韓國家畜繁殖學會誌 20(4) : 413~421(1996)
Korean J. Animal Reprod.

Tendency and Problems in Porcine *in-vitro* Fertilization

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돼지체외수정의 연구동향과 문제점

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요 약

체외에서 포유동물의 난자와 정자의 배양에 관한 연구는 난자의 성숙과정과 수정현상에 대한 많은 새로운 정보를 제공하였다. 동시에 체외수정의 연구로부터 얻은 결과는 또다른 의문을 제기하였다. 특히 동결융해정액을 이용한 돼지체외수정의 경우 낮은 정자의 침입율과 전핵형성율 및 높은 다정자침입(polyspermy)율은 아직도 해결해야할 문제점으로 남아있다. 돼지난자의 성숙에 관한 연구의 성과는 수정후 낮은 전핵형성율을 개선시켰으나 타동물종에 비하면 아직도 매우 낮게 나타나고 있다. 한편 동결정액의 처리를 위하여 caffeine이나 Ca^{2+}와 같은 물질을 수정용 배지내에 첨가하는 등 수정능력획득의 유기를 위하여 여러 가지 방법이 연구되고 있지만 정자의 침입율은 아직도 낮고, polyspermy의 발생율은 높게 나타내고 있다. 따라서 정자의 침입율을 향상시키고 polyspermy를 억제하기 위하여 난관세포와의 공동배양, 난포액을 첨가한 배양액 내에서 정자의 전배양 및 정자농도의 조절은 매우 효과적인 방법으로 이용되어왔다. 그러나 수정란의 체외생산성 향상과 이와 관련된 연구를 보다 효과적으로 수행하기 위해서는 위에서 지적한 문제에 영향을 미치는 요인에 대한 보다 근본적인 이해가 요구된다.

I. INTRODUCTION

The use of new reproductive technologies is progressing rapidly in many farm animal species. New applications to produce transgenic animals from *in vitro* produced embryos are relying on the development and understanding of the events from oocyte maturation to implantation. In 1974 Motlik and Fulka reported in-vivo fertilization of porcine oocytes that had been matured *in vitro*. Two years later Baker and Polge(1976) summarized experiments on in-vitro and in-vivo fertilization of porcine and predicted that if *in vitro* maturation - *in vitro* fertilization techniques can be developed in domestic animals, it should be possible to utilize at least a significant portion of the vast number of follicular oocytes that are normally lost through atresia. Important steps towards realizing this prediction in the porcine were made by Iritani et al. (1978) who reported the first successful *in vitro* maturation and fertilization in porcine using spermatozoa incubated in isolated female genital tracks, Cheng(1985) who obtained piglets from oocytes matured *in vivo* and then fertilized *in vitro* and Mattioli et al. (1989) who generated piglets from *in vitro* maturation and fertilization oocytes. Porcine oocytes obtained from the aspiration of small immature follicles are a ready source of female gamates since these oocytes mature *in vitro* to the Meta-

phase-Ⅱ stage, and they can be successfully fertilized *in vitro* with fresh boar semen or frozen-thawed cauda epididymal spermatozoa. However, polyspermy and male pronuclear formation still remain prominent problems for *in vitro* fertilization of porcine oocytes. In this paper, research tendency and problems on *in-vitro* fertilization of porcine will be suggested.

