Abeydeera et al., 1997a; Abeydeera et al., 1998a; Abeydeera et al., 1998b). Further evaluation and optimization of the porcine IVF and IVC culture systems should be investigated.

Very recently the phenomena of using live spermatozoa as a vehicle to transport exogenous DNA into oocytes has been revisited. Huguet and Esponda (2000) demonstrated that following injection of plasmid encoding GFP into mouse vas deferens and subsequent breeding with normal females in estrus, 4 out of 53 newborns were positive by PCR for the GFP gene. Kuznetsov et al. (2000) showed that when rabbit sperm cells were treated with a mixture of exogenous linear and circular DNA, production of transgenic animals increased dramatically above the efficiency of that

when pronuclear microinjection was used. The increase was from 1.4% with pronuclear injection to an average of 68% following sperm-DNA incubation and subsequent insemination. Chan et al. (2000a) observed the fate of a transgene during the ICSI and IVF procedures using rhodamine-tagged plasmid DNA and high resolution imaging. They observed that the transgene remained on the cell surface and was retained after ICSI, but it could not be detected after sperm penetration of the oocyte following IVF. This implies that the DNA was bound on mostly on the sperm surface and cytoplasmic enzymes including proteases may play a role in dissociation of the sperm-DNA binding (Chan et al., 2000b).

Thus, although considerable controversy has surrounded the issue of sperm-mediated gene transfer, increasing numbers of reports have confirmed that spermatozoa can be used a vectors to transfer foreign DNA into oocytes in a number of species whether through ICSI or IVF. Although low success in results is still prevalent, as future work is conducted, more defined experimental procedures and protocols for the different techniques will likely be developed and minimize this inconsistency observed between laboratories.

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APPENDIX A

TL-HEPES-PVA MEDIA (Funahashi et al., 1997)

Constituent	mM
NaCl	114.0
KCl	3.2
$NaHCO_3$	2.0
KH_2PO_4	0.34
Sodium lactate	10.0
$MgCl_2.6H_2O$	0.5
$CaCL_2.2H_2O$	2.0
HEPES	10.0
Sodium pyruvate	0.2
Sorbitol	12.0
Gentamicin	25 g/L
Penicillin G	65 g/L
Polyvinyl Alcohol	0.1 %

MATURATION MEDIA used in Chapter 3

BSA-free NCSU-23 (see below) supplemented with:
0.1 mg/ml cysteine
10% (v/v) follicular fluid
10 IU/ml equine chorionic gondaotropin*
10 IU/ml human chorionic gonadotropin*
10 IU/ml epidermal growth factor

*present for the first 22 h of maturation, absent for the second 22 h of maturation

MATURATION MEDIA used in Chapter 4

Tissue culture media 199 with earle's salts supplemented with:
5 μg/ml insulin
10 ng/ml epidermal growth factor
0.6 mM cysteine
0.2 mM sodium pyruvate
25 μg/ml gentamicin
10% porcine follicular fluid (v/v)
5 μg/ml FSH*
5 μg/ml LH*
*present for the first 22 h of maturation, absent for the second 22 h of maturation

FERTILIZATION MEDIA-modified Tris-buffered medium (mTBM; Abeydeeraand Day, 1997b)

Constituent	mM
NaCl	113.1
KCl	3.0
$CaCl_2$	10.0
Tris	20.0
Glucose	11.0
Sodium pyruvate	5.0
Caffeine	5.0
BSA	2.0 g/L

NCSU-23 MEDIA (Petters and Wells, 1993)

Constituent	mM
NaCl	108.7
KCl	4.78
$CaCl_2.2H_2O$	1.70
KH_2PO_4	1.19
$MgSO_4$	1.19
$NaHCO_3$	25.07
L-Glutamine	1.0
D(+)-Glucose	5.55
Taurine	7.0
Hypotaurine	5.0
Penicillin-G, K salt	0.05
Streptomycin sulfate	0.06
BSA	4 g/L

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