

Appearance, fate and utilization of abnormal porcine embryos produced by in vitro maturation and fertilization

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In vitro production (IVP) including in vitro maturation (IVM) and fertilization (IVF) is now an important technology for obtaining live piglets. However, there are still two significant obstacles to the efficient production of viable porcine embryos: (1) polyspermy and (2) fertilization of oocytes arrested at the immature stage. These phenomena relate to production of embryos with abnormal ploidy (polyploidy). To avoid these problems, careful selection of mature oocytes for IVF, and regular monitoring of normal and abnormal fertilization (polyspermy and/or lack of male pronucleus formation) are very important. In our recent studies, however, we have confirmed that some oocytes with abnormal ploidy after polyspermy can develop into diploid embryos with potentially normal developmental ability. The mechanism by which such fertilized polyploid oocytes develop to a normal state during embryo development is still not well understood. Attempts to clarify this mechanism would hopefully reveal data that are very useful for not only IVP but also other technologies such as the production of transgenic or cloned animals using IVM oocytes, including other species, also for human reproductive manipulation. In this review, we focus on studies of normality of IVM oocytes and ploidy of IVP embryos, and try to suggest practical ways of solving the problems mentioned above in pigs.

Normality of porcine zygotes produced in vitro

The in vitro developmental competence or viability of porcine in vitro-matured (IVM)–in vitro-fertilized (IVF) oocytes to the blastocyst stage was first confirmed and reported by Mattioli *et al.* (1989). Since then, live-born piglets have been obtained from IVM–IVF embryos after in vitro culture (IVC) to the 2- to 4-cell stages (Mattioli *et al.* 1989; Yoshida *et al.* 1993; Funahashi *et al.* 1996; Funahashi & Day 1997). Viable piglets have also been generated by transfer of in vitro-produced (IVP = IVM, IVF and IVC) embryos at the blastocyst stage (Marchal *et al.* 2001; Kikuchi *et al.* 2002). Over the last few years, IVC procedures have been improved, but IVM and IVF systems are still hampered by problems resulting in poor developmental ability, and low quantity and quality of the embryos produced. This leads to embryo loss even after their transfer to recipients. One of the major causes of this problem is abnormal ploidy of IVP embryos due to (1) polyspermy during IVF and (2) fertilization of oocytes arrested at the

immature stage. In addition, aneuploidy during oocyte maturation should be also noticed as a cause of embryo abnormality (Lechniak et al. 2007). It is essential to make every effort to achieve normality in IVP embryos after IVM and IVF. However, it is basically impossible to prevent precocious meiotic arrest or polyspermy in all cultured oocytes even using currently established technologies. Another approach for obtaining a good result (live offspring) under these conditions is to select fertilized oocytes or embryos that guarantee the developmental competence to piglets. It is generally accepted that only monospermic fertilized oocytes that have matured meiotically to the metaphase-II (M-II) stage can be guaranteed to show normal embryonic development. On the other hand, in comparison with sperm, oocyte sources are limited, especially those from rare genetic resources. To apply in vitro reproductive technologies in such animals, it is important to utilize as many oocytes as possible from a limited number of females. For this propose, assuming some ability to develop to term, abnormally generated embryos may be used to generate offspring. For half a century, mammalian embryos with abnormal ploidy have been known to be capable of surviving even into the post-implantation period (Piko & Bomsel-Helmreich 1960; Bomsel-Helmreich 1971; Han et al. 1999a). However, the details of the mechanisms involved have remained unclear.

In the present review article focusing on porcine IVP systems, we summarize the status and consequences of generating abnormal embryos obtained as a result of polyspermy and also by fertilization before completion of meiotic maturation. We also consider the use of abnormally generated embryos to improve porcine IVP efficacy.

Polyspermy

Status of efforts to reduce polyspermy

Polyspermy occurs as a result of simultaneous penetration of an oocyte by two or more spermatozoa. The problem of polyspermy in IVP porcine systems is significant, and has remained unsolved for many years. The main reason for polyspermic fertilization seems to be the presence of a large number of spermatozoa at the site of IVF in the absence of a regulatory effect of the female reproductive tract to control the quantity and quality of spermatozoa. An optimal sperm concentration and time interval for IVF are basic requirements for avoiding an extremely high incidence of polyspermy (Nagai et al. 2006). In our laboratory, we have chosen batches of frozen-thawed epididymal sperm showing good penetration ability to conduct IVF by co-incubation of 1×10^5 sperm/ml with oocytes for 3 h. Under these conditions, we are able to achieve reproducible sperm penetration beginning at 2 h post-insemination, the penetration rate reaching a plateau of around 80% at 4 h with a 60% polyspermy rate and an average of 3 spermatozoa per oocyte (Kikuchi et al. 2002, 2006). This means that only 20% of the oocytes subjected to IVF are monospermic. Another reason for polyspermy is considered to be the insufficient ability of IVM oocytes to block polyspermy due to a delayed or incomplete zona reaction and the imperfect characteristics of the zona caused by lack of exposure to oviduct fluid (Funahashi 2003).

