Selected molecular and microfluidic aspects of mammalian oocyte maturation-perspectives: a review

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ABSTRACT: Maturation of oocytes is the most important stage, which determines the subsequent successful fertilization, zygote formation, attainment of blastocyst stage, normal embryo growth and development, as well as appropriate implantation. Since in some species of mammals the efficiency of IVM maturation is still very low, many studies have focused on new combinations of media supplements. In some species of mammals, including pigs, mice, bovines, goats and dogs, EGF, BSA, and progesterone are successfully used as an enhancer of the IVC system. Application of the Lab-on-Chip system in reproductive biology opens new possibilities for the development of techniques available for the assessment of the developmental competency or potency manifested by mammalian oocytes and embryos. In most cases, the Lab-on-Chip system was used in mice. However, an increasing number of examples are available in which the system has been applied to evaluate porcine and bovine gametes and embryos. In this article, the role of EGF, BSA and progesterone is described in relation to maturation efficiency of mammalian oocytes. Moreover, the possibilities of using microfluidics (Lab-on-Chip) for the detection of oocyte developmental competency are also shown.

Keywords: microfluidics; Lab-on-chip; oocyte maturation; media supplementation

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1. Mammalian COC maturation

Maturation of the cumulus-oocyte complex (COC) is one of the most important factors which determines entry into metaphase II (MII), subsequent successful fertilization, as well as the ability of an embryo to undergo an appropriate growth and development (Bukowska et al., 2008; Kempisty et

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al., 2009; Antosik et al., 2010a,c). It was also suggested that COC maturation is correlated with the ability of oocytes and embryos to reach developmental competence or potential (Jaskowski et al., 2010; Kempisty et al., 2011b). Since in canine species only 20% of ovarian oocytes reach the MII stage, several well recognized media supplements are available which may increase the low number of oocytes that reach full maturity (Otoi et al., 2000; England et al., 2001; Mrazek and Fulka, 2003).

Due to the persisting low efficiency of in vitro maturation (IVM) of some mammalian oocytes, including oocytes of dogs, in vitro culture (IVC) conditions are increasingly being modified to contain new combinations of media supplements, which may increase the number of oocytes reaching the MII stage. In addition, the detection of apoptosis in mammalian oocytes and the surrounding follicular cells may lead to the recognition of IVC efficiency with a special relationship to applied media supplements.

Developmental competence or potency is defined as the ability of oocytes to grow and develop with special relation to maturation, successful monospermic fertilization, zygote formation, as well as a development of the embryo to the blastocyst stage. These important stages also influence proper implantation, foetal development and the birth of healthy offspring. Many authors suggest that COC maturation is the most important or critical stage for future embryo growth. During the long stage of COC maturation, oocytes, through several molecular and cellular modifications, reach full developmental competency.

The molecular modifications of COC during maturation include accumulation of mRNA and proteins, which originate from long stages of development during oogenesis and folliculogenesis (Kempisty et al., 2011b). In MII oocytes these accumulated molecules become a template for the synthesis of proteins during the consecutive steps of embryo growth.

Since it was shown that mammalian spermatozoa may be transferred into oocytes to obtain fertilization, it has been suggested that spermatozoal mRNA may play an important role during oocyte maturation and the early steps of embryo development following fertilization (Ostermeir et al., 2004). Therefore, identification of spermatozoal mRNA in early stage embryos in relation to fertilization and early embryogenesis is now of high significance. Using a hamster oocyte model, Ostermeier et al. (2004) showed that protamine 2 (PRM2) and clusterin (Clu) mRNA are transferred into oocytes by spermatozoa during fertilization. They suggested that these spermatozoal mRNAs reflect a toxic effect during early embryogenesis. These results were confirmed by Kempisty et al. (2008) in the porcine oocyte model. They detected the presence of spermatozoal mRNA encoding PRM2 and Clu in porcine zygotes and two-cell stage embryos. They detected no disturbances in early embryo development. However, further analyses using embryos at preimplantation stages needs to be performed. Thus, spermatozoal mRNA may be an important missing piece of the fertilization puzzle in those species of mammals (such as dogs) where IVF still suffers from very low efficiency.

In mammalian spermatozoa mRNA is synthesized during the early stages of spermiogenesis. Moreover, during the subsequent stages of spermatogenesis, mRNA is accumulated in cytoplasmic droplets or partially degraded (Kempisty, 2008). The RNA, also called "the RNA of long life", is accumulated in cytoplasmic droplets, is very stable, and is stored for a long time and present in mature spermatozoa. It is suggested that RNA, which is present in these cells collected after ejaculation, may be a marker of efficiency or expression of mRNA during the early stages of spermatogenesis. Therefore, the detected RNA fingerprint is a specific "historical record" of growth, development and differentiation of spermatogonial cells, mostly in humans, but also in other mammalian species including pigs (Kempisty, 2008). It is also suggested that there is no *de novo* synthesis of mRNA in mature spermatozoa, but that the detected RNA represents remains of RNA-storing cytoplasmic droplets (Gorska et al., 2006; Kempisty et al., 2006, 2007; Sikora et al., 2006; Depa-Martynow et al., 2007; Jedrzejczak et al., 2007; Bukowska et al., 2011). However, some reports indicate that small sections of mRNA may be actively synthesized on the mitochondrial fraction of ribosomes. The stable RNA in mature spermatozoa may be also delivered to oocytes during fertilization, where, as it was suggested, it may exert toxic effects on the already formed zygote and the growing embryo (Ostermeier et al., 2004). With respect to the fact that mRNA in oocytes is stored and the template detected in embryo is of a female gamete origin, it is suggested that these ribonucleic acid molecules may also reach a stable structure, which is partially proven by their long term accumulation.

