
THESES OF DOCTORAL (PhD) DISSERTATION

- SOMFAI TAMÁS -

**SYNCHRONIZATION OF IN VITRO
MATURATION OF PORCINE OOCYTES**

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1. INTRODUCTION AND OBJECTIVES

The success of in vitro maturation and fertilization (IVM/IVF) in pigs has been improved in many aspects in the last decade, but still worse than that in cattle. Beside the problem of polyspermy, insufficient cytoplasmic and nuclear maturation can cause low fertilisation and blastocyst rate. The success of nuclear and cytoplasmic maturation of oocytes is a crucial point of efficiency in IVM/IVF systems.

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). The meiotic competence of GV stage pig oocytes used for IVM/IVF varies within the population therefore some oocytes can start meiosis earlier than others causing heterogeneity in maturation status and ageing among the oocytes after IVM (Funahashi et al., 1997a). To overcome this phenomenon, a synchronization of nuclear maturation is necessary.

Before the initiation of meiosis, the follicle maintains a high level of intercellular cAMP in the oocyte, which controls the meiotic arrest of oocytes at GV stage (Bornslaeger et al., 1986). Spontaneous maturation occurs by the interruption of metabolism between the follicle components (somatic cells and follicular fluid) and the oocyte. Resumption of meiosis, germinal vesicle breakdown (GVBD) is associated with the reduction of cAMP level (Schultz et al., 1983). The artificial elevation of the intercellular cAMP during the first half of the IVM culture might overcome the problem of asynchronous maturation. This can be achieved by the use of dibutyryl cyclic AMP (dbcAMP), a membrane permeable cAMP analogue (Funahashi et al.

1997b), invasive adenylate cyclase (iAC) and 3-isobutyl 1-methylxanthine (IBMX) (Luciano *et al.*, 1999).

Not all the cultured oocytes complete their nuclear maturation; some of them remain at 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 (Kikuchi *et al.* 1999a). In several IVM/IVF systems, 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 (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). It was reported that mouse (Eppig *et al.* 1994; Polanski 1995) and porcine (Kikuchi *et al.* 1999a) oocytes arrested at M-I stage after a certain period of in vitro culture needed for nuclear maturation undergo cytoplasmic maturation and, in such oocytes, male and female pronuclei are formed after fertilization. However, up to our knowledge, there is no study about the possibility of embryonic development of such meiotically arrested immature porcine oocytes.

Cumulus cells are known to play an important role in regulation of meiotic progression of oocytes by transferring the inhibitor signal through gap junctions that elevates intercellular cAMP level in the oocytes. (Dekel and Beers, 1980) Regarding the somatic compartment of the follicle, not only the cumulus cells affect the oocyte nuclear maturation. The mechanism in regulation of oocyte maturation by granulosa cells has also been reported (Motlik *et al.*, 1991). During in vitro culture of porcine cumulus-oocyte complexes (COCs), we could observe different behaviours of somatic cells

enabling us to distinguish four morphological categories of COCs. Since there are morphological differences (colour, grade of expansion) between the somatic compartments of COCs from each category, we suggest a difference in the metabolic functions of such cells, which might affect nuclear and cytoplasmic maturation of the oocytes.

Objectives

1. **The first objective** was to examine the effect of intracellular cAMP elevation during oocyte collection and IVM 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 second objective** of our experiments was to study the embryonic development 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 find out if there is any correlation between the morphology and behaviour of somatic cells and the kinetics of nuclear and cytoplasmic maturation in cumulus-oocyte complexes (COCs) and granulosa-cumulus-oocyte complexes (GCOCs)

2. MATERIALS AND METHODS

2.1 Synchronisation of meiotic maturation by high level of intercellular cAMP

Intercellular cAMP in oocytes was elevated either by 0.1 µg/ml iAC and 0.5 mM IBMX (in collection medium) or 1mM dbcAMP (in maturation medium).