Up to now, many attempts to decrease polyspermy have been reported. There have been several attempts to imitate in vivo conditions during IVF in order to regulate the number of spermatozoa near the oocytes. Some procedures have been based on the concept of allowing a small number of functional or capacitated spermatozoa to approach the oocytes, and these include the climbing-over-a-wall (COW) method (Funahashi & Nagai 2000), straw IVF (Li et al. 2003) and the biomimetic microchannel IVF system (Clark et al. 2005). Other methods have aimed at improving the characteristics of oocytes (more specifically the zona pellucida: ZP), such as treatment of oocytes with oviductal glycoproteins before IVF (Kouba et al. 2000; McCauley

et al. 2003a), and/or modulation of sperm capacitation, acrosome status or sperm binding to the ZP by techniques such as exposure of spermatozoa and oocytes to oviductal cells (Nagai & Moor 1990), oviductal fluid (Kim et al. 1997), oviduct-specific glycoproteins (Kouba et al. 2000; McCauley et al. 2003a), follicular fluid (Funahashi & Day 1993) or hyaluronan (Suzuki et al. 2000) before or during IVF. Although these approaches have been able to moderate the frequency of polyspermy, they could not eliminate it completely. Furthermore, some of these methods require special equipment (e.g. the biomimetic microchannel) or involve undefined factors (e.g. co-culture systems), and thus cannot be applied routinely to porcine IVP systems. As a consequence, in currently used IVF systems, the incidence of polyspermic fertilization is still very high. Thus, polyspermy is still considered to be a major problem that reduces the productivity of IVP systems in the pig.

Selection of monospermic oocytes among IVF oocytes

The most reliable method for selecting monospermic oocytes is to observe a single penetrated sperm head or male pronucleus in the ooplasm directly. Observation of a pronucleus(ei) is quite reliable, because oocytes with two (male and female) pronuclei are normally the result of monospermy, whereas oocytes with three or more pronuclei are the result of polyspermy. Unfortunately, the ooplasm in large animals such as pigs and cattle contains a large number of lipid droplets that make direct observation of organelles difficult. Pronuclei in such oocytes can be visualized by either nuclear staining or centrifugation. Vital nuclear staining is usually performed using fluorochromes such as Hoechst 33342, but this has been proven to have a detrimental effect on embryo development (Ebert et al. 1985; Tsunoda et al. 1988; Smith 1993; Yang et al. 1990).

Centrifugation is an effective way to polarize lipid droplets and thus to visualize pronuclei (Cran 1987), and seems to be safer than nuclear staining with fluorescent dye. To study the effects of centrifugation on IVP zygotes, we have evaluated the survival and developmental ability of fertilized oocytes after centrifugation (Somfai et al. 2008). In pigs, pronucleus formation is evident from 5 h post-insemination and reaches a plateau after 8 h (Kikuchi et al. 2006). Oocytes emitting 2 polar bodies, and thus considered to be mature and activated by sperm penetration, were centrifuged 10 h after insemination at $10,000 \times g$ for 20 min. This method rendered pronuclei visible even under a stereo microscope, and also allowed the oocytes to remain viable. They were then cultured in vitro for 6 days. The cleavage rates on Day 2 (Day 0 = the day of IVF), and the rates of viable blastocysts and average numbers of cells within them on Day 6 did not differ significantly between the centrifugation and control groups. These results indicate that centrifugation itself does not affect developmental ability. After centrifugation, oocytes with 1, 2 or more pronuclei and unfertilized oocytes were separable under a stereo microscope by direct observation of pronuclei (Fig. 1). The efficacy of selection was verified by fixation and staining, and we found that, in most (75.0–82.8%) of the selected oocytes, the number of pronuclei assessed by direct observation matched that assessed by nuclear staining (Somfai et al. 2008). We therefore considered that the selection of mono- and polyspermic oocytes by centrifugation followed by direct nuclear observation at the pronucleus stage was acceptable for further experiments.

Development after polyspermic fertilization

When zygotes with different numbers of pronuclei were selected and cultured separately, we found that the cleavage rates of oocytes with one or two pronuclei (considered to be

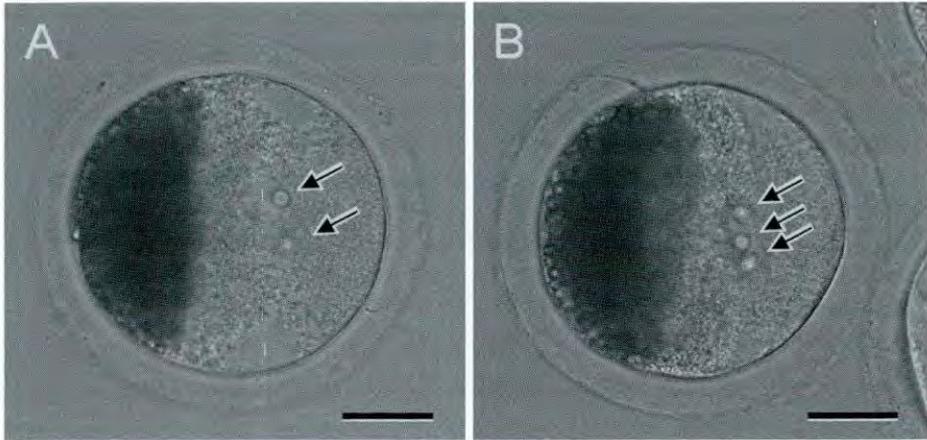


Fig. 1 Porcine oocytes after centrifugation treatment 10 h after IVF. Location and number of pronuclei are determined by the presence of nucleolus precursor bodies (arrows). A) Zygote with two pronuclei. B) Zygote with three pronuclei. Scale bar represents 30 μm .