During the long period of oocyte maturation and differentiation (folliculo-and oogenesis) large

amounts of mRNA and proteins become synthesized and accumulated. At the early steps of embryogenesis following embryo implantation appropriate amounts of these mRNAs are stored as a template for the synthesis of new proteins involved in regulatory mechanisms responsible for early embryo growth and development. These oocyte-specific molecules are also called "maternal factors", involved in sequential steps of embryogenesis. Li et al. (2010) described the maternal-effect genes as mRNA templates which appear between the last stages of oocyte maturation and embryonic genome activation, necessary for early embryo development. Recent studies, performed mostly in the mouse oocyte/embryo model, indicated the existence of maternally provided factors and multi-component mRNA and protein complexes that regulate preimplantation stages of embryo development. It was found in several studies that maternally derived mRNAs are degraded at the stage of meiotic maturation and ovulation. In many species of mammals new activation of the embryonic genome develops in one-cell stage and two-cell stage embryos (Vastenhouw et al., 2010). Early embryonic development is regulated at the stage of transition between fully matured oocytes and zygotes, also called the maternal-zygote transition (MZT). This mechanism was recently intensely investigated using experimental models, such as echinoderms, nematodes, insects, fish, amphibians and mammals (Tadros and Lipshitz, 2009). Generally, in all of these models MZT may be divided into two stages; the first is characterized by substantial degradation of maternal mRNA and proteins, whereas the second is associated with an increase in transcription during zygote formation, following successful fertilization. Although this mechanism is common to all these models, there exist significant differences in the timing and scale of these two events.

Molecular changes in COC include accumulation inside the cytoplasm of oocytes of appropriate amounts of several substrates, enzymes and other molecules involved in biochemical reactions. A normal level of hormones is also important for determining oocyte competency and quality; these are transferred into oocytes from the culture media or are actively synthesized in the cell cytoplasm. Biochemical changes have pronounced effects on the cell, which may be defined as the ability of oocyte to change the specific cell organellum activity in relation to modified culture conditions (e.g., using of transcription or translation activators or inhibitors). The specific transport of small peptide molecules or hormones is possible, since *gap junction* connections are formed. These connections build a specific "protein corridor", which is formed by proteins called connexins (Cx's), in most cases connexin 37 (Cx37), connexin 43 (Cx43), and connexin 45 (Cx45). Since the Cx's are responsible for transferring specific molecules into oocytes, it is suggested that these proteins are the most important players in the regulation of COC maturation, which finally leads to attainment of the MII stage. Antosik et al. (2011) showed that the expression of Cx43 was highly correlated with the size of follicles from which the COC were collected. Moreover, an association was also found between COC morphology (number of cumulus cell layers, colour and granularity of cytoplasm) and the expression of connexin, (Antosik et al., 2010c). Therefore, based on these results it may be suggested that the expression of Cx43 may indicate the COC quality as well as serving as a new marker of oocyte developmental competency.

Vitrification is one of the most frequently used procedures to preserve male and female germ line cells from various species of mammals. However, these procedures are often invasive or have a negative effect on subsequent fertilization or embryo growth and development. Moreover, the vitrified cells, especially oocytes, cannot reach such a full developmental competence as before vitrification. Tharasanit et al. (2009), using equine COC, analyzed the protective role of cumulus cells during oocyte vitrification. After using a plasma membrane impermeable DNA marker (ethidium homodimer-1) they examined the percentage and location of dead cumulus cells as well as the effect of dead cumulus cells on the IVM efficiency of oocytes. In addition, they labelled the oocytes for connexin-43 to check the formation of proper *gap junction* connections between the oocytes and surrounding cumulus cells during vitrification. They found that the cryopeservation of immature COC led to an increased number of dead cumulus cells (13 vs 2.6% for controls; $P < 0.05$). Surprisingly, the higher proportion of dead cumulus cells did not affect the post-thawing maturation rates (approximately 30% MII), which was presumably the result of death of cells localized at the periphery of the cumulus mass. Hence, the *gap junction* connections were not disrupted. It is suggested that cumulus cells protected *gap junction* connections upon cryopreservation,

and cumulus-enclosed MII oocytes preserved the quality of the meiotic spindle as compared to denuded oocytes (38.1 vs 3.1% normal spindles; $P < 0.05$). Nemcova et al. (2006) investigated the role of different phases of bovine follicular growth on the developmental competence of oocytes, measured using maturation markers, such as Cx43, Cx31 and Bax. In this experiment oocytes were recovered from small (2–5 mm) or medium (6–10 mm) follicles, in the growth/stagnation (G/S) or dominance/regression (D/R) stage of the first follicular wave. Then, the oocytes were successfully matured, and fertilized. Expression of Bax, Cx43 and Cx31 was analyzed in pooled blastocysts. The authors did not observe significant differences in Bax and Cx31 expression between D7 and D8 embryos derived from oocytes collected from medium or small follicles in the G/S or D/R stages. However, the expression of Cx43 was increased in D8 blastocysts derived from oocytes recovered from medium and small follicles in the G/S stage as compared to D/R stage. Taking into account these results, it is suggested that Cx43 may be used as a marker in determining oocyte and embryo developmental potential, because the higher expression of this gene was observed in oocytes of an increased competence.

