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Up date of *in vitro* production of porcine embryos

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1. ABSTRACT

There have been intensive attempts to establish reliable *in vitro* maturation (IVM) and fertilization (IVF) methods in pigs. Although a great deal of progress has been made, current IVM-IVF systems still suffer from a low rate and poor quality of *in vitro* produced embryos . In this review, we will review the recent studies about IVM-IVF of porcine oocytes and the *in vitro* culture (IVC) system, especially modified *in vitro* production (IVP) system that produces high quality of porcine blastocysts. We then try to suggest practical ways to solve the problems mentioned above in the pigs.

2. INTRODUCTION

Pigs have been playing an important role in meat production in many countries, especially in Asia. They produced over 25% of the energy and 5% of the protein that human obtained from animal sources in the world in early 1990s (1), and the percentages may be getting higher now because Bovine Spongiform Encephalopathy (BSE) in cattle and the bird influenza have the potential to ignite a human pandemic, which may reduce beef and chicken consume. Moreover, new applications of pigs outside animal industry are drawing attention in the field of human medicine. It is hoped that pig organs can be used as human organ replacement in the face of the serious shortage of human organs for transplantation (2). The use of pigs as experimental animals is also gaining ground; for example, they are useful models for studying arteriosclerosis (3). Thus, their application in fields other than traditional animal industry is expanding. The new technologies to produce transgenic and cloned pigs also provide us with the potential of very rapid genetic manipulation and dissemination. Furthermore, somatic cell cloning is expected to be a valuable method for conserving genetic resources in pigs. All such technologies involve manipulating oocytes and/or embryos *in vitro*. Thus the concept of *in-vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes in pigs has a particular importance now. Since Mukherjee (4) reported that mouse oocytes could be matured and fertilized *in vitro* and developed to term in 1972, there have been intensive attempts in pigs. Mattioli *et al*. (5) succeeded in getting piglets from IVM-IVF embryos in 1989. Although a great deal of progress has been made, our current IVM-IVF systems still suffer from two major problems: 1) a high rate of polyspermy, i.e. the penetration of porcine oocyte by more than one spermatozoon *in vitro*, and 2) a low rate of

development of IVM-IVF embryos to the blastocyst stage and the low quality of embryos as compared with *in vivo* produced embryos in pigs (6). Recently Kikuchi *et al.* (7), however, reported an excellent IVC system after IVM-IVF using a low oxygen tension during IVM and this modified IVC system produced high quality of blastocysts and piglets were obtained after transferring these blastocysts.

In this review, we first reviewed the studies about IVM-IVF-IVC of oocytes in pigs and then tried to suggest the practical ways to enhance the efficiency of these new reproductive technologies. More fundamental aspects of IVF in pigs have been reviewed by Sun and Nagai (8) and will not be reviewed here.

