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Review

Cytoplasmic Maturation of Porcine Oocytes for Successful Male Pronuclear Formation and Early Embryonic Development

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Successful *in vitro* production of porcine embryos requires a series of integrated, effective techniques for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). This paper reviews about cytoplasmic maturation associated with the efficiency of *in vitro* production of porcine embryos. Traditionally, the failure to form a male pronucleus have been reported as serious problems in producing porcine embryos following IVM and IVF. The problem of male pronuclear formation is currently considered to be mainly due to oxidative stress during IVM. More recently the developmental competence of embryos following IVM and IVF has been investigated through improvement of culture conditions for oocyte maturation. Currently, an acceptable rate of blastocyst formation and the birth of live piglets has been achieved by investigating affecting factors during IVM, IVF and IVC of porcine oocytes. Since the ovarian oocytes available for IVM are primarily those present in mid-size antral follicles of prepuberal gilts, more research is needed to gain an improved understanding of the factors associated with the developmental competence in oocytes from both preantral and antral follicles.

Key words : pig, oocyte, maturation, fertilization, developmental competence

Introduction

In vitro production of porcine embryos has been expected to provide an effective system for mass production of oocytes and/or embryos for *in vitro* manipulation such as production of transgenic pigs and for use in research studies on meiosis, fertilization, early embrynic development and early pregnancy loss. Efficient production of porcine embryos *in vitro* would reduce the cost for procurement of embryos for these purposes. Recent development of successful culture techniques for early development of porcine embryos from the 1-cell to blastocyst stages[1-3] further encourage us to develop a system for efficient *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes, with particular attention on the historical problems of a reduced incidence of male pronuclear formation and a high incidence of polyspermy. As recently reviewed by us[4-7], rapid progress toward the solution of traditional major problems has been made, and the efficiency of *in vitro* blastocyst production from follicular oocytes has been improved significantly by modification of conditions especially during IVM[8,9]. The present paper reviews about cytoplasmic maturation associated with successful IVM, IVF and *in vitro* culture (IVC)

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for early embryonic development in pigs.

Nuclear maturation

The continuation of the meiotic processes of oocyte chromatin is so-called nuclear maturation. Porcine oocytes remain in the dictyate stage of the first meiotic prophase until 18 h after hCG injection[10] and the interval from the onset of the LH surge to ovulation averages 44 ± 3 h[11]. Although porcine oocyte cumulus complexes (OCCs) have traditionally been matured *in vitro* without changes in the culture conditions, dramatic changes seem to occur *in vivo* in the nucleus and cytoplasm of oocytes, particularly during the period when the oocytes are at the germinal vesicle stage. Exposure of OCCs to gonadotropins for only the first 20 h period of IVM is adequate[12], and removing cumulus cells 24 h after the start of culture does not adversely affect nuclear maturation[13]. Porcine oocytes are sensitive to a heterogenous nuclear RNA synthesis inhibitor[14], a protein phosphorylation inhibitor, and a protein synthesis inhibitor[15], during the first 12 h of maturation *in vitro*. Therefore, transcriptional events, protein phosphorylation and protein synthesis activities associated with nuclear maturation seem to occur during a relatively early period of IVM. It has been suggested that external calcium influx is not a direct requirement for germinal vesicle breakdown but may be required by porcine oocytes for progression beyond metaphase I of meiosis[16]. Further, protein kinase A, protein kinase C, and calmodulin pathways modulate protein synthesis and phosphorylation activities in porcine oocytes and cumulus cells, and consequently appear to affect nuclear maturation[17].

Cytoplasmic maturation associated with male pronuclear formation

Cytoplasmic maturation is composed of the acquisition of factors which are needed for male pronuclear formation and, occasionally, early embryonic development. Cytoplasmic maturation (determined by the incidence of male pronuclear formation) abnormalities have been reduced by modifications of IVM conditions. Early IVM protocols resulted in detrimental interactions among the culture medium, cumulus cells and oocytes, which consequently affected low ability in oocytes to form a male pronucleus. In a recent study[18], it has been shown that polypeptides synthesized by porcine OCCs during IVM (in the presence of FSH and LH) correspond closely to those synthesized during *in vivo* maturation; however, there is a lag in the time of appearance and disappearance of polypeptides of OCCs matured *in vitro*. The ability of porcine oocytes to form a male pronucleus is affected by hormonal levels[12,19,20], follicular secretions[21-29], intracellular ionic strength[30-32] and especially oxidative stress[7]. In addition, recent evidence[30,33] has indicated that male pronuclear formation is affected by oocyte glutathione content at the end of maturation. Both the incidence of male pronuclear formation and glutathione content in porcine oocytes is increased during IVM when cysteine, which is a thiol, is added to the maturation medium; whereas, the content decreases if cysteine is absent[33]. The incidence of male pronuclear formation is also increased if other thiols such as cysteamine[34] and beta-mercaptoethanol (H. Funahashi and B. N. Day, unpublished observation) is supplemented during IVM. Therefore, the historical problem in male pronuclear formation following IVM-IVF appears to be mainly due to oxidative stress. Although the presence of cumulus cells surrounding the oocyte is required throughout IVM to maintain a high oocyte glutathione level[35], the presence of cysteine from 36 h after the start of IVM will maintain both a high oocyte glutathione content and a high incidence of male pronuclear formation, and the absence of cysteine from 36 h after the start of IVM induces a significant reduction in both of these[36]. Thus, the oxidative stress may be espe-

