In vitro aging of porcine oocytes

I. Petrová[1], M. Sedmíková[2], E. Chmelíková[2], D. Švestková[2], R. Rajmon[2]

[1]Grammar School, Říčany near Prague, Czech Republic
[2]Department of Veterinary Science, Faculty of Agronomy, Czech University of Agriculture, Prague, Czech Republic

ABSTRACT: Porcine oocytes matured *in vitro* develop in various ways if they are further cultivated. In our studies these oocytes were cultivated for 1 to 5 days (*in vitro* aging). During the 1st day of aging, most of them remained at the stage of metaphase II (98%). Then many oocytes underwent the spontaneous parthenogenetic activation. The portion of activated oocytes reached its peak after 2 or 3 days of aging *in vitro* (39 or 45%). The portion of fragmented oocytes peaked at the same time (28%). During subsequent aging *in vitro* (i.e. day 4 or 5 of aging), the portion of lysed oocytes significantly increased (30 or 37%). The highest portion of spontaneously activated parthenogenetic embryos at a pronuclear stage (35%) was observed during the 2nd day of aging *in vitro*. These pronuclear embryos had mainly one polar body with two pronuclei (47% of all pronuclear embryos) or two polar bodies with one pronucleus (38% of all pronuclear embryos). During the 3rd and 5th day of *in vitro* aging, there was a significant increase in the portion of parthenogenetic embryos cleaved to the 2-cell or 3-cell stage. When considering the prolonged *in vitro* culture of porcine oocyte, only the first day of aging should be taken into account, since beyond this time significant changes, i.e. parthenogenesis, fragmentation or lysis, occurred in oocytes under *in vitro* conditions.

Keywords: pig; oocyte; parthenogenesis; fragmentation

In vitro matured oocytes are used for biotechniques of different type, e.g. *in vitro* fertilization and embryo production, cloning by the transfer of nuclei from somatic cells or transgenesis.

During *in vitro* maturation, the oocyte resumes meiosis. It undergoes the germinal vesicle breakdown and enters the stage of metaphase I followed by the stages of anaphase I and telophase I. Finally, the oocyte enters metaphase II, when the meiosis in mammalian oocytes is blocked again (Wassarman, 1988). The exit from this meiotic block depends on the activating stimulus which induces the decay of molecules responsible for the maintenance of meiotic block. During fertilization, the activating stimulus is brought into the oocyte by the sperm (Yanagimachi, 1988).

Despite intensive efforts, the quality of *in vitro* matured oocytes remains lower. There are several ways for improvement, e.g. the modification of culture media (e.g. Coy and Romar, 2002) or the temporal blockage of oocytes at the meiotic prophase with various inhibitors (Lonergan *et al.*, 1998; Mermillod *et al.*, 2000). One possibility of improving the quality of oocytes is their prolonged culture *in vitro* (Chian *et al.*, 1992; Pavlok *et al.*, 1997).

However, the culture beyond the time necessary for the completion of maturation could be accompanied by complex events assigned as "aging". The aging of oocytes can result in several different outputs, e.g. parthenogenesis or fragmentation (Gable and Woods, 2001). Only oocytes that remained at the stage of metaphase II during their prolonged culture *in vitro* could be still used for further biotechnological interventions. Gable and Woods (2001) unsuccessfully attempted to improve the quality of *in vitro* matured equine oocytes from

Supported by the Ministry of Agriculture of the Czech Republic (Grant No. QD 00085), by the Grant Agency of the Czech Republic (Grant No. 523/03/H076), and by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 412100003).

48 to 96 or 144 hours. Unfortunately, only limited data are available on the prolonged culture and aging of pig oocytes *in vitro*. The aim of the present study was to follow the development of pig oocytes during the prolonged culture and to determine the interval during which the oocytes remain intact and arrested at the stage of metaphase II.

MATERIAL AND METHODS

The isolation of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrous cycle and transported to the laboratory within 1 h in a saline solution (0.9% of sodium chloride) at 39°C. Fully grown oocytes were collected from follicles by the aspiration of follicles measuring 2–5 mm in diameter with a 20-gauge needle. Only the oocytes with compact cumuli were chosen for further studies.