3. IMPROVEMENT OF IVM OOCYTE QUALITY

3.1 Control of anti-oxidant content in IVM oocytes

Several evidences suggest that glutathione (GSH) is a crucial intra-oocyte factor for events after maturation in both cattle and pigs. Yoshida *et al.* (9) reported that the concentration of GSH in porcine oocytes increased when the concentration of cysteine added to IVM medium was increased, and at the same time the rate of male pronucleus formation became higher. Cysteine is a critical component amino acid of GSH and GSH is a thiol tripeptide that is synthesized by the gamma–glutamyl cycle where availability of cysteine is crucial (10). GSH has an important role in providing cells with anti-oxidant thus protecting the cells against the toxic effect of oxidative damage (10). Therefore the promoting effect of GSH on male pronucleus formation after IVF is speculated to act synergistically in the following ways: GSH promotes breaking of disulfide bond (S-S) of protamine in penetrated sperm head by making oocyte cytoplasm in redox state; and/or the GSH reacts as a substrate of glutathione peroxidase to be a scavenger of free radicals in oocytes, enhancing their competence as a whole (1). The impact of high GSH content during porcine oocyte maturation was proven by the fact that Yoshida *et al.* (11) produced piglets derived from IVM-IVF oocytes by using a culture medium (TALP) of relatively simple components supplemented with cysteine for maturation. Yamauchi and Nagai (12) reported that cysteamine, a thiol with reducing function, increased the content of GSH and promoted male pronucleus formation even in cumulus denuded porcine oocytes (DOs) cultured in TCM 199. Probably in this case, cysteamine converted (reduced) cystine to cysteine in TCM 199, a cystine-rich medium. When DOs and cumulus-oocyte complexes (COCs) were cultured in the medium supplemented with cysteamine, the GSH content of COCs was significantly higher than that of DOs (12). Furthermore, Mori *et al.* (13) demonstrated elegantly that gap junctional communication between the porcine oocyte and cumulus cells might play an important role in regulating GSH inflow from cumulus cells, by using a gap junction inhibitor, heptanol. The importance of gap junctional communication, coupling between oocytes and follicular cells (corona cells), for normal maturation of porcine oocytes was reported (14, 15). Thus the GSH is probably one of the most crucial molecules to be transported through the junction. Considering these facts, the efficiency of GSH synthesis in COCs should be best when the maturation medium is supplemented with cysteine, because both oocytes and attached cumulus cells can uptake cysteine resulting in high GSH content which in turn improves the oocyte developmental competence (6).

The intercellular ascorbic acid (AsA) is another important anti-oxidant in oocytes (16). However, Tatemoto *et al.*, (17) reported a significantly lower content of intercellular AsA in COCs than in DOs after IVM in pigs. Thus there might be some cumulus cell derived factors, other than GSH and AsA, which can go through gap junctional communication and enhance the rate of male pronucleus formation in oocytes after IVF. Further studies are needed to clarify this point.

Furthermore most recently it was reported that porcine follicular fluid plays a critical role in protecting oocytes from oxidative stress through a higher level of radical scavenging activity elicited from superoxide dismutase isoenzymes, resulting in the enhancement of cytoplasmic maturation responsible for developmental competence after IVF (18).

Taken together, it is recommended to supplement IVM medium with both components that can increase the GSH content in oocytes and porcine follicular fluid.

3.2 IVM of oocytes under a low oxygen tension

In general, *in vitro* culture is maintained at 20% O_2 tension that is higher than that of the *in vivo* environment resulting in an increased production of reactive oxygen species (ROS) (19). ROS can cause lipid peroxidation and enzyme inactivation, causing cell damage by promoting hydroxyl radical formation (20). To decrease the production of ROS, it has been found that reduction of the oxygen tension during embryo culture from 20% to 5% has a beneficial effect on embryo development in pigs (21).

However, there have been only two reports investigating the effects of oxygen tension during maturation culture on the nuclear maturation and their subsequent developmental ability. Kikuchi *et al.* (7) cultured porcine COCs *in vitro* under 5% O_2 or 20% O_2 conditions, and IVM oocytes were fertilized *in vitro* and subsequently were cultured for 6 days *in vitro*. They found that nuclear maturation and blastocyst formation rates were not different, however, the quality of blastocysts measured by the total cell number in blastocysts was higher (mean cell number = 43.5) after IVM under 5% O_2 than that after 20% O_2 (37.8).

Iwamoto *et al.* (22) assessed the effects of (1) type of immature oocytes [COCs or parietal granulosa plus cumulus-oocyte complexes (GCOCs)]; (2) oxygen (O_2) tension (5% or 20%), on maturation, activation and parthenogenetic development. The rate of nuclear maturation in the GCOC group (73.0) was significantly higher than that in the COC group (60.6%), but the rates did not differ between the 5% and 20% O_2 tension groups. When oocytes were matured under 5% O_2 tension and stimulated, the rate of normal oocyte activation (a female

