Ph.D. DISSERTATION

- SOMFAI TAMÁS -

SYNCHRONIZATION OF IN VITRO MATURATION OF PORCINE OOCYTES

Supervisors:

Dr. Dr. habil. Iváncsics János †

Dr. Farkasné Dr. Bali Papp Ágnes

Ph.D. School for the Biological, Technological, Ecological, Feeding and Economical Questions of Animal Production.
Chairman of the PhD School: Prof. Dr. Schmidt János

Sub program: Improvement and Breeding Technology Considerations of Animal Production
Sub program leader: Prof. Kovácsné Dr. Gaál Katalin

UNIVERSITY OF WEST-HUNGARY

FACULTY OF AGRICULTURE AND FOOD SCIENCES

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IN VITRO FERTILIZATION OF PORCINE OOCYTES WITH CRYOPRESERVED OR FRESH SEMEN

Értekezés doktori (PhD) fokozat elnyerése érdekében

Írta:

Somfai Tamás

Készült a Nyugat-Magyarországi Egyetem "Az Állati Termék Előállítás biológiai, technológiai, ökológiai, takarmányozási és ökonómiai kérdései " Doktori Iskola

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Témavezetõ: Dr. Farkasné Dr. Bali Papp Ágnes

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CONTENT

1 ABSTRACT

In the present study, the effect of meiotic synchronisation by a transient meiotic arrest at GV stage on nuclear and cytoplasmic maturation, in vitro fertilization and subsequent embryonic development of in vitro matured (IVM) porcine oocytes was investigated.

Meiotic synchronisation was achieved by elevating the intercellular cAMP level of the oocyte during the first 22 h of the 46 h IVM period. Supplementation of maturation medium with 1 mM dbcAMP succesfully inhibited meiosis during the first 22 of IVM and synchronised maturation of the cultured oocytes that resulted in a higher rate of mature (M-II) oocytes and a higher rate of monospermic fertilization and blastocysts after IVF and IVC of the treated oocytes than that of the control. This suggests that synchronization of maturation using dbcAMP enhances meiotic potential of oocytes and results in a high developmental competence by monospermic fertilization.

In the second part of the study, we compared the developmental capacity of mature and meiotically arrested porcine oocytes derived from IVM. The most of the oocytes that failed to resume meiosis during IVM were arrested at GV and M-I stage while a remarkable proportion of oocytes also remained at proM-I stage. After IVF there was no difference in polyspermy and pronucleus formation between the M-II and M-I arrested oocytes while GV arrested oocytes showed a significantly higher frequency of polyspermy and failed to form pronuclei. After IVC, there was no significant difference in blastocyst rates between the M-II and M-I arrested oocytes, however the

blastocyst derived from M-I oocytes had less cells than that of from M-II oocytes. The ratio of inner cell mass and trophectoderm cells did not differ between the two groups. These results prove the developmental ability of M-I arrested oocytes to blastocyst stage and reveal their decreased cleavage speed, probably due to their abnormal ploidy.

In the third part of the study, the effect of somatic cells surrounding the oocyte on nuclear progression and cytoplasmic maturation was investigated. During IVM different morphologic classes of porcine COCs can be distinguished regarding the status and expansion of the cumulus cells. It was found that the frequency of normal cumulus expansion is higher when granulose cells are attached to the COCs. Nuclear progression of COCs was slightly accelerated without granulosa cells. Oocytes attached to the bottom of culture dish with dark, compact cumulus undergo precoccious nuclear and cytoplasmic maturation. The rate of monospermic fertilization after IVF of normal COCs showing normal cumulus expansion was higher than that of COCs attached to the dish. It was concluded, that diverse behavior of cumulus cells during in vitro culture affects both maturation and IVF of porcine oocytes. Granulosa cells promote normal cumulus expansion thus decrease heterogeneity in nuclear and cytoplasmic maturity amongst oocytes.

KIVONAT

Éretlen petesejtek in vitro érlelése nagy mennyiségű alapanyagot képes biztosítani különböző embrió-technológiai illetve manipulációs módszerekhez. Ismert jelenség azonban, hogy az in vitro maturáció (IVM) során a sertés petesejtek eltérő időben kezdik meg illetve fejezik be a meiotikus érést, így az IVM végére az érettség szempontjából a petesejtek jelentős szórást mutatnak, ami a manipulációs eljárások hatékonyságát jelentősen csökkenti.

Tanulmányunk első részében megvizsgáltuk, hogy sertés petesejtek érésének a meiózis átmeneti blokkolásával történő szinkronizálása milyen hatással van azok érési, termékenyülési illetve embrionális fejlődési képességére. A magi érés blokkolását a 46 órás maturáció első 22 órájában, a sejten belüli cAMP szint emelésével végeztük. A maturáció átmeneti blokkolását követően a kezelt csoport maturációs rátája, az in vitro fertilizációt (IVF) követően a monospermia, illetve a hólyagcsírák aránya magasabb volt, mint a kontrol csoportban, ahol jelentős mennyiségű petesejt rekedt meg M-I állapotban. Tehát a magi érés szinkronizálása javítja a petesejtek meiotikus és fejlődési potenciálját.

A tanulmány második részében az érett (M-II) petesejtek termékenyítést követő embrionális fejlődési képességét hasonlítottuk össze olyan petesejtekével, melyek a meiózis során éretlen állapotban megrekedtek. Az ilyen petesejtek főként GV vagy metafázis-I állapotban állnak meg de jelentős számú petesejt blokkolt le a prometafázis-I állapotában is. Az M-II és az M-I állapotú petesejtek között nem volt különbség a polispermia és a pronukleuszképződés tekintetében az IVF-et követően. Ezen felül, az IVF-et követő embriótenyésztés során nem különbözött a hólyagcsíra

állapotba jutott embriók aránya sem, mindamellett az M-I állapotú petesejtekből származó embriók alacsonyabb sejtszámmal rendelkeztek, mint az azonos korú, M-II állapotú petesejtekből származók. Az embrionális sejtcsomó és a trofektodermális sejtek aránya nem különbözött a két csoport között. Ezen eredmények egyértelműen bizonyítják, hogy a tartósan M-I állapotban megrekedt sertés petesejtek éppúgy képesek hólyagcsíra állapotig fejlődni, mint érett társaik, azonban osztódási sebességük alacsonyabb, feltehetően azok abnormális ploidiája miatt. Ez kihangsúlyozza az IVF-re használt petesejtek érettségi állapot alapján történő elbírálásának fontosságát.

A dolgozat harmadik részében azt vizsgáltuk, hogy milyen hatást fejtenek ki a petesejtet körülvevő testi sejtek a petesejtek spontán sejtmagi érésére az IVM során. Eredményeink azt mutatják, hogy a kumulusz sejtek tenyésztőedényhez történő kapcsolódása kiváltja a petesejtek spontán, idő előtti érését és a petesejtek öregedését is. Ezzel jelentősen megnő a polispermiás megtermékenyülés gyakorisága. Amennyiben azonban a petesejtet körülvevő kumulusz réteghez granulóza sejtek is kapcsolódnak, az IVM során a normális kumulusz expanzió nagyobb gyakorisággal következik be, kevesebb sejt kapcsolódik a tenyésztő edényhez és ezzel párhuzamosan az idő előtti érés gyakorisága lecsökken, tehát a granulóza sejtek természetes úton szinkronizálják a petesejtek érését.

2 OVERVIEW OF LITERATURE

2.1 Current status of in vitro embryo production in pigs

In vitro production (IVP) of mammalian embryos is an important supporting technology not only for basic sciences and medicine, but also for advanced animal husbandry and biotechnology which enables us to generate a large number of viable embryos. IVP includes three major technological steps; the in vitro maturation (IVM) of immature oocytes, in vitro fertilization (IVF) and in vitro culture (IVC) of the fertilized oocytes.

In vitro embryo production has several advances; it makes us possible to utilize at least a significant proportion of the vast number of follicular oocytes that are normally lost through atresia using slaughterhouse ovaries as basic material for oocyte collection that are normally abattoir-vaste. Thus IVP enables us to produce a larger number of embryos with less cost and in less time compared to in vivo embryo production in pigs. Besides, in vitro maturation of oocytes provide mature eggs that can be materials as recipient oocytes for other reproductive technologies such as ICSI (Naka*i et al.,* 2003) and cloning (Betthauser *et al.,* 2000; Boquest *et al.,* 2002; Iwamoto *et al.,* 2003). These technologies using IVM oocytes are now expected to be used to produce transgenic pigs. Moreover the successful embryo transfer in rare pig breeds (Rátky *et al.,* 2001) and the cryopreservation of in vitro produced pig blastocysts was reported recently (Dinnyés *et al.,* 2003) underlining the importance of in vitro technologies in gene banking of rare breeds, endangered species and precious individuals as well.

The improvement of IVP systems could enhance the efficiency of micromanipulation and gene transfer technologies in the porcine species. This may manifest its effect in pork production by creating new transgenic species with better body and meat characteristics or growth.

The history of in vitro reproduction in pigs started three decades ago. Motlik and Fulka (1974) first reported the ability of in vitro matured (IVM) porcine oocytes to be fertilized. The first successful in vitro fertilization (IVF) of IVM oocytes in pigs was reported by Iritani *et al.* (1978). Important steps towards were made by Nagai *et al.* (1988), who successfully used frozen-thawed pig spermatozoa for IVF. The ability of IVM/IVF oocytes to develop to blastocyst stage was first confirmed by Mattioli *et al.* (1989). Further, piglets were born after the transfer of IVM/IVF embryos that were cultured to the 2-4 cell stage (Yoshida *et al.,* 1993; Funahashi *et al.,* 1996) or to morula stage (Day *et al.,* 1998). However the first successful transfers of IVP embryos at blastocyst stage were reported only recently (Marchal *et al.,* 2001; Kikuchi *et al.,* 2002).

In spite of the improvements that have been made in many aspects of IVF procedures, porcine IVP still struggles with problems that remained unsolved during the years such as the high incidence of polyspermy which is still the major problem that decreases the efficiency of in vitro embryo production in pigs (Nagai, 1994). The phenomenon of polyspermy can be affected by several factors such as the concentration of spermatozoa and the time period of in vitro fertilization (Nagai, 1996). Polyspermy in porcine IVM-IVF oocytes has also been considered to result from an irregular distribution of cortical granules in oocytes matured in vitro (Cran and Cheng, 1986) and oocyte aging caused by the asynchronous meiotic progression of

porcine oocytes during in vitro maturation (Grupen *et al.,* 1997). Therefore it can be stated, that one of the main reasons of polyspermy is the inadequate in vitro maturation culture of the oocytes. However, it must be noted, that high incidence of polyspermy is a typical characteristic of porcine species that ranges from 5-35% even in vivo (Hancock, 1959; Hunter RHF, 1967; Hunter RHF 1972; Hunter RHF, 1973) and that many of the polyspermic fertilized oocytes can develop to the blastocyst stage (Han *et al.,* 1999a). Most of the fetuses resulted from the transfer of polyspermic embryos were found to be diploid (Han *et al.,* 1999b) suggesting the existence of a mechanism(s) in porcine oocytes that can neutralize the effect of polyspermy on embryo ploidy.

2.2 Importance of meiotic synchronisation during in vitro maturation

The success of nuclear and cytoplasmic maturation of oocytes is a crucial point of efficiency in IVM/IVF systems. Maturation conditions can be upgraded by coculturing oocytes with oviduct epithelial cells and follicle cells (reviewed by Nagai, 1994), however the need for defined culture conditions has inceased recently in order to industrialize in vitro embryo production. The metabolism of nuclear and cytoplasmic maturation is not completely understood yet thus the present IVM systems can not completely represent the conditions that exist inside the follicle before and during the maturation of oocytes in vivo. Antral follicles are known to keep the oocytes arrested at germinal vesicle stage until the occurance of the meiosis activating signal (Pincus and Enzmann, 1935; Eppig and Downs, 1984). During oocyte collection and in vitro culture, spontaneous maturation can start since germinal vesicle (GV) oocytes resume meiosis

spontaneously when removed from the follicle (Pincus and Enzmann, 1935). A large variation in nuclear morphology of GV stage pig oocytes was found just after collection (Funahashi *et al.,* 1997a; Nagai *et al.,* 1997) and after a certain time of culture (Funahashi *et al.,* 1997a). It has been shown that there are differences in GV configuration and polypeptide synthesis between oocytes obtained from different size antral follicles causing heterogeneity in overall developmental competence within oocytes, collected for IVM/IVF (Mcgaughey *et al.,* 1979). Since the source of oocytes may differ (from different individuals and different size follicles), the developmental competence of GV stage pig oocytes used for IVM/IVF can also vary within the population used in one experiment. Therefore some oocytes can start meiosis earlier than others causing heterogeneity in maturation status among the oocytes after IVM that may affect monospermic fertilization, and further embryonic development. Supporting this theory a large variation in morphology and cell number of IVM/IVF pig blastocysts was reported by Kikuchi *et al.* (2002). To overcome this phenomenon the synchronization of nuclear maturation is necessary which can be achieved by a transient inhibition of meiotic maturation during the first half of the 44-48 h of IVM. Inhibition of GVBD in oocytes prior to in vitro maturation may have another advantage since oocytes can increase their developmental competence during meiotic arrest (Downs *et al.,* 1986; Funahashi *et al.,* 1997; Hashimoto *et al.,* 2002). A possible reason of this phenomenon might be the fact that protein synthesis in oocytes does not stop completely when arrested at GV stage temporarily (Marchal *et al.,* 2001).The transient inhibition of GVBD can be acieved by supression of activity of metaphase (or maturation)

promoting factor (MPF) or by chemicals that elevate intercellular levels of cyclic adenosine monophosphate (cAMP).

2.3 Manipulation of MPF activity

Progression of meiosis is regulated by a certain fluctuation in the activity of metaphase promoting factor (MPF), a protein kinase that plays its key role in promoting M-phase in mammalian oocytes (Hashimoto and Kishimoto, 1988; Fulka Jr. *et al.,* 1992; Motlik *et al.,* 1998). MPF consists of a regulator subunit, cyclin B-1 and a catalytic subunit, $p34^{cdc2}$ and plays an important role in the brakedown of the germinal vesicle, which is the first morphologic step of meiotic maturation. MPF shows its highest activity at metaphase-I and metaphase-II stages and turns inactive during anaphase-I and telophase-I (Hashimoto and Kishimoto, 1988; Fulka Jr. *et al.,* 1992; Motlik *et al.,* 1998). Supression of MPF activity in isolated oocytes prevents meiotic maturation and keeps the oocytes at GV stage. The inhibition of protein synthesis is a possible way to achieve this, however the use of protein synthesis inhibitors such as puromycin (Motlik *et al.,* 1991) or cycloheximide (Kubelka et al., 1988; Lonergan *et al.,* 1998) block not only the synthesis of MPF proteins but stop protein synthesis in general inside the oocyte. This may cause side effects regarding the further meiotic and developmental competence of the oocyte (Lonergan *et al.,* 1998). Considering this, the use of phosphorylation inhibitors such as 6-dimethylaminopurine (6-DMAP) (Avery *et al.,* 1998) or specific protein-kinase inhibitors such as butyrolactone-I (BL-I), roscovitine (ROS) is more expedient. BL-I, a potent inhibitor of MPF is known to prevent the resumption of meiosis reversibly in bovine (Kubelka *et al.,* 2000; Lonergan *et al.,* 2000; Imai *et al.,* 2002) and porcine oocytes (Wu *et al.,* 2002; Hirao *et al.,*

2003) via engaging the ATP binding sites of $p34^{cdc2}$, the catalytic subunit of MPF, without affecting chromosome condensation activity, mitochondrial and microfilament dynamics. However a delay of cytoplasmic maturation in metaphase II stage porcine oocytes was observed when oocytes were arrested at germinal-vesicle stage using BL-I prior to in vitro maturation (Hirao *et al.,* 2003). Besides, some reports reveal possible side effects of BL-I on oocyte quality. Using high (100-300 μM) concentrations of BL-I for parthenogenetic activation resulted in an elevated rate of activated porcine oocytes with two female pronuclei and only one polar body (showing a disability to extrude second polar body) compared to oocytes treated with lower doses of BL-I suggesting a (dose dependent) detrimental effect of BL-I on cytoskeleton probably via a non-specific effect on the MAP kinase system (Dinnyés *et al.,* 2000). Recently a long term (40 h) cultivation of bovine COCs with a high concentration (100 μM) of BL-I was found to destroy the contact between cumulus cells and oocyte and have detrimental effects on cytoplasmic and nuclear morphology (Fair *et al.,* 2002).

ROS, another specific inhibitor of CDC2 protein kinase can also be used for transient inhibition of GVBD in mammalian oocytes (Marchal *et al.,* 2001) and recently a combination of BL-I and ROS at low concentrations was reported to be effective to inhibit GVBD in bovine, without any side effects (Ponderato *et al.,* 2001).

2.4 Manipulation of intracellular cAMP level

A high level of intercellular cAMP is responsible for activating cAMP dependent protein kinase (PKA) that controls meiotic arrest of oocytes at GV stage (Bornslaeger *et al.,* 1986; Cameron *et al.,* 1987). For elevating the level of cAMP within mammalian oocytes,

gonadotropins such as FSH and LH acting through follicular cells are responsible (Bornslaeger and Schultz, 1985; Mattioli *et al.,* 1994; Shimada *et al.,* 2003). Resumption of meiosis, germinal vesicle breakdown (GVBD) is associated with an irreversibile cascade starting with the reduction in intraoocyte cAMP that is followed by PKA inactivation and the activation of mitogen activated protein (MAP) kinase (Schultz *et al.,* 1983; Bornslaeger *et al.,* 1986; Sun *et al.,* 1999). Spontaneous maturation is supposed to occur by the interruption of metabolism between the follicle components (granulose cells and/or follicular fluid) and the oocyte in which cAMP is maintained at a high level.

The intercellular level of cAMP can be elevated artificially by different chemicals such as invasive adenylate cyclase (iAC), an enzyme that transfers ATP of the oocyte into cAMP or phosphodiesterase inhibitors like 3-isobutylmethyl-xanthine (IBMX) which prevents degradation of cAMP. Treatments with permeable and stabile compounds that are similar to cAMP can also be used. Addition of dibutyryl cyclic AMP (dbcAMP), a membrane permeable cAMP analogue, into IVM medium during the first 20 h of maturation inhibits GVBD and has a uniform effect on the nuclear stage of pig oocytes (Funahashi *et al.,* 1997b). The use of a combination of invasive adenylate cyclase (iAC) and 3-isobutyl 1-methylxanthine (IBMX) during oocyte collection increased the meiotic and subsequent embryonic developmental competence of IVM/IVF bovine oocytes (Luciano *et al.,* 1999) suggesting that changes in the level of intracellular cAMP during collection might affect further meiotic or developmental competence of oocytes.

2.5 Fertilization and development of immature oocytes

During IVM, not all the cultured oocytes complete their nuclear maturation; some of them remain at germinal vesicle (GV) stage or can be arrested at metaphase-I (M-I) stage even by the end of the culture period needed for the full nuclear maturation (Bae and Foote, 1980; Bagger *et al.,* 1987; Motlik and Fulka, 1986; Eppig *et al.,* 1994; Polanski, 1995; Kikuchi *et al.,* 1999).

The completion of nuclear maturation of porcine follicular oocytes is affected by cytoplasmic factors such as the capacity of oocytes to start or finish meiosis and the culture conditions. Therefore, after maturation culture, the nuclear status of oocytes results in various stages of meiosis. Some of the oocytes reach M-II stage, whereas the others remained at immature stages such as GV and M-I. Meiotic arrest can be caused by numerous factors such as insufficient meiotic competence affected by the follicle and oocyte diameter (Szybek, 1972; Sorensen and Wassarman, 1976; Motlik and Fulka, 1986; Eppig *et al.,* 1994) or stress caused by the inadequate culture conditions such as isolation and culture media (Bae and Foote, 1980; Bagger *et al.,* 1987; Kikuchi *et al.,* 1999). The age and the strain (in mice) of the donor animals also affects meiotic competence of GV oocytes: in pigs oocytes obtained from adult sows have an advanced competence to resume in vitro maturation than that of obtained from prepuberal gilts (Marchal *et al.,* 2001a). In mice oocytes of strain LT are known to exhibit a high incidence of arrest in the progression of meiosis at M-I stage (Hirao and Eppig, 1997).