Ⅱ. PROBLEMS OF *IN-VITRO* FERTILIZATION

1. Maturation conditions

The maturation of the oocyte can be divide into two different aspects: nuclear maturation and cytoplasmic maturation. The nuclear maturation in porcine was described by Edwards in 1965 and proceeded to the metaphase-Ⅱ stage in 43~46h *in vitro*. Similar results were obtained by many research, but many oocytes remained at metaphase-Ⅰ even after 48 h of culture. The duration of meiotic maturation is substantially longer in porcine(42~44h) than in cattle, sheep and goats(24 h). This delay observed *in vitro* reflects a comparable situation *in vivo* where the time between the LH surge and germinal vesicle breakdown is 20~24h. The importance of the maintenance of intercellular coupling between cumulus cells and the oocyte was emphasized by Moor et al. (1990), who concluded that the contribution made by follicle cells to cytoplasmic maturation is fully evident only when gonadotrophins are present in the IVM medium and when a non-static culture system is used. The addition of LH and FSH to the IVM medium was found to accelerate and faciliate meiotic maturation in several studies(Galeati et., 1990 ; Mattioli et al., 1991); LH selectively improved cytoplasmic maturation. Funashi et al(1994) also reported at least two different hormonal conditions during maturation *in vitro*, which are the presence of PMSG during the first 20 h of culture and the absence of PMSG and oestradiol during the second 20 h of culture, are beneficial to meiotic and cytoplasmic maturation of porcine oocytes.

Different media were tested for their capacity to promote nuclear maturation. If nuclear maturation appears to proceed normally in most oocytes in medium containing BSA or serum, the formation of a normal male pronucleus following fertilization is not completed normally in most oocytes. It has been reported that glutathione synthesis during oocyte maturation is an important factor for subsequent formation of a male pronucleus(Yoshida et al. 1993 ; Yoshida, 1993). An IVM medium containing a high concentration of cysteine was found to enhance the ability of porcine oocytes to form the male pronucleus(Yoshida et al., 1992, 1993). It is belived that the addition of cysteine to the IVM medium may be an important factor in the synthesis of glutathione by the porcine oocyte and in enhancing formation of the pronucleus after sperm penetration.

Results from a study by Ding and Foxcroft (1992) confirmed that follicular shells apparently secrete factors that support cytoplasmic, if not nuclear maturation. They also showed that male pronucleus formation rate, in sperm-penetrated oocytes, was correleate to diameter and shell weight of cocultured follicles; large follicles were more supportive of cytoplasmic maturation than small one. To determine the effect of culture system and presence of intact follicle shells on porcine IVM, porcine oocytes were cultured with different numbers of cumulus cells either in a static system or in a non-static system(Nagai et al., 1993). Elsewhere, Funahashi and Day(1993 a, b) showed that the removal of hormone supplements from IVM medium at 20h after culture enhanced cytoplasmic maturation

and cumulus expansion. They also found that a serum-supplemented IVM medium reduced the ability of porcine oocytes to form an male pronucleus formation; serum supplementation apparently promoted rapid progression of meiotic maturation. Maturation media are uaually supplemented with proteins, such as fetal calf serum(Zheng and Sirard, 1992), newborn piglet serum(Funahashi and Day, 1993a) and bovine serum albumin. However, when added to mKRB solution supplemented with FSH, fetal calf serum has been shown to inhibit maturation of porcine oocytes(Naito et al., 1988). The author suggest that serum in the IVM medium may not allow porcine oocytes sufficient opportunity for intercellular communication with cumulus cells, which may influence male pronucleus formation after sperm penetration. Lee et al. (1992) have shown that the maturation rate of porcine oocytes was increased by supplementation of the IVM medium hormones and 20% dialyzed porcine follicular fluid.

Among the growth factors currently implicated in the modulation of oocyte maturation are EGF, TGF-α, TGF-β, IGF-I and IGF-Ⅱ. The effect of growth factors on oocyte maturation has been examined in porcine by several investigators. Illera and Petters(1993) evaluated the addition of EGF+IGF-I to IVM medium in the presence of follicular fluid and hormones. Addition of the growth factors adversely affected oocyte quality in presence of hormones and porcine follicular fluid but had a favourable effect when oocytes were cultured without gonadotrophins or porcine follicular fluid. Singh et al. (1993) examined the effects of combinations of EGF, TGF-β and FSH, LH, oestradiol and androstenedione on maturation and cumulus expansion in porcine. Other studies reported by Singh and Amstrong(1994) support the view that EGF of follicular origin may play a role in the regulation of follicle development and oocyte maturation.