pronucleus formation and emission of the second polar body) was significantly higher (38.5%) than when oocytes were matured under 20% O_2 tension (24.5%). On the other hand, the rate of normal activation was not significantly different between the COC and GCOC groups. When COC and GCOC matured under 5% and 20% O_2 tension were stimulated and subsequently cultured *in vitro* for 6 d, the rate of blastocyst formation did not differ between the oocyte types and also between the O_2 tension groups. However, blastocyst quality as measured by mean total cell number was significantly higher in the 5% O_2 group (34.6 for COC; 33.8 for GCOC) compared with the 20% O_2 group (25.9 for COC; 27.0 for GCOC). These results suggest that low O_2 tension (5%) during IVM of porcine oocytes promotes their ability to be activated normally and improves the quality of parthenogenetic blastocysts, as reported in IVM-IVF oocytes (7).

Bing *et al.* (23) cultured porcine DOs and COCs in the medium supplemented with or without cysteamine under 5% O_2 or 20% O_2 conditions to measure GSH content of oocytes, and IVM oocytes were fertilized *in vitro* to assess their ability to form male pronucleus. Although the GSH content of DOs cultured in the absence of cysteamine was not affected by the oxygen tensions, DOs cultured under the low oxygen tension had a significantly higher rate of male pronucleus formation than that of DOs cultured under the high oxygen tension. In this report, the small (0.5 pmol/oocyte) non-significant increase in the GSH content of DOs cultured without cysteamine under 5% vs 20% oxygen may be sufficient to significantly increase in the rate of male pronucleus formation. Alternatively, culture under 5% O_2 may change the metabolism of oocytes so that they showed a different response to sperm penetration, promoting male pronucleus formation. Under 5% O_2 despite the male pronucleus formation rates of DOs cultured in the medium with and without cysteamine being the same, DOs cultured with cysteamine showed a significantly higher GSH content (6.63 pmol/oocyte) than that of DOs cultured in the medium without cysteamine (1.83 pmol/oocyte) ($P<0.05$). These results indicate that even in the absence of cysteamine the metabolism of DOs may be enhanced by culture under 5% O_2, resultingin improved male pronucleus formation.

Taken together, it is suggested that COCs should be cultured under 5% O_2 (7) in practical in IVP systems of porcine embryos.

3.3. Importance of cumulus cells during IVM of oocytes

It is generally accepted that cumulus cells during maturation period support IVM of oocytes to the metaphase-II stage and reduction of DNA fragmentation (24) of oocytes, and also that they are involved in the cytoplasmic maturation needed for developmental competence of post-fertilization such as male pronucleus formation (25) and development to the blastocyst stage in pigs (26). Without cumulus cells, porcine oocytes could not develop beyond the 4 cell stage even after maturing in the medium containing cysteamine and having a high content of GSH and a high rate of male pronucleus formation after IVF (12). Furthermore, Bing *et al.* (23) cultured porcine DOs and COCs in the medium supplemented with or without cysteamine under 5% O_2 or 20% O_2 conditions to compare GSH content of oocytes, and IVM oocytes were fertilized *in vitro* to assess their ability to form male pronucleus. The GSH content of DOs cultured in the presence of cysteamine was significantly higher than that of COCs cultured in the absence of cysteamine under both oxygen tensions (5% O_2: 6.63 vs 2.67 and 20% O_2: 5.85 vs 2.57 pmol/oocyte). However, in contrast with this result, cysteamine-treated DOs showed a significantly lower rate of male pronucleus formation than that of COCs under 5% O_2 (54.1% vs 76.7%), and the same rate as that of COCs without cysteamine treatment under 20% (67.0% vs 79.3%). In addition, when cultured in the absence of cysteamine, despite the GSH contents of COCs and DOs being similar under both oxygen tensions (5% O_2: 2.67 vs 1.83 and 20% O_2: 2.57 vs 1.32 pmol/oocyte), a significantly higher rate of male pronucleus formation was obtained for COCs than for DOs (5% O_2: 76.7% vs 41.0% and 20% O_2: 79.3% vs 24.4%). Although it should be noted that both DOs and COCs cultured under 20% O_2 condition showed significantly higher GSH contents and higher rates of male pronucleus formation when DOs or COCs were cultured with cysteamine than without cysteamine (23). Thus the absolute amount of GSH is not the sole determinant of male pronucleus formation. Therefore it is suggested that the existence of cumulus cells during IVM may be involved in enhancing male pronucleus formation in oocytes after IVF. Because porcine oocytes cultured *in vitro* with cumulus cells maintain gap junctional communication during the first 24-32 h (15), the factors that facilitate cytoplasmic maturation may be transported from cumulus cells to the oocytes through gap junctional communication. Although these factors have not been determined yet, some molecular substrates such as ions, nucleotides, amino acids and hormones have been reported to traverse from cumulus cells to oocytes via gap junctional communication (27-29). Alternatively, cumulus cells might neutralize the harmful effect of ROS during IVM to protect the oocytes from oxidative stresses and to improve oocyte maturation and subsequent development after IVF (30). Tatemoto *et al.* (30) reported that porcine DOs were highly susceptible to oxidative stress resulting in the oocyte degeneration, and that cumulus cells efficiently prevented oocytes from the cell damage.