The oocyte culture

Before cultivation the oocytes were washed three times in the culture medium. The oocytes were cultured in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland) containing sodium bicarbonate (0.039 ml of a 7% solution per ml of medium), calcium lactate (0.6 mg/ml), sodium pyruvate (0.25 mg/ml), gentamycin (0.025 mg/ml) HEPES (1.5 mg/ml), 13.5 IU eCG : 6.6 IU hCG/ ml (P.G.600 Intervet, Boxmeer, Holland) and 10% foetal calf serum (GibcoBRL, Life Technologies, Germany, Lot No. 40F2190F).

The oocytes were cultured in 3.5-cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3 ml of the culture medium at 39°C in a mixture of 5% CO_2 in air.

After 48 hours of maturation, the oocytes were transferred into P.G.600-free culture medium and cultured for further 1, 2, 3, 4 or 5 days.

The evaluation of oocytes

At the end of the culture, the oocytes were relieved of cumulus cells by repeated pipetting through a narrow glass pipette and their morphology was evaluated under an inverted microscope. Then the oocytes were mounted on slides, fixed with acetic alcohol (1 : 3, v/v) for at least 24 h and stained with 1% orceine. The oocytes were examined under a phase contrast microscope. The stages of maturation were determined according to the criteria published by Motlík and Fulka (1976).

The activation was considered to occur if the oocytes were in the pronuclear stage and a polar body was visible. The number of cells in parthenogenetic embryos was determined after mounting the embryos on slides, fixation with acetic alcohol for at least 24 hours, and staining with 1% orceine.

Statistical analysis

Data from all experiments were subjected to statistical analysis. Each experiment was carried out four times. The results were pooled for the presentation and evaluated by chi-square analysis (Snedecor and Cochran, 1957). The mean percentage of oocytes or embryos reaching the given stage of maturation or development in all experiments did not vary from the pooled percentage by more than 2.5%. A P value of less than 0.05 was considered significant.

RESULTS

In vitro culture of pig oocytes for 48 hours resulted in 96% of oocytes at the stage of metaphase II. The remaining oocytes were at the stage of metaphase I.

When these *in vitro* matured oocytes had been further cultured *in vitro*, their subsequent development was different. Some oocytes were spontaneously activated and started the parthenogenetic development, whereas others underwent fragmentation or lysis.

As it follows from our data (Table 1), the oocytes mainly remained at the stage of metaphase II during the first day of aging. However, there was a significant decrease in the rate of metaphase II oocytes and a significant increase in parthenotes or fragmented oocytes during the second day of aging. During the 3rd day of aging, there was another significant decrease in the number of metaphase II oocytes and a significant increase in fragmented oocytes. A significant decrease in the parthenogenotes and concomitant significant increase in lysed oocytes was observed on day 4 of aging. No significant changes occurred on day 5 of aging.

Table 1. The fate of *in vitro* aged pig oocytes. The oocytes matured *in vitro* for 48 hours and then they were cultured for further 1, 2, 3, 4 or 5 days

Type of oocyte	Days of *in vitro* aging				
	1	2	3	4	5
Metaphase II	98^{A}	35^{B}	22^{C}	16^{C}	23^{C}
Parthenotes	2^{A}	39^{BC}	45^{C}	30^{B}	34^{B}
Fragmented	0^{A}	17^{B}	28^{C}	24^{BC}	20^{BC}
Lysed	0^{A}	9^{A}	5^{A}	30^{B}	37^{B}
Total number of cultured oocytes	120	120	120	120	120

$^{A, B, C}$statistically significant differences ($P < 0.05$) between the oocytes of different age at the same developmental category (i.e. differences within the rows) are indicated by different superscripts

Table 2. Chromatin configuration in aging pig oocytes at the pronuclear stage

Chromatin configuration in embryo	% of the total number of pronuclear parthenogenetic embryos
1 polar body + 2 pronuclei	47^{A}
2 polar bodies + 1 pronucleus	38^{A}
1 polar body + 1 pronucleus	3^{B}
More than 2 pronuclei	12^{B}
Total number of pronuclear embryos	90

$^{A, B}$statistically significant differences are indicated by different superscripts