cially detrimental at relatively late stages of IVM when active intercellular coupling between the oocyte and cumulus cells is significantly reduced[20,37].

Recent studies using follicular secretions suggest that the ability of porcine oocytes to form a male pronucleus seems to be related to the steroid environment[27], especially the ratio of progesterone to estradiol[38]. Differences in effectiveness of exogenous hormone supplementation in various IVM systems using follicular secretions[19,21,24,28] may be associated with variable steroid concentrations in the follicular supplement. Although the mechanisms of oocyte glutathione regulation during maturation are not clear, follicular secretions with exogenous hormones may offer a more suitable environment for glutathione and protein synthesis in porcine OCCs.

Although porcine follicular fluid contains a relatively high concentration of salts (Na^+, 128-145 mM; Cl^-, 97.3 mM)[39], high NaCl concentration in the maturation medium detrimentally affects not only histone H1 kinase activity[40], microfilament organization[32] and glutathione content of porcine oocytes at the end of IVM, but also the incidence of male pronuclear formation[30] and *in vitro* development following IVM/IVF[31]. Supplementation of maturation media containing relatively high NaCl levels with organic osmolytes, such as taurine and sorbitol, reduces the severity of the detrimental effect[32]. Follicular secretions may contain organic osmolytes since organic osmolytes exist universally in cells and physiological fluid[41,42]. Therefore, a low NaCl concentration or the presence of organic osmolytes in the maturation medium seems to be required for normal porcine oocyte metabolism, and consequently for achievement of cytoplasmic maturation.

Male pronuclear formation

Potential role of glutathione during male pronuclear formation is shown in Figure 1. Male pronuclear formation in mammalian oocytes is completed after reduction of disulfides in the sperm protamine, replacement of sperm protamine by oocyte histones, and DNA synthesis[43-45]. Glutathione is believed to be associated with the reduction of the disulfide bond cross-linking of sperm protamine[45]. As described above, synthesis of sufficient glutathione during IVM of porcine oocytes appears to be required for sperm decondensation and male pronuclear formation[30,33,46,47]. Oocyte glutathione content is known to decrease following sperm penetration[33,48,49], but not following electrical stimulation[48] or microinjection of a G-protein stimulator[50]. The decrease of glutathione at fertilization seems to be due to gamma-glutamyltranspeptidase of spermatozoa[50]. Since IVF following microinjection of gamma-glutamyltranspeptidase into porcine IVM oocytes yielded a

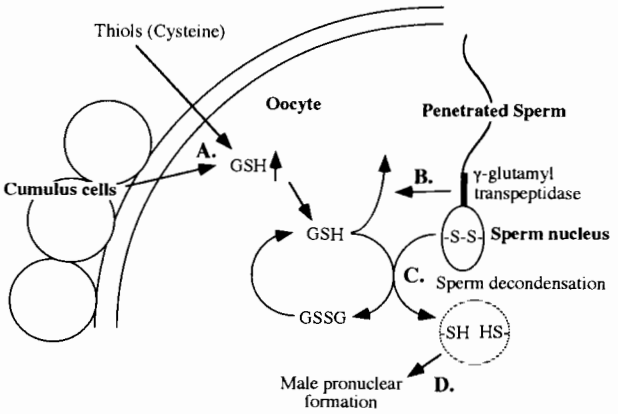

Fig. 1 Hypothesis for changes of oocyte glutathione (GSH) content during maturation and fertilization and the potential role of GSH in sperm decondesation. During IVM, oocyte GSH content (A) is affected by cumulus cells and the presence of thiols such as cysteine. After sperm penetration, oocyte glutathione content appears to be decreased by gamma-glutamyltranspeptidase of spermatozoa (B). However, sperm decondensation is dependent on glutathione content after sperm penetration, and is taken place (C). Other factors, such as kinase and (or) phosphatase activities, are thought to contribute to male pronuclear formation following spern decondensation (D) GSSG indicates glutathione disulfide (Modified from Day and Funahashi[5]).

reduced incidence of male pronuclear formation[50], the ability of oocytes to form a male pronucleus appears to be dependent on oocyte glutathione level following sperm penetration. A low rate of male pronuclear formation in oocytes penetrated by a large number of spermatozoa[51] may be due to an abnormally low level of oocyte glutathione induced by polyspermic penetration.