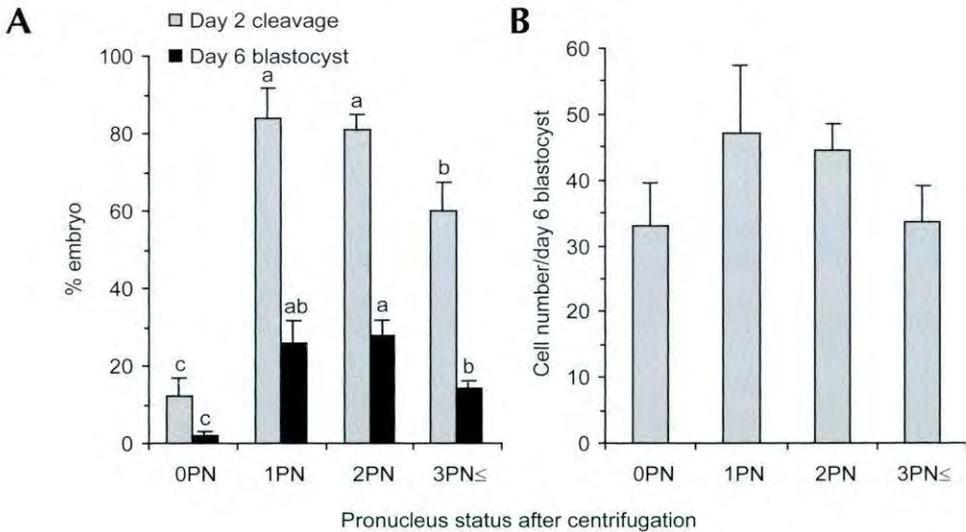


Fig. 2 In vitro development of in vitro-produced porcine zygotes with different numbers of pronuclei visualized by centrifugation. (A) Rates of cleaved embryos on Day 2 and blastocysts on Day 6. (B) Average cell number of blastocysts on Day 6. 0PN; oocytes without pronucleus, 1PN; oocyte with one pronucleus, 2PN; oocytes with two pronuclei, and 3PN \leq ; oocytes with three or more pronuclei. Mean \pm SEM are presented. ^{a-c} Percentages with different superscripts differ in each category ($P < 0.05$). Data is from Somfai et al. (2008).

parthenogenetic or monospermic, respectively) were significantly higher than those of oocytes with three or more pronuclei (considered to be polyspermic) (Fig. 2) (Somfai et al. 2008). The rate of development to the blastocyst stage was higher for zygotes with two pronuclei (monospermy) than for those without any pronucleus or with three or more pronuclei (unfertilized oocytes

or polyspermy, respectively), although it was not significantly different from that of zygotes with one pronucleus (parthenotes). The numbers of cells did not differ significantly among the oocytes thus classified. We analyzed the ploidy of blastocysts that had developed from putative monospermic and polyspermic zygotes (Table 1), and found that 73.8% of blastocysts that developed from monospermic oocytes were diploid (2n) whereas 12.5% of them were mixoploid containing a diploid cell(s). Thus, after calculation, 86.3% of the blastocysts that had developed from monospermic oocytes were found to contain diploid cells. On the other hand, 31.3% and 14.5% of polyspermic oocytes were found to develop into diploid blastocysts and mixoploid blastocysts containing a diploid cell(s), respectively. Calculation revealed that 45.8% of the blastocysts from polyspermic oocytes contained a diploid cell(s). These results suggest that not only monospermic oocytes but also a proportion of polyspermic oocytes may have the ability to develop to diploid embryos or fetuses.

Table 1. Ploidy of in vitro-cultured porcine blastocysts developing from zygotes bearing two or multiple pronuclei.

Pronuclear status after centrifugation	% ploidy of blastocyst					% blastocyst with 2n cells		% blastocyst with 2n cells
	n	2n	3n	4n	4n <	Mixoploid with 2n cells	Mixoploid without 2n cells	
2PN	8.9 ± 4.4	73.8 ± 11.9 ^a	0	4.7 ± 4.7	0	12.5 ± 12.5	0	86.3 ± 0.5 ^a
3PN ≤	17.0 ± 11.9	31.3 ± 10.1 ^b	12.1 ± 6.5	6.0 ± 3.2	3.7 ± 3.7	14.5 ± 7.2	15.1 ± 2.6	45.8 ± 9.4 ^b

PN; pronuclei.

Mean ± SEM are presented.

^{a,b} Percentages with different superscripts differ in each column ($P < 0.05$).

Table is modified from Somfai *et al.* (2008).