The diverse behavior of cumulus cells during *in vitro* culture affects nuclear and cytoplasmic maturation of porcine oocytes, which also affects IVF results (31). The frequency of cumulus expansion reported to be higher in case of granulosa-cumulus-oocyte complexes (GCOCs) (82.8%) than that of COCs (65.9%) after maturation for 48 h. The percentage of M-II stage oocytes significantly increased progressively until 48 h of IVM in the GCOC, while in the COC group this value reached its plateau (65.6 %) at 42 h of IVM and did not change significantly by 48 h of culture. Especially, relationship among oocytes, cumulus cells and culture conditions (attachment to the bottom of the dish) might affect the maturity and fertilization ability of the oocytes. The oocytes attached to the bottom of culture dish with dark, compact cumulus

underwent nuclear maturation earlier (58.8% at 36 h) than that of the floating oocytes showing normal cumulus expansion (31.6% at 36 h). The rate of monospermic fertilization after IVF of COCs that show normal cumulus expansion was higher (44.1%) than that of COCs attached to the dish (9.1%). It can be concluded that granulosa cells may promote normal cumulus expansion thus decreasing heterogeneity in nuclear and cytoplasmic maturation amongst oocytes. In fact, the condition of cumulus cells during maturation culture alters meiotic spindle morphology, which is considered to be one of the indices of cytoplasmic maturation and affects on the normal pronuclear formation after IVF (32).

3.4. Step-wise IVM systems

Currently, to improve the developmental competence of IVM oocytes, some reversible inhibitors of meiotic resumption, such as butyrolactone-1 (33), cycloheximide (34), dibutyryl cAMP (35, 36) and roscovitine (37-39), were added to IVM media during preincubation or the first 20 h of IVM. Mainly these experiments have been carried out to expect some accumulation of developmental factors in oocytes through communication with cumulus cells before their meiotic resumption. Although all of these chemicals prevented the germinal vesicle breakdown and synchronized the progress of oocyte maturation, cell cycle dependent kinase inhibitors such as butyrolactone-1 and roscovitine, did not improve the developmental competence of the oocytes. Only dibutyryl cAMP and protein-synthesis inhibitor, cycloheximide, were reported to improve the development of the oocytes to the blastocyst stage after IVF (34-36).

Utilization of step-wise IVM systems with cycloheximide or dibutyryl cAMP could be beneficial for oocyte developmental competence in pigs.

4. PREVENTION OF ABNORMAL FERTILIZATION

Although various approaches have been made to reduce the incidence of polyspermic penetration into IVM oocytes, this persistent difficulty still constitutes a major obstacle to the production of normal porcine embryos *in vitro* (40-42), probably due to a slow zona reaction and simultaneous sperm penetration (43, 44). To solve this problem, there seems to be some rooms for further improvement in the efficiency by reducing the simultaneous initiation of acrosome reaction which triggers a sperm race for penetration or by improving the quality of IVM oocytes (42).