Table 3. The parthenogenetic development of *in vitro* aged pig oocytes

The stage of parthenogenetic development (% of the total number of oocytes)	Days of aging *in vitro*				
	1	2	3	4	5
Pronuclear	2^{A}	35^{C}	22^{B}	17^{B}	14^{B}
2–3 blastomeres	0^{A}	4^{AB}	15^{B}	10^{AB}	15^{B}
4–7 blastomeres	0^{A}	0^{A}	7^{A}	3^{A}	4^{A}
8 blastomeres	0^{A}	0^{A}	0^{A}	0^{A}	1^{A}
Total number of cultured oocytes	120	120	120	120	120

$^{A, B, C}$statistically significant differences ($P < 0.05$) between the oocytes of different age at the same developmental category (i.e. differences within the rows) are indicated by different superscripts

When parthenotes entered the pronuclear stage, they exhibited several types of pronuclear and polar body configurations (Table 2). The majority of oocytes had one extruded polar body and two pronuclei (47%) or two extruded polar bodies and one pronucleus (38%). A significantly lower portion of oocytes had one polar body and one pronucleus or more than 2 pronuclei (12%).

A significant cleavage of parthenogenetic embryos was seen from day 3 of aging (Table 3). The embryos usually reached the 2-cell-stage (44 of 149 parthenogenetic embryos, i.e. 30%). Only 9% of all

parthenogenetic embryos proceeded beyond the 4-cell stage and only 1 embryo (i.e. 0.7%) reached the 8-cell stage.

DISCUSSION

Under our culture conditions almost all oocytes matured to the stage of metaphase II within 48-hour culture *in vitro*. Only 4% of oocytes were at the stage of metaphase I. It can be concluded that these metaphase I oocytes were just delayed in their maturation, because after another day of *in vitro* culture (i.e. after the 1st day of aging), the observed oocytes were either at the stage of metaphase II or very rarely at the pronuclear stage after spontaneous parthenogenetic activation (2%). We assume that the oocytes at the stage of metaphase I after 48 hours of *in vitro* maturation complete their maturation to the stage of metaphase II during the 1st day of aging.

The oocytes remained at the stage of metaphase II during the 1st day of aging. The meiotic arrest occurring at this stage of maturation was maintained by elevated levels of the maturation-promoting factor (MPF). This activity is attributed to the complex of cycline B and cycline-dependent kinase $p34^{cdc2}$ (Motlík and Kubelka, 1990). The phosphorylation of this complex resulted in the so called "pre-MPF" which has no activity of MPF. During the aging of pig oocytes, the transition of MPF to pre-MPF was observed by Kikuchi *et al.* (1999, 2000). The spontaneous parthenogenetic activation of *in vitro* aged pig oocytes could be due to this mode of MPF inactivation. But it could also be due to the destruction of cycline B induced by changes in intracellular calcium levels, because the intracellular calcium signalling is significantly altered in aged oocytes (Takahashi *et al.*, 2000, 2003).

The ratio of aged oocytes spontaneously activated *in vitro* is generally low (with maxima up to 45%) and is lower than the activation rate after the artificial parthenogenesis with various artificial stimuli in pig oocytes matured *in vitro* under our culture conditions (Jílek *et al.* 2000, 2001; Petr *et al.*, 1995, 2000, 2002). Both diploid parthenogenetic embryos (i.e. embryos with two pronuclei and one polar body) and haploid parthenogenetic embryos (i.e. embryos with one pronucleus and two polar bodies) resulted from the aging of pig oocytes *in vitro*. This output of aging is similar to the results of artificial parthenogenetic activation of aged mouse oocytes (Pinyopummin *et al.*, 1993), which also resulted in the rise of both diploid and haploid embryos. On the other hand, the artificial induction of parthenogenesis in non-aged pig oocytes matured *in vitro* could result in the dominant development of diploid embryos (Jílek *et al.*, 2000, 2001). A limited number of oocytes with one polar body and one pronucleus could represent diploid parthenogenetic embryos which underwent the syngamy of pronuclei. On the other hand, this sole pronucleus could also be the result of direct decondensation of chromosomes of metaphase II plate with nonfunctioning meiotic spindle. The disruption of the meiotic spindle and the dispersion of chromosomes in aging oocytes could result in the formation of polypronuclear parthenogenetic embryos. The disruption of spindle in aged oocytes was demonstrated by Mailhaes *et al.* (1998).