In several IVM/IVF systems in pigs, cumulus-oocyte complexes are inseminated instead of denuded oocytes to enhance better acrosome reaction of the spermatozoa (and thus fertilization) by the presence

of cumulus cells which are known to initiate acrosome reaction (reviewed by Van Soom *et al.,* 2002). In such systems, all the cultured oocytes are inseminated including ones that are arrested at the immature stage before the achievement of nuclear maturation to metaphase-II (M-II). However less is known about the embryonic development of fertilized immature oocytes.

It has been reported, that porcine oocytes fail to form both female and male pronuclei when they are penetrated by spermatozoa at GV stage (Wang *et al.,* 1994; Wang and Niwa, 1997). In such situation the GV remains intact and the heads of the penetrating spermatozoa remain condensed. The rate of penetrating spermatozoa increases due to the lack of cortical granule distribution which is a characteristic of the immature porcine oocytes (Wang *et al.,* 1997).

Maturing mammalian oocytes penetrated by spermatozoa at M-I stage are known to be able to complete their nuclear maturation to M-II (Chian *et al.,* 1992; Polanski, 1995; Kikuchi *et al.,* 1999). However, formation of pronuclei can not be observed because due to the elevated activity of MPF at M-I and M-II stages the female chromatin remains at metaphase stage and the head of the penetrating sperm cells remain compact or the male chromatin form abnormal clusters or metaphase chromosomes that can be incorporated into the maternal metaphase plate (Kikuchi *et al.,* 1999).

However it was reported that mouse (Eppig *et al.,* 1994; Polanski, 1995) and porcine (Kikuchi *et al.,* 1999) oocytes permanently arrested at M-I stage undergo cytoplasmic maturation after a certain period of in vitro culture needed for nuclear maturation and, in such oocytes, male and female pronuclei are formed after fertilization. Up

to our knowledge, - there is no study about the embryonic developmental ability of such M-I arrested porcine oocytes.

2.6 Importance of somatic cells around the oocyte

Cumulus cells play an important role on oocyte maturation since they provide and transfer several known and unknown factors that are essential for normal meiotic and cytoplasmic maturation and further embryonic development after fertilization such as glutathione (GSH), which is an important factor for subsequent formation of male pronucleus (Yoshida *et al.,* 1993). Cumulus cells can incorporate cystine, the oxidised form of L-cysteine which can not be utilized by the oocyte, to synthesize GSH. Glutathione produced in the cumulus cells may enter the oocytes through the corona-oocyte complexes (reviewed by Nagai, 1994; Nagai, 2001) (Figure 1).

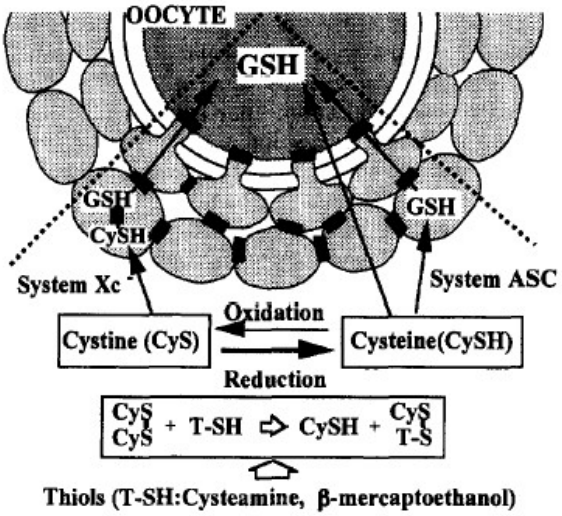


Fig 1. Glutathione synthesis in the cumulus cells and the oocyte and its transport from the cumulus cells to the oocyte through gap junctions (Nagai, 2001).

Another substrate which can not be metabolized by the oocyte itself is glucose. Cumulus cells metabolize glucose to pyruvate that can pass to the oocyte and enhance its quality (Downs and Utecht, 1999). Moreover, cumulus cells are known to play an

important role in regulation of meiotic progression of oocytes. During the growth and capacitation of oocytes (before initiation of meiosis) cumulus cells are responsible for maintenance of oocyte nucleus in GV stage via elevating intercellular cAMP level of the oocyte (Dekel and Beers, 1980; Racowsky 1984; Eppig and Downs, 1984; Tanghe *et al.,* 2002; Shimada *et al.,* 2003) by transferring an inhibitor signal trough gap junctions. Initiation of meiosis is also related to cumulus-function, there are evidences that cumulus cells secrete a meiosis-inducing factor (Guliang *et al.*, 1994; Xia *et al.,* 2000; Downs, 2001). However, the interruption of the meiosis-arresting signal, (e.g. the disruption or occlusion of gap junctions between the oocyte and the surrounding somatic compartment) also initiate the meiotic maturation in the oocytes (Larsen *et al.,* 1986; Isobe *et al.,* 1996; Isobe and Terada, 2001). Regarding the somatic compartment of the follicle, not only the cumulus cells affect the oocyte nuclear maturation. The transient inhibitoric effect of granulose cells on nuclear maturation of oocytes has also been published (Motlik *et al.,* 1991; De Loos *et al.,* 1994), suggesting a possible role of granulose in regulation of oocyte maturation.

A relationship between the LH induced changes of COC morphology and the nuclear progression during in vivo maturation of porcine oocytes has already been reported (Torner *et al.,* 1998). However, during the in vitro culture of porcine cumulus-oocyte complexes (COC) a different behavior of somatic cells can be observed enabling us to distinguish four morphological categories of COCs. Since there are morphological differences (colour, grade of expansion) between the somatic compartment of COCs from each categories we suggest a difference in the metabolic functions of such cells, that might affect nuclear and cytoplasmic maturation of the oocytes.

3 EXPERIMENTS

3.1 Objectives

The experiments presented in the present study were made according to three major objectives

1. **The first objective** was to examine the effect of intracellular cAMP during oocyte collection and in vitro culture on nuclear maturation, fertilization and subsequent embryonic development of porcine oocytes. Maturation media supplemented with or without IBMX and iAC were used for oocyte collection and following oocyte maturation culture was performed in the presence or absence of dbcAMP.

2. Without meiotic synchronisation a remarkable amount of oocytes remained arrested at M-I stage in our first study. The cytoplasmic maturation of such oocytes was reported by Kikuchi et al. (1999) but without any information about their developmental competence. **The second objective** of our experiments was to study the developmental potential of porcine oocytes that were permanently arrested before M-II stage during IVM. The nuclear status of oocytes with (PB+) and without (PB-) was investigated after 48 h of IVM. Pronuclear formation, monospermy rates and developmental ability to blastocyst stage after IVF and IVC of M-II stage and meiotically (GV or M-I stage) arrested oocytes was compared.

3. **The third aim** of the present study was to investigate the possible correlation between the morphology and functional acivity of somatic cells. The kinetics of nuclear and cytoplasmic

maturation in cumulus-oocyte complexes (COCs) and granulose-cumulus-oocyte complexes (GCOCs) was studied as well.

3.2 Synchronisation of meiotic maturation by high level of intercellular cAMP

3.2.1 MATERIALS AND METHODS

3.2.1.1 Oocyte Collection and In Vitro Maturation

Prepuberal porcine ovaries from cross-bred gilts (Landrace x Large White) were obtained from the local abattoir and carried to the laboratory in Dulbecco's Phosphate Buffered Saline (PBS) within 2 h at 35°C. Dissection of follicles in 3-6 mm diameter and collection of cumulus oocyte complexes (COCs) were performed in a collection medium supplemented with or without iAC and IBMX: The basic collection medium (BCM) (used as control) was NCSU37 (Petters and Wells, 1993) supplemented with 50 µM β-mercaptoethanol (Sigma Chemical Co., St Luis, MO, USA, M-7522), 25 mM HEPES, 1 mg/ml polyvinyl alcohol (PVA) (Sigma, P-8136), 100 unit/ml penicillin G potassium (Sigma, P-7794), and 0.1 mg/ml streptomycin sulfate (Sigma, S-9137). The osmolarity was adjusted to 0.285 osmol/kg, the pH was regulated to 7.3. Complete collection medium (CCM) was BCM supplemented with 0.5 mM IBMX (Sigma, I-7018) and 0.1 µg/ml iAC (adenylate cyclase toxin; Alexis Biochemicals, Lausen, Switzerland, 630-088). The entire procedure of oocyte collection took about one hour each time.

COCs were cultured in a maturation medium, which was modified NCSU-37 containing 10% (v/v) pig follicular fluid (PFF), 50 µM β-mercaptoethanol, 0.6 mM cysteine, 10 IU/ml PMSG (PMS 1000 IU, Nihon Zenyaku Kogyo, Koriyama, Japan), 10 IU/ml hCG (Puberogen 500 unit, Sankyo, Tokyo, Japan) and 1mM dbcAMP (Sigma, D-0627). Some COCs matured without dbcAMP were used as control. After the

first 22 hours of maturation, the COCs were transferred into 500µl maturation medium without any hormonal and dbcAMP supplement and cultured for additional 24 h. The COCs were cultured in batches of 20-30 in 500 µl of maturation medium (without covering by mineral oil) in four-well dishes at 39°C under 5% O_2 (adjusting CO_2 and N_2 to 5% and 90%, respectively).

3.2.1.2 In Vitro Fertilization (IVF) and In Vitro Culture (IVC)

IVF and IVC were carried out as described previously (Kikuchi *et al.,* 2002). After 46 h of maturation culture, COCs were transferred into 100 µl droplets of fertilization medium, which was Pig-FM (Suzuki *et al.,* 2002) modified with 2 mM caffeine and 5 mg/mL bovine serum albumin (BSA, Fraction V, Sigma), covered by mineral oil. About 25 oocytes per 100 µl medium were fertilized by frozen-thawed (Kikuchi *et al.,* 1998) and preincubated (for 15 min, Nagai *et al.,* 1988) epididymal spermatozoa from a Landrace boar where the final concentration was 1×10^5/ml.

After coincubation of gametes for 3 h, cumulus cells and attached spermatozoa were removed from the oocytes by pipetting through a fine glass pipette. They were transferred into IVC medium. Two types of IVC medium were prepared (Kikuchi *et al.,* 2002). The basic IVC medium was NCSU-37 modified with addition of 0.4% (w/v) BSA and 50 µM β-mercaptoethanol. IVC-PyrLac (basic IVC medium plus 0.17 mM sodium pyruvate and 2.73 mM sodium lactate) was used from Day 0 (the day of IVF was defined as Day 0) to Day 2 and IVC-Glu (basic medium plus 5.55 mM glucose) was used from Day 2 to Day 6. IVM-IVF oocytes were cultured at 38.5°C under 5% O_2.

3.2.1.3 Oocyte and embryo evaluation with orcein staining

For evaluation of meiotic stage of oocytes, IVF results and total number of cells in blastocysts, oocytes or embryos were mounted on glass-slides and fixed with acetic ethanol (1:3) for at least three days and then stained with 1% orcein (in 45% acetic acid) and examined under a phase-contrast microscope.

Evaluation of oocytes: Meiotic progression starts with the chromatin condensation in GV stage oocytes and leads to the breakedown of the GV. According to the status of chromatin and the integrity of GV membrane four types of GV can be distinguished (Motlik and Fulka, 1976).

GV-I: The GV membrane is intact. The compact chromatin is arranged in ring or horseshoe shape around the nucleolus. The nucleoplasm is unstained, fine and granular (Figure 2 A)

GV-II: The GV membrane is still intact, the granulation of the nucleoplasm and the integrity nucleolus is still unaffected, however, a few orcein-positive zones (chromocenters) appeared on the nuclear membrane (Figure 2 B). In late GV-II stage the delocalisation of chromatin from the nucleolar part to the periphery of the nucleoplasm can be observed (Figure 2 C).

GV-III. This stage is still characterised by an intact GV membrane however the nucleoplasm loses its granulation. The chromatin is distributed in separate well-stained clumps localised mainly around the visible nucleolus in early GV-III (Figure 2 D) and later it forms a homogenous network of the decondensed chromatin filaments in the nucleoplasm (Figure 2 E).

GV-IV. The nuclear membrane becomes less distinct in the early GV-IV (Figure 2 F). Later, the nucleolus disappears completely. The chromatin can still form an irregular network (Figure 2 F) sometimes with distinguishable individual filamentous bivalents. Later, the chromatin shows an intensive condensation and reorganization

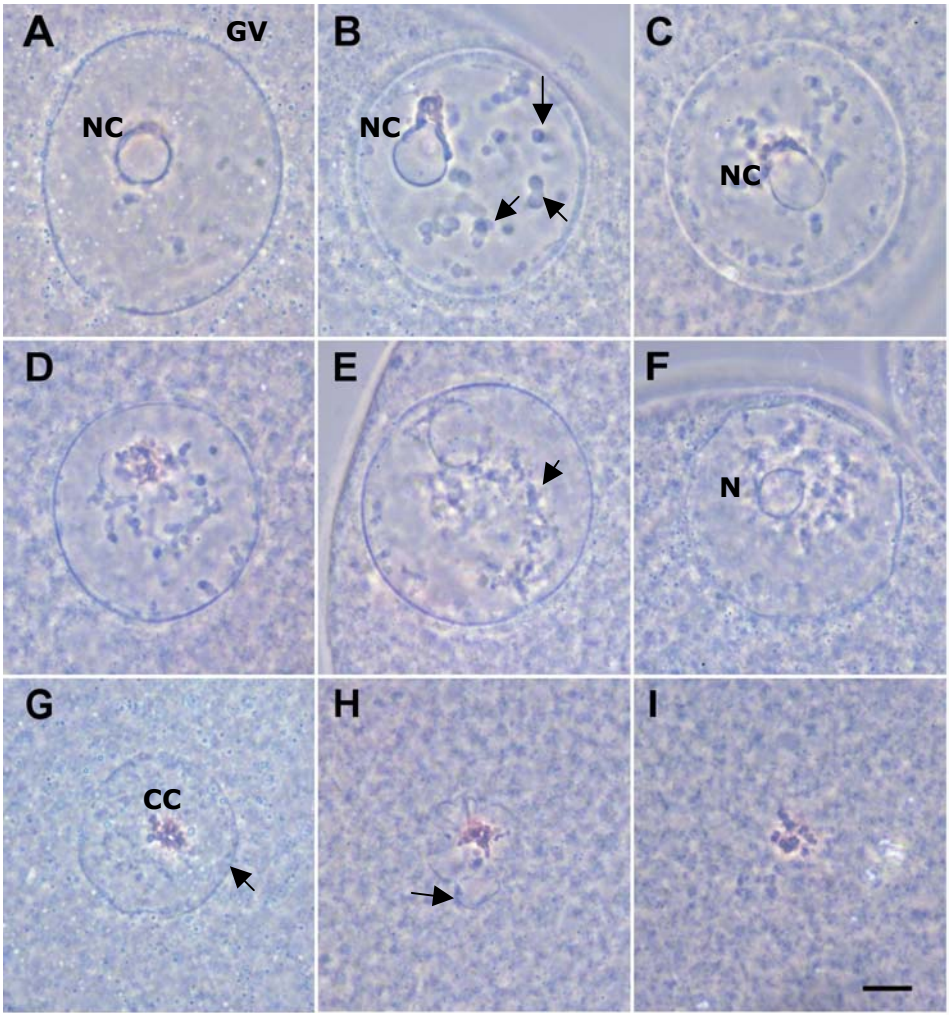


Fig 2. Nuclear progression of porcine oocytes during IVM: The breakdown of the germinal vesicle. **A**: GV-I; **B**,**C**: GV-II, arrow shows the chromocenters; **D**,**E**; GV-III, arrow shows decondesed chromatin filaments; **F,G,H**: GV-IV, arrow shows disrupting GV membrane; **I**: GVBD. Scale bar represents 10 µm. Abbreviations: GV= germinal vesicle; NC= nucleolus surrounded by chromatine; N= nucleolus without chromatin; CC= condensed chromatin.

around the centre of the GV, while the nuclear membrane shrinks (Figure 2 G) and starts to get damaged (Figure 2 H).

GVBD or **diakinesis**: The nuclear membrane is no longer visible. The chromatin is condensed into single lumps or discrete fragments. Individual chromosomes and microtubules have not appeared yet (Figure 2 I). The brakedown of the germinal vesicle is the first major morphological step of the meiotic progression that leads to the condensation of chromosomes and the formation of meiotic spindle.

At **prometaphase-I** microtubuli of the future meiotic spindle appear and the chromosomes start to form the metaphase plate, however the chromosome pairs have not separated from each other completely as individuals, many of them are still attached (Figure 3 A and B). At the definite **metaphase-I** stage the chromosome pairs are completely separated as individuals and align on an equatorial plate of the meiotic spindle (Figure 3 C and D). As the division begins in **anaphase-I**, the chromosomes are more or less distinguishable (Figure 3 E), however, later at **telophase-I**, during the extrusion of the first polar body they tend to form compact masses of condensed chromatin (Figure 3 F). At the end of nuclear maturation, oocytes are at **metaphase-II** stage showing a meiotic spindle with metaphase chromosomes and the completely extruded first polar body (Figure 3 G and H).

Fig 3. Nuclear progression of porcine oocytes during IVM.

A: Prometaphase-I stage (lateral view)

B: Prometaphase-I stage (frontal view)

C: Metaphase-I stage (lateral view)

D: Metaphase-I stage (frontal view)

E: Anaphase-I stage (lateral view)

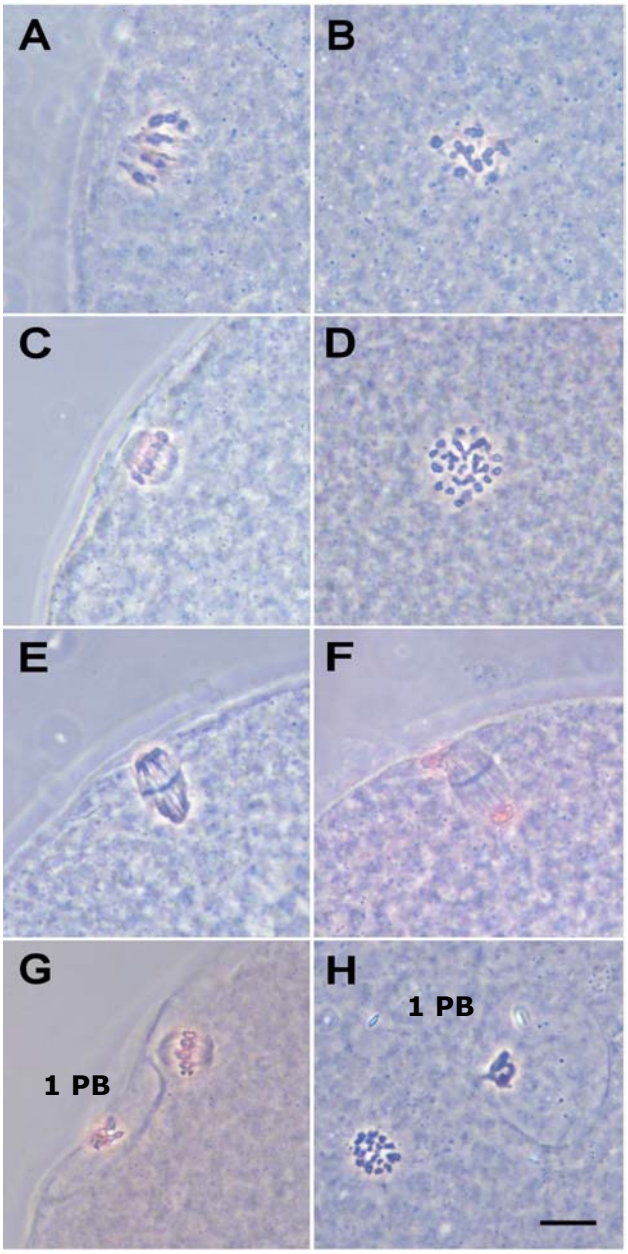
F: Telophase-I stage (lateral view)

G: Metaphase-II stage (lateral view)

H: Metaphase-II stage (frontal view).

1 PB = first polar body

Scale bar represents 10 µm.



Explanation for Fig 3:

M: metaphase plate
F: Frontal view of metaphase plate.
L: lateral view of metaphase plate.

Besides, abnormal nuclear morphology of oocytes can also be observed. The most common among them is the **degeneration** of oocytes which can be characterised by the damage of the germinal vesicle stage nucleus and the sponge-like texture of the usually **small sized** (approximately 90-100 µm in diameter) oocyte (Figure 4 A). Small sized oocytes in general remain arrested at GV-II or GV-III stage (Figure 4 B). The abnormality of an intact germinal vesicle is rarely seen such as ones having an **extra nucleolus** (Figure 4 C). The meiotic and developmental potential of such oocytes is unknown.