To evaluate the effects of elevated intercellular cAMP level on nuclear progression and oocyte maturation, COCs were collected and cultured in vitro in NCSU 37 with or without cAMP stimulators for 22 hours, then culture was followed without cAMP supplement. Nuclear progression during IVM was evaluated after fixation at 12, 22, 36 and 46 h of culture by orcein staining. Chromatin condensation (GV stage) in the oocytes was classified according to Motlik and Fulka (1976).

To study the effect of cAMP supplement in collection medium and maturation medium on fertilization parameters, COCs were collected and matured as described above were fertilized in vitro with 1×10^5 /ml frozen-thawed epididymal spermatozoa. Ten hours after insemination, oocytes were fixed, stained with acetic orcein and investigated under phase-contrast microscope. 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.

Oocytes collected and matured as described in experiment 1 were fertilized in vitro and cultured for 6 days. 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.

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

COCs were collected from slaughterhouse ovaries and matured for 48 h in vitro then oocytes were denuded. Oocytes with (PB+) or without (PB-) a visible polar body were separated under a stereo microscope and their nuclear status was evaluated after fixation and staining with acetic orcein.

To evaluate their ability of being fertilized, after 48 h of maturation culture, PB+ and PB- oocytes were separated and IVF was performed. Inseminated oocytes were fixed 10 h after IVF stained with acetic orcein and examined for fertilization status (sperm penetration and pronuclear formation) under a stereo microscope. Oocytes were judged to be penetrated when they had one or more male pronuclei and/or sperm heads with corresponding sperm tails.

The embryonic development (blastocyst rates) of in vitro fertilized PB+ and PB- oocytes was compared after 6 days of in vitro culture. The total cell number and the ratio of ICM and TE cells of blastocysts from PB+ and PB- oocytes were evaluated after differential staining of the cell nuclei with propidium iodide and Hoechst under an epifluorescence microscope.

2.3 The relationship between cumulus morphology and oocyte maturation

GCOCs and COCs were collected from slaughterhouse ovaries and matured in vitro in the absence of any cAMP supplement for 48 h. Four types of COCs can be distinguished according to the characteristics of somatic compartment from 30h of IVM.

Type 1: The COC is floating in the maturation medium; the oocyte is surrounded by a light coloured fully expanded cumulus mass.

Type 2: The COC is floating in the maturation medium; the oocyte is surrounded by a dark brown coloured compact or semi-compact somatic compartment.

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.

Type 4: The COC is attached to the bottom of the culture dish, the oocyte is partially denuded, and the loss of cumulus cells ranges at least the 30% of the oocyte surface. The remaining cumulus cells are dark coloured and compact.

Experiment 1: COCs and GCOCs were classified according to the characteristics of their somatic compartment as described above at 30, 36, 42 and 48h 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), and then nuclear status of oocytes was evaluated using a phase-contrast microscopy.

Experiment 2: The ability of oocytes to form female pronucleus was assessed in order to estimate the capacity of the cytoplasm to

potentiate oocyte activation. M-II oocytes from COCs and GCOCs of each morphologic type were collected separately at 42h of IVM, and then parthenogenetic activation was performed by electric stimulation with 1.0 kV/cm for duration of 100 μ sec. Ten hours after the stimulation, oocytes were fixed and stained with aceto-orcein. Activated status was evaluated using a phase-contrast microscopy.

Experiment 3: Oocytes from COCs and GCOCs of each morphologic type were subjected to IVF after 48 h of IVM. Inseminated oocytes were fixed 10 h after IVF and stained with acetic orcein to examine sperm penetration and pronuclear formation.

2.4 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 ($P < 0.05$) using GLM procedures of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Data of PGA and IVF and IVC results were analysed by Chi-square test ($P < 0.05$).

