Improvement of developmental competence of aged porcine oocytes by means of the synergistic effect of insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF)

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ABSTRACT: There is a constant increase in demand for large numbers of high-quality oocytes of domestic mammals for various reproductive biotechnologies. Prolonged *in vitro* culture represents one of the many possible ways of meeting this demand. However, oocytes further cultured *in vitro* after reaching the stage of metaphase II undergo the complex process called "ageing", which decreases the quality of oocytes. The aim of the present study was to improve the culture conditions for *in vitro* matured porcine oocytes by supplement of the epidermal growth factor (EGF) or the insulin-like growth factor 1 (IGF-1) in order to reduce the adverse effects of ageing. Supplement of either EGF or IGF-1 had no significant effect on the ageing of porcine oocytes. Significant effects were demonstrated when the oocytes were cultured with both EGF and IGF-1. A combination of 10 μg EGF/ml with 25 ng IGF-1/ml was the most effective. Oocytes aged under these conditions retained very good developmental competence. We observed development to the morula (21%) or blastocyst (25%) stage in oocytes aged for 1 day with EGF and IGF-1. Porcine oocytes matured *in vitro* are more resistant to the ageing when cultured in the presence of both EGF and IGF-1 and these conditions retain an elevated developmental competence for a certain time.

Keywords: epidermal growth factor; insulin-like growth factor 1; oocyte; ageing; pig

Contemporary reproductive biotechnologies depend on rich sources of high-quality oocytes. Oocytes matured *in vitro* are often used for *in vitro* fertilization, for cloning using the transfer of nuclei from somatic cells or for transgenesis. During maturation, the oocytes undergo the germinal vesicle breakdown followed by the stages of metaphase I, anaphase I and telophase I. The oocytes then enter the stage of metaphase II, when meiosis is spontaneously arrested (Wassarman, 1988). Further progress of meiosis beyond this spontaneous blocking depends on the activation stimulus which induces decay and inactivation of the molecules responsible for maintenance of the meiotic block at the stage of metaphase II. Under natural conditions, this stimulus is brought into the oocyte by the sperm (Yanagimachi, 1988).

The quality of oocytes matured *in vitro* is compromised to various extents. Sometimes, this obstacle is overcome by the prolonged *in vitro* culture

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of oocytes after they have reached the stage of metaphase II. Such oocytes were used for *in vitro* fertilization (Chian et al., 1992; Pavlok et al., 1997), cloning (Stice et al., 1994; Tanaka and Kanagawa, 1997) or for production of parthenogenetic embryos (Jolliff and Prather, 1997).

Prolonged *in vitro* culture of oocytes is accompanied by complex processes called ageing. In porcine oocytes, the ageing was observed to induce changes in the oocyte cytoskeleton (Kim et al., 1996; Suzuki et al., 2002) and activity of various enzymes (Kikuchi et al., 1999, 2000). These changes can result in spontaneous parthenogenetic activation, in fragmentation or in lysis in aged porcine oocytes (Petrová et al., 2004). It is obvious that all these ageing-related events compromise the quality of oocytes and of used reproductive biotechnologies.

Various methods have been tested for the suppression of ageing-related phenomena, e.g. Kikuchi et al. (2000) described caffeine as a potent inhibitor of spontaneous parthenogenetic activation and fragmentation in porcine oocytes, aged *in vitro*. Tarin et al. (1998) decreased the fragmentation and increased the developmental competence in ageing mouse oocytes using dithiothreitol. However, these compounds are not natural for mammalian cells. Moreover, caffeine is able to induce aneuploidy in mammalian oocytes (Mailhes et al., 1996) and dithiothreitol is able to induce aneuploidy in mammalian zygotes under certain conditions (Szczygiel and Ward, 2002). Based on these data it is clear that we need more physiological treatments to protect the oocytes from detrimental ageing-related changes.

Even *in vivo*, a substantial part of the oocyte population in the ovary is spontaneously dying and their survival can be maintained using so called survival factors (Johnson et al., 2004). Among others, the growth hormone, a follicle-stimulating hormone, a luteinizing hormone, progesterone, oestradiol, insulin, activin and interleukin 1-beta all belong to these survival factors. A significant role among survival factors is also played by growth factors, e.g. the insulin-like growth factor 1 (IGF-1), the epidermal growth factor (EGF) or the basic fibroblast growth factor (Markström et al., 2002). Many of these survival factors also protect oocytes and embryos cultured under *in vitro* conditions. Significant antiapoptotic effects of IGF-1 (Fabian et al., 2004) or EGF (Cui and Kim, 2003) on embryos developing *in vitro* were described.