2. Capacitation of porcine spermatozoa

For *in vitro* fertilization in porcine, it is clearly essential to have a means of artificially capacitating sperm ; over the past 20 years, much time and effort have been devoted to this problem. Part of the artificial capacitating procedure involves the removal of seminal proteins and the other substances that coat the sperm membrane of ejaculated semen. Some workers have sought to establish the role of substances in seminal plasma by using epididymal porcine sperm which have not been coated with accessory gland proteins. It is clear that differences in the capacitating ability of porcine sperm do exist.

The successful capacitation of porcine spermatozoa *in vitro* has been demonstrated for the first time by Nagai et al. (1984). Epidymal spermatozoa were preincubated at high concentrations in a simple medium for 4h prior to insemination of oocytes matured *in vitro* and 49~84% of zona-intact oocytes were penetrated. Similarly, a capacitation method employing a 4~5 h preincubation of ejaculated spermatozoa at a high concentration but with elevated levels of Ca^{2+} and the high pH of the preincubation medium, modified tissue culture medium, was used by Cheng(1985) to obtain penetration(100%) of *in vivo* and *in vitro* matured oocytes. The preincubated spermatozoa were transferred into a modified TCM 199 that had been supplemented with 2 mM caffeine. There is ample evidence to suggest that the porcine oviduct may play a key role in sperm capacitation by providing an environment that facilitates both this process and fertilization. Certainly, there is much information now available to show that the porcine oviduct does not function simply as a site for sperm retention, but probably participates in

the physiological maintenance of sperm fertilizing capacity and consequently contributes to the fertilization of the oocyte. There is evidence that porcine oviductal cells secrete factors that are capable of supporting and maintaining porcine sperm viability and motility for *in vitro* fertilization(Park and Sirard, 1995 ; Dubuc and Sirard, 1995). It is known that secretory epithelial cells of the ungulate oviduct produce a wide array of glycoproteins, and that this production is mediated by the hormones of the oestrous cycle (Erickson-Lawrence et al., 1989). Prior to fertilization, sperm and oocytes are exposed to the oviductal fluid, which contains secretions from these secretory cells as well as serum transudate.

In porcine, spermatozoa preparation and capacitation as employed by Mattioli et al. (1989) involved prolonged incubation(24~48 h) in a commercial semen diluent, with subsequent washing and use of Percoll layers to provide a highly motile fraction. This was a modification of procedures previously reported by Cheng et al. (1986). In Korea, Park et al. (1990) suggested that storage of boar semen at 20℃ for 10~12 h and preincubation at a high sperm concentration was a suitable method of including capacitation of sperm in that species. Combination treatments with ionophore A23187, heparin and caffeine have been examined by several workers(Wang et al., 1991 ; Lei et al., 1992). No capacitation procedure comparable to the relatively simple heparin procedure used in cattle is currently available for use in pigs.