3. RESULTS

Synchronisation of meiotic maturation by high level of intercellular cAMP

- 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.
- The treatment of oocytes with 1mM dbcAMP during the first 22 h of IVM completely inhibited GVBD, while more than 40% of the control oocytes underwent GVBD by 22h IVM. The percentage of GVBD in control and the cAMP treated oocytes reached its maximum at 36 h IVM.
- 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 (12 h and 10 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 22 h, respectively).
- By the end of the IVM period a significantly higher amount of oocytes reach M-II stage in the dbcAMP treated group than in the control, while more oocytes remain arrested at M-I stage without the dbcAMP treatment.
- The treatment of oocytes with cAMP did not affect the total fertilization rates, however the usage of dbcAMP for 22 h IVM resulted in a significantly higher monospermy rate, compared to the control. cAMP supplementation during oocyte collection did not affect any fertilization result.

- The treatment of oocytes with cAMP during IVM resulted in a higher blastocyst formation rate after IVF/IVC than that of without control. cAMP supplementation during oocyte collection had no effect on blastocyst rates. The quality (total cell number) of embryos did not differ between the treatment groups.

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

- After 48 h IVM, the vast majority (91.4%) of the oocytes with a visible polar body was found to be at M-II stage, the rest were at telophase-I or spontaneously activated.
- The most of the oocytes without a visible polar body were arrested at GV stage (41.6%) and M-I stage (34%), the rest were at M-II (8.3%), proM-I (6.4%) anaphase-I or telophase-I (3.3%), abnormal (2.6 %) and degenerated (3.4%).
- There was no difference in total and monospermic fertilization rates between the PB+ and the PB- oocytes after IVF.
- A significantly higher proportion of the PB+ oocytes developed to blastocyst stage than that of PB- group after IVF (34.6% and 20.7%). Considering the facts, that only 8.3% of the oocytes were at M-II stage in the PB- group, and that the GV oocytes can not form embryo, after penetration, this results indicate that the most of the blastocyst in PB- group were resulted from the fertilization of M-I arrested oocytes.
- After differential staining of the blastocyst it was found, that the BP+ blastocysts contained a significantly higher number of cells in total than that of the PB- ones (and) however the ratio of ICM and

trophectoderm cells did not differ between the two experimental groups.

The effect of cumulus morphology on nuclear and cytoplasmic maturation

- The proportion of floating oocytes with expanded somatic compartment was significantly higher in the GCOCs than in the COCs at 30, 36, 42 and 48 h of culture.
- There was no difference in GVBD rate between the GCOC and the COC groups during the culture when examined regardless of somatic compartment morphology. 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, while in the COC group this value reached its plateau at 42 h of IVM and did not change significantly by 48 h of culture.
- When nuclear progression of oocytes from different morphological COC classes was examined it was found, that oocytes attached to the bottom of the culture dish with a compact (Type 3) or degraded (Type 4) cumulus reach the maximum of their maturation rates (M-II) earlier than that of the floating oocytes with expanded or compact cumulus. The earlier nuclear maturation of bottom-stuck oocytes suggests their earlier ability of being activated, due to their precocious ageing.
- After parthenogenetic activation of oocytes at 42h IVM, it was found, that oocytes attached to the culture dish (Type 3 and 4)

underwent normal activation (female pronucleus formation) in a much higher frequency than the floating oocytes, which remained at M-II stage or showed abnormal activation (M-III) without pronucleus formation. This finding supports our previous suggestion about the pre-cocciuous ageing of the bottom attached oocytes.

- After IVF of oocytes, a significantly higher rate of polyspermy was observed in Type 4 and Type 3 groups than in other categories. Since the high polyspermy is a characteristic of the aged oocytes, this result also underlines the early ageing of oocytes which are attached to the culture dish.

4. NEW SCIENTIFIC RESULTS

1. During oocyte collection a remarkable fall of intercellular cAMP level does not occur, 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 have a low cell number, 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 precocious 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. Granulosa cells prevent the attachment of cumulus cells to the bottom of culture dish, and thus pre-ocious nuclear and cytoplasmic maturation of oocytes.

5. SUGGESTIONS

(The use of new findings for theoretical and practical purposes)

Synchronisation of meiotic maturation by high level of intercellular cAMP.