The aim of our study was to test improved culture conditions which could maintain the quality of porcine oocytes cultured *in vitro* after they have reached the stage of metaphase II. We used two survival factors for this purpose – the growth factors EGF and IGF-1. The quality of oocytes was determined on the basis of their morphology and was based on their developmental competence *in vitro* after parthenogenetic activation using an artificial activating stimulus (Jílek et al., 2001).

MATERIAL AND METHODS

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from immature gilts and transported to the laboratory within 1 h in a saline solution (0.9% sodium chloride) at 39°C. Fully-grown oocytes were collected by aspirating follicles that were 2–5 mm in diameter with a 20-gauge needle. Only oocytes with compact cumuli were chosen for further study. Before culture the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland) containing sodium bicarbonate (0.039 ml of a 7.0% solution per millilitre of the medium), calcium lactate (0.6 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).

The oocytes were cultured for 48 hours in 3.5-cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3.0 ml of the culture medium at 39°C in a mixture of 5.0% CO_2 in air.

For ageing, the oocytes were denuded of their cumulus cells and placed in a M199 culture medium without foetal calf serum and supplemented with bovine serum albumin (10 mg/ml, Sigma, Aldrich). During each experiment, the medium for ageing was further supplemented with either EGF or IGF-1 (Sigma, Aldrich) or with a combination of both growth factors.

Oocyte activation and culture of embryos

The oocytes were activated using the method described by Jílek et al. (2001). Briefly, oocytes matured *in vitro* and denuded of their cumulus cells were subjected to a 5-minute treatment with 25 μM calcium ionophore A23185 (Sigma, Aldrich). Calcium

ionophore was diluted in modified M199 medium without foetal calf serum and without bovine serum albumin. After the treatment, the oocytes were washed in a M199 medium supplemented with bovine serum albumin and cultured for 2 hours in a NCSU23 culture medium (Peters and Wells, 1993) supplemented with 2 mM 6-dimethylaminopurine (DMAP, Sigma, Aldrich). The oocytes were then washed carefully and cultured in a DMAP-free NCSU23 medium in four-well Petri dishes (Nunc, Roskilde, Denmark), each well containing 1.0 ml of the culture medium. The eggs were cultured at 39°C in a mixture of 5.0% CO_2 in air for 7 days.

Evaluation of oocyte activation and embryo cleavage

At the end of the culture, the oocytes or embryos were mounted on slides, fixed with acetic alcohol (1 : 3, v/v) for at least 24 h, and stained with 1.0% orcein (Sigma, Aldrich). The oocytes and embryos were examined under a phase contrast microscope. Activation was considered to have occurred if the oocytes were in the pronuclear stage. Oocytes remaining at metaphase II or arrested at anaphase II or at telophase II were not considered as activated. The number of nuclei in parthenogenetic embryos was counted at the end of the culture (i.e. 7 days after activation).

Statistical analysis

Data from all experiments were subjected to statistical analysis. Each experiment was performed four times. The results were pooled for presentation and evaluated by chi-square analysis (Snedecor and Cochran, 1980). The mean percentage of oocytes or embryos reaching the given stage of maturation or development in all trials did not vary from the pooled percentage by more than 2.5%. P value less than 0.05 was considered significant.

RESULTS

After 48-hour culture *in vitro*, 98% of the oocytes matured to the stage of metaphase II. The remaining oocytes were observed at the stages of metaphase I, anaphase I or telophase I. The course of oocyte ageing *in vitro* is shown in Table 1. A majority of the oocytes remained at the stage of metaphase II after 1 day of ageing. On the second day of ageing, there was a significant decrease in the percentage of oocytes remaining at the stage of metaphase II and a significant increase in the percentage of oocytes which had undergone either spontaneous parthenogenetic activation or fragmentation. On the third day of ageing, the percentage of oocytes at the stage of metaphase II further decreased and the percentage of fragmented oocytes increased. On the fourth day of ageing, there was a significant decrease in parthenogenetically activated oocytes and the percentage of lysed oocytes increased. We did not observe any significant changes on the 5th day of ageing.