Our data agree with previous studies that found high frequencies of chromosomal abnormalities (mainly polyploidy) in IVP porcine embryos, which were thought to have resulted from polyspermic fertilization (McCauley *et al.* 2003b; Somfai *et al.* 2005). Polyspermic porcine embryos reportedly have an ability to develop to the blastocyst stage in vitro (Han *et al.* 1999a). This suggests that a considerable proportion of IVP blastocysts in those studies resulted from polyspermic fertilization. It has also been reported that, when polyspermic zygotes are transferred into recipients, most of the fetuses on Day 40 are diploid. This indicates that, in many polyspermic embryos, the effect of abnormally high numbers of penetrating spermatozoa on embryo ploidy is neutralized during in vivo development (Han *et al.* 1999b).

Possible mechanism of ploidy correction in polyspermic embryos

The mechanisms involved in the correction of polyploidy to a normal diploid status in polyspermic oocytes are still unclear. The ploidy of porcine embryos resulting from polyspermic fertilization is thought to depend on the location of the pronuclei before the first cleavage (Han *et al.* 1999b). This phenomenon is believed to be related to the different cleavage patterns of polyspermic embryos (including abnormal tripolar and tetrapolar cleavage), which can result in diploid, polyploid and also mixoploid embryos with a wide variety of blastomere ploidy after the first cell cleavage, including polyploids, diploids, and aneuploids (Han *et al.* 1999b; Funahashi 2003). It is believed that, during early development of embryos, blastomeres with abnormal ploidy fail to develop to the fetal stage whereas those with diploid blastomeres are able to do so. Thus, mosaic embryos having diploid cells may have the ability to develop to term (Han *et al.* 1999b, Funahashi *et al.* 2003). Other authors have suggested the involvement

of a different mechanism; Kola et al. (1987) found that tripolar cleavage of polyspermic human oocytes always resulted in abnormal numbers of chromosomes, whereas the appearance of a protrusion in 2 cell embryos was associated with diploid status in polyspermic oocytes. Similar results were obtained recently in in vitro-produced bovine embryos (Somfai et al. 2009). Funahashi (2003) has mentioned another possible mode of ploidy correction, when the chromosome compartment (a male pronucleus or a decondensed sperm head) from one sperm does not contribute to syngamy and the formation of the mitotic metaphase. Such a male nucleus may remain in the cytoplasm of one of the blastomeres of the resulting 2-cell embryo, and later this extra chromatin is disrupted and disappears through a mechanism that involves lysosomes. In current IVP systems, a reasonable proportion (15–30%) of porcine embryos can develop to the blastocyst stage, but with a wide variety of morphological appearances including partially fragmented embryos characterized by both nuclear and anuclear blastomeres (Kikuchi et al. 2002; Somfai et al. 2005; Somfai et al. 2008). Our recent study (Somfai et al. 2008) has also revealed that at 36 h after IVF a higher rate of cleaved monospermic than polyspermic embryos developed to the two-cell stage, whereas the proportion of embryos containing four or more blastomeres was significantly higher in the polyspermic than in the monospermic group. Nuclear staining revealed that the frequency of embryos containing bi- or multi-nuclear blastomere(s) did not differ between the monospermic and polyspermic groups, whereas the proportion of embryos containing at least one blastomere without any nucleus (considered as a sign of a fragmented cytoplasm) was significantly higher in polyspermic than in monospermic embryos. The mean number of blastomeres was significantly higher in polyspermic than in monospermic embryos, whereas the mean number of nuclei per embryo did not differ between the two groups (Table 2). These results suggest that polyspermic fertilization is associated with partial embryo fragmentation; however, it is not clear if this phenomenon is related to ploidy correction during early embryonic development.

Table 2. Ploidy of in vitro-cultured porcine blastocysts developing from zygotes bearing two or multiple pronuclei.

Pronuclear status after centrifugation	% ^a cleaved	% ^b embryos by microscopic evaluation			Nuclear staining			
		2 cell	3 cell	4 cell ≤	% ^b embryos with multiple nuclei	% ^b embryos with anuclear blastomere	Mean no. blastomeres per embryo	Mean no. nuclei per embryo
2PN	77.7 ± 6.0 ^c	56.0 ± 6.0 ^c	22.0 ± 6.1	24.9 ± 7.0 ^c	6.9 ± 3.4	34.4 ± 3.1 ^c	2.8 ± 0.1 ^c	2.3 ± 0.1
3PN ≤	59.7 ± 6.4 ^d	26.7 ± 4.1 ^d	25.8 ± 3.9	47.7 ± 6.6 ^d	13.9 ± 1.3	57.6 ± 3.8 ^d	3.4 ± 0.1 ^d	2.5 ± 0.1

PN; pronucleus(ei).

All embryos were evaluated or fixed at 36 h after onset of IVF

^a Percentage to the examined embryos

^b Percentage to the cleaved embryos

Mean ± SEM are presented.

^{a,b} Percentages with different superscripts differ in each column (P < 0.05).

Table is modified from Somfai et al. (2008).