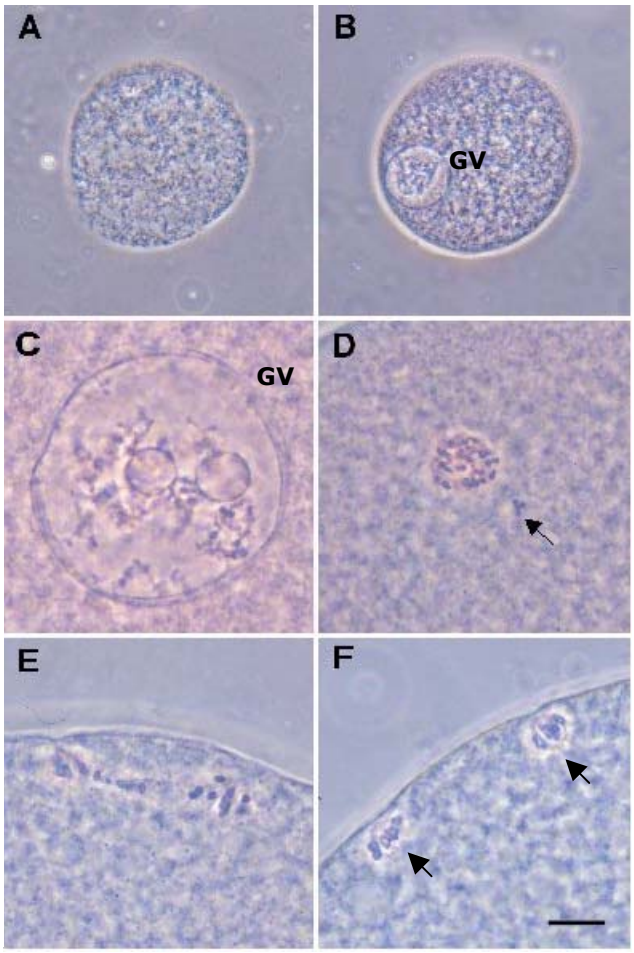


Fig 4. Nuclear progression of porcine oocytes: abnormal nuclear morphology.
A: Degeneration
B: Small oocyte arrested at GV stage.
C: GV with two nucleoli.
D: Chromosome plate at M-I stage. A single chromosome (marked with an arrow) failed to get incorporated into the M-I plate.
E: The complete failure of metaphase plate formation: scattered chromosomes.
F: The failure of first polar body extrusion: an oocyte with two metaphase plates (marked with arrows).
Scale bar represents 50 µm in **A, B** and 10 µm in **C**,**D**,**E**,**F**.

Anomalies of metaphase plate formation can also occur such as the **missing** of **chromosomes** from the metaphase plate (Figure 4 D) or the complete **failure of metaphase plate formation** (Figure 4 E) which might be related to a suggested problem of meiotic spindle organization. The effect of these anomalies on the future developmental competence of these oocytes is not known, it is suggested, that the lack of single chromosomes from the metaphase plate might cause aneuploidy. In some cases, the nuclear division of the oocyte occurs without the extrusion of the first polar body resulting **oocytes with two metaphase plates** (Figure 4 F). Fertilization of oocytes with two metaphase plates might result in digyny. The developmental ability of such oocytes has not been proved yet.

Evaluation of zygotes: To study the effect of different reatments on male pronucleus formation, fertilization rates and monospermic fertilization rates, inseminated oocytes were fixed 10 h after IVF and stained as described above. After staining, different stages in the transformation of sperm heads into male pronucleus can be distinguished. Right after penetration, the **sperm head** is **compact** with a more or less uniform dark coloration (Figure 5 A). In case of penetration of oocytes at GV stage or with high intercellular MPF activity, the sperm head remains compact even several hours later (Wang *et al.,* 1994; Kikuchi *et al.,* 1999). In case of optimal cytoplasmic maturity of the oocyte, the head of the fertilizing spermatozoa swells. **Swollen sperm head**s (Figure 5 B) are usually evenly stained and show a smooth, grey coloration. The next stage is the **recondensation** (Figure 5 C) of the swollen spermatozoa which is characterised by the uneven coloration of the shrunk, round-shaped sperm head after staining. Finally, this recondensed mass

start its **decondensation** (Figure 5 D) and develop into an unevenly stained enlarged membrane-surrounded vacuolum that leads to the development of the **male pronucleus** (Figure 5 E), an expanded,

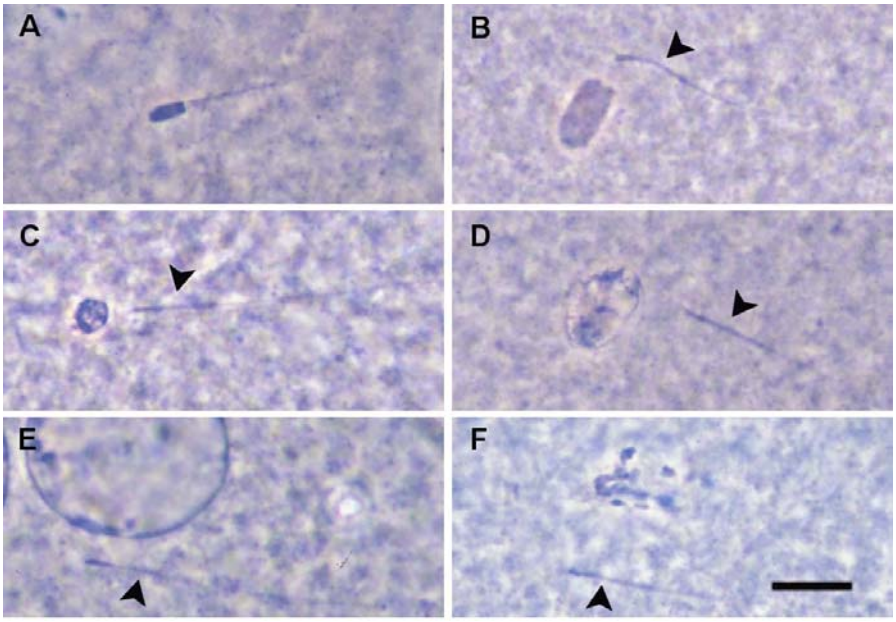


Fig 5. Fertilization of porcine oocyte: male pronucleus formation. **A**: condensed sperm head; **B**: swollen sperm head; **C**: re-condensed sperm head; **D**: decondensed sperm head; **E**: male pronucleus;**F**: male chromatine transformed into metaphase chromosomes. Arrow heads show corresponding sperm tails. Scale bar represents 10 μm.

completely round shaped vesicle including the decondensed filaments of the male genome and a nucleolus (or nucleoli). In case of inadequate cytoplasmic maturity (high MPF activity) of the oocyte the penetrated spermatozoon fails to form male pronucleus, remains condensed or under the influence of the active MPF, the sperm head transforms into **metaphase chromosomes** (Figure 5 F) (Kikuchi *et al.,* 1999). In the present study, only the oocytes with male pronucleus(ei) and/or decondensed sperm head(s) with

corresponding sperm tails were judged as fertilized. Normal monospermic fertilization is characterised by the **extrusion of the second polar body** (Figure 6 A) and the existance of **the female pronucleus and a single male pronucleus** (Figure 6 B; Figure 7 A). Oocytes with more than one male pronucleus were considered as **polyspermic zygotes** (Figure 7 B).

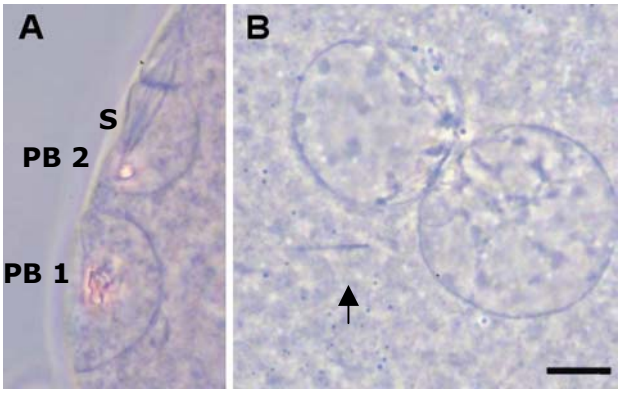


Fig 6. Fertilization of porcine oocyte: extrusion of the second polar body (**A**); male and female pronucleus formation (**B**). **PB 1**: first polar body; **PB2**: second polar body with the spindle (**S**).The arrow shows a sperm tail. Scale bar represents 10 μm.

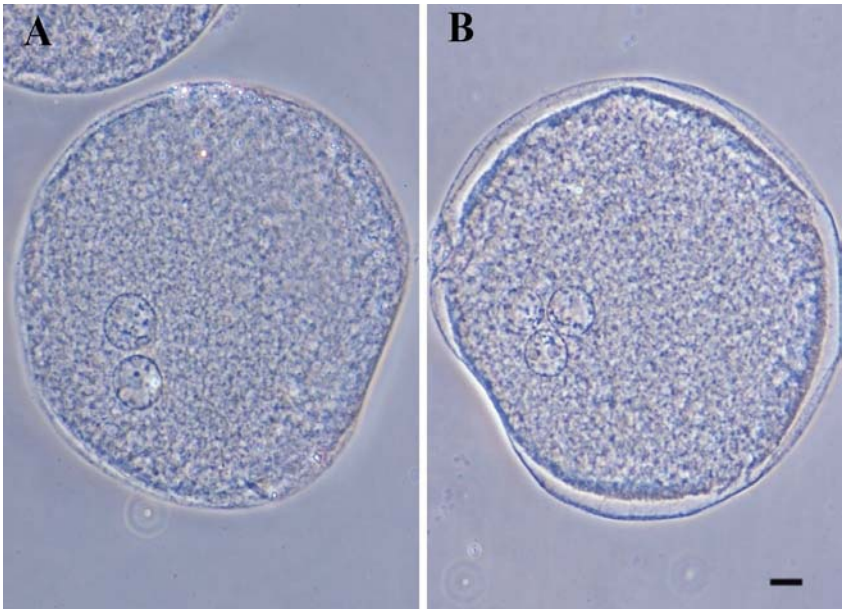


Fig 7. Fertilization of porcine oocyte: monospermy (**A**) with two (one ♂ and one ♀) and polyspermy (**B**) with three pronuclei. Oocytes were fixed 10 h after IVF. Scale bar represents 10 μm.

Evaluation of embryos: On Day 6 of IVC, IVM/IVF embryos were fixed and and stained as described above. Only embryos with a blastocoel and not less than ten blastomers were considered as **blastocyst** (Figure 8 A, B and C). In case of **partially living embryos** (Figure 8 C) only the embryos with not less than fifty percent living part were donsidered as normal blastocyst while embryos with blastocoel but with less than fifty percent living part and/or less than 10 live blastomers (Figure 8 D) were excluded form this category. Besides the blastocysts a remarkable proportion of embryos remains arrested at four cell stage, usually showing signs of fragmentation (Figure 8 E) or fail to divide (Figure 8 F).

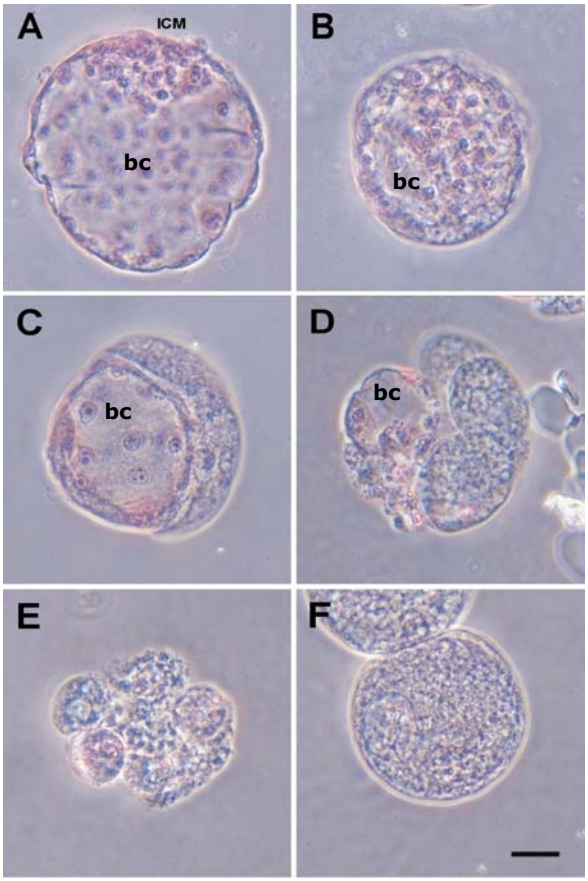


Fig 8. Morphology of porcine IVP embrios on day 6 IVC after staining with orcein.

A: Expanded blastocyst with an explicit inner cell mass (ICM).

B: Expanding blastocyst.

C: Partially living (half) blastocyst with approximately 10 cells.

D: Partially living (quarter) embryo with a blastocoel but less than 10 cells.

E: Embryo arrested at an early stage (4-6 cells) showing the signs of fragmentation.

F: Undivided zygote.

bc: blastocoel

Scale bar means 50 µm.

3.2.1.4 Statistical analysis

Each treatment of each experiment was replicated at least three times. Statistical analyses of IVM data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.01$) using GLM procedures of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Data of IVF and IVC results were analysed by Chi-square test ($P < 0.05$). Data are expressed as mean ± SEM.

3.2.2 Experimental Design

Experiment 1: To evaluate the effects of IBMX and iAC in collection medium and dbcAMP in maturation medium, respectively, on nuclear progression and oocyte maturation, COCs were collected using BCM and CCM collection media, and were cultured in vitro with or without 1mM dbcAMP for 22 hours, then culture was followed in the medium without hormones and dbcAMP supplement. Nuclear progression during IVM was evaluated after fixation at 12, 22, 36 and 46 h of culture. Chromatin condensation (GV stage) in the oocytes was classified according to Motlik and Fulka (1976).

Experiment 2: To study the effect of IBMX and iAC in collection medium and dbcAMP in maturation medium on fertilization parameters, COCs were matured in the presence or absence of 1 mM dbcAMP, and then fertilized in vitro. The inseminated oocytes were fixed at 10h after the insemination. Only the oocytes with male pronucleus(ei) and/or decondensed sperm head(s) with corresponding sperm tails were judged as penetrated. Zygotes with one female and one male pronucleus (or decondensed sperm head) and with two polar bodies were classified as normally (monospermic) fertilized oocytes.

Experiment 3: Effect of IBMX and iAC in collection medium and dbcAMP in maturation medium for oocytes on their subsequent embryonic development after IVF. On Day 6, all of the IVM/IVF embryos were fixed and evaluated for the rate of blastocyst formation and their cell number in each blastocyst.

See results from page 48.

3.3 In vitro fertilization and development to blastocyst stage of immature porcine oocytes arrested before metaphase-II stage

3.3.1 MATERIALS AND METHODS

3.3.1.1 Oocyte collection and in vitro maturation

Ovaries from prepuberal cross-bred (Landrace × Large White) gilts (their body weigth was approximately 100 kg) were collected at the local slaughterhouse and were carried to the lab in PBS at 35-37 °C. Cumulus-oocyte complexes (COCs) were collected by scraping of from 3-5 mm follicles in Medium 199 supplemented with 10% fetal bovine serum (Gibco, Life Technologies Inc., Grand Island, USA), 20mM HEPES, 100 unit/mL penicillin G potassium (Sigma Chemical Co., St. Louis, MO, USA, P-7794) and 0.1 mg/mL streptomycin sulfate (Sigma, S-9137). To obtain a remarkable number of oocytes arrested at M-I stage, a one-phase IVM was performed without the synchronisation of nuclear maturation. The basic medium was M199 which was reported to result better oocyte quality than NCSU 37 (which we used previously) regarding the cumulus expansion (Abeydeera *et al.,* 1998; Abeydeera *et al.,* 2000). Maturation culture was performed in Medium199 (prepared with 20mM HEPES; Gibco) supplemented with 10% porcine follicular fluid (pFF), 0.91 mM sodium pyruvate (Sigma, S-3362), 10 IU/ml PMSG, 10 IU/ml hCG, 20 ng/ml epidermal growth factor (EGF, Sigma), 150 μM cysteamine (Sigma) and antibiotics in 4-well dishes (Nunclon Multidishes, Nalge Nunc International, Denmark) in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39°C. The pH was adjusted to 7.3. At the end of the

culture period, COCs were denuded using a fine glass pipette after a brief treatment with 0.1% hyaluronidase.

3.3.1.2 IVF and IVC of porcine oocytes

IVF and in vitro culture (IVC) were carried out according to the previous report by Kikuchi et al. (2002) with slight modifications. Briefly, after denuding of COCs, oocytes with and without a visible first polar body (PB) were separated under a stereo microscope into PB+ and PB- groups, respectively. About 20 oocytes were transferred into 100 µl Pig-FM (Suzuki *et al.*, 2002) droplets covered by paraffin oil. They were coincubated then with 1×10^5/ml frozen-thawed epididymal spermatozoa (Kikuchi *et al.,* 1998) for 3 h at 39°C under 5% CO_2, 5% O_2 and 90%N_2. The day of IVF was defied as Day 0. After removal of spermatozoa attached to the surface of zona pellucida by gentle pipetting with a fine glass pipette, IVC was performed in IVC-PyrLac for Days 0-2 and in IVC-Glu for Days 2-6.

3.3.1.3 Oocyte and embryo evaluation with orcein staining

For evaluation of meiotic stage of oocytes, IVF results, pronucleus formation and total number of cells in blastocysts, oocytes or embryos were mounted, fixed, stained and evaluated as described in chapter *3.2.1.3*.

3.3.1.4 Blastocyst evaluation with differential staining

Differential nuclear staining of inner cell mass (ICM) and trophectoderm (TE) cells were performed according to Macháty *et al.*, (1998) with a small modification. Briefly, after digestion of zona pellucida with 0.5% (w/v) pronase, embryos were washed in IVC-PyrLac buffered with 20 mM-HEPES and supplemented with 4 mg/ml

polyvinyl alcohol (IVC-PyrLac-HEPES), in which osmolairity was adjusted to 285 osmol/kg, and then they were exposed to 1:5 rabbit anti-pig whole serum (Sigma, P-3164) for 40 min at 39°C. Then they were washed three times in IVC-PyrLac-HEPES and incubated in 1:15 dilution of guinea pig complement (Sigma, S-1639) supplemented with 20 µg/ml propidium iodide (Sigma, P-4170) and 20 µg/ml Hoechst-33342 (Sigma, B-2261) for 30 min at 39°C. Then embryos were briefly fixed in 50% ethanol, and mounted individually in glycerol on a glass-slide covered with a coverslip. The blastocysts were examined under UV light using an Axioplan 2 epifluorescence microscope (Carl Zeiss Jena GmbH). The ICM nuclei labelled with Hoechst appeared blue and TE nuclei labelled by both Hoechst and propidium iodide appeared pink to red (Figure 9). Numbers of ICM and TE nuclei were counted directly under the microscope.

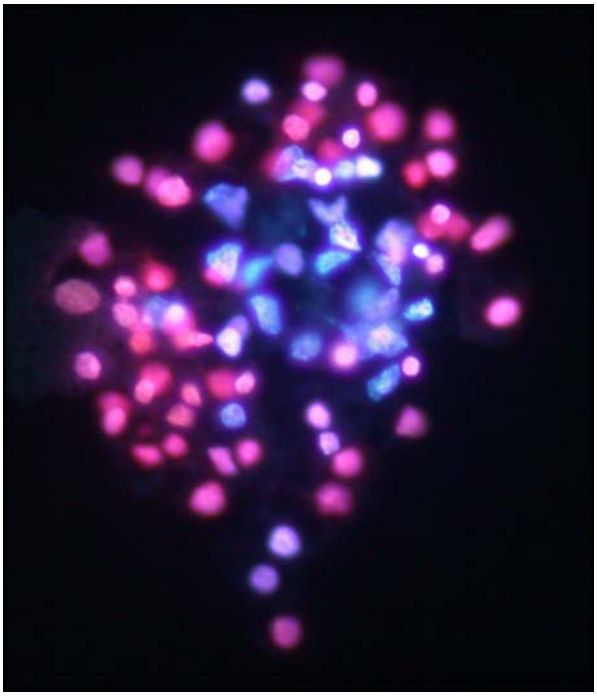


Fig 9. Porcine IVM blastocyst after differential staining with Hoechst 33342 and propidium iodide. ICM nuclei are blue. TE nuclei are pink. The photograph was taken at magnification 400 ×.