The morphological changes in COC include the cytoplasm, *zona pellucida* and meiotic spindle modification during oocyte maturation. Since the entire transcriptional and translational machinery is activated during COC maturation, the colour of the oocyte's cytoplasm may be changed; indeed, this occurs during COC culture-maturation *in vitro*. After 48 h of IVM using porcine COC, the oocyte cytoplasm may look contracted and dark as compared to *in vivo* collected COC. This could be associated with the induction of degenerative processes inside the oocyte cytoplasm, directing the cell toward the apoptotic pathway.

2. The role of EGF, BSA and progesterone as media supplements during mammalian COC maturation

Since the first in vitro maturation (IVM) system was applied, several studies have shown differential IVM efficiency in various species of mammals, including cattle, pigs, goats, mice, cat, dogs and humans. Use of different IVM media as well as different media supplements leads to an increase in the number of oocytes which reach the MII stage, which in turn increases the number of cells which attain the full maturation status.

Epidermal growth factor (EGF), bovine serum albumin (BSA) and progesterone were frequently used as media supplements in other species of mammals, such as cattle and pigs. EGF is described as a factor which has several effects on different types of cells, on their differentiation *in vivo* and is a potent mitogenic factor for a variety of *in vitro* cultured cells of both ectodermal and mesodermal origin. Expression of EGF was detected in various types of tissues including reproductive tissues, such as ovary, endometrium and uterus (Antosik et al., 2010). BSA represents another media supplement used in the culture of oocytes collected from various species of mammals. Cui et al. (2006) showed that cultivation of canine oocytes in media supplemented with EGF (100 ng/ml) and BSA (0.4%) increases the resumption of meiosis by canine oocytes *in vitro*. In contrast to these results, previous results by Bolamba et al. (2002) documented no differences in canine oocytes that reach the MI and MII stages in media supplemented with BSA (3 mg/ml) with or without 20% foetal bovine serum (FBS) or synthetic oviduct fluid (SOF). These results suggested that supplementation of media with BSA is not essential for canine oocytes to reach full maturity. The steroidogenic activity of COC and ovarian tissues is recognized as one factor which influences the acquisition of oocyte developmental competence (Grupen and Armstrong, 2010). Otoi et al. (1999) showed an increased estradiol and progesterone concentration in oestrous bitch serum which, as suggested by others, had been found to support nuclear maturation of canine oocytes (Nickson et al., 1993; Otoi et al., 1999). However, the positive influence of progesterone on canine oocyte nuclear maturation *in vitro* is controversial and requires further investigation (Willingham-Rocky et al., 2003; Kim et al., 2005).

In the majority of mammalian species, EGF, BSA and progesterone are the most frequently used media supplements. The effect of EGF on *in vitro* maturation efficiency was investigated in studies on several species of mammals, including dogs, pigs and cattle. It was shown that supplementation of maturation media with EGF (10 ng/ml EGF for 72 h as compared to 0 and 30 ng/ml EGF) significantly increased attainment of the MII stage by canine oocytes (Song, 2010). The authors concluded that EGF used as a media supplement positively influenced the progress of canine COC maturation to

the MII phase of IVM. In a similar study, Tkachenko et al. (2010) focused their attention on the influence of EGF on *in vitro* maturation and on the cleavage rate of marmoset monkey oocytes. In this study they cultured COC in media supplemented with 10 ng/ml EGF in combination with 1 or 10 IU/ml of gonadotrophins (FSH/hCG 1 : 1 ratio). In contrast to previous studies, they showed that COC cultured with the addition of EGF did not differ in maturation or fertilization rates. The addition of EGF led to the degeneration of COC and decreased the first cleavage rate; in addition, in the EGF groups some embryos cleaved faster than in the group without supplementation. It was suggested that EGF might negatively influence COC maturation, which was especially detectable in the presence of low doses of gonadotropins, used as supplements. Moreover, the same authors showed that EGF may play a protective role against the effects of higher doses of gonadotropins. In contrast to these results, Lott et al. (2010) investigated the role of EGF as a TCM-199 media supplement in combination with IGF-I and cysteine in bovine embryo development. They showed that supplementation of media with EGF combined with cysteine and IGF-I led to an increase in the percentage of cleaved embryos, while the rates of blastocyst development were the same in the two types of culture. In the same experiment they showed that the proportion of embryos reaching the morula stage increased in media supplemented with cysteine.

The effect of EGF supplementation on IVM efficiency as compared to the effect of gonadotropins (FSH) was also studied (Uhm et al., 2010). It was shown that EGF mimicked the effect of FSH on the IVM of porcine COC and had a synergistic effect with this gonadotropin on the cytoplasmic maturation of porcine oocytes. These results suggest that the addition of EGF to the maturation system can optimize the beneficial effects of FSH on the cytoplasmic maturation of porcine COC and opens new perspectives for using EGF as a factor, which mimics the actions of gonadotropins. The results of studies investigating the role of EGF on COC maturation or embryo cleavage are contradictory in most of the cases. Therefore, unambiguous experimental proof for a positive or negative role of EGF is still required.