Fertilization before completion of meiotic maturation

Cytoplasmic maturity of "metaphase-I-arrested" oocytes

In our previous IVM system (Kikuchi et al. 1999a), matured oocytes at the M-II stage appeared at 30 h and the maturation rate became maximal after 36 h of IVM. About 35% of the cultured oocytes failed to reach the M-II stage and about 25% of them were arrested while still immature

(Fig. 3). These oocytes were characterized by a typical metaphase plate without a first polar body. This phenomenon is often referred to as “metaphase-I (M-I) arrest”. In general, meiotic arrest at the M-I stage can be caused by a number of factors, such as insufficient meiotic competence because of an inadequate follicular phase or oocyte diameter (Szybek 1972; Sorensen & Wassarman 1976; Motlik & Fulka 1986; Eppig *et al.* 1994) or stress caused by inappropriate culture conditions, such as the use of inadequate isolation or culture media (Bae & Foote 1980; Bagger *et al.* 1987; Kikuchi *et al.* 1999b). Intracellular calcium regulation reported to affect this phenomenon (Lechniak *et al.* 2005). As well as in pigs, in certain mouse strains (LT/Sv and LT-related strains), it is known that oocytes undergo meiotic arrest during the M-I stage (Hirao & Eppig 1997; 1999).

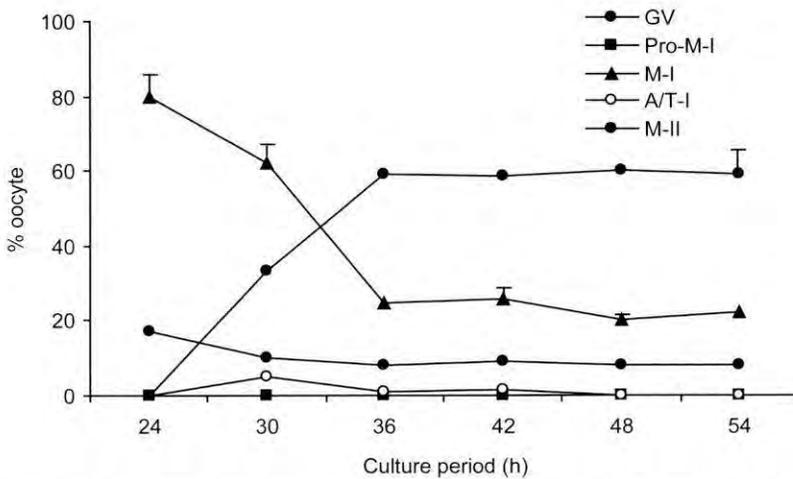


Fig. 3 Nuclear transition during in vitro maturation of porcine follicular oocytes. Oocytes were cultured and their nuclear status examined every 6 h from 24 to 54 h. Data are presented as means \pm SE. GV, germinal vesicle; Pro-M-I, pro-metaphase-I; M-I, metaphase-I; A/T-I, anaphase-I or telophase-I; M-II, metaphase-II. The figure is modified from Kikuchi *et al.* (1999a).

Mature (M-II) oocytes can be distinguished from immature ones by direct observation of a first polar body with minimum error [less than 6% in the polar body (PB) (+) group, Kikuchi *et al.* 1999b]. Comparing with selection after staining with a fluorescent dye, this method is very easy to perform and does not seem to affect developmental ability (Ebert *et al.* 1985; Tsunoda *et al.* 1988; Smith 1993; Yang *et al.* 1990). Our previous study (Kikuchi *et al.* 1999b) showed that “M-I-arrested” oocytes completed their cytoplasmic maturation just like M-II oocytes, and were capable of activation to form male and female pronuclei after IVF and also the female pronucleus after parthenogenetic stimulation. Mature oocytes having a first polar body emitted a second polar body after oocyte activation, whereas “M-I-arrested” oocytes without a polar body were activated with emission of a single polar body (Table 3). The pronucleus status (timing of its formation and morphological features) did not differ between activated “M-I-arrested” and M-II oocytes (Somfai *et al.* 2005). These results suggest that cytoplasmic maturation for oocyte activation can be completed after 48 h, even when nuclear maturation has not completed the M-II stage. On the other hand, maturing oocytes with insufficient cytoplasmic maturity [PB(-) oocytes after culture for 24 h, considered to be “fresh” M-I] did not respond to parthenogenetic stimulation and only a few of them formed pronuclei after IVF. This difference was explained by a high level of maturation-promoting factor in “fresh” M-I oocytes and a reduced level of

this factor in oocytes that were cultured for 48 h, regardless of the existence of a polar body (Kikuchi et al. 1999b). In fact, sperm heads that had penetrated into the immature cytoplasm of “fresh” M-I oocytes did not undergo transformation to a male pronucleus, but recondensed or transformed to metaphase-like chromosomes (Fig. 4). This also confirms the insufficient cytoplasmic ability of M-I oocytes at an earlier maturational stage. These results suggest that, unlike maturing oocytes, oocytes arrested at an immature stage after germinal vesicle breakdown undergo cytoplasmic maturation that allows oocyte activation and male pronucleus formation after IVF, similar to the case of matured (M-II) oocytes.

Table 3. Activation of porcine oocytes classified on the basis of the presence of a polar body.