The most significant changes occurred on the 3rd day of ageing when oocytes at the stage of metaphase II decreased to the minimum and the percentage of fragmented or parthenogenetically activated oocytes was maximal. The percentage of

Table 1. The course of *in vitro* ageing of porcine oocytes. Oocytes were matured *in vitro* for 48 hours and then further cultured *in vitro* for another 1, 2, 3, 4 or 5 days

Type of oocyte (%)	Ageing *in vitro* (days)					
	0*	1	2	3	4	5
Metaphase II	98^{A}	98^{A}	35^{B}	22^{C}	16^{C}	23^{C}
Parthenogenotes	0^{A}	2^{A}	39^{BC}	45^{C}	30^{B}	34^{B}
Fragmentation	0^{A}	0^{A}	17^{B}	28^{C}	24^{BC}	20^{BC}
Lysis	0^{A}	0^{A}	9^{A}	5^{A}	30^{B}	37^{B}
Total number of oocytes	120	120	120	120	120	120

^{A,B,C}statistically significant differences between oocytes of the same category at different ages (i.e. differences within lines) are indicated by different superscripts

*remaining oocytes (2%) were observed at the stages of anaphase I, metaphase I or telophase I

lysed oocytes had not increased significantly yet. For these reasons we decided to use ageing after 3 days as a marker for the antiapoptotic effects of growth factors EGF or IGF-1.

EGF at concentrations from 5 to 25 μg/ml of culture medium had no significant effect on the oocyte aged for 3 days *in vitro*. The percentage of oocytes at the stage of metaphase II, parthenogenotes or fragmented oocytes was not changed when compared with the ageing without EGF. When used at a concentration of 50 μg/ml of the culture medium, EGF significantly decreased the percentage of spontaneously activated parthenogenetic oocytes (Table 2).

Similarly, we did not observe any significant effect of IGF-1 at a concentration from 10 to 100 ng/ml of the culture medium on the percentage of fragmented or lysed oocytes and oocytes remaining at the stage of metaphase II. At a concentration of 50 ng/ml IGF-1 the percentage of spontaneously activated parthenogenetic oocytes significantly increased (Table 3).

Surprising effects were observed in experiments performed to test the combined effects of EGF and IGF-1 (Table 4). When we combined the concentrations of EGF and IGF-1, which were not effective in each respective growth factor alone, we observed a significant effect on the oocytes after 3 days of ageing *in vitro*. High concentrations of both factors (20 μg EGF/ml and 50 ng IGF-1/ml; 45 μg EGF/ml and 100 ng IGF-1/ml) increased the percentage of lysed oocytes. Low concentrations (5 μg EGF/ml and 10 ng IGF-1/ml) did not prevent increased fragmentation of the oocytes aged for 3 days *in vitro*. It seems that an optimal combination was 10 μg EGF/ml and 25 ng IGF-1/ml which allowed 70% of oocytes to remain at the metaphase II stage after 3 days of *in vitro* ageing. After this treatment, 24% of the oocytes underwent spontaneous parthenogenetic activation, 6% lysed and no oocyte underwent fragmentation.

Table 2. Effects of EGF on the ageing of porcine oocytes. Oocytes were matured for 48 hours *in vitro* and were then further cultured *in vitro* in a medium supplemented with EGF for 3 days

Type of oocyte (%)	Concentration of EGF (μg/ml)				
	0.0	5.0	10.0	25.0	50.0
Metaphase II	22^{A}	21^{A}	24^{A}	24^{A}	30^{A}
Parthenogenotes	45^{A}	46^{A}	38^{AB}	39^{AB}	30^{B}
Fragmentation	28^{A}	26^{A}	34^{A}	36^{A}	36^{A}
Lysis	5^{A}	7^{A}	8^{A}	1^{A}	4^{A}
Total number of oocytes	120	120	120	120	120

^{A,B}statistically significant differences between oocytes of the same category after different treatment with EGF (i.e. differences within lines) are indicated by different superscripts

Table 3. Effects of IGF-1 on the *in vitro* ageing of porcine oocytes. Oocytes were matured *in vitro* for 48 hours and were then further cultured *in vitro* in a medium supplemented with IGF-1