The disrupted cytoskeletal organization in aged pig oocytes (e.g. disruption of actin microfilaments in the cortical region of oocyte) is also connected with the susceptibility of oocytes to the fragmentation (Suzuki *et al.*, 2002). The fragmentation of aged oocytes is generally thought to be the result of apoptosis (Perez and Tilly, 1997; Perez *et al.*, 1999). This is not surprising because it is well known that the apoptosis is induced by changes in intracellular calcium levels (Orrenius *et al.*, 2003) and that calcium signalling is severely disturbed in aged oocytes (Takahashi *et al.*, 2000, 2003).

These changes along with the adverse effects of reactive oxygen species and metabolites generated during *in vitro* culture could result in the final destruction of oocyte through lysis.

We can conclude on the basis of our data that *in vitro* aged oocytes remained intact mainly during the first day of aging. During this interval, the majority of oocytes were blocked at the stage of metaphase II. After this interval, the oocytes underwent parthenogenesis, fragmentation or lysis. Further studies are needed to investigate the possibilities of the prolonged culture of pig oocytes *in vitro*. Especially the culture in media supplemented with various inhibitors of the cell cycle is worth considering.

REFERENCES

Chian R.C., Nakahara H., Niwa K., Funahashi H. (1992): Fertilization and early cleavage *in vitro* of aging bovine oocytes after maturation in culture. Theriogenology, *37*, 665–672.

Coy P., Romar R. (2002): *In vitro* production of pig embryos: a point of view. Repris. Fertil. Dev., *14*, 275–286.

Gable T.L., Woods G.L. (2001): Increasing culture time from 48 to 96 or 144 hours increased the proportion of equine cumulus oocyte complexes with negative or fragmented nucleus morphology. Theriogenology, *55*, 1549–1560.

Jílek F., Hüttelová R., Petr J., Holubová M., Rozinek J. (2000): Activation of pig oocytes using calcium ionophore: effect of protein synthesis inhibitor cycloheximide. Anim. Reprod. Sci., *63*, 101–111.

Jílek F., Hüttelová R., Petr J., Holubová M., Rozinek J. (2001): Activation of pig oocytes using calcium ionophore: effect of the protein kinase inhibitor 6-dimethyl aminopurine. Reprod. Dom. Anim., *36*, 139–145.

Kikuchi K., Naito K., Noguchi J., Shimada A., Kaneko H., Yamashita M., Tojo H., Toyoda Y. (1999): Inactivation of $p34^{cdc2}$ kinase by accumulation of its phosphorylated forms in porcine oocytes matured and aged *in vitro*. Zygote, *7*, 173–179.

Kikuchi K., Naito K., Noguchi J., Shimada A., Kaneko H., Yamashita M., Aoki F., Tojo H., Toyoda Y. (2000): Maturation/M-phase promoting factor: A regulator of aging in porcine oocytes. Biol. Reprod., *63*, 715–722.

Lonergan P., Fair T., Khatir H., Cesaroni G., Mermillod F. (1998): Effect of protein synthesis inhibition before or during *in vitro* maturation on subsequent development of bovine oocytes. Theriogenology, *50*, 417–431.

Mailhaes J.B., Young D., London S.N. (1998): Postovulatory ageing of mouse oocytes *in vivo* and premature centromere separation and aneuploidy. Biol. Reprod., *58*, 1206–1210.

Mermillod P., Tomanek M., Marchal R., Meijer L. (2000): High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. Mol. Reprod. Dev., *55*, 89–95.

Motlík J., Fulka J. (1976): Breakdown of germinal vesicle in pig oocytes *in vivo* and *in vitro*. J. Exp. Zool., *198*, 155–162.

Motlík J., Kubelka M. (1990): Cell-cycle aspects of growth and maturation of mammalian oocytes. Mol. Reprod. Dev., *27*, 366–375.

Orrenius S., Zhivotovsky B., Nicotera P. (2003): Regulation of cell death: The calcium-apoptosis link. Nature Rev. Molec. Cell. Biol., *4*, 552–565.

Pavlok A., Kaláb P., Bobák P. (1997): Fertilisation competence of bovine normally matured or aged oocytes derived from different antral follicles: morphology, protein synthesis, H1 and MBP kinase activity. Zygote, *5*, 235–246.

Perez G.I., Tilly J.L. (1997): Cumulus cells are required for the increased apoptotic potential in oocytes of aged mouse. Human Reprod., *12*, 2781–2783.