Culture [h]	Polar body*	Treatment	% oocytes	% activated oocytes	
				With one polar body	With two polar bodies
24	–	IVF	6.2 ± 1.5 ^b	80.0 ± 16.7 ^a	20.0 ± 16.7 ^b
48	–	IVF	68.6 ± 10.8 ^a	90.0 ± 1.7 ^a	10.0 ± 1.7 ^b
48	+	IVF	61.8 ± 13.8 ^a	5.2 ± 2.3 ^b	94.9 ± 2.3 ^a
24	–	ES	0 ^b	–	–
48	–	ES	52.9 ± 5.0 ^a	73.0 ± 9.7 ^a	13.5 ± 4.7 ^a
48	+	ES	80.5 ± 4.8 ^a	8.9 ± 2.6 ^b	79.0 ± 6.7 ^b

*Examined by Nomarski differential interference contrast microscopy before treatment.

IVF; in vitro fertilization, ES; electrical stimulation

^{a,b} Percentages with different superscripts differ in each treatment (IVF or ES) ($P < 0.05$)

Table is modified from Kikuchi et al. (1999b).

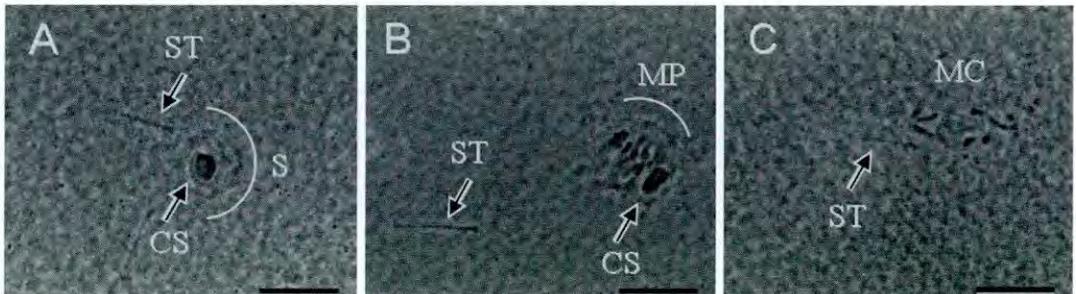


Fig. 4 Sperm heads penetrating an immature porcine oocyte at the metaphase-I stage. Oocytes were cultured for 24 h, fertilized in vitro and subsequently cultured for up to 36 h. Arrows indicate the tails of the penetrated spermatozoa. After initial decondensation, sperm heads were recondensed into a mass (Cs), being surrounded by a spindle (S) or incorporated into the maternal metaphase plate (Mp) (A and B, respectively), or transformed into metaphase-like chromosomes (C). Scale bars represent 10 μm . The figure is modified from Kikuchi et al. (1999b).

Developmental competence of “M-I-arrested” oocytes

“M-I-arrested” porcine oocytes seem to have some developmental ability, as they were shown to develop to the blastocyst stage after IVF, similarly to M-II oocytes (Somfai et al. 2005). In mice also, “M-I-arrested” oocytes have been shown to be capable of developing to the blastocyst stage (Eppig et al. 1994). The developmental competence of meiotically arrested porcine oocytes is rather poor, resulting in low rates of formation of blastocysts, which contain low numbers of cells (Table 4). Chromosomal analysis of blastocysts resulting from IVF of “M-I-

arrested" porcine oocytes has revealed a high frequency of chromosome numerical abnormality in blastomeres, especially an increased rate of triploidy (Table 5). Similar results have already been reported in mice (Eppig *et al.* 1994). Fertilization of "M-I-arrested" oocytes is suggested to result in the development of digynic triploid embryos. As reported by Kaufman *et al.* (1989), triploid diandric mouse embryos appear morphologically normal, but are smaller than normally fertilized diploid embryos.

Table 4. Blastocyst formation after IVF of porcine oocytes with/without a polar body after culture for 44 h.

Polar body*	% blastocysts	% blastocysts categorized morphologically as			Cell number per blastocyst
		Excellent	Good	Poor	
-	20.7 ± 2.8 ^b	60.0 ± 5.1	20.0 ± 8.5 ^b	20.0 ± 2.0 ^a	29.1 ± 1.4 ^b
+	34.6 ± 2.4 ^a	68.1 ± 5.1	23.0 ± 3.8 ^b	8.7 ± 1.4 ^b	52.0 ± 2.5 ^a

*Examined by stereo microscopy before IVF.

IVF; in vitro fertilization, ES; electrical stimulation

^{a,b} Percentages with different superscripts differ in each column (P < 0.05).

Table is modified from Somfai *et al.* (2005).

Table 5. Chromosome analysis of blastomeres in Day 6 blastocysts generated from porcine oocytes with/without a polar body.

Polar body	No. of blastocysts examined	No. of metaphases prepared (per blastocyst)	No. (%) of metaphases analyzed as				
			Total ^a	Haploid ^b	Diploid ^b	Triploid ^b	Tetraploid ^b
-	66	132 (2.0)	100 (78.8) ^c	16 (16.0)	44 (44.0) ^d	34 (34.0) ^c	6 (6.0)
+	122	241 (2.0)	155 (64.3) ^d	29 (18.7)	108 (69.7) ^c	13 (8.4) ^d	5 (3.2)

^a Percentage relative to the number of prepared metaphases

^b Percentage relative to the number of analyzed metaphases

^{c,d} Percentages with different superscripts differ in each column (P < 0.05)

Table is modified from Somfai *et al.* (2005).