Type of oocyte (%)	Concentration of IGF-1 (ng/ml)				
	0.0	10.0	25.0	50.0	100.0
Metaphase II	22^{A}	25^{A}	28^{A}	19^{A}	24^{A}
Parthenogenotes	45^{A}	47^{A}	44^{A}	56^{B}	56^{B}
Fragmentation	28^{A}	25^{A}	21^{A}	23^{A}	19^{A}
Lysis	5^{A}	3^{A}	7^{A}	2^{A}	1^{A}
Total number of oocytes	120	120	120	120	120

^{A,B}statistically significant differences between oocytes of the same category after different treatment with IGF-1 (i.e. differences within the lines) are indicated by different superscripts

Table 4. The combined effects of EGF and IGF-1 on *in vitro* ageing of porcine oocytes. Oocytes were matured for 48 hours *in vitro* and were then cultured *in vitro* in medium a supplemented with EGF and IGF-1 for another 3 days

Type of oocyte (%)	Concentrations of IGF-1 (ng/ml) a EGF (μg/ml)				
	EGF 0.0 IGF-1 0.0	EGF 5.0 IGF-1 10.0	EGF 10.0 IGF-1 25.0	EGF 20.0 IGF-1 50.0	EGF 45.0 IGF-1 100.0
Metaphase II	22^{A}	29^{AB}	70^{D}	50^{C}	39^{B}
Parthenogenotes	45^{A}	47^{A}	24^{B}	25^{B}	56^{B}
Fragmentation	28^{B}	25^{B}	0^{A}	0^{A}	1^{A}
Lysis	5^{A}	9^{A}	6^{A}	25^{B}	21^{B}
Total number of oocytes	120	120	120	120	120

^{A,B,C,D}statistically significant differences between oocytes of the same category after different treatment with EGF and IGF-1 (i.e. differences within the lines) are indicated by different superscripts

Table 5. The course of ageing in porcine oocytes under the effects of combined treatments with EGF and IGF-1. Oocytes were matured *in vitro* for 48 hours and then further cultured *in vitro* for another 1, 2, 3, 4 or 5 days in a medium supplemented with EGF (10.0 μg/ml) and IGF-1 (25.0 ng/ml)

Type of oocyte (%)	Ageing of oocytes in medium with 25.0 ng IGF-1/ml and 10.0 μg EGF/ml (days)						
	0*	1	2	3	4	5	6
Metaphase II	98^{A}	100^{A}	92^{A}	70^{B}	60^{BC}	58^{C}	42^{D}
Parthenogenotes	0^{A}	0^{A}	8^{A}	24^{B}	28^{B}	27^{B}	40^{C}
Fragmentation	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	4^{A}	8^{A}
Lysis	0^{A}	0^{A}	2^{AB}	6^{AB}	12^{B}	10^{AB}	10^{AB}
Total number of oocytes	120	120	120	120	120	120	120

^{A,B,C,D}statistically significant differences between oocytes of the same category and different age (i.e. differences within the lines) are indicated by different superscripts

*remaining oocytes (2%) were observed at the stages of metaphase I, anaphase I or telophase I

In a further experiment we determined the course of ageing in oocytes cultured under the influence of the treatment which appeared optimal in our previous experiment (i.e. ageing in a medium supplemented with 10 μg EGF/ml and 25 ng IGF-1/ml). The results are shown in Table 5. We clearly demonstrated that under this treatment the percentage of lysed or fragmented oocytes was not significantly increased even after 6 days of ageing. There is a continuous decrease in the percentage of oocytes remaining at the stage of metaphase II and simultaneously in the percentage of oocytes undergoing spontaneous parthenogenetic activation.

To check the viability of oocytes aged under the effects of combined treatment with 10 μg EGF/ml and 25 ng IGF-1/ml we activated these oocytes by an artificial stimulus (Table 6). Oocytes were first treated with 25 μM of calcium ionophore A23187 for 5 minutes and subsequently treated with 2 mM 6-DMAP for 2 hours. Artificially induced parthenogenetic development proceeded up to the stage of expanded blastocyst in oocytes aged for 1 day. Oocytes activated artificially after 2-day ageing did not develop beyond the morula stage. Oocytes aged for 3 or 4 days did not cleave to more than 16 blastomeres.