The influence of cysteine, EGF and IGF-I on the development of *in vitro*-fertilized bovine embryos was investigated by Lott et al. (2011). In these experiments, bovine oocytes were cultured in TCM-199 culture media with or without the addition of 0.6 mm cysteine at 0 or 12 h of IVM. After *in vitro* fertilization the embryos were cultured with the supplementation of foetal calf serum (FCS, 4%) alone, IGF-I (100 ng/ml); EGF (10 ng/ml); IGF-I + EGF (100 + 10 ng/ml). They authors found no differences in blastocyst development between the treatment groups, although there was an increased proportion of embryos attaining morula stage after cysteine addition to culture media. Moreover, the recovery of embryos was higher when the cysteine was added in combination with growth factors. It was suggested that the addition of cysteine to culture media significantly increased the maturation and fertilization ability of oocytes as well as the developmental potential of embryos. Furthermore, supplementation with cysteine in combination with growth factors could effectively be used as a replacement for FCS. In other experiments, Sirisathien and Brackett (2003) investigated the influence of IGF-I and EGF supplementation on bovine embryo development and nuclear DNA fragmentation as indices of embryo quality. The zygotes obtained by IVF were randomly cultured in either control (with no added growth factor) or treatment groups, i.e., with 50 ng/ml IGF-I (experiment 1) or 5 ng/ml EGF (experiment 2). The percentage of blastocysts formed was significantly higher in the IGF-I treatment group as compared to the control (38.0% vs. 28.5%). The proportion of blastocysts was also increased in the EGF treatment group, as compared to the control (38.5% vs. 30.7%). In both treatment groups (IGF-I and EGF) lower percentages of TUNEL-positive cell nuclei or no TUNEL-positive cell nuclei at all were observed. Similarly to the results quoted above, the authors found that supplementation with IGF-I in combination with EGF significantly increased the developmental potential of bovine embryos.

The effect of BSA on the maturation efficiency of COC has recently been the subject of intense focus using canine female gametes. Cui et al. (2006) investigated the role of BSA combined with EGF supplementation in the IVM of canine oocytes. They showed that the addition of 0.4% BSA and 100 ng/ml EGF led to an increased number of MII oocytes, as compared to the control. Moreover, EGF supplementation in the presence of BSA increased the meiotic resumption of oocytes. The effect of macromolecule (BSA) supplementation was also examined using goat oocytes and embryos. In experiments performed by Herrick et al. (2004), the

influence of BSA on embryonic development was investigated. In this study the cleavage, blastocyst development, hatching, and total cell number of goat embryos were examined. The varying concentrations of BSA (2.5, 8.0, or 20.0 mg/ml) used did not affect the developmental potency of the investigated embryos. Moreover, it was suggested that BSA may be successfully used for the maturation of oocytes and development of embryos *in vitro*, as a goat-specific media supplement. Similarly to these results, Bolamba et al. (2002) used canine COC as a model for investigating the effect of BSA supplementation on IVM efficiency. In this study, supplementation with BSA was at a concentration of 3 mg/ml BSA, in combination with SOF (synthetic oviductal fluid) + 20% FBS (foetal bovine serum). The percentages of oocytes at MI to MII stages did not vary between the treatments. However, the addition of BSA and FBS to culture media significantly increased the number of MI and MII oocytes as compared to the control only after 72 h of culture. Furthermore, the number of MI and MII oocytes did not differ at 24, 48 and 72 h of culture, as compared to the control. Thus, under defined culture conditions supplementation with BSA had no effect on IVM efficiency. It may be inferred from these data that supplementation with BSA is not always essential for successful IVM and may exert a species-specific effect.

Varga et al. (2011), using bovine oocytes and embryos as a model, investigated the role of BSA used at different concentrations (3 and 8 g/l) as well as the effects of freezing and lyophilisation as procedures for preserving the SOF during bovine embryo development. They observed that supplementation of culture media with BSA (3 g/l) led to increased Day 7 blastocyst expansion rates (18.3 ± 1.6 vs. 14.4 ± 0.7; $P < 0.05$). Moreover, it was observed that the addition of FCS to SOFaa (SOF with amino acids) medium supplemented with sodium citrate (SOFaaci) at 48 h and at 72 h post-insemination led to higher rates of Day 6 bovine embryos, as compared to FCS supplementation at 18 or 96 h post-insemination. However, they did not observe differences in hatching rates between the treatment groups. Moreover, Choi et al. (2002) found that culture of bovine blastocysts in medium supplemented with BSA for 72 h and then in FBS-containing medium for the subsequent 96 h resulted in an increased rate of blastocyst formation (49% versus 25–36%) as compared to other groups (BSA to BSA or PVA to PVA, BSA or FBS). Taking into account both of the mentioned above results, it was suggested that supplementation with BSA alone or in combination with FBS significantly increases bovine blastocyst formation rate and might be a factor which promotes embryo growth and development. However, the enhanced development of bovine embryos *in vitro* may have no influence on pregnancy outcome after embryo transfer in this species of mammals.