3. Control of polyspermy

Under *in-vivo* conditions, fertilization of porcine oocytes occurs soon after ovulation and is monospermic in over 95% of case(Hunter, 1973). In contrast to *in-vivo* fertilization, in *in-vitro* fertilized oocytes are exposed to a large number of capacitated spermatozoa for a longer period prior to male pronuclear formation frequently leading to polyspermy(Nagai and Moor, 1990). In addition to the polyspermy problem encountered with *in vitro* fertilization, it is common to observe large variations from individual to individual in terms of penetration and rates of polyspermy. Both ejaculated and epididymal spermatozoa have been used *in vitro* resulted in porcine(Kikuchi et al., 1997 ; Park et al., 1997). Percoll gradient centrifugation had been successfully used to separate highly motile spermatozoa from fresh boar semen with some reduction of the polyspermy, and piglets have been produced following embryo transfer(Mattioli et al., 1989). However, polyspermy is again high compared with *in vivo* fertilization or *in vitro* fertilization in other species. Wang et al. (1991) successfully obtained *in vitro* penetration with frozen thawed ejaculated boar spermatozoa, but the concentration of spermatozoa needed to achieve a high penetration rate was 25×10^6 cells/ml and no preincubation was used. The principal explanation for such a large number of cell is related to the low motility(5~20%) of cells at thawing. The quality of frozen semen is influenced not only by the donor but also, to a greater degree, by the freezing procedure used. Variations in cooling velocity, thawing velocity and cryoprotectant concentration may greatly influence the survival of spermatozoa after thawing. When optimal cooling and warming rates are used, increasing the glycerol concentration improved motility, but, at the same time, the percentage of spermatozoa with a normal apical ridge gradually decreases(Fiser and Fairfull, 1990). The concentration of glycerol did not significantly influence the normal apical ridge rate after thawing, but the separation procedure did (Zheng et al., 1992).

The importance of oviduct secretions and mo-

tility on the control of polyspermy has been studied and reviewed in detail by hunter(1991). An abnormal number of sperm cells at the site of fertilization induces polyspermy, reflecting the sensitivity of this species to critical sperm : oocyte ratio. This cannot be explained by abnormal migration of cortical granules, since contralateral oocytes were normally fertilized. It would seem that time is critical in regulating the number of spermatozoa entering the oocyt and a large number of spermatozoa at the site of fertilization would decrease the time between the first encounter and the second or the third and not leave sufficient time to properly install the blocking procedure. The oviduct could regulate the state of capacitation and the number of spermatozoa coming from the uterus, to ensure that the right conditions are present at the fertilization site. It is known that oviduct cells can bind to spermatozoa under the influence of estradiol and can release motile spermatozoa about 44 h later. This capacity is observed more readily with tissues from the isthmus than from the ampulla (Raychoudhury and Suarez, 1991). This capacity of oviduct cells is maintained when cells are cultured as vesicles in suspension(Dubuc and Sirard, 1995). It is also known that the oviduct secretes a viscous product at estrus which would trap sperm cells in the isthmus and indirectly act against polyspermy. This product would be present in smaller quantities during the luteal phase or in progesterone-treated females, resulting in higher polyspermy rates on those occasions(Hunter, 1991). This suggests that conditions of fertilization might be more important to achieve a high percentage of normal fertilization than the conditions of oocyte maturation.

4. Pronucleus formation

There are a number of recent studies in which different procedures were applied during *in vitro* maturation of porcine oocytes to study the development of the ability to form normal pronucleus but also to prevent excessive polyspermy during *in vitro* fertilization. In general, high penetration rates result in high polyspermy rates and even if male pronucleus formation is present, more than on male pronucleus can result in polyploidy. Recently, Ding and Foxcroft(1992) used follicular shells from follicles isolated 36 or 72 h after equine chorionic gonadotrophin injection. They observed a significant effect of follicular size on male pronucleus formation but no specific effect of the time following equine chorionic gonadotrophin. The effect of the follicle on the capacity to form a pronucleus does not seem to be associated with an effect in reducing polyspermy. In another study, the addiition of porcine follicular fluid to maturation media was effective for enhancing the rate of nuclear maturation, however, porcine follicular fluid was not effective for promoting male pronucleus formation. While these results are consistent with previous observations (Yoshida et al. , 1992), they conflict with the report of Naito et al. (1988), which incubated that porcine follicular fluid enhanced male pronucleus formation. These conflicting results might be due to differences in culture conditions or to interactions of porcine follicular fluid with specific components that make up the media used in the experiments. In Canada, Zheng and Sirard(1992) suggested that the effects of different sources of serum, follicular fluid or portion of the follicular wall were compared to evaluate their effects on penetration, polyspermy and pronuclear formation. In the majority of species soon after incorporation of the sperm nucleus into the oocyte cytoplasm, cytoplasmic factors react with sperm chromatin to induce molecular changes, including cleavage of disulphide bonds, partial enzy-