3.3.1.5 Statistical analysis

Each treatment of each experiment was replicated at least three times. Statistical analyses of IVF and IVC results were analysed by

ANOVA followed by Duncan's multiple range test ($p < 0.05$). Percent data were transformed into arcsin before the statistical analysis. Data are expressed as mean ± SEM.

3.3.2 Experimental design

Experiment 1. To evaluate meiotic progression during maturation culture, COCs were cultured for 36, 48 and 60 h. They were denuded, fixed and stained then their nuclear status was evaluated.

Experiment 2. To evaluate meiotic potential of oocytes, COCs were cultured for 48 h then oocytes were denuded. PB+ and PB- oocytes were separated and their nuclear status was evaluated after fixation and staining.

Experiment 3. Following 48 h of maturation culture, PB+ and PB- oocytes were separated and IVF was performed. Inseminated oocytes were fixed 10 h after IVF and examined for fertilization status (sperm penetration and pronuclear formation). Oocytes were judged to be penetrated when they had one or more male pronuclei and/or sperm heads with corresponding sperm tails.

Experiment 4. After IVF, PB+ and PB- oocytes were cultured for 6 days to examine their ability to develop to the blastocyst stage (PB+ blastocyst and PB- blastocyst, respectively). Embryos with clear blastocoel and with at least ten living cells were considered to be blastocysts. In order to characterize embryo quality, the total number of cells (nuclei) in each blastocyst and blastocyst morphology were evaluated after fixation and staining. Blastocyst morphology was evaluated according to the proportion of living and dead/degenerated parts inside the zona. The following three types of blastocysts were distinguished: *Type 1*, live blastocyst, the proportion of

dead/degenerated part is less than 10 percent (Figure 10 A), *Type 2*, the proportion of dead part is between 10 and 30 percent (Figure 10 B), and *Type 3,* the proportion of dead part is between 30 and 50 percent (Figure 10 C).

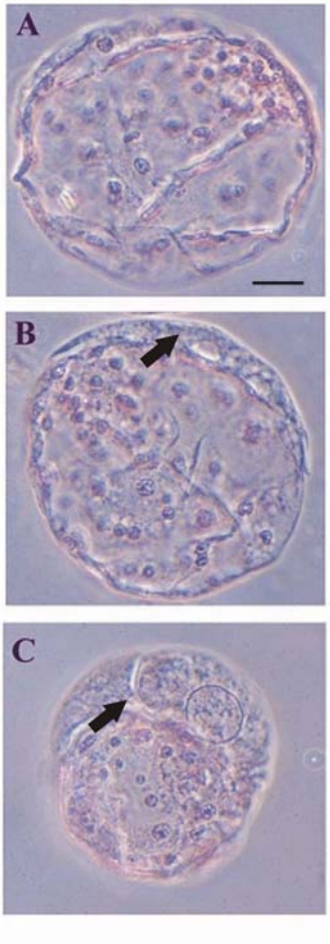


Fig 10. Porcine blastocysts produced in vitro after fixation on day 6 and staining with aceto-orcein. (**A**) *Type 1* blastocyst, the proportion of dead/degenerated part is less than 10 percent. (**B**) *Type 2* blastocyst, 10-30% of the embryo is degenerated. (**C**) *Type 3* blastocyst, 30-50% of the embryo is degenerated. All photographs were taken with a phase-contrast microscope at the same magnification. Arrows indicate the portion of the dead blastomeres less stained with orcein. Scale bar represents 50 μm.

Experiment 5. The number and ratio of ICM and TE cells of blastocysts from PB+ and PB- oocytes were evaluated after differential staining.

See results from page 62.

3.4 The relationship between cumulus morphology and oocyte maturation

3.4.1 MATERIALS AND METHODS

3.4.1.1 Oocyte collection and in vitro maturation

Pig ovaries were obtained from prepubertal cross-bred gilt (Landrace × Large White, approx. 100 kg body weight) and transported to the laboratory in PBS at 35°C.

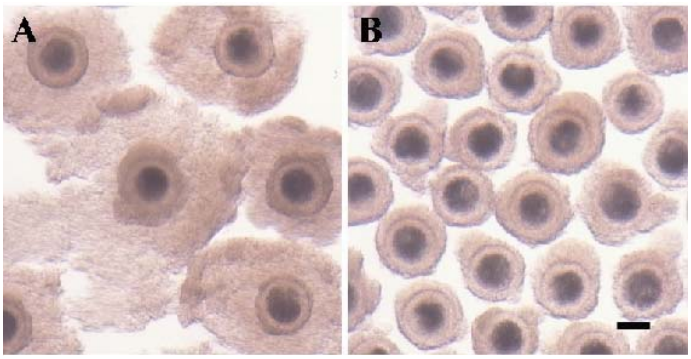


Fig 11. Sources of oocytes. (A): Granulosa-Cumulus-Oocyte Complexes (GCOCs); (B) Cumulus-Oocyte Complexes (COCs). Photographs were taken under a stereomicroscope at the same magnification. Scale bar represents 100 µm.

GCOCs (Figure 11 A) and COCs (Figure 11 B) of similar morphology were collected by dissection of 3-5 mm non-antral follicles in Medium 199 supplemented with 10% fetal bovine serum (Gibco), 20mM HEPES, 100unit/mL penicillin G potassium (Sigma Chemical Co., St. Louis, MO, USA, P-7794) and 0.1mg/mL streptomycin sulfate (Sigma, S-9137). A two-phase IVM was performed: COCs and GCOCs were transferred to first maturation medium (IVM1), which was Medium199 (prepared with 20mM HEPES; Gibco) supplemented with 0.91 mM sodium pyruvate (Sigma, S-3362), 10 IU/ml PMSG, 10 IU/ml hCG, 20 ng/ml epidermal growth factor (EGF, Sigma), 150 µM cysteamine (Sigma) 100 unit/ml penicillin G potassium (Sigma), 0.1

mg/ml streptomycin sulfate (Sigma) and 0.1% polyvinyl alcohol (PVA, Sigma) and incubated separately, in groups of 25-30 (COC) and 15-20 (GCOC) in 4-well dishes (Nunclon Multidishes, Nalge Nunc International, Denmark) in an atmosphere of 5% CO_2 and 5% O_2 in air at 39 °C. After 20 h of culture COCs and GCOCs were transferred into second maturation medium (IVM2) which was prepared in the absence of PVA but contained 10% porcine follicular fluid (pFF), otherwise identical to IVM1 and IVM was performed continuously under the conditions as described above. pFF was collected in advance by aspiration with a syringe and cetrifuged at 1,800 × *g* for 1.5 h and the supernatant was stored at –20 °C. Then enough amount of the stock were once thawed, mixed, centrifugated again and stored at –20 °C as the single batch until use.

3.4.1.2 Classification of COCs

Four types of COCs can be distinguished according to the characteristics of somatic compartment from 30 h of IVM.

Type 1: The COC is floating in the maturation medium, the oocyte is surrounded by a light coloured fully expanded cumulus mass (Figure 12 A).

Type 2: The COC is floating in the maturation medium, the oocyte is surrounded by a dark brown coloured compact or semicompact somatic compartment (Figure 12 B).

Type 3: The COC is attached to the bottom of the culture dish, the oocyte is surrounded by a dark brown coloured compact somatic compartment (Figure 12 C).

Type 4: The COC is attached to the bottom of the culture dish, the oocyte is partially denuded, the loss of cumulus cells ranges at least

the 30% of the oocyte surface. The remaining cumulus cells are dark coloured and compact (Figure 12 D).

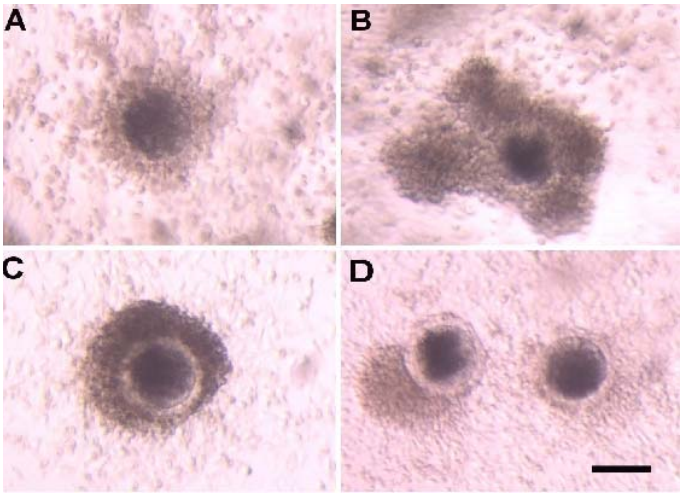


Fig 12. Morphological classes of complexes at 30 h of IVM according to the behaviour of the somatic compartment. (A) Type 1: floating oocyte surrounded by expanded cumulus; (B) Type 2: floating oocyte surrounded by dark, compact cumulus; (C) Type 3: the complex is attached to the bottom of the culture dish, the oocyte is surrounded by a dark, compact cumulus; (D) Type 4: the complexes are attached to the bottom of the culture dish, the oocyte is partially denuded, the loss of cumulus cells ranges at least 30% of the oocyte surface. The remaining cumulus cells are dark coloured and compact. Photographs were taken under a stereo microscope at the same magnification. Scale bar represents 100 μm.

3.4.1.3 Parthenogenetic activation (PGA) of IVM oocytes

At the end of IVM, oocytes were denuded using a fine glass pipette after a brief treatment with 0.1% hyaluronidase. Denuded oocytes with the first polar body -considered as matured oocytes- were harvested under a stereomicroscope. Matured oocytes were transferred into activation solution which consisted of 0.28 M d-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) BSA and washed once. Then they were stimulated with direct current (D.C.) pulse of 1.0 kV/cm for duration of 100 µsec using a somatic hybridizer (SSH-2, Shimadzu, Kyoto, Japan). After electric pulse oocytes were transferred into 500 µl droplets of in vitro culture (IVC) medium which is a modified NCSU-37 medium containing 4 mg/ml BSA and 50 µM βmercaptoetanol, 2.73 mM sodium lactate and 0.165 mM sodium pyruvate. They were subsequently cultured for 8-10 h at 39°C under 5% O_2 tension, and then fixed. After staining, activation status (pronuclear formation, extrusion of a second polar body or fragmentation) was evaluated under a phase-contrast microscope.

3.4.1.4 IVF and IVC of porcine oocytes

IVF and in vitro culture (IVC) were carried out as described above (chapter *2.2.1.2*) with slight modifications. Briefly, after denuding of COCs, oocytes with a visible first polar body (PB) were selected under a stereo microscope and used for IVF. About 20 oocytes were transferred into 100 µl Pig-FM (Suzuki *et al.,* 2002) droplets covered by paraffin oil. They were coincubated then with 1×10^5/ml frozen-thawed epididymal spermatozoa (Kikuchi *et al.,* 1998) for 3 h at 39°C under 5% CO_2, 5% O_2 and 90%N_2. After removal of spermatozoa

attached to the surface of zona pellucida by gentle pipetting with a fine glass pipette, IVC was performed in IVC-PyrLac for 10 h.

3.4.1.5 Oocyte and embryo evaluation with orcein staining

For evaluation of meiotic stage of oocytes, parthenogenetic activation and IVF results, oocytes or embryos were mounted, fixed and stained as described in chapter *3.2.1.3*.

Evaluation of nuclear status, fertilization rates and blastocyst formation was happened as described in chapter *3.2.1.3*.

Evaluation of parthenogenetic activation: In the present study, the oocytes beyond the anaphase-II stage were defined as being activated and the percentages of activated oocytes; 1) **normal activation** (characterized by a female pronucleus formation with the first and second polar bodies Figure 13 A), 2) **fragmentation** (abnormal cleavage characterized by unequal blastomeres Figure 13 B), 3) **Metaphase-III** (M-III) stage oocytes (characterized by metaphase stage female chromosomes (Figure 13 D) - often

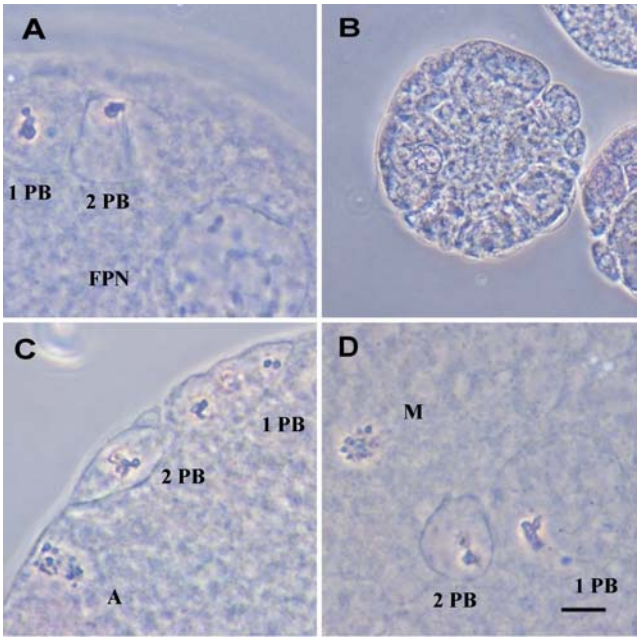


Fig 13. Nuclear morphology of porcine oocytes 8 hours after parthenogenetic activation. A: normal activation; B: fragmentation; C: M-III stage with abnormal chromosome arrangement; D: M-III stage.
1 PB: first polar body; **2 PB**: second polar body;**FPN**: female pronucleus;**A**: abromally arranged anaphase-like chromosomes;**M**: metaphase plate
Scale bar represents 10 µm.

arranged abnormally (Figure 13 C) - with the first and second polar bodies), were scored. Unactivated oocytes (remaining at Metaphase-II: M-II, stage) were also scored.

3.4.1.6 Statistical analysis

Each treatment of each experiment was replicated at least three times. Statistical analyses of IVM data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$) using GLM procedures of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Data of PGA and IVF results were analysed by Chi-square test ($P < 0.05$).

3.4.2 Experimental design

Experiment 1: The relation between the morphology of somatic compartment and the kinetics of nuclear maturation was studied in case of COCs and GCOCs. COCs and GCOCs were classified according to the characteristics of their somatic compartment as described above at 30, 36, 42 and 48 h of IVM, and then oocytes were denuded using a fine glass pipette after a brief treatment with 0.1% hyaluronidase. The denuded oocytes were fixed in acetic ethanol (1:3 v/v) for 3-5 days and stained with 1% aceto-orcein (Sigma), then nuclear status of oocytes was evaluated using a phase-contrast microscopy as described in chapter *3.2.1.3*.

Experiment 2: The ability of oocytes to form a female pronucleus was assessed in order to estimate the capacity of the cytoplasm to potentiate oocyte activation. Oocytes from COCs and GCOCs of each morphologic type were collected separately at 42 h of IVM, and then parthenogenetic activation was performed as described above. Eight hours after the stimulation, oocytes were fixed in acetic ethanol (1:3

v/v) for 3-5 days and stained with 1% aceto-orcein (Sigma). Activated status was evaluated using a phase-contrast microscopy as described in chapter *3.4.1.4*.

Experiment 3: Oocytes from COCs and GCOCs of each morphologic types were subjected to IVF after 48 h of IVM. Inseminated oocytes were fixed 10 h after IVF and stained as described in chapter *3.2.1.3* to examine sperm penetration and pronuclear formation. The oocytes were considered to be penetrated when they had one or more male pronucleus(ei) and/or swollen sperm head(s) with a corresponding sperm tail(s). Zygotes with one female and one male pronucleus (or decondensed sperm head) and with two polar bodies were classified as normally (monospermic) fertilized oocytes.

See results from page 78.

4 RESULTS

4.1 Synchronisation of meiotic maturation by high level of intercellular cAMP

Experiment 1. No significant difference in chromatin condensation between oocytes collected and/or matured in the presence or absence of cAMP was observed at 12 h of culture (Figure 14).

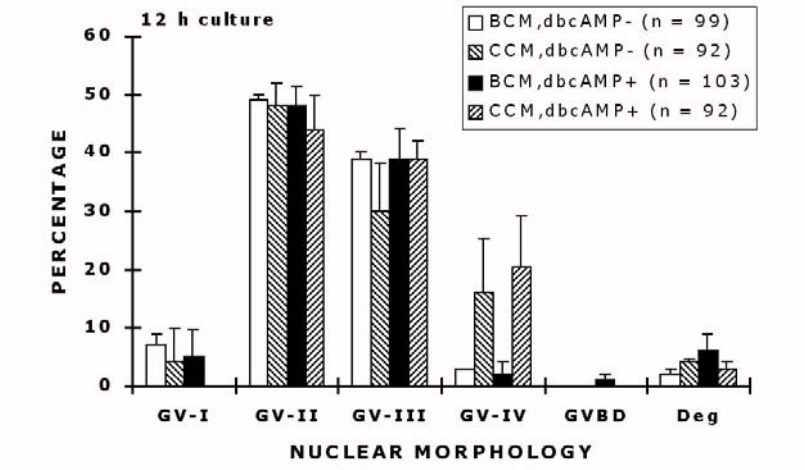


Fig 14. Nuclear morphology (mean ± SEM) of oocytes after 12 h of culture of four different treatments. Abbreviations: BCM= Basic Collection Medium; CCM= Complete Collection Medium; dbcAMP- = COCs cultured in the absence of 1 mM dbcAMP; dbcAMP+ = COCs cultured in the presence of 1 mM dbcAMP. Numbers of oocytes examined in different treatment groups are given in parentheses.

Both in the dbcAMP- and dbcAMP+ groups almost all the oocytes remained at GV stage, where GV-II (48.6 ± 5.8% and 47.6 ± 8.0%, respectively) and GV-III (39.3 ± 4.6 and 38.6 ± 5.2%, respectively) were dominant. Only a very few remained at GV-I or reached more

condensed stages of chromatin (GV-IV). The rate of degenerated oocytes was the same between the groups.

Significant differences in nuclear progression of oocytes matured with or without dbcAMP were detected at 22 h of culture (Figure 15).

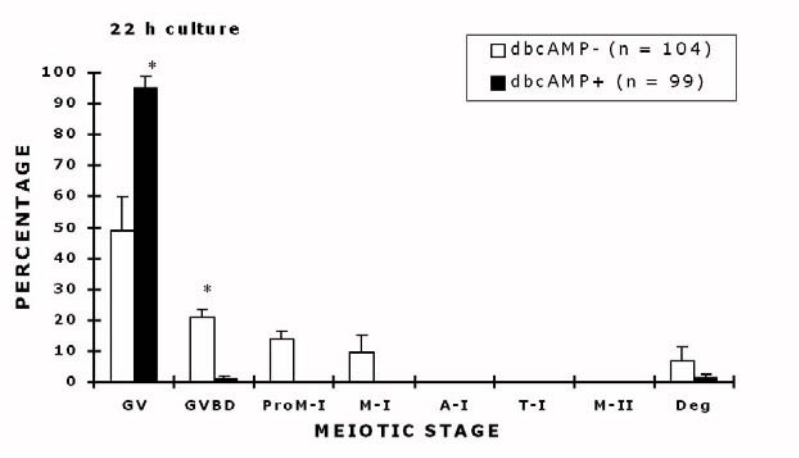


Fig 15. Distribution (mean ± SEM) of meiotic stage of porcine oocytes after 22 h culture with or without 1 mM dbcAMP. Asterisk above the bars mean significant differences ($p < 0.01$). Numbers of oocytes examined in different treatment groups are given in parentheses.

By this time, GVBD occurred in a high rate (44.3 ± 8.1%) of oocytes that were matured in the absence of dbcAMP, the rest of them remained at GV stage and 9.6 ± 5.2 % of oocytes already reached metaphase-I (M-I) phase. The nuclear status of oocytes that were cultured with dbcAMP was at GV stage, which is similar to that at 12 h of culture with an unremarkable rate (1.0 ± 1.0%) of oocytes that underwent GVBD. The rate of degenerated oocytes in this period of culture was the same in the treatment groups. No difference in nuclear stage between oocytes collected with different levels of cAMP was observed at this period (data not shown).

By 36 h of culture, the most of oocytes underwent GVBD in both dbcAMP- and + groups (89.3 ± 2.4% and 93.6 ± 3.4%, respectively). A considerable rate (38.0 ± 6.4%) of the oocytes matured in the absence of dbcAMP reached metaphase-II (M-II) by this time and a significant proportion showed M-I (16.6 ± 5.3%) (Figure 16).