Progesterone (P4), as the steroid hormone most important for reproduction, plays a central role during oestrus in several species of mammals. It was also suggested that P4 is actively synthesized by reproductive organs and germ line cells, especially in relation to oocytes (Dressing et al., 2010; Shimizu et al., 2010). Wen (2010) investigated the production of this hormone by follicular fluid and granulosa cells in IVF patients. They found no significant association between oocyte recovery and steroid levels in follicular fluid (FF). Also, no association was found between FF steroid levels for those oocytes that did or did not fertilize. Moreover, they found increased concentrations of P4 in granulosa cells; however, this was not dependent on follicular size. They concluded that progesterone levels were not associated with the maturation or fertilization ability of human oocytes. The influence of P4 on cumulus cell viability and function, and porcine oocyte nuclear and cytoplasmic maturation, was investigated by Grupen and Armstrong (2010). They recorded the concentration of progesterone in spent IVM medium and the level of apoptosis in cumulus cells, as well as the expansion of cumulus cells, the ability to reach MII stage and the formation of blastocysts after IVF. The results indicated that increased P4 production led to higher cumulus expansion and a decreased incidence of apoptosis. A negative association was found between P4 synthesis by cumulus cells and the induction of apoptosis. Furthermore, oocytes recovered from FF with higher concentrations of P4 (+FF, +FF) displayed an increased ability to reach the MII stage and increased blastocyst formation, as compared to the group with a low concentration of this steroid (–FF, –FF). The results suggested that FF and a high content of P4 display several positive effects on porcine oocyte IVM, and exert protection against oxidative stress while apoptosis induction in cumulus cells is strongly associated with the steroidogenic activity of these cells. It was also argued that P4 in FF has an important influence on the acquisition by oocytes of developmental competence. While the role of P4

production and expression of progesterone receptor were intensively investigated by several teams, another important factor is the progesterone receptor-membrane component (PGRMC1), associated with P4 action in various tissues. Luciano et al. (2010) examined the role of PGRMC1 expression in bovine oocytes and embryos in relation to embryo developmental competence. Using western-blot assays, they described the expression of this protein in germinal vesicle (GV)- and MII-stage oocytes as well as in zygotes and blastocysts. Moreover, a significant association was also found between PGRMC1 expression and male and female pronucleus formation. Confocal microscopic observation revealed differential PGRMC1 protein localization in various maturation stages of bovine oocytes. Taken together, these results indicate that P4, its receptor and the membrane component have an important impact on mammalian oocyte developmental competence as well as providing an important mechanism (such as apoptosis) for controlling the reproductive potency of mammals.

In other studies using the bovine blastocyst model, a differential effect of P4 on culture efficiency was observed. In addition, Larson et al. (2011), using bovine oocytes fertilized *in vitro*, investigated the influence of P4 in two experiments (LO, 1 ng/ml or HI, 100 ng/ml during either the first (Culture-1, Day 1 to 3) or the second (Culture-2, Day 4 to 7) phase of culture, on the *in vitro* developmental efficiency of the embryo, embryo metabolism or blastocyst cell number. They found no differences between cleaved oocytes, the percentage of cleaved embryos that developed to the morula or subsequent stages, the blastocyst, or subsequent stages or the hatched blastocyst stage, among the treatment groups. The quantities of glucose metabolized per blastocyst per hour did not differ among the groups, although the blastocysts from Culture-2 metabolised more glucose ($P = 0.03$), as compared to other groups. They also observed a correlation between the embryos that received low doses of progesterone and the ability to metabolize glucose. The number of cells per blastocyst in the control group was increased as compared to both treatments ($P = 0.039$). It was suggested that supplementation of the embryo culture with P4 did not improve the efficiency or quality of the obtained bovine blastocysts. However, the effect of P4 supplementation seems to be different depending on the stage of embryos development. It was recently reported that supplementation of the embryo culture system with P4 had a positive effect on the early stages of mammalian embryo development (Aparicio et al., 2011). In contrast to these results, Pereira et al. (2009) suggested that P4 may affect embryo *in vitro* culture. In these experiments presumptive zygotes were transferred into embryo growth medium supplemented in Experiment 1 with bovine oviduct epithelial cells (BOEC) ($n = 378$) and BOEC plus onapristone (OP), ($n = 325$); in Experiment 2 with granulosa cells (GC), ($n = 514$); GC plus OP ($n = 509$); BOEC ($n = 490$); BOEC plus P4 ($n = 500$); BOEC plus P4 and OP ($n = 502$). They found no differences between BOEC and BOECOP ($P > 0.05$) in relation to the development rates of embryos, quality or developmental stages. Supplementation with P4 negatively influenced embryo development rates in the BOEC system (BOECP4 = 16.8 ± 2.6% vs. BOEC = 23.7 ± 1.7%, $P = 0.02$). However, this effect was abolished when the OP was added into the culture system. It was suggested that supplementation of embryo cultures with high concentrations of P4 was not directly associated with embryo survival at early stages of development. However, it is also possible that P4 may act via several other mechanisms, which are related to the co-culture of embryos with other cell types.