When parthenogenetic activation was artificially induced in oocytes which had aged for 1 day without growth factors, their parthenogenetic development did not differ from the developmental rate of oocytes activated artificially immediately after completing their maturation (i.e. activation in oocytes matured for 48 hours *in vitro* and not subjected to any ageing). However, the oocytes aged

Table 6. Parthenogenetic developmental competence of porcine oocytes aged in the presence of EGF and IGF-1. Oocytes were matured for 48 hours *in vitro* and were then cultured *in vitro* in a medium supplemented with EGF (10.0 μg/ml) and IGF-1 (25.0 ng/ml) for 0, 1, 2, 3 or 4 days. They were then activated by an artificial activating stimulus (treatment with 25 μM of calcium ionophore A23187 for 5 min and then treatment with 2 mM DMAP for 2 hours) after which they were cultured in a NCSU23 medium for another 7 days

Stage of parthenogenetic development (%)	Ageing with EGF and IGF-1 (days)				
	0	1	2	3	4
No cleavage	11^{A}	19^{AB}	27^{BC}	33^{C}	28^{BC}
2–3 blastomeres	15^{A}	14^{A}	33^{B}	42^{B}	66^{C}
4–7 blastomeres	33^{A}	12^{C}	27^{AB}	17^{BC}	4^{C}
8–16 blastomeres	11^{A}	9^{A}	8^{A}	8^{A}	2^{A}
Morula	15^{A}	21^{A}	5^{B}	0^{B}	0^{B}
Blastocyst	15^{A}	25^{A}	0^{B}	0^{B}	0^{B}
Total number of oocytes	120	120	120	120	120

^{A,B,C}statistically significant differences between embryos of the same developmental stage after a different length of oocyte ageing (i.e. differences within the lines are indicated by different superscripts)

Table 7. The competence to develop beyond the morula stage in oocytes aged under different conditions. Oocytes were matured for 48 hours *in vitro* and were then cultured *in vitro* in a medium supplemented with EGF (10.0 μg/ml) and IGF-1 (25.0 ng/ml) or in a medium without growth factors for 1 day. Control oocytes were not subjected to ageing *in vitro* (control without ageing). These oocytes were then activated by an artificial activating stimulus (treatment with 25 μM of calcium ionophore A23187 for 5 min and then treatment with 2 mM DMAP for 2 hours) and were then cultured in a NCSU23 medium for another 7 days

Stage of parthenogenetic development (%)	Type of ageing		
	Control without ageing	1 day without EGF or IGF-1	1 day with EGF and IGF-1
Morula	12^{A}	15^{A}	21^{A}
Blastocyst	16^{A}	15^{A}	25^{A}
Morula + blastocyst	28^{A}	30^{A}	46^{B}
Total number of oocytes	120	120	120

^{A,B,C}statistically significant differences between embryos of the same developmental stage after different types of oocyte ageing (i.e. differences within the lines) are indicated by different superscripts

for 1 day with 10 μg EGF/ml and 25 ng IGF-1/ml exhibited significantly higher development beyond the stage of morula (46%) than oocytes aged for 1 day without growth factor (30%) or oocytes activated without ageing (28%). These data are shown in Table 7.

DISCUSSION

In the present study we confirmed our previous results which showed that porcine oocytes matured *in vitro* underwent ageing during further *in vitro* culture. This process resulted in spontaneous parthenogenetic activation, fragmentation or lysis in aged porcine oocytes (Petrová et al., 2004). Studies on oocytes of various mammalian species indicate that the changes in aged oocytes are due to alterations in the cytoskeleton (Kim et al., 1996; Suzuki et al., 2002), changes in the enzymatic activity of ooplasm (Kikuchi et al., 1990, 2000; Gordo et al., 2000; Papandile et al., 2004), damage to the mitochondria (Fissore et al., 2002) or to disturbed intracellular signalling (Jones and Whittingham, 1996;

Igarashi et al., 1997; Gordo et al., 2000; Takahashi et al., 2000).

Besides the spontaneously occurring parthenogenetic activation, we also observed a very marked percentage of fragmentation in oocytes aged under our culture conditions. This phenomenon is due to apoptosis, which is induced in aged oocytes by complex changes in the cytoskeleton, intracellular signalling and activity of the caspases (Fissore et al., 2002).