matic degradation of nuclear proteins, the release of chromatin-associated protamines and their immediate replacement by oocyte-derived histones. The oocyte cytoplasmic activity that controls male pronucleus formation has been defined as the male pronucleus growth factor(Thibault and Gerard, 1973). The essential cytoplasmic factor appears in the oocyte during the final maturation phase(Ding et al., 1992). The predominantly asynchronous formation between male and female pronucleus in porcine IVM-IVF oocytes is thought to result from inadequate oocyte maturation.

Ⅲ. PERSPECTIVES AND CONCLUSIONS

The great deal of knowledge accumulated by many investigators has greatly enhanced improvements in porcine IVM-IVF system. Yoshida et al. (1993) reported that full developmental potential has been demonstrated of porcine embryos derived from oocytes using very basic procedures : aspiration followed by maturation in a static system, in simple medium supplemented with porcine follicular fluid and cysteine, followed by insemination *in vitro* in mTALP containing oligopeptides with freshly ejaculated spermatozoa. However, the number of piglets obtained from IVM-IVF oocytes is extremely low. It is quite obvious that the conditions prevailing *in vivo* must be better understood to achieve a high number of viable oocytes and embryos with the objective to introduce new techniques in reproduction management in porcine. Hormones like FSH, LH and estradiol 17β and some growth factors such as EGF, TGFα, TGFβ, IGF-I and protein sources and porcine fillicular fluid are important for the *in vitro* survival and maturation of porcine oocytes. *In vitro* studies culturing bovine oocyte with pieces of their own follicular wall((Carbonneau and Sirard, 1994 ; De Loos et al., 1994) and thecal cells (Richard and Sirard, 1996) evidently shows the involvement of the follicular wall in the regulation of oocyte maturation presumably via the hormones and growth factors mentioned. As Bevers et al. (1997) point out, further improvements for IVM-IVF require more basic research to ascertain the cellular physiology of gamate maturation. Whether the *in vitro* observations reflect the regulation of *in vivo* oocyte maturation is questionable. From the foregoing part it can be concluded that cumulus oocyte complexes released from the follicular environment can be apparently maximally stimulated by a single hormone or growth factor to achieve optimal maturation.

The high polyspermy rates encountered in porcine are probably the result of a precarious equilibrium between the number of spermatozoa released in the ampulla and the speed of reaction of the oocyte to the first cell fusing with the plasma membrane. The vulnerability of individual oocytes to polyspermy is variable *in vivo* as one out of three oocytes will be affected if an excess of spermatozoa is present or if ageing of the oocyte is induced. This variability is amplified during *in vitro* culture and factors present in the follicle are acting during the maturation process both to decrease polyspermy and to enhance normal sperm decondensation. The use of cumulus and oviductal cells *in vitro* might help to regulate a normal interaction between spermatozoa and oocyte, by increasing capacitation through specific binding and by the same way limiting the concentration of spermatozoa avariable per unit of time.

Ⅳ. SUMMARY

In vitro culture has provided new information

on the mechanisms involved in fertilization how sperm and oocyte fuse together. At the same time, results obtained *in vitro* have led to new questions. Techniques for *in vitro* maturation of porcine oocytes have progressed such that the problem of the low rate of pronucleus formation with *in vitro* matured oocytes after *in vitro* fertilization has been nearly improved. On the other hand, porcine spermatozoa have been shown to be capacitated if the fertilization medium contains caffeine and Ca^{2+}, but the incidence of polyspermy in IVM-IVF oocytes is still high. To prevent polyspermy, co-culture with oviductal cells, sperm preincubation with porcine follicular fluid or control of sperm concentration, have been examined with significant effects but still remarkably high rates of polyspermy. The understanding of these influences is a prerequisite to enhancing *in vitro* production of porcine embryos.

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