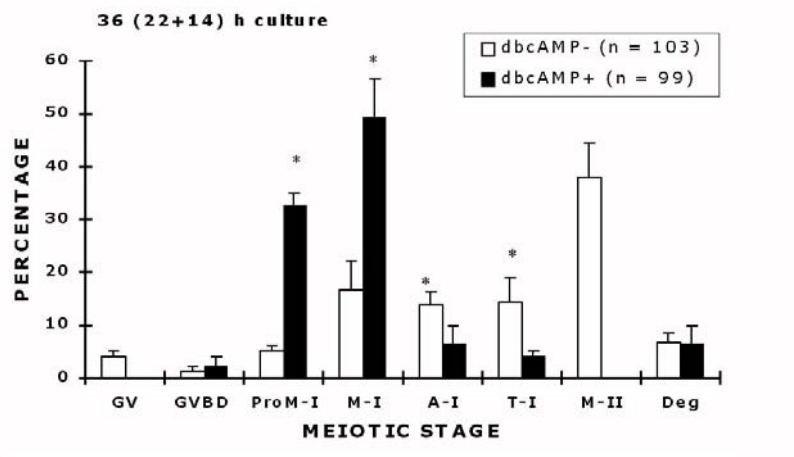


Fig. 16. Distribution (mean ± SEM) of meiotic stage of porcine oocytes after an additional 14 h cultivation following 22 h culture (36 h in total) with or without 1 mM dbcAMP. Asterisk above the bars mean significant differences ($p < 0.01$). Numbers of oocytes examined in different treatment groups are given in parentheses.

The remaining oocytes that underwent GVBD were at prometaphase-I (proM-I) (5.0±1%), telophase-I (T-I) (14.3 ± 4.6%), or anaphase-I (A-I) (13.6 ± 2.6%). In contrast, in the dbcAMP+ group a significantly higher proportion of oocytes were at M-I (49.3±7.3%) or proM-I (32.6±2.3) stage but none of the oocytes showed M-II phase nucleus (Figure 16). No difference in nuclear stage between oocytes collected with different levels of cAMP was observed at this period of culture (data not shown).

By the end of maturational period (at 46 h of culture), the majority (56.6 ± 7.8%) of the oocytes that were matured without dbcAMP reached M-II phase, while a remarkable proportion (30.6 ± 8.4%) of the oocytes remained arrested at M-I (Figure 17).

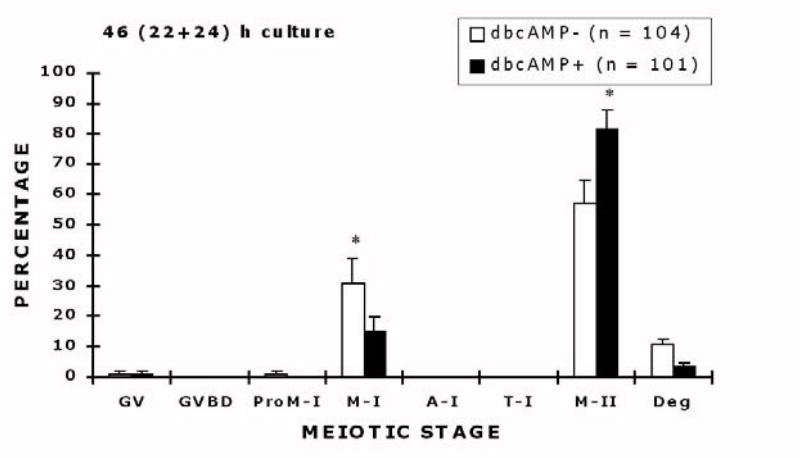


Fig. 17. Distribution (mean ± SEM) of meiotic stage of porcine oocytes after an additional 24 h cultivation following 22 h culture (46 h in total) with or without 1 mM dbcAMP. Asterisk above the bars mean significant differences ($p < 0.01$). Numbers of oocytes examined in different treatment groups are given in parentheses.

A higher rate (81.0 ± 6.5%) of oocytes treated with dbcAMP during the first 22 h of culture reached M-II phase and a significantly lower rate (15.0 ± 4.5%) of oocytes remained at M-I phase by the end of culture (Figure 17). No significant difference in the rate of degenerated oocytes was observed between the dbcAMP- and + groups by the end of the culture period (10.6 ± 1.6 and 3.0 ± 1.7%, respectively). No difference in nuclear morphology was observed between the oocytes that were collected with different levels of cAMP (data not shown).

Regarding the meiotic progression of oocytes it can be noted, that after releasing from the meiotic block, oocytes synchronised by 1 mM dbcAMP undergo GVBD within a shorter period of time than that of without dbcAMP treatment (14 h and 24 h, respectively) and similarly, following synchronisation the maximum percentage of M-II oocytes reaches its maximum within a shorter period of time than that of without treatment (10 h and 24 h, respectively) (Figure 18).

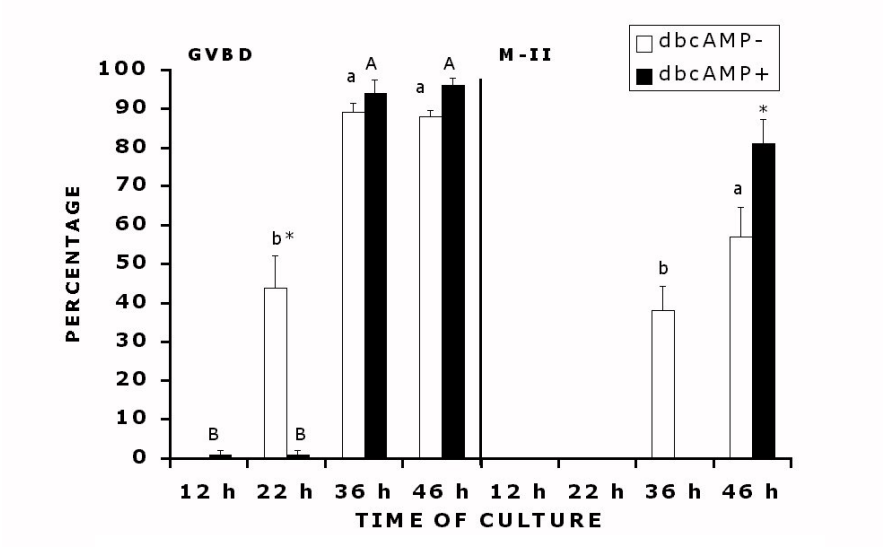


Fig. 18. Nuclear progression of oocytes treated with or without dbcAMP to GVBD and M-II stages during IVM. Values with different letters (A,B) represent a significant difference within the dbcAMP+ group. Values with different letters (a,b) represent a significant difference within the dbcAMP- group. Asterisk above the bars mean significant differences between the dbcAMP- and dbcAMP+ groups ($p < 0.05$).

Experiment 2. The penetration rate in the control (BCM, dbcAMP-) group was 45.6 ± 7.7% and no difference in penetration rate was observed between the treatment groups (Figure 19). The rate of monospermic fertilization was 9.3 ± 1.4% when no iAC in the

collection medium and/or dbcAMP in IVM medium was used. A significant increase (20.6 ± 3.0%) of normal monospermic fertilization rate was observed when dbcAMP was used during IVM.

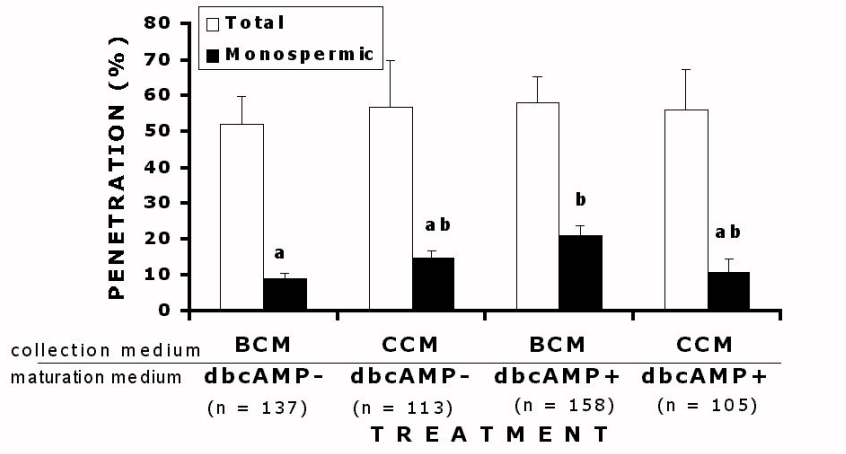


Fig. 19. Fertilization results 10 h after IVF of oocytes obtained by different collection media and cultured in the absence or presence of 1 mM dbcAMP for the first 22 h of the total 46 h maturation period. Abbreviations: BCM= Basic Collection Medium; CCM= Complete Collection Medium; dbcAMP- = COCs cultured in the absence of 1 mM dbcAMP; dbcAMP+ = COCs cultured in the presence of 1 mM dbcAMP. Data are presented as mean ± SEM. Different letters above the bars represent significant differences ($p < 0.05$). Numbers of oocytes examined in different treatment groups are given in parentheses.

There was no significant difference in monospermic fertilization rates between the treatment groups of different concentrations of cAMP in collection medium when dbcAMP was absent (9.3 ± 1.4% and 15.2 ± 1.6%) or present (20.6 ± 3.0% and 11.5 ± 3.6%) in maturation medium. The number of penetrating spermatozoa/oocyte was 2.11 in the control (BCM, dbcAMP−) group, and this value did not change

significantly when iAC or dbcAMP was used (2.02 and 2.06, respectively).

Experiment 3. The blastocyst rate of the oocytes collected with BCM and matured in the presence of dbcAMP was significantly higher than that of oocytes collected with BCM and matured without dbcAMP (32.1 ± 5.7% and 20.6 ± 2.9%, respectively) (Figure 20).

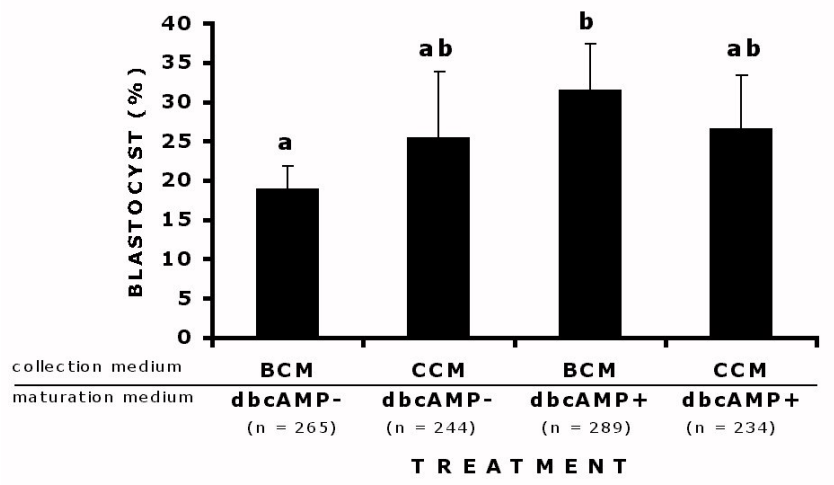


Fig. 20. In vitro developmental rates to blastocyst stage of porcine oocytes obtained by different collection media and cultured in the absence or presence of 1 mM dbcAMP for the first 22 h of the total 46 h maturation period. Abbreviations: BCM= Basic Collection Medium; CCM= Complete Collection Medium; dbcAMP- = COCs cultured in the absence of 1 mM dbcAMP; dbcAMP+ = COCs cultured in the presence of 1 mM dbcAMP. Data are presented as mean ± SEM. Different letters above the bars represent significant differences ($p < 0.05$). Numbers of oocytes examined in different treatment groups are given in parentheses.

The supplement of collection medium with iAC and IBMX resulted in no difference in the rate of blastocysts (25.7 ± 8.4%). The combination of iAC in collection medium and dbcAMP in maturation medium did not cause significant change in blastocyst rate (26.7 ± 6.8%). There was no significant difference in blastocyst quality

considering the number of cells in blastocysts obtained by the different oocyte collection and maturation methods (Figure 21).

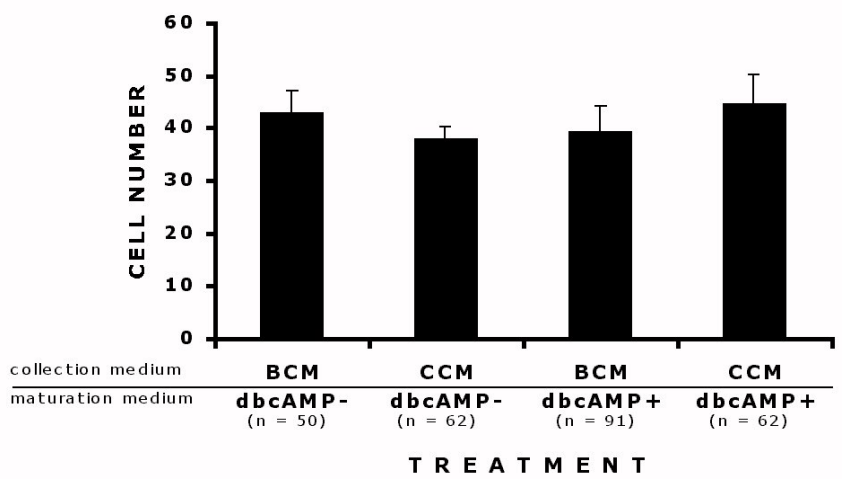


Fig. 21. Number of cells in blastocysts after in vitro culture of IVF oocytes obtained by the different oocyte collection and maturation methods. Abbreviations: BCM= Basic Collection Medium; CCM= Complete Collection Medium; dbcAMP- = COCs cultured in the absence of 1 mM dbcAMP; dbcAMP+ = COCs cultured in the presence of 1 mM dbcAMP. Data are presented as mean ± SEM. Numbers of oocytes examined in different treatment groups are given in parentheses.

Discussion. The present results confirm, also in the porcine species, that intercellular cAMP can cause meiotic arrest of mammalian oocytes at GV stage. To elevate intercellular cAMP level artificially, we used both phosphodiesterase inhibitors such as IBMX and iAC. Recently, the use of 0.5 mM IBMX was reported to arrest GVBD in porcine oocytes without any negative effect on the formation of LH receptors (Shimada *et al.,* 2003). iAC is an is an enzyme purified from the bacteria *Bordetella pertussis* (Wolff *et al.,* 1980). It enters and elevates intercellular cAMP content of mammalian cells effectively

(Confer *et al.,* 1984). The successful use of iAC dialyzed urea extract to elevate cAMP level in rat oocytes was reported (Aberdam *et al.,* 1987). It is also demonstrated that iAC can inhibit meiosis of both cumulus-enclosed and cumulus-free bovine oocytes in a dose dependent manner through accumulating intercellular cAMP and without decreasing the developmental competence (Aktas *et al.,* 1995). The successful use of a combination of iAC and IBMX to enhance developmental competence of bovine oocytes was reported when these chemicals were added to oocyte collection medium (Luciano *et al.,* 1999), suggesting that intracellular level of cAMP during collection might also affect further developmental competence of oocytes. In the present study in the porcine species, however, the chromatin condensation, nuclear maturation and further developmental competence of oocytes to the blastocyst stage did not differ when collection media supplemented with or without IBMX and iAC were used regardless of usage of dbcAMP during IVM (Figure 14). This result indicates that a decreased concentration of cAMP in the medium during oocyte collection did not affect maturation of porcine oocytes and subsequent development after IVF, consequently, the initiation of spontaneous maturation might occur during the incubation of the oocytes in our IVM system. Otherwise a difference in nuclear status between the oocytes collected with or without cAMP supplement would have been detected after 12 h of incubation.

During the initial stage of maturation in vivo, LH elevates intercellular cAMP in porcine oocytes (Mattioli *et al.,* 1994) and similar phenomenon occurs in vitro if COCs are exposed to FSH prior to LH (Shimada *et al.,* 2003). Since FSH and LH act positively upon IVM of porcine oocytes (Mattioli *et al.,* 1991), a transient meiotic arrest maintained by cAMP seems to be beneficial for normal maturation of

oocytes. The fact that LH enhances IVM in pigs more effectively when oocytes are exposed to this hormone only during the first 20 h of maturation culture (Funahashi *et al.,* 1994) suggests that timing of meiotic arrest must be crucial and should be maintained during the first half of maturation. Moreover, dbcAMP maintained meiotic arrest during the first 20 h of IVM of porcine oocytes successfully synchronized the nuclear maturation and resulted in an increased rate of blastocyst formation after IVF but without affecting the final rate of matured oocytes and the rate of monospermic fertilization (Funahashi *et al.,* 1997b). However dbcAMP and forskolin are known to increase GVBD rate of in vitro matured mouse oocytes through stimulating cumulus cells to secrete a diffusible meiosis-inducing substrate (Guoliang *et al.,* 1994) and similar results were reported when porcine oocytes were exposed to FSH or forskolin (Xia *et al.,* 2000). When we used 1mM dbcAMP to synchronize nuclear maturation of oocytes, no difference was observed in the nuclear morphology of oocytes after culture for 12 h irrespective of dbcAMP supplementation. This result suggests a certain synchronization of nuclear maturation of oocytes that were cultured in the absence of dbcAMP. A possible reason of this phenomenon might occur due to the gonadotroph hormones (PMSG and hCG) that were present both in the maturation medium of the dbcAMP treated and the control oocytes, too. Gonadotroph hormones are known to elevate the intracellular cAMP level of mammalian oocytes (Bornslaeger and Schultz, 1985; Mattioli *et al.,* 1994; Shimada *et al.,* 2003).

The present results suggest clearly that synchronization of meiosis with dbcAMP during the first 22 h of maturation resulted in a higher maturation rate and an increased proportion of monospermic zygotes (in the other word, reduced polyspermy) after IVF. In parallel with

the result of Funahashi *et al.,* (1997b) we also found a more synchronized GVBD with the use of dbcAMP while the nuclear status of oocytes cultured without dbcAMP showed heterogeneity during IVM, and no difference in GVBD rate was observed between the control and the dbcAMP treated groups. We suggest that dbcAMP seems to manifest its beneficial effect on meiotic competence during M-I to M-II transition, since a higher rate of M-II oocytes was observed when dbcAMP was added during the first 22 h of IVM and without this drug more oocytes remained at M-I phase by the end of culture (Figure 16). This outcome is in accordance with the results of Shimada and Terada (2002) who found that cAMP plays an important role in the regulation of meiotic progression beyond the M-I stage in porcine oocytes. In the other hand, we had a higher rate of monospermic fertilization when meiotic process was synchronized by an elevated level of cAMP (Figure 19). The reason of this outcome is unclear at present, however, there could be possibilities as follows: There seems to be a difference in the diversity of nuclear status and cytoplasmic maturity between oocytes treated with or without dbcAMP. Distribution of cortical granules (CG) in porcine oocytes during in vitro maturation is in tune with nuclear maturation (Wang *et al.,* 1997) suggesting relationship between nuclear and cytoplasmic maturation. Moreover it was shown that cytoplasmic maturation changes (e.g. CG distribution) occur in accordance with the meiotic resumption (Sun *et al.,* 2001). According to this phenomenon, a higher rate of full-mature oocytes might manifest in a higher percentage of normal fertilization, while without synchronization of meiosis a more heterogene population might be obtained including immature and aged oocytes thus resulting in a higher rate of abnormal fertilization such as polyspermy. Asynchronous meiotic

maturation of porcine oocytes cultured in vitro is known to occur resulting in a considerable population of aged oocytes that are susceptible to polyspermic fertilization (Grupen *et al.,* 1997). After maturation for 36 h, in the present study, a high incidence of nuclear maturation was observed among the oocytes that were cultured without dbcAMP, a remarkable proportion (38 ± 6.42%) has already finished meiotic process. These oocytes might have been aged after culture for 46 h at the time of IVF resulting heterogeneity in cytoplasmic maturation among oocytes at M-II phase. Contrary to this, oocytes after a certain term of meiosis synchronization showed more homogeneity in term of nuclear maturation and presumably cytoplasmic maturation as well, which could result in a lower rate of polyspermy. Other possible ways of dbcAMP to affect monospermic fertilization should also be considered. Increasing the intercellular cAMP content in cumulus oocyte complexes might affect the oocyte cytoplasmic maturation directly as well, through enhancing metabolism between the cumulus cells and the oocytes. Flagg-Newton *et al.,* (1981) reported that mammalian cells exposed to cAMP and dbcAMP show increased junctional permeability. FSH and dbcAMP are known to elongate the term of coupling and metabolic co-operation between cumulus cells and oocyte during in vitro culture in mice (Salustri and Siracusa, 1983). It has also been suggested that a certain level of intercellular cAMP might affect developmental competence of bovine oocytes through enhancing communication between the oocyte and cumulus cells (Modina *et al.,* 2001). Increased permeability of gap junctions between cumulus cells and oocyte might promote normal fertilization since cumulus cells are known to support the oocyte with an unknown factor(s) that is (are) necessary for normal cytoplasmic maturation, fertilization and further

embryonic development. Moreover, a direct effect of cAMP on block to polyspermy is also conceivable. dbcAMP was found to stimulate activity of tissue-type plasminogen activator (tPA) in porcine COCs during in vitro culture (Kim and Menino, 1995). tPA is synthetised in mouse and rat oocytes during meiosis (Haurte *et al.,* 1985) and released during activation of rat oocytes suggesting its possible role in zona reaction (Zhang *et al.,* 1992), which may affect the block of polyspermy in pigs. Taking these into consideration, we suggest that the elevated rate of blastocyst formation might have been caused by the higher rate of monospermic fertilization after meiotic synchronisation of oocytes with dbcAMP. Funahashi *et al.,* (1997b), however, found a higher rate of blastocyst formation following IVF of dbcAMP treated oocytes but without any effect on monospermic fertilization. In parallel with those results, an increased level of intercellular cAMP in porcine COCs by LH did not affect IVF rate and monospermic fertilization but resulted in an improved developmental competence of oocytes to the blastocyst stage after IVF (Shimada *et al.,* 2003). These results are in discrepancy and futher experiments are needed to clarify the relashionship between monospermy caused by cAMP treatment and subsequent development to the blastocyst stage.