3. Determination of the developmental competence of mammalian oocytes and embryos using microfluidics

In recent times, microfluidics has been frequently used in the chemical industry, to determine drug concentration and delivery, as well as to assess the danger of bioterrorist agents. In the past four years particularly there has been increased interest in microfluidics and the Lab-on-Chip systems in reproductive biology, and in the determination of gamete viability and gamete and embryo developmental competence (Szczepanska et al., 2009, 2010; Jaskowski, 2010; Walczak et al., 2010; Kempisty et al., 2011). The Lab-on-Chip system, used by Walczak et al. (2010) for the evaluation of bovine, pig, and canine oocytes and embryos consists of a silicon-glass sandwich with two glass optical fibres positioned "face-to-face". The dimensions of the microchannels are adjusted to the average size of an oocyte (110–140 μm). The total optical path length is 340 μm. The two optical fibres determine the "measurements chamber" within which the oocytes/embryo are located and

spectrophotometrically evaluated. The first optical fibre is aligned to the edge of the inlet microfluidical channel; the second fibre forms a "trap" for the oocytes/embryo, ensuring fluid flow but immobilizing the oocyte in the "measurement chamber". During the measurement light is transmitted from the source by the first optical fibre which passes through the culture medium, and is used to characterize the oocytes/embryo. It is then collected by another optical fibre, connected to the miniaturized spectrometer. After a short measurement (few seconds) the oocytes/embryo are flushed back for future treatment. The spectral characteristics of analyzed oocytes/embryos are recorded, normalized, and processed using Origin (USA) software. For each measurement, the spectral characteristics of the oocyte/embryo are standardized according to the culture media used for transferring the cells onto the chip.

Szczepanska et al. (2009) explored the possibility of using Lab-on-Chip systems to assess bovine and porcine oocyte potency. They introduced a new device, which assessed the characteristic microspectrophotometric features of mammalian gametes. The subsequent studies of this team have investigated the relationship between oocyte morphology as well as the follicular size and spectral characteristics of analyzed cells (Szczepanska et al., 2010; Walczak et al., 2010). Their results have shown that the morphology of porcine oocytes, according to the scale proposed by Jackowska et al. (2009), is highly associated with the spectral (spectral shift of analyzed cell) images of investigated cells. They conclude that the physical characterization of oocytes is strongly associated with their biological features and that the Lab-on-Chip system may be an important tool in the evaluation of the developmental competence of porcine and bovine oocytes. In similar experiments they focused on the non-invasiveness of this method, and analyzed embryo viability using the Lab-on-Chip system before transfer to recipients (pigs and cattle). They reported a of successful pregnancy rate of 50% in pigs and cattle after the Lab-on-Chip assessment (unpublished data). Thus, this method can be said to be non-invasive and has no influence on the ability of embryos to undergo successful implantation.

Although there are still comparatively few studies on the possibility of using the Lab-on-Chip device in reproductive biology, reports which describe its non-invasiveness and the possibility of using it for describing oocytes and the developmental competence of embryos mean that this number should rise in the near future.

4. Non-invasive methods for quality assessment of oocytes/embryos

The non-invasive assessment of the quality of oocytes/embryos is based on cellular, biochemical and metabolomical methods. Cellular methods of oocyte quality evaluation include assessment of first polar body quality, perivitelline space, *zona pellucida* structure, colour and granularity of the cytoplasm. It is well known that oocytes with clear, light cytoplasm manifest an increased ability to reach maturity *in vitro*, and to undergo subsequent fertilization and early stage embryo development (Montag et al., 2011). It has also been suggested that the structure of the *zona pellucida* is an important determinant of successful fertilization, especially in relation to the structure and diameter of the *zona pores*. Santos et al. (2008), et al., using scanning electron microscopy, investigated the developmental competence, *in vitro* fertilization outcome and incidence of polyspermy in bovine oocytes. They found that the diameter of *zona pores* had an important influence on the fertilization rate and was also associated with the developmental competence of oocytes. In addition, Antosik et al. (2010b), showed that the expression of the *zona pellucida* and integrin genes and proteins were associated with the morphological quality of porcine oocytes. Moreover, the morphology of porcine oocytes was associated with the differential distribution of *zona pellucida* protein 3 (ZP3) and integrin beta 2 (ITGB2) between the zona, membrane and oocyte cytoplasm.

The brilliant cresyl blue test (BCB) is the most frequently used test for biochemical evaluation of oocyte developmental competence, and is performed in several species of mammals, including bovine, pigs, goats and dogs (Catala et al., 2011). Using this test blue-stained oocytes are characterized by an increased developmental competence, whereas the unstained female gametes manifest lower competence. It was observed in several studies that blue-stained mammalian oocytes have an increased ability to mature, become fertilized and to undergo early embryo development (Silva et al., 2011). It was shown by Kempisty et al. (2011a) that the results of the BCB staining test had an impor-

tant influence on the fertilization ability of porcine oocytes. Moreover, they found that single or double exposure to the BCB test influenced the expression of zona genes as well as the distribution of zona proteins within the oocytes. However, Wongsrikeao et al. (2006) showed that double exposure to BCB test may exert a toxic effect on oocytes and leads to fertilization failure.