4.1. Control of polyspermic fertilization from sperm side by changing IVF procedure

In a majority of traditional porcine IVF systems (5, 45-47), boar spermatozoa are exposed to caffeine, which is generally known as a molecule that inhibit cyclic nucleotide phosphodiesterase, resulting in an increase in intracellular cyclic adenosine 3', 5'-monophosphate (48, 49), and induction of capacitation and spontaneous acrosome reaction of boar spermatozoa (49-51). However, it has been suggested that the acrosome reaction induced by caffeine achieves sperm penetration by 6 h after insemination, accompanied by an increase in polyspermic fertilization (52, 53). Recently, polyspermic penetration has been partially reduced, regardless of the presence of caffeine, by adding adenosine or fertilization-promoting peptide (FPP: pGlu-Glu-ProNH_2) in IVF medium (50, 51) or spermatozoa preincubation medium (54). In these studies, the analysis of the functional state of spermatozoa showed that adenosine and FPP stimulated capacitation but inhibited the spontaneous acrosome reaction. Furthermore, hyaluronic acid was also reported to induce a similar functional change as adenosine or FPP (55). Same as adenosine, hyaluronic acid that is present in the intraluminal fluid of the oviduct (56) was also found to be secreted by porcine COCs during IVM (57). Recently, it has been shown that oligosaccharide derived from chondroitin sulfate A has an ability to inhibit hyaluronidase activity of spermatozoa and improves the incidence of monospermic penetration by reducing the number of zona-binding spermatozoa (58). Utilization of molecules existing in the place of fertilization *in vivo* would be a way to overcome the problem of polyspermy.

On the other hand, the period of gamete co-culture during IVF also seems to affect the incidence of polyspermic fertilization. In traditional porcine IVF systems, although sperm penetration takes place around 3 h after insemination and is completed by 6 h, the incidence of polyspermy continues to increase when the co-culture duration is extended (50, 51). Therefore, majority of laboratories studying porcine IVF use the duration of gamete co-culture for about 6 h (7, 59, 60). Recently, a new strategy has been proposed to improve the efficiency of IVP of normal porcine embryos by reducing the gamete co-culture period to 10 min and then maintaining the oocytes with the zona-binding spermatozoa in fresh IVF medium, with caffeine but without swimming spermatozoa around, for 5 h (61). However, when shorter co-culture periods were applied with frozen-thawed spermatozoa, the penetration and polyspermy rates were not affected, and the overall efficiency of IVP of normal embryos was not improved (62). Although the efficiency in this strategy may be greatly affected by the type (fresh or frozen-thawed) and concentration of spermatozoa, a transient co-incubation (for 5 min) with fresh spermatozoa followed by an additional culture in caffeine-free and sperm-free fertilization medium for 8 h significantly improved the monospermic fertilization (54). Furthermore, pretreatment of spermatozoa with adenosine for 90 min increased both the incidence of capacitated cells and the monospermic rate in this transient co-culture system (54). *In vivo*, only spermatozoa that were capacitated in the oviduct can penetrate into oocytes following the AR on the zona pellucida. Additional attentive steps to mimic IVF should be required to establish successful porcine IVF systems. It was demonstrated that the presence of beta-mercaptoethanol prevents stimulating effect of caffeine on sperm capacitation and spontaneous AR and that the presence of beta-mercaptoethanol during IVF procedures, except at a transient co-culture period of gametes in the presence of caffeine, has a beneficial effect to maintain the function of gametes, the incidence of normal fertilization, and consequently the quality of IVF embryos (63).

However, since this strategy has been stayed at a limited improvement, additional modifications would be needed to improve the effectiveness of this new strategy.