Although sperm penetration in immature porcine oocytes is not different from that in matured oocytes if cumulus cells are removed before insemination[52,53], sperm nuclei that penetrate oocytes at the germinal vesicle stage decondense 16 h after insemination and then recondensed or formed metaphase chromatin 44 h after insemination[54]. Electrical activation of IVM porcine oocytes before or just after sperm penetration *in vitro* significantly decreases the incidence of male pronuclear formation[55]. Therefore, decondensation of the sperm nucleus and male pronuclear formation appear to be affected not only by oocyte glutathione but also by cell cycle -dependent factor(s). Although a high positive correlation between histone H1 kinase activity in IVM porcine oocytes and the incidence of male pronuclear formation has been reported[40,56], a high protein phosphatase activity is also required to dephosphorylate a 25 kDa polypeptide to a 22 kDa polypeptide[57] necessary for pronuclear formation. The time of male pronuclear formation is delayed in porcine IVM oocytes as compared with those matured *in vivo*[58]. Since asynchrony between male and female pronuclear formation occurs even in porcine oocytes with an improved glutathione content[48], delayed male pronuclear formation may be due to unknown factor(s) regulating replacement of sperm protamine by oocyte histone and(or) other cell cycle-dependent factor(s).

Developmental competence of porcine embryos following IVM-IVF

Culture of IVM-IVF embryos for early embryonic development

As reviewed previously[3,59], recent technological progress in culture of 1-cell porcine embryos matured and fertilized *in vivo* has been achieved by the use of simple media such as modified Whitten's medium, NCSU-23 or NCSU-37 media and modified Tyrode medium. Further, replacement of BSA with FBS in BECM-3 medium by the morula stage[60] or transfering embryos from a simple medium containing BSA (CZB medium) to modified Eagle's minimal essential medium containing 20% FBS[61] has been known to improve both the number of blastomeres and the incidence of hatched blastocysts. The successful culture of porcine embryos to the blastocyst stage have made it possible to examine the developmental ability of IVM/IVF porcine embryos. However, IVM and IVF porcine oocytes develop to the blastocyst stage in simple media with a very low efficiency, even with improved male pronuclear formation and monospermic penetration[31,62]. An improved early development of IVM/IVF embryos to develop to the blastocyst stage has been shown by culture in the amniotic fluid of developing chick embryos[63] and in co-culture systems with porcine cumulus cells[64], trophoblastic cells[64] or oviduct epithelial cell aggregates[65]. The incidence of blastocyst formation in simple media after IVM/IVF has also been improved by modification of IVM conditions such as using a medium containing a reduced concentration of sodium chloride[31] and supplementation of maturation medium with cysteamine[34] or organic osmolytes[32]. In pigs, embryo survival seems to be associated with a close synchrony between the peak concentration of oestradiol and the onset of the LH surge[11,66] or oestrus[66]. It has also been suggested that a longer time interval between onset of oestrous and

ovulation is important for the high rate of embryo survival in the Meishan pig[67]. Therefore, the steroid conditions surrounding oocytes before the start of IVM may play an important role in obtaining the competence of oocytes for embryonic development. Piglets from IVM/IVF embryos have been produced in only a few laboratories[9,32,68,69].

COCs with uniform ooplasm and a compact cumulus cell mass have usually been collected from antral follicles of slaughtered prepubertal gilts for *in vitro* production of porcine embryos. It has been reported that glutamine metabolism of IVM oocytes from prepubertal sheep is lower than that of oocytes from adult sheep and that the mitochondria and cortical granules of IVM oocytes from prepubertal sheep differ from those of IVM oocytes from adult sheep[70]. Further, the low developmental competence of calf oocytes as compared to cow oocytes would appear to be due not to difference in oocyte protein patterns[71], but to a low sensitivity of the inositol 1,4,5-trisphosphate receptor[72]. Therefore the mechanism for signal transduction in oocytes of pigs as well as sheep and cattle may not be completed until around the time of puberty or during the follicular phase of the oestrous cycle.

Further, the size of follicles which are selected for *in vitro* production of porcine embryos differs among investigators[5]. A large variation in the dictyate stage of the first meiotic prophase among oocytes has been observed when oocytes are collected from follicles of slaughtered gilts[8,9,73] and consequently seems to cause an increased range in the meiotic stage at the end of the maturation culture[9]. Since histone H1 kinase activity of aged oocytes at the metaphase-II stage is significantly lower with time in culture[74], extended culture duration to obtain a higher incidence of matured oocytes from meiotically asynchronized population may reduce the oocyte competence for early embryonic development. In contrast, the morphology of germinal vesicle of oocytes collected from gilts 72 h after injection of equine chorionic gonadotropins is closely synchronized[75]. An oocyte population derived from follicles of different ages may be expected to have an increased variation in range of quality of the oocytes. The ability of follicles to secrete steroids and support cytoplasmic maturation of the oocyte seems to be dependent on age rather than size of follicles[76].