For a non-invasive assessment of oocytes/embryo quality, evaluation of small molecular weight substances (metabolites) in the culture media or follicular fluid is often performed. In addition, Bender et al. (2010) suggested that the metabolites present in the follicular fluid might be used as markers of oocyte maturation ability as well as of the subsequent embryo development. They investigated the metabolomic profile of follicular fluid obtained from the dominant follicle of lactating cows and heifers using gas chromatography mass spectrometry. They suggested that the follicular microenvironment in cows negatively influenced the developmental potential of oocytes as compared to heifers, which might have an important influence on fertilization ability and outcome in these animals. Low-molecular weight metabolites, as end products of regulatory, maturation and differentiation processes, reflect the ability of oocytes/embryos to grow and develop. Glycolytic intermediates and amino acids are often used as markers when follicular fluid or culture media metabolites are non-invasively tested to quantify oocyte and embryo metabolism. It was found that the assessment of these markers may be used as a useful predictor of pregnancy outcome following embryo transfer in mammals (Singh and Sinclair, 2007; Revelli et al., 2009; Royere et al., 2009). In conclusion, morphometry analyses and the assessment of metabolomic markers are at present the most frequently used methods to detrmine embryo viability.

5. Perspectives

Although in several species of mammals IVM efficiency is satisfactory, in others, such as dogs, this efficiency is still very low. Therefore, in these species IVM should be enhanced with new media supplements or new combinations of supplements to improve IVC systems. Some reports have reported that the application of EGF, BSA and progesterone increases IVM efficiency in several species of mammals; however, others publications, in turn, refute these results. Therefore, it seems reasonable to assume that these media supplements have effect IVM efficiency in a species-specific manner. Since the Lab-on-Chip system was used in reproductive biology for the first time, there is a growing requirement to prove its non-invasiveness and to determine its potential for assessing oocyte and embryo developmental competence. Moreover, we still await the birth of healthy offspring after the transfer of embryos originating from Lab-on-Chip assessment. In the last 20 years molecular methods appear to be the most objective and precise for assessing the developmental potential of oocytes/embryos but there is an increasing requirement new methods that will be objective and in addition, non-invasive. Therefore, for the evaluation of oocyte/embryo viability, metabolomic markers are often used. Moreover, the analysis of genetic and metabolomic markers is now of high significance. We suggest that only a combination of both these approaches with microfluidics may precisely determine (without destruction of the analyzed cell) the biological features of mammalian oocytes and embryos.

6. REFERENCES

Antosik P, Kempisty B, Jackowska M, Bukowska D, Wozna M, Lianeri M, Brussow KP, Jaskowski J (2010a): Dfferential expression of genes encoding EGF, IGF-I, GF β1, TGF β2 and TGF β3 in porcine endometrium uring estrus cycle at different ages. Medycyna Weterynaryjna 66, 618–621.

Antosik P, Kempisty B, Jackowska M, Bukowska D, Lianeri M, Brussow KP, Wozna M, Jaskowski J (2010b): The morphology of porcine oocytes is associated with zona pellucida glycoprotein 3 and integrin beta 2 protein levels. Veterinarni Medicina 55, 154–162.

Antosik P, Kempisty B, Jackowska M, Piotrowska H, Bukowska D, Wozna M, Lianeri M, Brussow KP, Jaskowski J (2010c): Assessment of transcript and protein levels contributing to cell cycle control and gap junction connections in morphologically variable groups of porcine cumulus-oocyte complexes. Veterinarni Medicina 55, 512–521.

Antosik P, Kempisty B, Jackowska M, Piotrowska H, Wozna M, Bukowska D, Bryja A, Lianeri M, Brussow KP, Jaskowski JM (2011): Are the levels of Cdk4 and Cx43 proteins of porcine oocytes associated with follicular size? Animal Biology 61, 211–224.

Aparicio IM, Garcia-Herreros M, O'Shea LC, Hensey C, Lonergan P, Fair T (2011): Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. Biology of Reproduction 84, 910–921.

Bender K, Walsh S, Evans AC, Fair T, Brennan L (2010): Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction 139, 1047–1055.

Bolamba D, Russ KD, Olson MA, Sandler JL, Durrant BS (2002): In vitro maturation of bitch oocytes from advanced preantral follicles in synthetic oviduct fluid medium: serum is not essential. Theriogenology 58, 1689–1703.

Bukowska D, Kempisty B, Antosik P, Jaskowski JM, Olechnowicz J (2008): Selected aspects of canine oocytes maturation, fertilization and embryo development in dogs (in Polish). Medycyna Weterynaryjna 64, 617–736.

Bukowska D, Kempisty B, Sikora J, Wozna M, Jackowska M, Zaorska K, Antosik P, Jaskowski JM (2011): Functional and molecular analysis of mammalian spermatozoa in the assesment of male fertility potential (in Polish). Medycyna Weterynaryjna 67, 29–33.

Catala MG, Izquierdo D, Uzbekova S, Morato R, Roura M, Romaguera R, Papillier P, Paramio T (2011): Brilliant Cresyl Blue (BCB) stain selects largest oocytes with highest mitochondria activity, MPF activity and embryo development competence in prepubertal sheep. Reproduction 10, doi: 10.1530/REP-10-0528.

Choi YH, Lee BC, Lim JM, Kang SK, Hwang WS (2002): Optimization of culture medium for cloned bovine embryos and its influence on pregnancy and delivery outcome. Theriogenology 58, 1187–1197.