Perez G.I., Tao X.J., Tilly J.L. (1999): Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. Molec. Human Reprod., *5*, 414–420.

Petr J., Grocholová R., Rozinek J., Jílek F. (1995): Activation of *in vitro* matured pig oocytes by combined treatment of ethanol and cycloheximide. Theriogenology, *45*, 1473–1478.

Petr J., Rozinek J., Jílek F., Urbánková D. (2000): Activation of porcine oocytes using cyclopiazonic acid, an inhibitor of calcium-dependent ATPases. J. Exp. Zool., *287*, 304–315.

Petr J., Urbánková D., Tománek M., Rozinek J., Jílek F. (2002): Activation of *in vitro* matured pig oocytes using activators of inositol triphosphate or ryanodine receptors. Anim. Reprod. Sci., *70*, 235–249.

Pinyopummin A., Takahashi Y., Hishimuna K., Kanagawa H. (1993): Development of haploid and diploid mouse parthenotes – effect of oocyte aging *in-vivo*. Jap. J. Vet. Res., *41*, 81–87.

Snedecor G.W., Cochran W.G. (1957): Statistical methods, Iowa: Iowa State University Press.

Suzuki H., Takashima Y., Toyokawa K. (2002): Cytoskeletal organization pf porcine oocytes aged and activated electrically or by sperm. J. Reprod. Dev., *48*, 293–301.

Takahashi T., Saito H., Hiroi M., Doi K., Takahashi E. (2000): Effects of aging on inositol 1,4,5-triphosphate-induced Ca^{2+} release in unfertilized mouse oocytes. Molec. Reprod. Dev., *55*, 299–306.

Takahashi T., Takahashi E., Igarishi H., Tezuka N., Kurachi H. (2003): Impact of oxidative stress in aged oocytes on calcium oscillations at fertilization. Molec. Reprod. Dev., *66*, 143–152.

Wassarman P.M. (1988): The mammalian ovum. In: Knobil E., Neill J. (eds.): The Physiology of Reproduction. Raven Press, New York. 69–102.

Yanagimachi R. (1988): Mammalian fertilization. In: Knobil E., Neill J. (eds.): The Physiology of Reproduction. Raven Press, New York. 135–185.

Received: 03–11–05
Accepted: 04–02–02

ABSTRAKT

***In vitro* stárnoucí prasečí oocyty**

In vitro dozrálé prasečí oocyty po další kultivaci mají několik odlišných osudů. V naší studii jsme oocyty kultivovali po dobu jednoho až pěti dnů (oocyty stárnoucí *in vitro*). První den stárnutí v drtivé většině setrvávají ve stadiu metafáze II (98 %). Poté podléhá značná část oocytů spontánní partenogenetické aktivaci. Podíl aktivovaných oocytů dosahuje maxima po dvou až třech dnech stárnutí in vitro (39 a 45 %). Ve stejné době kulminoval i podíl fragmentovaných oocytů (28 %). V průběhu dalšího stárnutí *in vitro* (tj. 4. a 5. den stárnutí) významně stoupá podíl oocytů, jež podlehly lýze (30 a 37 %). Nejvyšší podíl spontánně aktivovaných partenogenetických embryí v pronukleárním stadiu (35 %) byl pozorován během druhého dne stárnutí. Tato pronukleární embrya měla většinou jedno pólové tělísko s dvěma prvojádry (47 % všech embryí v pronukleárním stadiu) nebo dvě pólová tělíska s jedním prvojádrem (38 % všech embryí v pronukleárním stadiu). Třetí a pátý den stárnutí *in vitro* jsme pozorovali nárůst podílu partenogenetických embryí dělících se do dvou až tří blastomer. Pokud zvažujeme prodlouženou kultivaci prasečích oocytů *in vitro*, pak má smysl uvažovat jen jeden den stárnutí oocytů *in vitro*, protože za touto hranicí podléhají oocyty kultivované *in vitro* výrazným změnám, jako je partenogeneze, fragmentace nebo lýza.

Klíčová slova: prase; oocyty; partenogeneze; fragmentace

Corresponding Author

Ing. Mgr. Markéta Sedmíková, PhD., Česká zemědělská univerzita, Agronomická fakulta, 165 21 Praha 6-Suchdol, Česká republika
Tel. +420 224 382 933, fax +420 234 381 841, e-mail: sedmikova@af.czu.cz