In the present study we inseminated COCs instead of denuded oocytes in order to achieve better fertilization results. There might have been a certain proportion of oocytes arrested at M-I stage among the fertilized oocytes according to the nuclear status of each treatment group and this fact might have affected the blastocyst rates. On the base of M-II rates at the end of IVM and presuming that M-I arrested oocytes can not form blastocyst, we re-calculated the blastocyst rates. It was found that the blastocyst rate of dbcAMP

treated oocytes is still significantly higher than that of the control group (43.4% and 33.0%, respectively). However porcine oocytes arrested at M-I stage can be activated (Kikuchi *et al.,* 1999) suggesting their ability to form embryo.

An increased meiotic potential and developmental competence of dbcAMP treated oocytes is reported in this study. Porcine oocytes with a lower meiotic and/or developmental competence can upgrade their meiotic and developmental ability due to the elongated period of metabolism between the cumulus cells and the oocyte through the maintenace of meiotic arrest at GV stage by dbcAMP treatment.

4.2 In vitro fertilization and development to blastocyst stage of immature porcine oocytes arrested before metaphase-II stage

Experiment 1. In the present culture conditions, about 75% of the oocytes had completed nuclear maturation until 36 h, and the incidences of nuclear status did not change significantly until 60 h (Figure 22). The oocytes which failed to complete meiosis were at immature stage, where oocytes at the metaphase-I stage were dominant.

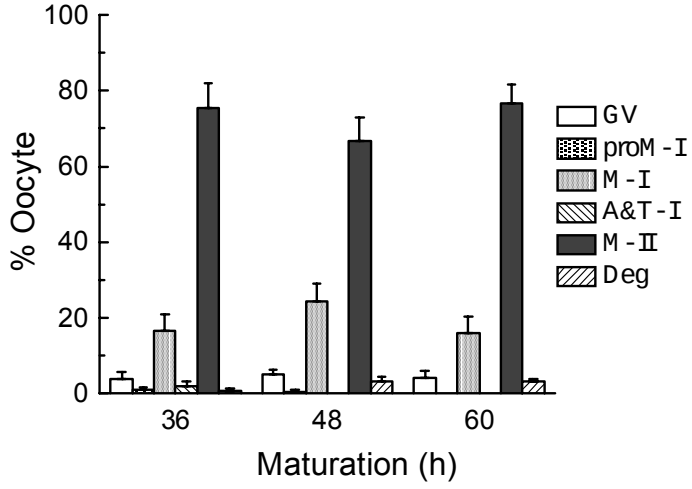


Fig. 22. Nuclear maturation during in vitro maturation culture for 36 to 60 h. After the statistical analysis, the status was found to be not different in each category. A total four replicated trials using 219 – 230 oocytes for each duration were carried out. GV; Germinal vesicle stage, M-I; metaphase-I, A&T-I; anaphase- and telophase-I, M-II; metaphase-II, and Deg; Degenerated.

Experiment 2. As shown in Table 1, after maturation culture for 48 h, 75.4% (442 of 586) of oocytes had a visible polar body and classified as PB+, whereas the remains were categorized as PB-. Nuclear status of oocytes classified as PB+ and PB- was also shown. The most (91.4%) of the PB+ oocytes were at M-II stage and the rest of them were at telophase (T-I) stage (2.9%) or showed the sign of spontaneous activation beyond M-II stage such as anaphase/telophase-II and metaphase/anaphase-III stages (5.5%). The most of the PB- oocytes were arrested at germinal vesicle (GV; 41.6%) and metaphase-I (M-I; 34.0%) stages, and the others were at M-II (8.3%), prometaphase-I (proM-I; 6.2%) and anaphase-I (A-I; 2.7%) stages and only one oocyte (0.6%) was at telophase-I (T-I) stage. The total proportion of abnormal oocytes in the PB- group was 6.0% including oocytes with two metaphase chromosomes (2.0%), degenerated ones (3.4%) and one with scattered female chromosomes (0.6%).

Experiment 3. The results of IVF of PB+ and PB- oocytes were shown in Table 2. When total 340 PB+ and 116 PB- oocytes were inseminated, there was no significant difference between two groups in the percentage of oocytes penetrated by a spermatozoon(zoa) (75.5 ± 6.5% and 79.0 ± 2.8%, respectively), monospermy (24.6 ± 2.2% and 24.3 ± 4.9%, respectively). There was no significant difference in average number of penetrated spermatozoa between the PB+ and PB- groups (2.4 ± 0.3 and 2.5 ± 0.1, respectively). The rate of fragmentation in the PB+ group was significantly higher than that of the PB- (3.2 ± 1.2% and 0%, respectively).

Table 1. Nuclear status of oocytes with (PB+) or without (PB-) a visible polar body after maturation culture for 48 h. Data are expressed as total number and percentage.

Oocyte type	Total	GV	ProM-I	M-I	A-I	T-I	M-II	Spontaneous activation		Abnormal chromosome distribution		Deg
								A-II or T-II	A-III or M-III	2 chromosome plates	Scattered chromosomes	
PB+	442					13 (2.9%)	404 (91.4%)	22 (4.9%)	3 (0.6%)			
PB-	144	60 (41.6%)	9 (6.2%)	49 (34.0%)	4 (2.7%)	1 (0.6%)	12 (8.3%)			3 (2.0%)	1 (0.6%)	5 (3.4%)

GV: Germinal vesicle; ProM-I: prometaphase-I; M-I: metaphase-I; A-I: anaphase-I; T-I: telophase-I; M-II: metaphase-II; A-II: anaphase-II; T-II: telophase-II; A-III: anaphase-III; M-III: metaphase-III

Table 2. In vitro fertilization results at 10 h after insemination of oocytes with (PB+) and without (PB-) a visible polar body. Data are presented as total number and mean ± SEM in parentheses.

Oocyte type	*No. of inseminated oocytes*	*Oocytes penetrated by a spermatozoon(zoa)*		*Sperm No./oocyte*
		Total	*Monospermy*	
PB+	340	249 (75.5 ± 6.5%)	87 (24.6 ± 2.2%)	2.4 ± 0.3
PB-	116	91 (79.0 ± 2.8%)	26 (24.3 ± 4.9%)	2.5 ± 0.1

Table 3. Sperm penetration and oocyte activation of inseminated PB- oocytes arrested at GV or presumably arrested at M-I (MI*) or M-II (MII*) stages and morphological changes of penetrated sperm heads.

Oocyte type	*Inseminated oocytes*	*Penetrated*	*Monospermic*	*Sperm/oocyte*	*Activated*	*Status of penetrated spermatozoa*			
						All sperm.	*Condensed chromosomal*	*Enlarged or recondensed*	*Decondensed or pronuclear*
GV	38	29 (76.3%[b])	6 (15.7%[a])	3.5 ± 0.5[a]	0 (0%[b])	103	93 (90.2%[a])	10 (9.7%[a])	0 (0%[b])
M-I*	56	42 (75.0%[b])	14 (25.0%[a])	1.8 ± 0.1[b]	40 (95.2%[a])	82	4 (4.9%[b])	0 (0%[a])	78 (95.1%[a])
M-II*	19	17 (89.4%[a])	3 (15.7%[a])	2.7 ± 0.2[ab]	17 (100%[a])	47	0 (0%[c])	0 (0%[a])	47 (100%[a])

Data are presented as total number and percentage (in parentheses). Sperm/oocyte data are presented as mean ± SEM .

M-I*; Unfertilized oocytes arrested at M-I stage, penetrated unactivated M-I stage oocytes and penetrated activated oocytes with one polar body were included in this group. M-II*; Unfertilized oocytes at M-II stage, penetrated unactivated M-II stage oocytes and penetrated activated oocytes with two polar body were included in this group.

[a,b]Percentage or value with different superscripts is significantly different in the category (p<0.05).

According to the nuclear status and the number of polar bodies at 10 h after IVF, PB- oocytes could be divided into three categories; 1) oocytes arrested at GV stage, 2) presumably M-I arrested (M-I* = unfertilized oocytes arrested at M-I stage, penetrated but non-activated M-I stage oocytes, and penetrated and activated oocytes with one polar body were included in this group), and 3) presumable M-II arrested (M-II* = including unfertilized oocytes at M-II stage, and penetrated and activated oocytes with two polar bodies) at the time of fertilization (Table 3). The appearance of pronuclear zygotes without polar body was so seldom that we neglected such embryos. The percentage of penetrated oocytes and the monospermy did not differ between the GV and the M-I* groups but it was higher in the M-II* group. However, the average number of penetrating spermatozoa was significantly higher in the GV arrested oocyte than that of the M-I* group.

Experiment 4. Embryonic development to blastocysts of PB+ and PB- oocytes after IVF and subsequent IVC for 6 days are shown in Table 4.

Table 4. Blastocyst formation, number of blastomers and blastocyst quality after IVF of PB+ and PB- oocytes on day 6 of IVC.

Oocyte type	*Oocytes inseminated*	*Blastocysts*	*Cell No*	*Embryo morphology*		
				Type 1	*Type 2*	*Type 3*
PB+	265	91 (34.6 ± 2.4%)[a]	52.0 ± 2.5[a]	62 (68.1%)	21 (23.0%)	8 (8.7%)[b]
PB-	121	25 (20.7 ± 2.8%)[b]	29.1 ± 2.8[b]	15 (60.0%)	5 (20.0%)	5 (20.0%)[a]

Data are presented as mean ± SEM. [a,b]Percentage or value with different superscripts is significantly different in the category ($p<0.05$). *Type 1* blastocyst: the proportion of dead/degenerated part is less than 10 percent of the embryo; *Type 2* blastocyst: 10-30% of the embryo is degenerated; *Type 3* blastocyst: 30-50% of the embryo is degenerated.

The rate of blastocysts in the PB+ group was significantly higher than that in the PB- group (34.6 ± 2.4% and 20.7 ± 2.8%, respectively). The number of all blastomer nuclei in PB+ blastocysts (52.0 ± 2.5) was significantly higher than that of PB- blastocysts (29.1 ± 2.8). Concerning the morphology of the blastocyst, there was no difference between the PB+ and PB- blastocysts in the proportions of *Type 1* blastocysts (68.1% and 60.0%, respectively) and *Type 2* blastocysts (23.0% and 20.0%, respectively), while the percentage of *Type 3* blastocysts was higher in the PB- group than that in PB+ (20.0% and 8.7%, respectively).

***Experiment 5*.** The result of the differential staining of blastocysts is shown (Table 5).

Table 5. The number of inner cell mass (ICM) and trophoectoderm (TE) cells in 6 day blastocyst obtained from IVF/IVC of PB+ and PB- oocytes.

Oocyte type	No Blastocysts examined	Cell Number in embryos		
		ICM	TE	Total
PB+	42	15.46 ± 0.5[a] (33.59%)	30.56 ± 1.9[a] (66.4%)	46.02 ± 5.2[a]
PB-	20	9.12 ± 1.5[b] (30.72%)	20.56 ± 4.0[a] (69.27%)	29.68 ± 5.0[b]

Data are presented as mean ± SEM.

[a,b]Value with different superscripts is significantly different in the category (p<0.05).

The number of ICM cells in the PB+ group was significantly higher than that of the PB- group (15.46 ± 0.5 and 9.12 ± 1.5, respectively), while there was no difference in the number of TE cells

between the two groups (30.56 ± 1.9,and 20.56 ± 4.0, respectively). However proportion of ICM and TE cells in PB+ and PB- blastocysts did not differ significantly, when the ratio were calculated as both ICM/TE (1:1.9 and 1:2.2, respectively) and ICM/Total cell number (1:2.9 and 1:3.2, respectively). The number of all blastomers in the PB+ group was higher than that of the PB- group (46.02 ± 5.2 and 29.68 ± 5.0, respectively), which is in accordance with the result of *Experiment 3*.

Discussion. The completion of nuclear maturation of porcine follicular oocytes is affected by cytoplasmic factors such as the capacity of oocytes to start or finish meiosis and the culture conditions. Therefore, after maturation culture, the nuclear status of oocytes results in various stages of meiosis. Some of the oocytes matured to M-II stage, whereas the others remained at immature stages such as GV and M-I. Such nuclear status did not change after a certain period (from 36 h to 60 h of culture; Figure 22), which was also confirmed in the previous studies using a different maturation system (Kikuchi *et al.*, 1999a; Kikuchi *et al.*, 1999b). To generate normal zygotes with a normal ploidy by in vitro procedures, preparation of mature oocytes with a haploid genome before IVF is important. For this purpose, selection of mature oocytes from the immature ones according to the presence or absence of a first polar body seems to be a reliable method other than the establishment of a completed IVM system showing 100% of maturation rate. Our results confirmed that the vast majority (91.4%) of the oocytes having the visible first polar body were at M-II stage and that 2.9% were just before finishing nuclear maturation at T-I. Surprisingly, the rest (5.5%) of the PB+ oocytes showed the signs of spontaneous activation. Although the reason of this phenomenon is unclear, the

early nuclear and cytoplasmic maturation of such oocytes may be probable. During maturation culture, some oocytes undergo precocious nuclear maturation and start ageing earlier than the rest of the population, where metaphase promoting factor (MPF) activity decreases enabling the oocytes to be activated spontaneously (Kikuchi *et al.* 2000). In the present study, porcine oocytes completed nuclear maturation by 36 h. Thus some oocytes with advanced maturation/ageing of the cytoplasm seemed to be activated spontaneously by the end of the maturation culture for 48 h.

The nuclear status of PB- oocytes appeared to be even more heterogeneous. The most of the oocytes that failed to undergo nuclear maturation remained at GV stage or were arrested at M-I stage in accordance with previous reports (Motlik and Fulka 1986; Kikuchi *et al.* 1999a). However, a certain amount (6.2%) of PB- oocytes were at proM-I stage. Since most of the oocytes complete GV brakedown (GVBD) by 36 h of IVM in the present culture system (Figure 22), these oocytes might have been arrested at proM-I stage. Up to our knowledge, there is no report of such a phenomenon. The reason of meiotic arrest at proM-I stage is not clear yet. One possible explanation is the insufficient ability of protein synthesis needed for nuclear maturation in the arrested oocytes. As reported in mouse oocytes by Hashimoto and Kishimoto (1988), the induction of GVBD and chromosome condensation (the initiation of meiosis) were inducted by a primary MPF which is initially produced independently of protein synthesis, and then the appearance of a secondary MPF, which requires protein synthesis, is necessary to achieve the definite metaphase showing the condensed chromosomes aligning on an equatorial plate of the meiotic spindle. We suggest that the failure of this new MPF synthesis could have caused the meiotic arrest at proM-

I stage in pigs. Besides the meiotically arrested and abnormal oocytes, the PB- group contained a remarkable amount (8.3%) of oocytes at M-II stage. As in vitro matured porcine M-II oocytes have much smaller perivitelline space and the size of polar body is usually smaller than that of the in vivo matured oocytes (Wang *et al.* 1998), we could not always detect the first polar body during the observation under a stereo microscope resulted in failure of the complete selection of mature oocytes, as reported previously (Kikuchi *et al.* 1999a).

In consistence with Kikuchi *et al.,* (1999a), no difference was observed in penetration and monospermic fertilization rates between the PB+ and PB- oocytes (Table 2). A slight, but significant difference was found in the percentage of fragmented embryos between the PB+ and PB- groups (3.2% and 0%, respectively, data not shown) at 10 h post-IVF. The PB+ (M-II) oocytes may be easily fragmented after the oocyte activation because their MPF activity is lower than that of PB- (M-I) oocytes (Kikuchi *et al.* 1999a and 2000). Another possible explanation for this phenomenon could be a suggested difference between the PB+ and PB- embryos in the actin filament distribution, that is known to affect subsequent embryo development regarding their capability to undergo early fragmentation (Wang *et al.* 1999). We observed that, after an electric pulse, almost all of the activated PB+ oocytes resulted in zygotes with one female pronucleus and two polar bodies at 10 h (Somfai *et al.* unpublished observation). However, IVF of PB- oocytes resulted in various types of zygotes concerning the number of pronuclei and polar bodies. The zygotes with 2 polar bodies might have been generated by fertilization of a small population of M-II oocytes that were included in the PB- group, while zygotes with one polar body were created by the fertilization of

oocytes arrested before M-II, because no delayed maturation was observed at/after the time of insemination (48 h; Fig 22). It seems to be necessary to discuss about fertilization status of PB- oocytes according to the nuclear status and the number of polar bodies 10 h after IVF. In the present study, three categories of oocytes could be distinguished within the PB- group (GV, M-I* and M-II* as described in Table 3). The results indicate the similar rates of penetrated oocytes and the monospermy between the GV and the M-I* groups, while the rates were significantly higher for the M-II* group, and also significantly more spermatozoa penetrated into the GV group than the M-I* group. This suggests that there may be a difference in the quality of zona pellucidae (ZP) among GV arrested and M-I or M-II arrested oocytes. These findings confirm the results presented by Wang *et al.*(1994) and might reflect the lack of cortical granule distribution, which is essential for the prevention of polyspermy, in the GV arrested oocytes. Although a significant difference was observed between the MI* and MII* oocytes in the rate of monospermic fertilization, the number of spermatozoa in oocytes did not differ. We assume that there is no difference in the quality of ZP of M-I and M-II arrested oocytes revealing the similar cytoplasmic characteristics and ability to block polyspermy in the two groups. None of the GV arrested oocytes were activated and almost all of the penetrating spermatozoa remained condensed (90.2%), enlarged or recondensed (9.7%), no male pronucleus formation was observed in this group. This is in agreement with the report described previously (Wang and Niwa 1997). There was no difference in the activation rate of penetrated oocytes between the MI* and MII* groups (95.2% and 100%, respectively) and the percentage of penetrating spermatozoa that formed male pronucleus also did not differ significantly (95.1%

and 100%, respectively) suggesting the similar cytoplasmic characteristics of M-I and M-II arrested oocytes confirming the previous results in mice (Eppig *et al.* 1994) and those in pigs (Kikuchi *et al.* 1999a). Almost all of the penetrated PB- oocytes, except for GV stage oocytes, were activated at 10 h after IVF. It was suggested that the cytoplasmic maturation has already completed even when their nuclear status was arrested at M-I stage. It has been reported that bovine (Chian *et al.* 1992) and murine (Polanski 1995) oocytes arrested at M-I stage during maturation culture completed their nuclear maturation to M-II stage when they were fertilized and subsequently cultured. However, the present study demonstrated clearly that nuclear status did not change during the time of fertilization, and also it was reported that M-I arrested porcine oocytes form a female pronucleus and extrude only one polar body after sperm penetration without completion of their nuclear maturation (Kikuchi *et al.* 1999a). It is of interest that, in the present study, pronuclear status of PB+ oocytes (e.g. timing of formation and morphological features) was similar to that of PB- oocytes at 10 h after IVF without any delay or failure in pronucleus formation in the PB- group. This suggested that activation of M-I arrested oocytes occurred without achieving nuclear maturity.