The ploidy of porcine embryos, as suggested previously, might be related to their developmental competence. The existence of a ploidy correction mechanism like that operating in polyspermy has not yet been confirmed in zygotes or embryos from "M-I-arrested" oocytes. Nevertheless, diploid cells have been found in blastocysts that have developed from "M-I-arrested" oocytes, suggesting that the embryos are diploid, or are mixoploid but contain diploid cells. Detailed chromosome analysis will be needed to clarify the nuclear competence of such embryos.

Possible mechanism of "M-I arrest" before completion of oocyte maturation

We have compared the chromosome configurations of "M-I-arrested" oocytes with those of M-I oocytes at 33 h of IVM ("real M-I" oocytes) and with those of M-II oocytes at 48 h of IVM (Somfai *et al.* 2006) (Fig. 5). In a series of experiments, we matured porcine oocytes in the presence of the actin polymerization inhibitor cytochalasin-B (CB), a drug that inhibits actin filament polymerization. This drug has been reported to induce meiotic arrest of mammalian oocytes at the M-I stage (Wassarman *et al.* 1976). Constant tracking of nuclear progression during maturation revealed that segregation of homologous chromosomes did, in fact, occur in CB-treated oocytes; however, extrusion of the first polar body failed. This caused the formation of two sets of segregated homologous chromosomes inside the oocyte. Later, these sets united and formed a single, tetraploid metaphase plate (Somfai *et al.* 2006) (Fig. 6). Similar observations

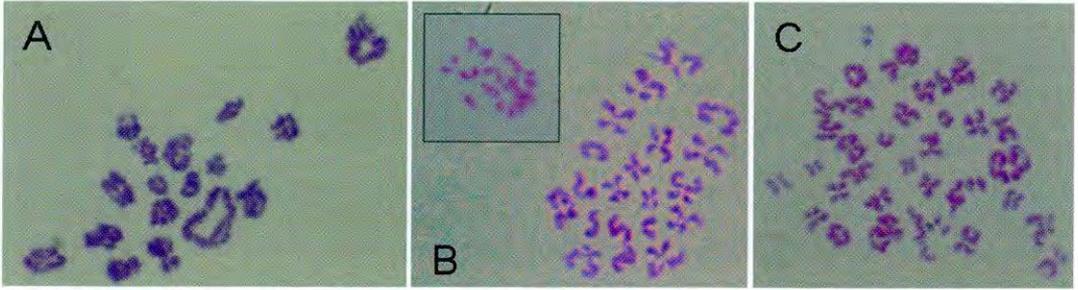


Fig. 5 Morphology of porcine oocyte chromosomes. (A) chromosomes prepared from oocytes at the metaphase-I (M-I) stage (33 h IVM), where 19 ring-shaped, symmetrical complexes of the homologous chromosomes from bivalents are observed; (B) chromosomes prepared from oocytes at the metaphase-II (M-II) stage (44 h IVM). Insert: chromosomes incorporated by a first polar body; and (C) chromosomes prepared from oocytes cultured in the presence of cytochalasin-B (44 h IVM). Both M-II- and CB-treated oocytes displayed similar separate chromosomes with two chromatids; M-II oocytes had 19 chromosomes, whereas the number of chromosomes in the CB-treated oocytes was double (38) compared with M-II oocytes. The figure is modified from Somfai et al. (2006).