Our results show, that in porcine IVP systems, the artificial synchronisation of oocyte maturation improves the efficiency of the system, considering the number of blastosysts. Since no negative side effect of dbcAMP was observed in the present study, the practical use of this drug during the first 20-22 h of IVM culture to synchronise oocyte maturation could be used in any porcine IVP system.

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

Without the artificial synchronisation of oocyte maturation, a remarkable amount of oocytes remain arrested at M-I (diploid) stage. As presented in this study, these oocytes develop to blastocyst stage. However with a low cell number, which reveals their abnormal ploidy. According to this result, the selection of oocytes at M-II stage (oocytes with polar body) for IVF is a prerequisite in order to exclude such embryos, especially in those IVP systems, where oocyte maturation is not synchronised.

Besides, the finding, that M-I arrested oocytes undergo cytoplasmic maturation, and are able to develop to blastocyst stage shows their potential to be used as recipient cells for nuclear transfer or for further investigations to study the development of polyploidy or gynogenetic embryos.

The effect of cumulus morphology on nuclear and cytoplasmic maturation.

The finding that cumulus morphology has a relationship with oocyte maturation and thus oocyte ageing underlines the possibility and importance of the selection of oocytes with homogenous nuclear and cytoplasmic characteristics for IVF according to the cumulus morphology. According to our results, oocyte from floating COCs with expanded cumulus should be used for further experiments in order to avoid high polyspermy or early fragmentation. The importance of such preselection is especially high when oocyte maturation is not synchronised artificially.

The fact, that the presence of granulosa cells enhances normal cumulus expansion suggests the superiority of GCOCs to COCs in porcine IVP systems. However only a very limited number of GCOCs can be obtained by the traditional oocyte collection methods thus the use of COCs in in vitro systems is more convenient. This application requires further improvements of the present IVP systems, especially regarding the high frequency of polyspermy.

6. PUBLICATIONS

1. ORIGINAL PAPERS PUBLISHED IN SCIENTIFIC JOURNALS

1.1 In English

1.1.1 Somfai T, Bodó Sz, Nagy Sz, Papp ÁB, Iváncsics J, Baranyai B, Gócza E, Kovács A (2002). Effect of swim up and percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. *Reproduction in Domestic Animals* **37**(5):285-290

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* 1.1.4. Somfai T, Kikuchi K, Onishi A, Iwamoto M, Fuchimoto D, Bali Papp Á, Sato E, Nagai T (2003). Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. *Zygote* **11**: 199-206

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1.2 In Hungarian

1.2.1 Somfai T, Bali-Papp Á, Iváncsics J (1999). A sertés petesejtek aspirációja és in vitro maturáltatása. *Acta Agronomica Ovariensis* **41(1):101-112**

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1.2.2. Bodó Sz, Nagy Sz, Baranyai B, Somfai T, Gócza E, Kovács A (2000). Spermaminőség jellemzése swim up előtt és után. *Állattenyésztés és Takarmányozás* **49**(6):581-583

2. ORAL PRESENTATIONS

2.1 Somfai T, Kikuchi K, Onishi A, Bali Papp Á, Dinnyés A, Sato E, Nagai T (2003). Effect of meiotic arrest at germinal vesicle stage by butyrolactone-I on meiotic competence, activation, fertilization and embryonic development of porcine oocytes. V. Magyar Genetikai Kongresszus, Siófok 2003. április 13-15. (proceeding, 58. page).

3. POSTER PRESENTATIONS

3.1 In English

3.1.1. Bali Papp Á, Somfai T, Petz B, Nánássy, L, Pécsi T, Iváncsics J (2001). How can influence the different treatment of frozen/thawed boar spermatozoa viability BOKU International Kongress, 18-21. November, Wien, Austria 181.

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* 3.1.3. Somfai T, Kikuchi K, Onishi A, Iwamoto M, Fuchimoto D, Bali-Papp Á, Sato E, Nagai T (2003). Effects of invasive adenylate cyclase,

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3.2 In Hungarian

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