In the present study, we investigated the embryonic development of porcine oocytes arrested at M-I stage at the time of IVF. Porcine oocytes at GV stage form pronucleus directly from interphase when matured with Na-EDTA and such activated oocytes have the ability to form blastocyst indicating that completion of nuclear maturation is not a prerequisite for blastocyst formation (Azuma *et al.* 2001). The ability of blastocyst formation in M-I arrested in vitro maturing mouse oocytes was reported by Eppig *et al.* (1994). In their study, the

blastocyst formation ability of M-II oocytes was higher than that of the M-I arrested ones. Although also in our study the blastocyst formation rate of in vitro fertilized PB- porcine oocytes during IVC was significantly lower than that of the PB+ oocytes, the rate is still remarkably high (20.7 ± 2.8% and 34.6 ± 2.4%, respectively). Regarding that a high proportion (41.6%) of PB- oocytes were arrested at GV stage and could not be activated and consequently could not form blastocyst, we recalculated the blastocyst rate of PB- oocytes after excluding the presumptive number of the oocytes arrested at GV stage. It was clear that, without the GV arrested oocytes, the blastocyst rate of PB- group would have been 35.3% which did not differ significantly from that of the PB+ group (34.6%) suggesting that the ability to form blastocyst was the same in M-II and M-I arrested porcine oocytes. It must be, however, noted that the PB- group always contained a remarkable amount (8.3%) of M-II oocytes. Even so, according to the blastocyst formation rate of PB+ oocytes and presuming that none of the oocytes arrested meiotically at such as the GV or M-I stage can form blastocyst, only 2.9% blastocysts could be generated only from the M-II oocytes in the PB- group. In the present study, however, the incidence of blastocysts in PB- group (20.7%) was higher than the calculated value (2.9%). Thus it can be concluded that the majority of the PB- blastocysts were resulted from the fertilization of M-I arrested oocytes. Up to our knowledge, this is the first report describing about the ability of meiotically arrested porcine oocytes to form blastocyst. The ability of porcine oocytes arrested at different stages of meiosis to from blastocyst following fertilization is not clarified yet since there is no reliable method to distinguish the nuclear status of porcine oocytes without affecting their further viability and developmental capacity.

The possibility of proM-I arrested and some abnormal oocytes to form embryo after fertilization should also be considered. Oocytes with two chromosome plates (Figure 4 F; page 30) usually appear in our IVM systems but in a very low proportion, possibly due to the failure of first polar body extrusion at T-I stage. There is a possibility that these oocytes might also have the ability to be activated and form digynic embryos after IVF.

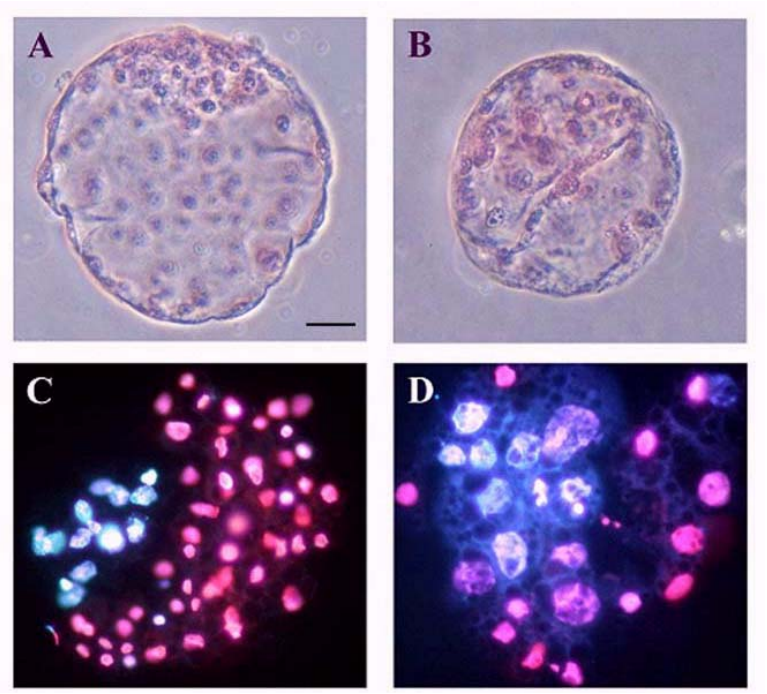


Fig. 23. Morphology of porcine blastocysts produced in vitro on day 6. Blastocyst derived from IVF/IVC of PB+ oocytes shows bigger size and higher cell number after staining with orcein (**A**) and differential staining (**C**), than that of PB- oocytes (**B**, **D**). Micrographs **A** and **B** were taken with a phase-contrast microscope at the same magnification. Micrographs **C** and **D** were taken with an epifluorescence microscope. Scale bar represents 50 μm in (**A**) and (**B**), 40 μm in (**C**) and 20 μm in (**D**).

Although there was no difference in their morphology, the total number of cells in PB- blastocyst was significantly lower than the PB+ blastocysts. This phenomenon may reflect their size too, the PB- blastocysts were smaller than that of the PB+ ones (Figure 23 A and B). Similarly, diandric triploid mouse embryos were reported to be morphologically normal but smaller than normal fertilized embryos (Kaufman *et al.* 1989). This phenomenon might be related to the abnormal ploidy of the PB- embryos. Fertilized and activated M-I or proM-I arrested oocytes or oocytes with two chromosome plates must be polyploid since they have two sets of the maternal genomes. The report that 75% of embryos obtained from IVF of M-I arrested mouse oocytes appeared to be triploid (Eppig *et al.* 1994) supports our hypothesis. Mammalian embryos with abnormal ploidy are known to have the ability to survive even into the postimplantation period (Piko and Bomsel-Helmreich 1960; Bomsel-Helmreich 1971; Han *et al.* 1999). Polyspermic porcine embryos with abnormal ploidy are known to have the developmental ability to the blastocyst stage but with lower numbers of ICM cells than that of the normal diploid embryos (Han *et al.* 1999). However, in the present study the ratio of ICM and TE cells was the same between PB+ and PB- blastocysts (Table 5, Figure 23 C and D) suggesting that the difference in total cell number might be due to the slower development of PB- embryos. This theory was supported by the fact that triploid mouse embryos were found to cleave more slowly, than diploid ones (Takagi and Sasaki 1976). To have better understanding of the development of meiotically arrested and fertilized porcine oocytes, further studies on their ploidy would be necessary, however, the high incidence of polyspermy that occurs in pig IVF might make such investigations very complicated.

It can be suggested that porcine oocytes arrested and aged at M-I stage develop to the blastocyst stage after IVF. Meiotic arrest at M-I stage can be caused by numerous factors such as insufficient meiotic competence affected by the follicle and oocyte diameter (Szybek 1972; Sorensen and Wassarman 1976; Motlik and Fulka 1986; Eppig *et al.* 1994) or stress caused by the inadequate culture conditions such as isolation and culture media (Bae and Foote 1980; Bagger *et al.* 1987; Kikuchi *et al.* 1999a). Near the end of growth phase, mammalian oocytes develop their ability of meiotic progression to M-II. In parallel with this, oocyte cytoplasm acquires the competence to undergo embryonic development referred as "cytoplasmic maturation". During IVM the frequency of both events increase as oocytes are originated from larger follicles in pigs (Marchal *et al.* 2002). In this study, porcine oocytes were collected from follicles of 3-6 mm in diameter, which were reported to be large enough for the oocytes to be capable of undergoing both meiotic maturation and preimplantation development (Marchal *et al.* 2002). Considering this, the arrest of meiotic progression at M-I in oocytes with competence to develop to the blastocyst stage might be due to the insufficient culture conditions for maturation rather than intrinsically inadequate meiotic competence.

4.3 The effect of cumulus morphology on nuclear and cytoplasmic maturation

Experiment 1. The proportion of floating oocytes with expanded somatic compartment was significantly higher in the GCOCs that in the COCs at 30 (82.8±2.5% and 65.9±3.1%, respectively), 36 (78.8±4.4% and 61.7±5.3%, respectively), 42 (77.0±5.1% and 55.6±4.1%, respectively) and 48 h (74.3±9.9% and 48.3±3.2%, respectively) of culture (Figure 24).

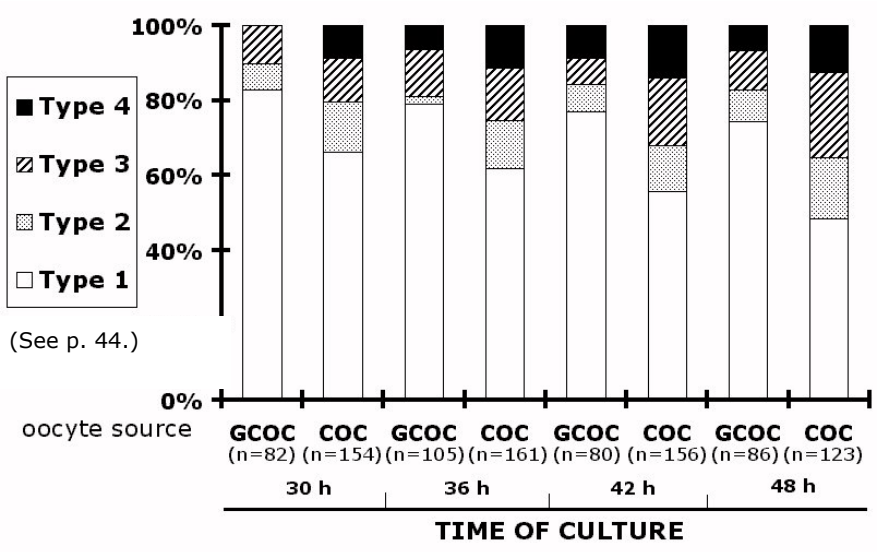


Fig. 24. Percentage of different morpologic categories of complexes during IVM of GCOCs and COCs. Numbers of oocytes examined at different culture periods are given in parentheses. Data are presented as mean.

There was no difference in GVBD rate between the GCOC and the COC groups during the culture when examined regardless of somatic

compartment morphology (Figure 25). The percentage of M-II stage oocytes also did not differ at 30, 36, 42 and 48 h of IVM, however there was difference in the nuclear progression: the percentage of M-II stage oocytes significantly increased progressively until the 48 h of IVM in the GCOC (74.5 ± 11.5%), while in the COC group this value reached its plateau (65.6 ± 4.1%) at 42 h of IVM and did not change significantly by 48 h of culture.

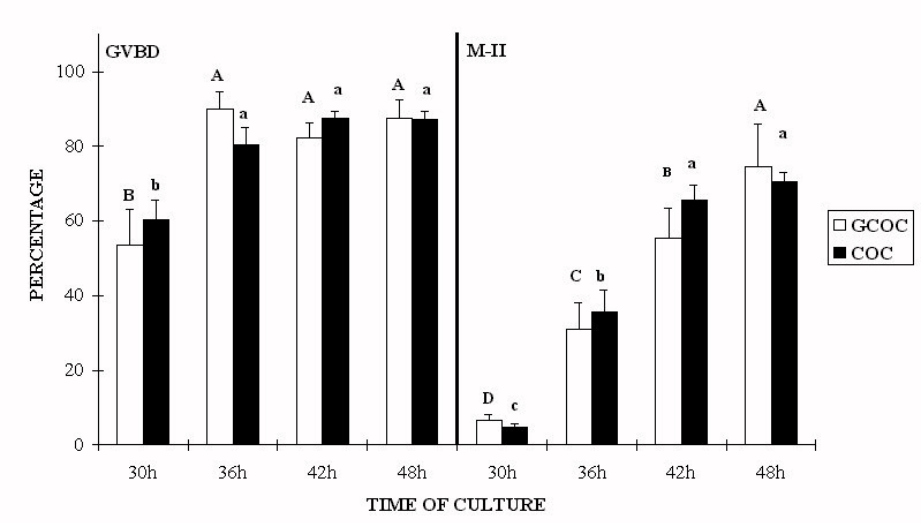


Fig. 25. Nuclear progression of GCOCs and COCs to GVBD and M-II stages during IVM culture when examined regardless of somatic compartment morphology. Values with different letters (A,B,C,D) represent a significant difference within the GCOCs category. Values with different letters (a,b,c,d) represent a significant difference within the COCs category. ($p < 0.05$).

GVBD: Germinal Vesicle Breakdown

M-II: Metaphase-II

Since the number of GCOCs were limited after collection from ovaries and only a low proportion of them showed the characteristics of Type 2, Type 3 or Type 4 groups by the end of the culture period, we concentrated our further investigations using oocytes from all COC categories and only Type 1 oocytes from the GCOC group.

There was no significant difference in GVBD between the Type 1, Type 2, Type 3, Type 4 COC and Type 1 GCOC oocytes at 30 h, 36 h, 42 h and 48 h (Figure 26).

However, there was a significant difference in the nuclear progression of each group (except Type 4): the percentage of GVBD oocytes significantly increased by the 42 h of IVM in the Type 2 and Type 3 oocytes while the progression of this value reached its plateau at 36 h in the Type 1 oocytes both in the GCOC and COC groups (Figure 26) ($p< 0.05$). The proportion of oocytes underwent GVBD did not change in the Type 4 oocytes during the culture.

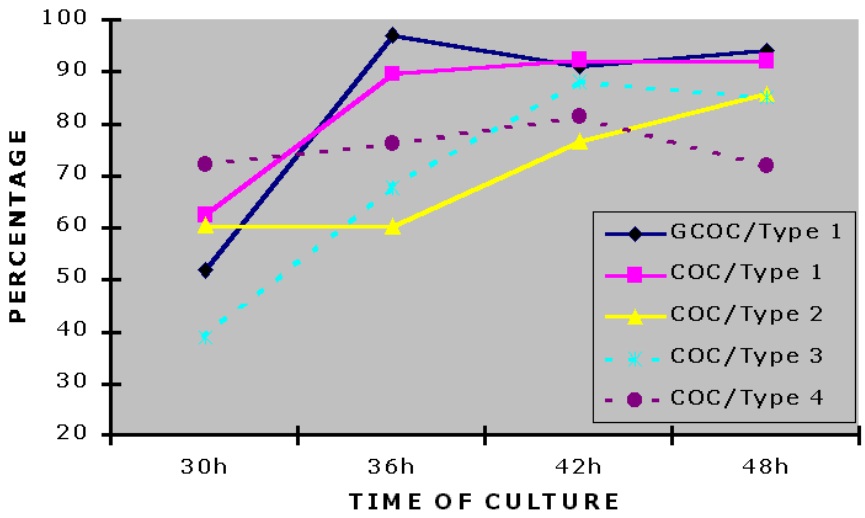


Fig. 26. Nuclear progression to GVBD of oocytes from morphologically different complexes during IVM culture. Data are presented as mean.

Regarding the percentage of M-II stage oocytes the differences were more spectacular between the different morphologic types (Figure 27).

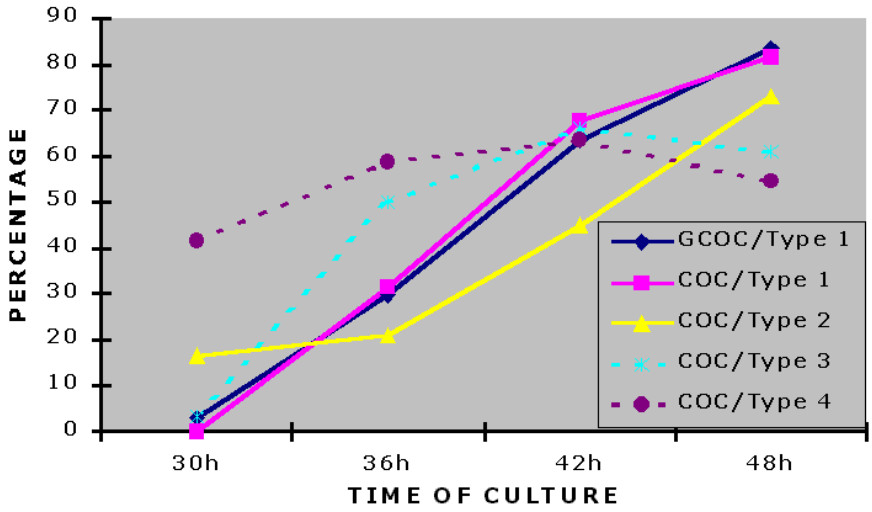


Fig. 27. Nuclear progression to M-II stage of oocytes from morphologically different complexes during IVM culture. Data are presented as mean.

At 30 h of IVM, the percentage of M-II oocytes was significantly higher in the Type 4 oocytes (41.6 ± 14.1%), than that of the Type 2, Type 3, Type 1 COC and Type 1 GCOC oocytes (0 to 16.6%). At 36 h of IVM, the percentage of M-II oocytes was significantly higher in the Type 4 (58.8 ± 10.4%) oocytes than that of the Type 2, Type 1 COC and Type 1 GCOC oocytes (21.0 to 31.6%) and did not differ from the Type 3 oocytes (50.0 ± 2.5%). At 42 h of IVM, there was no difference in M-II rate between the Type 4, Type 3, Type 2 Type 1 COC and Type 1 GCOC oocytes (44.9 to 67.7%). Finally at 48 h of IVM, there was no significant difference in M-II rate between he

studied groups. The nuclear progression to M-II stage significantly increased from the 30 h until the 48 h of IVM in the GCOC Type 1, the COC Type 1 and Type 2 oocytes (Figure 27). The M-II rate in the Type 3 oocytes reaches its plauteu at 36 h of IVM and shows no significant change until 48 h of IVM, while in the Type 4 group the percentage of M-II stage oocytes does not change significantly from 30 h to the 48 h of IVM.

Experiment 2. As shown in Figure 28, when electric stimulation was given to the matured oocytes after culture for 42 h, the proportion of oocytes having female pronucleus was similar in the Type 3 and Type 4 oocytes (85.4 ± 14.5% and 82.5 ± 3.1%, respectively) but was significantly higher than that of the Type 1 COC and GCOC oocytes (16.7 ± 4.4% and 15.6 ± 3.7%, respectively).

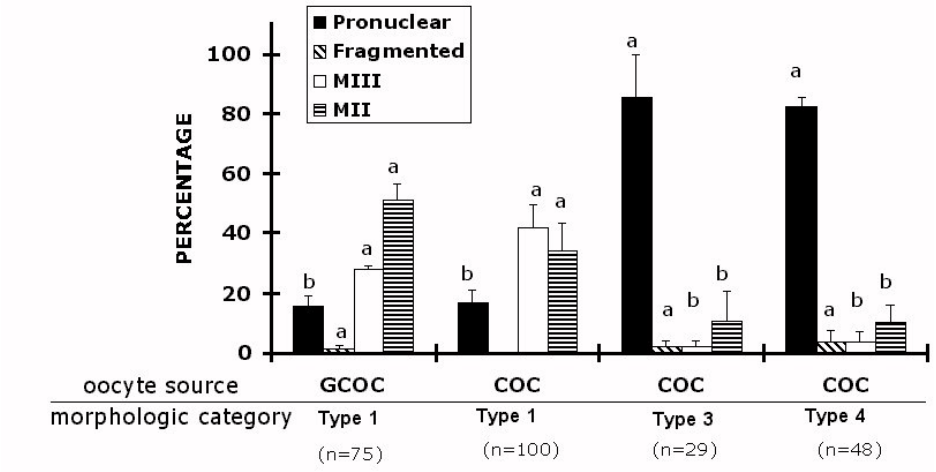


Fig. 28. Distribution (mean ± SEM) of different activation categories following parthenogenetic activation after 42 h IVM of morphologically different complexes. a,b differ significantly (p<0.05) within each activation category. Numbers of oocytes examined in different treatment groups are given in parentheses.

The rate of fragmented oocytes did not differ between the studied categories. The percentage of another category of abnormal oocyte activation (M-III) was significantly higher in the GCOC and COC Type 1 group (28.0 ± 14.5% and 41.6 ± 7.8%, respectively) than that of the Type 3 and Type 4 oocytes (2.0 ± 2.0% and 3.5 ± 3.5%, respectively). The proportion of unactivated oocytes, remaining at M-II stage, was also higher in the GCOC and COC Type 1 group (51.1 ± 5.5% and 33.9 ± 9.5%, respectively) than that of the Type 3 and Type 4 oocytes (10.4 ± 10.4% and 10.1 ± 5.7%, respectively).