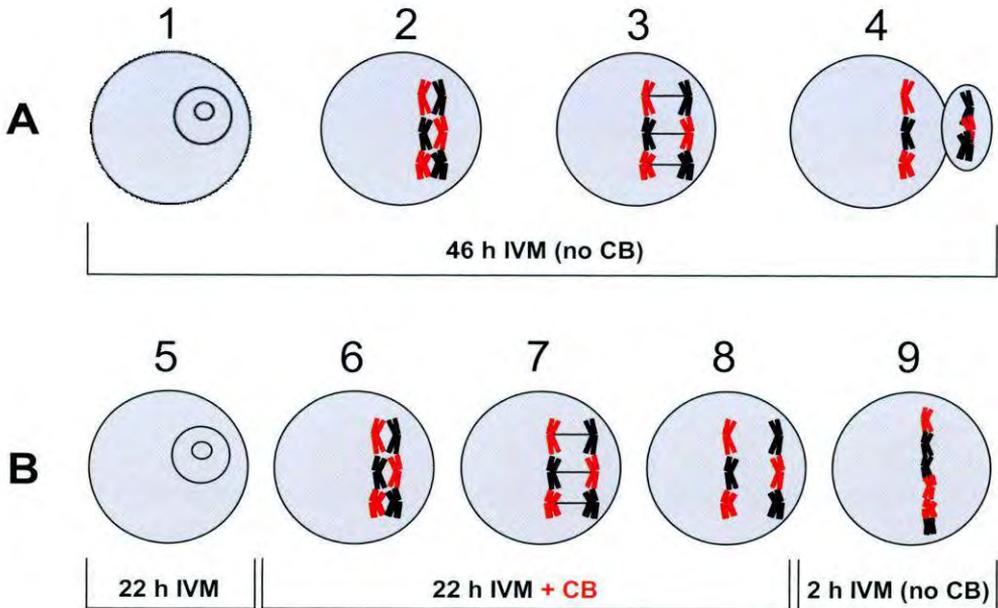


Fig. 6 Scheme of meiotic maturation without (A) or with (B) cytochalasin-B (CB). (A) Germinal vesicle (GV) oocytes (1) are cultured in vitro without CB for 46 h. After the GVBD (around 33 under these culture conditions), the homologous paternal and maternal chromosomes form bivalents [metaphase-I (M-I) stage (2)]. At this stage, the oocyte is tetraploid. Later segregation of the homologous chromosomes occurs (3) with extrusion of the first polar body [metaphase-II (M-II) stage (4)], so the oocyte becomes diploid. (B) After culture of GV oocytes for 22 h in CB-free medium (5), they are subsequently matured in the presence of CB for 22 h. The M-I stage (6) is followed by segregation of homologous chromosomes (7), but extrusion of the polar body is inhibited, so that all chromosomes remain in the oocytes (8). During the last phase of maturation culture, the segregated chromosomes undergo rearrangement into an M-I-like metaphase plate (9). The figure is modified from Somfai et al. (2006).

were published previously by Kubiak *et al.* (1991), who cultured mouse oocytes in the presence of cytochalasin-D, a drug similar to CB. The metaphase plate of porcine oocytes after treatment with CB contained $2n$ (38) segregated chromosomes, unlike those of “real M-I” oocytes that contained 19 pairs of homologous chromosomes forming bivalents and those of M-II oocytes that contained n (19) segregated chromosomes with 2 chromatids. Therefore the structure of the metaphase plates in “M-I-arrested” oocytes generated by CB treatment appears similar to that of M-II oocytes; however, it contains a double (diploid) set of 2 chromatids. Therefore such a nuclear stage cannot be considered a “real M-I” stage, but rather an “M-I-like” stage.

Previously, we compared the chromosome complements of CB-treated oocytes with those of “M-I-arrested” oocytes that were blocked spontaneously during IVM, and found exactly the same chromosome configurations (Fig. 7) (Somfai *et al.* unpublished results). This suggests that, during meiotic progression from the M-I to M-II stages, an as-yet unclarified mechanism gives rise to a similar actin-depolymerization-related phenomenon of chromosome reunion and rearrangement, even after successful segregation of the homologous chromosomes. The resulting nuclear stage is characterized by a single metaphase plate without any polar body, and has been referred to as “M-I arrest” in our previous papers (Kikuchi *et al.* 1999b; Somfai *et al.* 2005). This abnormality during porcine oocyte maturation occurs with increased frequency as culture is prolonged (Sosnowski *et al.* 2003). This suggests the malfunctions of the spindle during maturation culture. Further studies of these phenomena will be important not only in porcine IVP system, but also in human reproduction, because incompleteness of oocyte maturation before the M-II stage is considered to be an important cause of human infertility (Mrazek & Fulka 2003).

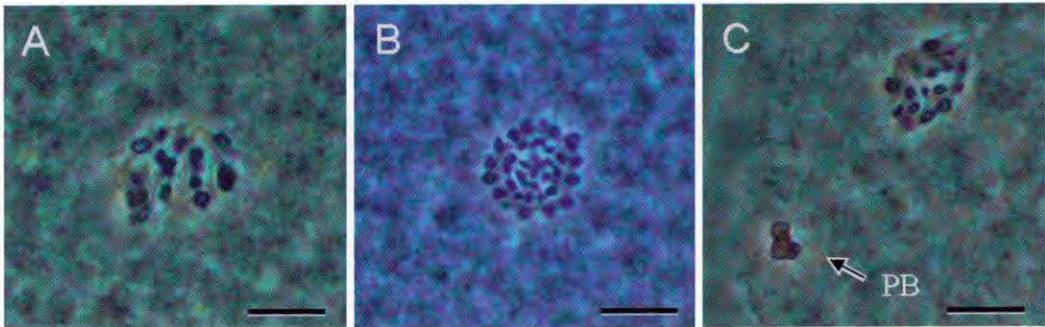


Fig. 7 Chromosome configurations of (A) metaphase-I (cultured for 33 h), (B) “metaphase-I-arrested” (for 48 h) and (C) metaphase-II porcine oocytes. Specimens were prepared after fixation and staining with aceto-orcein, and examined by phase-contrast microscopy. Scale bars represent 10 μm .

Conclusion

Our results have clearly revealed that development to the blastocyst stage is not a perfect indicator of embryo quality in porcine IVP systems, since polyploid embryos can develop to the blastocyst stage. Careful selection of M-II oocytes for IVF, and regular monitoring of fertilization and polyspermy rates are very important in order to obtain reliable results, not only in porcine IVP systems but also in other technologies using IVM oocytes. On the other hand, it is necessary to know more about the mechanism by which fertilized oocytes with abnormal ploidy can develop to a normal state during embryo development. Further improvements of IVM-IVF systems are necessary to increase the degree of nuclear and cytoplasmic maturity and to reduce the incidence of polyploidy in porcine IVP systems.

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