Reducing morphological variation in the germinal vesicle of oocytes appears to enhance the developmental competence of porcine oocytes. Preincubation of COCs in maturation medium without gonadotropins for 12 h before exposing them to gonadotropins reduces the variation in the morphology of germinal vesicle and enhances the developmental competence following IVM/IVF[8]. Further, exposure of COCs to dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) for the first 20 h period of IVM does not affect the incidence of nuclear maturation of oocytes at 44 h after the start of IVM or sperm penetration after IVF but does increase the homogeneity of oocyte nuclear maturation[9]. This treatment also improves the early development to the blastocyst stage of porcine embryos after IVF, and piglets have been produced with a high efficiency after embryo transfer of the IVM-IVF embryos at the 2-4-cell stages[9]. Treatment with hypoxanthine may be expected to produce similar effects during IVM because hypoxanthine is believed to maintain the oocyte arrest by modulating cAMP level through its inhibitory action on cAMP-phosphodiestrase[77]. Since increasing and decreasing cAMP per se stimulates oocyte phosphorylation via signal transduction pathway, the stimulatory effect of cAMP may also enhance the competence of oocytes to develop to the blastocyst stage. Inhibiting all tyrosine phosphorylation with the tyrosine-specific inhibitor prevents changes in the morphology of germinal vesicle[15]. A 42-kD protein in porcine oocytes that increases in amount after 12 h of maturation culture is

localized to condensing and condensed chromosomes[78].

The incidence of blastocyst formation of IVM/IVF porcine embryos is also improved by modification of IVM conditions after the germinal vesicle stage. The presence of tissue inhibitor of metalloproteinase-1 (TIMP-1) from 20 to 44 h after the start of IVM of porcine oocytes enhances the oocyte competence to the blastocyst stage without affecting factors associated with fertilization[79]. A combination of techniques using dbcAMP during the first 20 h of IVM and TIMP-1 from 20 to 44 h improves the ability of IVM-IVF embryos to develop to the blastocyst stage to a more acceptable level (34%) for *in vitro* production of embryos[79]. TIMP-1 is a major secretory protein of porcine preovulatory granulosa cells after hCG administration[80]. Following the preovulatory gonadotropin surge, the concentration of TIMP-1 mRNA increases and localizes to the granulosa cells[80]. Therefore, TIMP-1 appears to play an important role for oocytes to obtain the full ability for early embryonic development. However, an understanding of how TIMP-1 promotes the ability of porcine oocytes to develop to the blastocyst stage without affecting fertilization has still not been achieved. Co-culture of porcine COCs with follicular shell pieces improved early embryonic development to the blastocyst stage after IVF[81]. It is also interesting to speculate if the effect of the follicular shell pieces is mainly due to TIMP-1 secretion from granulosa cells.

Conclusion

Failure in male pronuclear formation has been overcome by reducing the oxidative stress of oocytes during IVM. The developmental competence of oocytes matured and fertilized *in vitro* has been enhanced through modification of culture conditions during IVM. Oocyte competence for early embryonic development appears to be achieved by mimicking active communications between the oocyte and follicular cells. In the most current IVM-IVF system, more than 80% of porcine oocytes that were matured and fertilized normally developed to the blastocyst stage.

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前核形成および初期胚発生に関係する豚卵胞卵子の細胞質成熟

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豚受精卵の体外生産を成功させるためには，体外成熟，体外受精および体外発生に必要な効率的な一連の技術の積み重ねが必要である．本総説は，効率的な豚受精卵の体外生産に関係する体外成熟中の諸要因について論じる．長い間，体外成熟卵子の体外受精後の雄性前核の形成不全は，体外受精卵を生産するための深刻な問題として，多くの研究者によって長い間研究されてきた．しかし現在では，雄性前核形成に関するこの問題は，すでに解決され，主に体外成熟中の酸化ストレスが原因であると考えられている．さらに最近，体外成熟・受精卵の初期発生能力は，卵成熟期間中の培養条件の改善を通じて飛躍的に改善されており，現在では，豚受精卵の初期発生に影響を及ぼす卵子成熟中の諸要因についての研究によって，産業的に利用可能な胚盤胞期受精卵作出効率および産仔の生産効率が得られている．体外成熟可能な卵胞卵子は，通常春機発動以前の屠畜雌豚の卵巣に存在する中型成熟卵胞から回収されている．そこで，今後の研究において，春機発動以前の雌豚および性成熟を経た雌豚のそれぞれに存在する卵胞卵子の発生能力に影響を及ぼす要因のさらなる理解が必要とされる．