Experiment 3. As shown in Figure 29, when the GCOC Type 1, the COC Type 1, Type 2 Type 3 and Type 4 oocytes were fertilized in vitro, the total penetration rates 10 h after IVF of these 5 categories were not different (70.0 to 89.8%), however there was a significant

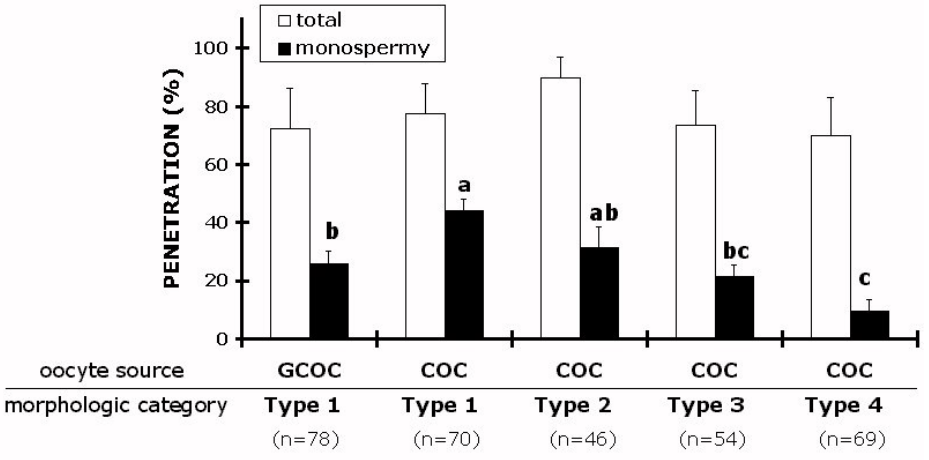


Fig 29. Fertilization results 10 h after IVF of IVM oocytes obtained from morphologically different complexes. (Note: all of the penetrated oocytes in each groups formed at least one male pronucleus). Data are presented as mean ± SEM. Different letters above the bars represent significant differences ($p < 0.05$). Numbers of oocytes examined in different treatment groups are given in parentheses.

difference in their monospermic fertilization rates ($p < 0.05$). The rate of monospermic fertilization was 44.1 ± 4.1% in the COC Type 1 group, it was significantly higher than those of the GCOC Type 1 (25.8 ± 4.2%), COC Type 3 (21.2 ± 4.4%) and Type 4 (9.6 ± 3.9%) groups, but did not differ significantly from that of COC Type 2 (31.3 ± 7.2%). The lowest rate of monospermic fertilization was obtained in the Type 3 and Type 4 groups (21.2 ± 4.4% and 9.6 ± 3.9%, respectively).

Discussion. Diverse behavior of cumulus cells during in vitro culture of COCs and GCOCs is reported in this study. During the IVM of such complexes, first signs of somatic compartment attacching to the bottom of the culture dish could be observed from 20-22 h of culture in our IVM system, however the four morphological classis, described in this study could be clearly distinguished from the 30 h of culture. A significant difference in the nuclear status of IVM oocytes obtained from morphologically different COCs was observed. Oocytes attached to the bottom of culture dish with dark, compact cumulus underwent nuclear maturation earlier than those of the floating ones (Figure 26, Figure 27) while the final maturation rates did dot differ significantly. A possible reason for this phenomenon most likely is the inadequate function of cumulus cells to maintain GV during in vitro culture in the bottom-attaced complexes. During IVM cumulus cells are known to maintan the oocyte nucleus at GV stage (Dekel and Beers, 1980; Isobe *et al.,* 1996; Tanghe *et al.,* 2002). A fact that removing of cumulus cells during IVM initiates nuclear progression beyond GV stage suggests that an inadequate cumulus function might be a possible reason for precoccious nuclear maturation (Isobe et al., 1996). A similar phenomenon was observed in case of human, other primate and horse oocytes obtained from atretic follicles that are

known to resume nuclear maturation in an advanced manner (Gougeon and Testart, 1986; Lefevre et al., 1987; Lefevre et al., 1988; Hinrichs and Williams, 1997). In atretic granulosa and cumulus cells, the mitotic activity ceases and the function of these cells to maintain meiotic arrest in oocytes fails resulting in an earlier spontaneous maturation when compared to COCs showing normal cumulus function and expansion (Lefevre et al., 1987; Lefevre et al., 1988; Hinrichs and Williams, 1997). Oocytes from atretic follicles are usually surrounded by a dark cumulus compartment and they can be distinguished during oocyte collection. However, in the present study, we collected similar looking oocytes surrounded by a clear and compact cumulus (Figure 11), and the morphological differences of somatic compartment were first detected after 20-22 h of maturation culture and fully expressed after 30 h of culture (data not shown). We suggest that some COCs collected from 3-6 mm non-atretic follicles start morphological and functional changes similar to atresia during the culture making the cumulus function insufficient to maintain meiotic arrest thus resulting in an earlier initiation of nuclear maturation of such oocytes.

The relationship between the somatic compartments bottom-attached status and the suggested inadequate cumulus function is also not clarified yet. A possible mechanism is the steroid-synthesis of the somatic cells attached to the bottom. Follicle cells are known to secrete progesterone when attached to the bottom of the culture dish (Nagai et al., 1993; Nagai, 2001) and progesterone secretion from cumulus cells was also observed in the presence of FSH/LH in the maturation media (Shimada et al., 2003). Progesterone causes the interruption of gap junctional communication by the reduction of connexin-43 in porcine cumulus cells (Shimada et al., 2002) resulting

in the initiation of nuclear maturation in porcine oocytes (Shimada et al., 2001). However, the hypothesis that progesterone secreted by somatic cells attached to the culture dish induces precoccious nuclear maturation of Type 3 and Type 4 oocytes in the present study is not supported by the fact, that Type 4 COCs appeared from the 20-22 h of culture and regarding their maturation status at 30 h of IVM they must have started maturation before their attachment, consequently it is not the suggested progesterone production that triggered their spontaneous maturation. The difference in nuclear status between the Type 3 and Type 4 oocytes might be related to the loss of cumulus cells in the Type 4 group.

Besides, it must be noted, that the grade of attachment of somatic cells to the culture dish might be affected by several factors, such as the culture system (static or non-static) or the characteristics of the culture dish (with or without specially coated surface).

The cytoplasmic maturation of oocytes seemed also to be affected by the somatic compartment behavior. After parthenogenetic stimulation of M-II stage oocytes at 42 h IVM – when M-II rate was the same in the stuck and floating oocytes (Figure 27) – the rate of female pronucleus formation was significantly higher in the oocytes attached to the bottom of the culture dish, than that of the floating ones (Figure 28) suggesting that the cytoplasm acquires its capacity to support activation earlier in the bottom-attached oocytes than in the floating ones.

The reason of this phenomenon might be the aging of the bottom-attached oocytes caused by the earlier nuclear maturation. Following nuclear maturation, in order to be able to be activated oocytes must acquire a certain cytoplasmic capacity, which can be characterised by

the decreasing activity of the metaphase promoting factor (MPF) and mitogen-activated protein kinase (MAPK) and accelerated ability for oocyte activation even with a weak stimulation, during the aging of the oocytes (Kikuchi et al., 1995; Liu et al., 1998; Kikuchi et al., 2000). In the present study, the bottom-attached (Type 3 and Type 4) oocytes showed significantly higher pronuclear formation rates than the floating ones ($p< 0.05$). They might start aging and thus might achieve their ability to be activated earlier while at the M-II stage for Type 1 oocytes, because their MPF and MAPK activity might be higher at the same culture period. After parthenogenetic stimulation at 42 h IVM, a high incidence of abnormal activation (M-III) was observed in the floating GCOC and COC oocytes besides the unactivated ones suggesting that in such oocytes the initiation of activation (extrusion of the second polar body) occurs normally after parthenogenetic activation but the formation of female pronucleus is inhibited and the female chromatin remains to form metaphase chromosomes. This phenomenon suggests a decreased activity of MPF but a high level of active MAPK in such oocytes (Liu et al., 1998).

After IVF no difference in male and female pronucleus formation was found between the examined groups (data not shown) however, the incidences of polyspermy were significantly higher in the bottom-attached (Type 3 and Type 4) than that of the floating oocytes (Type 1 and Type 2). This result also reveal the advanced ageing caused by the earlier nuclear maturation of the bottom-attached oocytes since as reported by Grupen *et al.,* (1997) aged oocytes are known to be more capable to polyspermic ferilization during IVF.

The effect of granulose cells attached to COCs on the dynamics of changes in cumulus morphology during in vitro culture was also studied. In previous studies, the effect of granulosa cells to maintain

meiotic arrest of the oocyte at GV stage was reported (Motlik *et al.,* 1991; De Loos *et al.,* 1994). In our study no difference was observed between the Type 1 GCOC and COC oocytes in their nuclear progression (Figure 26 and 27) and their ability of being activated (Figure 28). On the other hand, the incidence of normal cumulus expansion was significantly higher in the GCOC group than in the COC group and the nuclear progression to M-II stage was more accelerated in the COCs than in the GCOCs when examined regardless the somatic compartment morphology (Figure 25). These results lead us to the conclusion, that granulosa cells attached to the COC affect nuclear (and thus cytoplasmic) maturation of oocytes by maintaining normal cumulus function around them. They prevent cumulus function to turn atretic, thus decrease heterogeneity in nuclear progression, ageing and thus the ability to be activated amongst oocytes. Supporting our suggestion, follicular secretions were reported to maintain intercellular metabolic communication between cumulus cells and oocytes in pigs (Mattioli *et al.,* 1988).

Heterogene nuclear and cytoplasmic maturation of IVM oocytes caused by the diverse behavior of cumulus cells is reported in this study. Although in the same morphological category of Type 1, a significantly lower rate of monospermic fertilization was obtained for GCOC oocytes than COCs. The reason of this difference is not clear. Perhaps it might be related to a difference in cytoplasmic maturation of GCOC and COC oocytes, including the proper movement of cortical granules of which exocytosis leads to the block polyspermic fertilization through zona reaction. The movement of cortical granules was suggested to be controlled by a sub-plasmalemmar layer of actin (Cran, 1989), and its synthesis was suggested to depend on the presence of an active intercellular cooperation of cumulus cells and

oocytes (Mattioli et al 1988). The present results indicated that the nuclear progression to M-II stage reached its plateau at the same time for Type 1 oocytes of both COCs and GCOCs. However considering our observation, that granulosa cells promoted cumulus expansion of oocytes in earlier stage of maturation the active intercellular cooperation of cumulus cells and oocytes might be lost earlier in Type 1 GCOCs than Type 1 COCs resulting in low level of actin synthesis.

Synchronisation of porcine oocyte maturation by different chemicals in vitro is already established (Funahashi *et al.,* 1997; Wu *et al.,* 2002). However, this study reveales the importance of the use of oocytes with similar cumulus morphology in case of one phase IVM systems in order to provide the homogeneity of oocyte quality.

5 SUMMARY

Porcine oocytes are known to show a heterogeneous nuclear morphology during IVM. However the diverse nuclear – and thus cytoplasmic – maturity of oocytes is a non-expedient phenomenon in the IVM related technologies such as IVF, ICSI or nuclear transfer. This reveals the necessity of meiosis synchronisation during IVM of porcine oocytes. Synchronisation of the first meiotic division can be achieved by a transient arrest of the re-initiation of nuclear maturation, the germinal vesicle brakedown. An elevated intercellular level of cAMP is known to inhibit GVBD of oocytes reversibly. Intercellular cAMP level can be elevated either by invasive adenylate cyclase (iAC) enzyme, 3-isobutyl 1-methylxanthine (IBMX) or dibutyryl cyclic AMP (dbcAMP).

We investigated effects of iAC, IBMX and dbcAMP on porcine oocyte in vitro maturation (IVM), in vitro fertilization (IVF) and subsequent embryonic development. Porcine oocytes were collected in Hepes-buffered NCSU-37 medium supplemented with or without 0.1 µg/ml iAC and 0.5 mM IBMX. IVM was performed in a modified NCSU-37 supplemented with or without 1 mM dbcAMP for 22 h and then without dbcAMP for an additional 24 h. After IVF, they were cultured in vitro for 6 days. After 12 h of IVM, no difference in nuclear status was observed irrespectively of supplementation with these chemicals during collection and IVM. At 22 h, most (95%) of the oocytes cultured with dbcAMP remained at germinal vesicle (GV) stage, whereas 44.3% of the oocytes cultured without dbcAMP underwent GV breakdown. At 36 h, oocytes with dbcAMP were progressed to prometaphase-I or metaphase-I (M-I) (32.6% and

49.3%, respectively), whereas non-treated oocytes were more progressed to anaphase-I, telophase-I or metaphase-II (M-II) (13.6%, 14.3% and 38.0%, respectively). At 46 h, the rate of matured oocytes at M-II was higher when cultured with dbcAMP (81%) than that without dbcAMP (57%), while the proportion of oocytes arrested at M-I was lower when cultured with dbcAMP (15%) than without dbcAMP (31%). The rate of monospermic fertilization was higher when cultured with dbcAMP (21%) than without dbcAMP (9%) with no difference in total penetration rates (58% and 52%, respectively). The blastocyst rate was higher when cultured with dbcAMP (32%) than without dbcAMP (19%). These results suggest that a change of intracellular level of cAMP during oocyte collection does not affect maturational and developmental competence of porcine oocytes and that synchronization of meiotic maturation using dbcAMP enhances meiotic potential of oocytes by promoting M-I to M-II transition and results in high developmental competence by monospermic fertilization.

As presented above, a remarkable amount of IVM oocytes remains arrested at certain stages of meiosis (mostly at M-I) by the end of the maturation culture, especially when nuclear maturation is not synchronised artificially. In our culture system, the rate of mature (M-II) and immature oocytes did not change from 48 h to 60 h of IVM which proves that oocytes at immature stages (GV, proM-I or M-I) at 48 h IVM are not caused by slow or late-resumed meiosis but resulted in from a permanent arrest of the meiotic cell division. However, the developmental competence of such oocytes has not been studied until now. After in vitro maturation (IVM) for 48 h of cumulus-oocyte complexes, 75.4% of them extruded a visible polar body (PB). The most of the oocytes with a polar body (PB+ group)

found to be at metaphase-II (M-II) stage (91.4%), the rest were at the telophase-I (T-I) or pronuclear oocytes after spontaneous activation. Most of the oocytes without visible polar body (PB- group) appeared to be arrested at germinal vesicle (GV) (41.6%) and first meiotic metaphase (M-I) (34.0%) stages, the remains were at M-II (8.3%), prometaphase-I (6.2%), anaphase-I (2.7%), telophase-I stages (0.6%) or had abnormal arrangements of chromosomes (6.0%). After IVF of oocytes, there was no significant difference between PB+ and PB- groups in the penetration rates (75.5% and 79.0%, respectively), and also in monospermy (24.6% and 24.3%, respectively). There was no difference in the activation rate of PB+ and PB- groups (95.2% and 100%, respectively) at 10 h after IVF. After the subsequent in vitro culture for 6 days, a significantly higher rate of inseminated oocytes developed to the blastocyst stage in the PB+ group than in the PB- group (34.6% and 20.7%, respectively), however after recalculating the blastocyst rates by neglecting the GV stage oocytes from the PB- group, it was found that there is no difference in blastocyst rates between the M-I arrested and M-II oocytes (35.3% and 34.6% respectively). The number of blastomer nuclei in embryos obtained from PB+ group (52.0) was significantly higher than that of the PB- group (29.1). Morphological appearance regarding the proportion of degenerated part of the blastocysts seemed to be the same in the PB+ and PB- groups however PB- blastocysts appeared smaller sized. Although the quality of PB+ embryos is better than that of the PB- group, the proportion of ICM and TE cells in PB+ and PB- blastocysts did not differ significantly (1:1.9 and 1:2.2, respectively). These results indicate that porcine oocytes arrested at M-I stage undergo cytoplasmic maturation during maturation culture and have the same ability to form blastocyst after

IVF as M-II oocytes, however with a lower cell number which might be caused by the abnormal ploidy of M-I obtained embryos.

Based on the morphology and expansion of the cumulus cells, several different classes of porcine cumulus-oocyte complexes (COCs) can be distinguished, during their maturation in vitro. The goal of the present study was to find out the rate of each morphologic category in case of COCs and granulosa-cumulus-oocyte complexes (GCOCs), the caharacteristics of their nuclear progression, cytoplasmic maturation and the frequency of monospermy after IVF. It was found that the frequency of cumulus expansion is higher in case of GCOCs than that of COCs. Nuclear progression of COCs was more accelerated than that of GCOCs. Oocytes attached to the bottom of culture dish with dark, compact cumulus underwent nuclear maturation earlier than oocytes showing normal cumulus expansion. After parthenogenetic activation at 42 h IVM, a significantly higher proportion of the bottom-attached oocytes developed female pronucleus than the floating oocytes, suggesting the earlier cytoplasmic maturation of the bottom-attached oocytes. This phenomenon might be caused by advanced aging of the bottom-attached oocytes, which is resulted in by their precocious nuclear maturation. After IVF at 48 h IVM the rate of monospermic fertilization of normal COCs showing normal cumulus expansion was higher than that of COCs attached to the dish. This also reveals the advanced aging of the bottom-attached oocytes. These results suggest that diverse behaviour of cumulus cells during in vitro culture affects nuclear and cytoplasmic maturation of porcine oocytes, which also affects IVF results, especially in culture systems, where oocyte maturation is not synchronised artificially. It can be concluded that granulosa cells promote normal cumulus expansion thus decrease

heterogeneity in nuclear and cytoplasmic maturation amongst oocytes.

Our results reveal the necessity of oocytes with similar nuclear and cytoplasmic characteristics for embryo technologies. To achieve homogenous and improved oocyte quality, artificial synchronisation of the first meiotic division by dbcAMP is a possible way. Besides, the careful selection of oocytes showing the signs of metaphase II stage (the existence of the first polar body) for further use is essential in order to decrease the frequency of abnormal ploidy amongst the embryos. Since the surrounding somatic cells affect the nuclear progression of the oocytes, in order to provide the homogeneity of oocyte quality the use of oocytes with similar cumulus morphology is important, especially in one-phase IVM systems, when meiosis is not synchronised artificially. These findings might help us to improve the efficiency of IVP systems and thus micromanipulation and gene transfer technologies in the porcine species.

6 NEW SCIENTIFIC RESULTS

1. During oocyte collection a remarkable fall of intercellular cAMP level does not occure, thus oocyte collection does not trigger spontaneous maturation.
2. During synchronisation of maturation by a transient inhibition of GVBD with cAMP, porcine oocytes increase their potential to undergo meiotic maturation and monospermic fertilization. This phenomenon results in a higher blastocyst rate after IVF/IVC.
3. Porcine oocytes arrested at proM-I or M-I stages undergo cytoplasmic maturation and are able to form blastocyst after IVF/IVC. However these embryos show a delayed development, probably due to their abnormal ploidy.
4. Attachment of cumulus cells to the bottom of the culture dish triggers nuclear maturation of porcine oocytes during IVM of COCs causing their precoccious nuclear and cytoplasmic maturation and thus heterogeneity in their ability of being activated. This phenomenon necessitates the artificial synchronisation of nuclear maturation amongst oocytes.
5. Graulosa cells prevent the attachment of cumulus cells to the bottom of culture dish, and thus heterogenous nuclear and cytoplasmic maturation of oocytes.

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9 APPENDIX

9.1 Nomenclature of abbreviations

A-I	anaphase-I
A-II	anaphase-II
ATP	adenosine triphosphate
BCM	basic collection medium
BL-I	butyrolactone-I
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCM	complete collection medium
CG	cortical granule
COC	cumulus-oocyte complex
dbcAMP	dibutyryl cyclic adenosine monophosphate
6-DMAP	6-dimethylaminopurine
EGF	epidermal growth factor
FPN	female pronucleus
FSH	follicle stimulating hormone
GCOC	granulose-cumulus-oocyte complex
GSH	glutathione
GV	germinal vesicle
GV-I	germinal vesicle-I stage
GV-II	germinal vesicle-II stage
GV-III	germinal vesicle-III stage
GV-IV	germinal vesicle-IV stage
GVBD	germinal vesicle breakedown
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
hCG	human chorion gonadotrophin

iAC	invasive adenylate cyclase
IBMX	3-isobutyl 1-methylxanthine
ICSI	intracytoplasmic sperm injection
IVC	in vitro culture
IVC-Glu	IVC medium supplemeted with glucose
IVC-PyrLac	IVC medium supplemeted with pyruvate and lactate
IVF	in vitro fertilization
IVM	in vitro maturation
IVP	in vitro pruduction
LH	luteinizing hormone
M-I	metaphase-I
M-II	metaphase-II
M-III	metaphase-III
MAP	mitogen activated protein kinase
MPF	metaphase promoting factor
MPN	male pronucleus
PB	polar body
PBS	Dulbecco’s phosphate buffered saline
pFF	porcine follicle fluid
PGA	parthenogenetic activation
PKA	cAMP dependent protein kinase
PMSG	pregnant mare’s serum gonadotrophin
proM-I	prometaphase-I
PVA	polyvinyl alcohol
ROS	roscovitine
T-I	telophase-I
T-II	telophase-II
tPA	tissue-type plasminogen activator