In the present study we used growth factors EGF or IGF-1 for the suppression of fragmentation in aged porcine oocytes. However, we did not demonstrate any significant effect of each respective growth factor on the apoptosis (i.e. fragmentation) in aged oocytes. Each growth factor significantly influenced the rate of spontaneously activated parthenogenotes but their effects were opposite. EGF significantly decreased the rate of spontaneously activated oocytes, IGF-1 significantly increased the percentage of spontaneously activated parthenogenotes in aged oocytes.

In our study we used much higher doses of EGF that those used for antiapoptotic effects on pig parthenogenetic embryos (Cui and Kim, 2003), embryos obtained after *in vitro* fertilization (Wei et al., 2001), porcine oocyte maturation (Lie et al., 2002; Herrick et al., 2003) or follicle *in vitro* culture (Mao et al., 2004). It is not clear why EGF acts only in these high concentrations under our conditions. It could be due to the source of EGF but it could also be relevant to the fact that we used aging oocytes which can react differently from *in vitro* matured oocytes or embryos. IGF-1 levels used in our study were comparable to those used for the antiapoptotic effect in mouse embryos by Fabian et al. (2004).

Since we used denuded porcine oocytes, there arises a question about the presence of EGF and IGF-1 receptors in the oocyte. Both types of receptors are present in follicles in mammalian ovary and also in ovarian follicles in nonmammalian species (fish – Wang and Ge, 2004; amphibian – Grigorescu et al., 1994; bird – Van Nassauw and Harrisson, 2000). EGF receptors and their mRNA were detected in bovine (Yoshida et al., 1998), goat (Gall et al., 2004), hamster (Garnett et al., 2002) and human (Qu et al., 2000a) oocytes. Moreover, Lonergan et al. (1996) described the effect of EGF on denuded bovine oocytes and suggested that EGF can act directly on the oocyte. The situation is more complicated in the porcine oocytes. EGF receptors and their mRNA are clearly present in the somatic compartment of ovarian follicle (Vaughan et al., 1992; Singh et al., 1995; Procházka et al., 2003). However, Vaughan et al. (1992) observed mRNA for EGF receptors in porcine oocytes and Singh et al. (1995) described very weak presence of mRNA for EGF receptors and EGF receptors in porcine oocytes. IGF receptors were found in bovine (Yoshida et al., 1998; Armstrong et al., 2002), rat (Zhao et al., 2002) and human (Qu et al., 2000b) oocytes. They were also detected in porcine oocytes (Okamura et al., 2001). Based on these data it is possible that nonsignificant effects of EGF could be due to the low level of EGF-receptors.

There is no clear reason for the stimulatory effects of IGF-1 on the spontaneous parthenogenetic activation in aged porcine oocytes. This can be due to the fact that IGF-1 is able to activate PI 3-kinase (Grothey et al., 1999). This kinase is deeply involved in the regulation of meiosis in porcine oocytes and under certain conditions it can be involved in the exit of the oocyte from the metaphase II stage (Shimada et al., 2001).

Surprisingly, we observed a significant synergistic effect of EGF and IGF-1 on the ageing of porcine oocytes. The concentrations ineffective for each respective growth factor exhibited a significant effect when EGF was used in combination with IGF-1. We observed an increased percentage of oocytes which remained at the stage of metaphase II during 3 days of ageing, and we also observed a significant decrease in the percentage of oocytes undergoing spontaneous parthenogenetic activation or fragmentation.

Based on our data, we cannot specify the principles of observed synergism between EGF and IGF-1, however, similar synergisms of EGF and IGF-1 were often observed during the induction of various physiological processes in a wide spectra of somatic cells, e.g. astroglia (Han et al., 1992), keratinocytes (Marikovsky et al., 1996), oesophageal epithelial cells (Quereshi et al., 1997), prostate cancer cells (Putz et al., 1999), retina epithelial cells (Kaven et al., 2000) or mammary epithelial cells (Woodward et al., 2000). The synergism of EGF and IGF-1 is reached by various mechanisms in these cells. In astroglia, EGF induces additional synthesis of IGF-1 in cells (Han et al., 1992). In mammary epithelial cells, EGF decreased the concentration of the IGF-1-binding protein 2, which is otherwise bound on IGF-1 and blocked its physiologic effects (Woodward et al., 2000). In prostate cancer cells, EGF and IGF-1 activate a signal cascade of p42/ERK2 kinase or a signal cascade of protein kinase A (Putz