Recently, Yoshioka *et al.* (60) have developed a chemically defined, porcine gamete medium (PGM) that was devised on the basis of the concentrations of inorganic elements and energy substrates in the pig oviduct. They reported that the addition of theophylline, adenosine and cysteine to PGM (PGMtac) enhanced the monospermy in porcine IVF and the subsequent embryo development. This IVF system using PGMtac had widespread utility with both frozen-thawed and liquid-stored spermatozoa (64). In their study, a gradient centrifugation method using Percoll, which was effective to collect and concentrate motile sperm (65), has been applied to wash frozen-thawed spermatozoa. Furthermore, Funahashi and Nagai (66) invented a new IVF system designated as a climbing-over-a–wall (COW) IVF method in which highly motile spermatozoa can be selected physically, and demonstrated that COW-IVF method can increase the normal penetration of frozen-thawed boar spermatozoa into IVM oocytes without any reduction in the sperm penetration rate. Similar unique IVF system, using a cryopreservation straw (67), has also been reported to reduce the incidence of polyspermic penetration in pig IVM/IVF. The principle of these systems is similar, because spermatozoa need to swim some distance to reach oocytes. Additional unique contrivances in IVF systems may be required not only to select motile spermatozoa but also to disperse the time for spermatozoa to reach the surface of oocytes.

4.2. Control of polyspermic fertilization from oocyte and oviduct sides

A weak and delayed cortical granule release, which is important for zona reaction to prevent polyspermic fertilization, was present in porcine IVM-IVF oocytes (68) and has been thought to be the main reason for a high rate of polyspermic fertilization (69). Recently, it has been found that the degree of cortical granule exocytosis of IVM oocytes could be significantly improved in the presence of beta-mercaptoethanol (63).

Whereas when spermatozoa were co-cultured with oviduct cells for 2.5 h and then used for IVF of IVM oocytes on oviduct cell monolayer, polyspermic rates, maintaining high sperm penetration rates, were reduced by 40-50% as compared with control (70). And the use of oviduct fluid from sows on the 20th or 21st day of estrus (added to maturation medium in 10, 30, or 100% concentration) to culture oocytes for 1.5 hours before IVF reduced the polyspermic fertilization rate without lowering the IVF rate (71). The observations of IVM-IVF oocytes by laser microscope revealed that IVM oocytes exposed to oviduct fluid and ovulated oocytes have a similar cortical granule reaction (71). Furthermore, it was suggested that the ability of porcine oocytes to undergo the zona reaction is not fully developed until their exposure to the oviduct (72). Therefore the high rate of polyspermic fertilization in IVM-IVF of porcine oocytes is not only related to the cytoplasmic maturation but also to the modification of zona pellucida and/or cytoplasm of oocytes in the oviduct after ovulation. However, considering the difficulty of obtaining a large amount of oviduct fluid from pigs, *in vitro* synthesis of active ingredients that can be obtained by isolation and purification from oviduct fluid and their application to the IVM-IVF system were expected. Actually when hyaluronan, which is the major glycosaminoglycan found in the oviductal fluid, was added to IVF medium, the rate of polyspermic fertilization was decreased (73). Most recently it was reported that exposure of either *in vitro*-matured oocytes or spermatozoa to porcine oviduct-specific glycoprotein pOGP decreased polyspermy and spermatozoa binding while maintaining high penetration rates of pig oocytes fertilized *in vitro* (74).

It was reported that prefertilization incubation of spermatozoa in a suitable concentration of follicular fluid reduced polyspermy (75). And Snap-frozen follicular fluid from medium size follicles in IVF medium reduced polyspermy (76).

4.3. Sperm penetration into immature oocytes

Another possibility to produce the abnormal embryos is considerable by sperm penetration into immature oocytes. During fertilization, meiosis completes by the second polar body emission after the sperm penetration. The ploidy of oocyte genome is reduced to "n" during meiosis and zygotic genome turns to "2n" again by sperm penetration. One of the problems causes by oocyte side is the meiotic arresting at immature stages such as metaphase-I or the failure of maturation to metaphase-II stage. Sperm can penetrate into such immature oocytes and activates the oocytes resulting in formation of male and female pronuclei (77). Furthermore, these zygotes derived from immature oocytes can develop to the blastocyst stage (78). The mechanisms transiting from the first to second cell division and arresting before the mature stage are not well understood. Such cases might be seen in other species such as human. Further studies to clarify the mechanisms of oocyte arresting and activation should be progressed.