et al., 1999). The two signal cascades are active in mammalian oocytes and are involved in the regulation of their meiosis. The role of protein kinase A in the regulation of meiosis in mammalian oocytes was described e.g. by Kovo et al. (2002), Rodriguez et al. (2002) and Webb et al. (2002). The role of p42/ERK2 kinase in the regulation of meiosis in mammalian oocytes was described e.g. by Liu et al. (1998), Liu and Yang (1999), Sun et al. (1999), Lee et al. (2000) and Tian et al. (2002). If the kinase p42/ERK2 was regulated through EGF and IGF-1 in porcine oocytes, it could be the reason for the decrease in the percentage of spontaneous parthenogenetic activation in ageing oocytes treated with EGF and IGF.

The high concentration of EGF and IGF-1 increased the lysed oocytes among the aged ones. Again, the reason for this effect is not clear, but a very similar effect was seen in somatic cells. EGF alone is able to induce cell death (Morrazzani et al., 2004) or potentiates the cytotoxic effect of other compounds (Qi et al., 2003). We cannot exclude the direct negative effects of both growth factors at the concentrations used in our study on aged oocytes. It is also possible that both these growth factors potentiate the adverse effects of some metabolites or free radicals, which are inevitably produced during *in vitro* culture.

In our study, we also demonstrated that the oocytes aged under the synergistic effect of optimal concentration of EGF and IGF-1 retained high developmental competence for a certain time. This was shown in the experiment where oocytes aged *in vitro* were artificially activated using a calcium ionophore and then cultured *in vitro*. The percentage of oocytes which developed beyond the morula stage was significantly higher in oocytes aged for 1 day with EGF and IGF-1 than the percentage of parthenogenetic embryos developing beyond the morula stage from oocytes artificially activated immediately after maturation (i.e. after 48 hours of *in vitro* culture) or from oocytes which aged for 1 day in a medium not supplemented with EGF and IGF-1.

This can partly be due to the fact that aged oocytes are more prone to parthenogenetic activation when compared with freshly matured oocytes. This sensitivity to the activating stimulus is a consequence of the decay and deactivation of enzymes, which maintained matured mammalian oocytes in the spontaneous meiotic block at the stage of metaphase II (Kikuchi et al., 1999, 2000). However, we observed a significant difference between the developmental capacity of oocytes aged in the presence of EGF and IGF-1 and oocytes aged without the growth factor. This indicates that a significant effect on the viability and developmental capacity of aged oocytes is exerted by EGF and IGF-1.

There are contradictory data on the effects of oocyte ageing on the developmental competence during pre-implantation embryonic development. Usually, the effects of the short-term ageing, which lasted only a few hours, were under investigation. Jolliff and Prather (1997) did not observe any decrease in the developmental competence of parthenogenetic embryos obtained from aged oocytes compared to parthenogenetic embryos generated from oocytes activated immediately after reaching the stage of metaphase II. Stice et al. (1994) even described improved development of the embryos originating from the transfer of nuclei from somatic cells to the cytoplasm of aged oocytes. On the other hand, Tanaka and Kanagawa (1997) described impaired development of embryos originating from the transfer of blastomere nuclei to the cytoplasm of aged oocytes. Chian et al. (1992) also reported impaired development of embryos originating from fertilization of aged bovine oocytes. A long-term culture of equine oocytes was tested by Gable and Woods (2001). These authors prolonged the culture time for oocyte maturation from 48 to 96 or 144 hours (i.e. ageing for 2 or 4 days). This prolongation of culture comparable to ageing in our experiments had a detrimental effect on the aged oocytes, which largely underwent lysis or fragmentation.

Based on our data we can conclude that porcine oocytes matured *in vitro* are more resistant to the ageing when cultured in the presence of both EGF and IGF-1. The oocytes cultured under these conditions retain an elevated developmental competence for a certain time. We suggest a prolonged culture in the presence of both EGF and IGF-1 as a method for the short-term maintenance of porcine oocytes for various reproductive biotechnologies.

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