5. IVC OF IVM-IVF OOCYTES

Our previous reports (79, 80) suggest that quality of IVP blastocyst were low, emphasizing that the IVC system is not optimal because only one or two days-IVC of IVM/IVF embryos resulted in a low developmental competence to term after transfer to the recipients. To optimize IVP system, many laboratories are trying to modify the culture system to obtain the term development after transfer of blastocysts to recipients. However, limited cases have been reported (7, 60, 81).

According to Kikuchi *et al.* (7), when porcine IVM-IVF oocytes were cultured in a IVC medium supplemented with pyruvate and lactate for the first 2 days and then in the medium containing glucose for subsequent 4 days under 5% O_2, the rate of blastocyst formation (25.3%) was higher as compared with that of glucose supplement for the first 2 days (14.5%). They also found that the cell number was significantly higher in IVC medium containing pyruvate and lactate (48.7) than that of glucose supplement (35.4). This result clearly indicates that

glucose in IVC medium for the first 2 days of culture is detrimental to the development of embryos. Furthermore, when conditioned medium, which was obtained from supernatant of the medium co-cultured with oviductal epithelial cells for 2 days and supplemented with pyruvate and lactate, was used as IVC medium during the first two days of embryo culture, it had a significant effect on the cell number of the blastocyst (58.3) as compared with the control (48.4), When expanding blastocysts after 5 days of culture (cell number: 49.7) were transferred to an estrus-synchronized recipient (50 blastocyst per a recipient), the recipient was pregnant and farrowed 8 normal piglets. On the other hand, expanded blastocysts after 6 days of culture (cell number: 80.2) were transferred to 2 estrus-synchronized recipients, both were pregnant and farrowed a total of 11 piglets.

Recently, Wongsrikeao *et al.* (82) suggested that the utilization of another hexose, fructose, for *in vitro* culture during days 3 to 6 have an advantage for the embryonic development and reduction of DNA fragmentation in blastomeres. Further discussion will be made on the energy supplementation to culture media in relation to embryo quality.

Yoshioka *et al.* (60) have demonstrated that pig IVM oocytes can be fertilized and develop to the blastocysts stage in chemically defined, protein-free media and that the IVP blastocysts are developmentally competent to full term after embryo transfer. In their study, when presumptive zygotes were cultured in porcine zygotes medium (PZM) containing pyruvate and lactate at the physiological levels of the pig oviduct fluid and no glucose, for 5 days, approximately 25% of zygotes developed to blastocysts in which mean number of cells was approximately 40. Furthermore, following transfer of 90 Day-5 blastocysts into four recipients, all of the recipients became pregnant and farrowed a total of 21 piglets. These results suggest that excellent embryos production system is established by modifying the IVP system. These improved systems produce high quality of blastocysts, and are useful for generating cloned and transgenic pigs.

6. PERSPECTIVE

Although the developmental potential of IVM oocytes is lower than that of *in vivo* matured oocytes (83, 84), the IVM has the advantage over oocyte collection from live animals. More oocytes with low cost can be obtained by IVM. Considering the fact that the most advanced biotechnology, such as somatic cell cloning, needs an extremely large number of oocytes (85), using of the IVM oocytes will be the successful and efficient method to produce somatic cell cloned piglets.

Furthermore it will be a great advantage to collect immature oocytes from small size of preantral follicles in pig ovaries and grow (IVG) and mature these oocytes *in vitro* (86). If a reliable IVG method is established and it can be combined with IVM-IVF method thus the efficiency of IVP of embryos will be greatly improved. In addition, a brand-new culturing method, micro-channel system, has been reported for *in vitro* embryo production (87-89). This system gives a merit to improve embryo development or to evaluate the embryo quality when the detection devise is introduced in the system (90). This system may have potential in the future (91).

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