Cui XS, Jin YX, Shen XH, Lee JY, Lee HS, Yin XJ, Kong IK, Kim NH (2006): Epidermal growth factor enhances meiotic resumption of canine oocytes in the presence of BSA. Theriogenology 66, 267–274.

Depa-Martynow M, Kempisty B, Lianeri M, Jagodzinski PP, Jedrzejczak P (2007): Association between fertilin beta, protamines 1 and 2 and spermatid-specific linker histone H1-like protein mRNA levels, fertilization ability of human spermatozoa, and quality of preimplantation embryos. Folia Histochemica Cytobiolgica 45, 79–85.

Dressing GE, Goldberg JE, Charles NJ, Schwertfeger KL, Lange CA (2010): Membrane progesterone receptor expression in mammalian tissues: a review of regulation and physiological implications. Steroids 76, 11–17.

England GC, Verstegen JP, Hewitt DA (2001): Pregnancy following in vitro fertilisation of canine oocytes. Veterinary Record 148, 20–22.

Gorska I, Kempisty B, Jagodzinski PP (2006): Epigenetic modification in germ line cells (in Polish). Ginekologia Praktyczna 3, 2–5.

Grupen CG, Armstrong DT (2010): Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. Reproduction Fertility and Development 22, 1100–1109.

Herrick JR, Behboodi E, Memili E, Blash S, Echelard Y, Krisher RL (2004): Effect of macromolecule supplementation during in vitro maturation of goat oocytes on developmental potential. Molecular Reproduction and Development 69, 338–346.

Jackowska M, Kempisty B, Antosik P, Bukowska D, Budna J, Lianeri M, Rosinska E, Wozna M, Jagodzinski PP, Jaskowski JM (2009): The morphology of porcine oocytes is associated with zona pellucida glycoprotein transcript contents. Reproductive Biology 9, 79–85.

Jaskowski JM, Kempisty B, Wozna M, Walczak R, Szczepanska P, Dziuban J, Antosik P (2010): Selected methods of developmental competence and selection of bosine oocytem and embryos (in Polish). Medycyna Weterynaryjna 66, 740–744.

Jedrzejczak P, Kempisty B, Bryja A, Mostkowska M, Depa-Martynow M, Pawlaczyk L, Jagodzinski PP (2007): Qantitative assessment of transition proteins 1, 2 spermatid-specific linker histone H1-like protein transcripts in spermatozoa from normozoospermic and asthenozoospermic men. Archives of Andrology 53, 199–205.

Kempisty B, Jedrzejczak P, Jagodzinski PP (2006): Structure and role of protamines 1 and 2 in spermatogenesis and male infertility. Ginekologia Polska 77, 238–245.

Kempisty B, Depa-Martynow M, Lianeri M, Jedrzejczak P, Darul-Wasowicz A, Jagodzinski PP (2007): Evaluation of protamines 1 and 2 transcript contents in spermatozoa from asthenozoospermic men. Folia Histochemica Cytobiologica 45, 109–113.

Kempisty B, Antosik P, Bukowska D, Jackowska M, Lianeri M, Jaskowski JM, Jagodzinski PP (2008): Analysis of selected transcript levels in porcine spermatozoa, oocytes, zygotes and two-cell stage embryos. Reproduction Fertility and Development 20, 513–518.

Kempisty B, Antosik P, Bukowska D, Jackowska M, Lianeri M, Jaskowski JM, Jagodzinski PP (2009): Assessment of zona pellucida glycoprotein and integrin transcript contents in porcine oocytes. Reproductive Biology 9, 71–78.

Kempisty B, Jackowska M, Piotrowska H, Antosik P, Wozna M, Bukowska D, Brussow KP, Jaskowski JM (2011a): Zona pellucida glycoprotein 3 (pZP3) and

integrin β2 (ITGB2) mRNA and protein expression in porcine oocytes after single and double exposure to brilliant cresyl blue test. Theriogenology 75, 1525–1535.

Kempisty B, Walczak R, Antosik P, Szczepańska P, Wozna M, Bukowska D, Zaorska K, Dziuban J, Jaskowski J.M (2011b): Mammalian oocyted nad embryos developmental competence evaluation systems based on the microfluidic technology Lab-on-Chip (in Polish). Medycyna Weterynaryjna 67, 25–28.

Kim MK, Fibrianto YH, Oh HJ, Jang G, Kim HJ, Lee KS, Kang SK, Lee BC, Hwang WS (2005): Effects of estradiol-17beta and progesterone supplementation on in vitro nuclear maturation of canine oocytes. Theriogenology 63, 1342–1353.

Larson JE, Krisher RL, Lamb GC (2011): Effects of supplemental progesterone on the development, metabolism and blastocyst cell number of bovine embryos produced in vitro. **Reproduction, Fertility, and Development** 23, 311–318.

Li L, Zheng P, Dean J (2010): Maternal control of early mouse development. Development 6, 859–870.

Lott W, Anchamparuthy V, McGilliard M, Mullarky I, Gwazdauskas F (2010): Influence of cysteine in conjunction with growth factors on the development of in vitro-produced bovine embryos. Reproduction in Domestical Animals 46, 585–594. doi: 10.1111/j.1439-0531.2010.01706.x.

Luciano AM, Lodde V, Franciosi F, Ceciliani F, Peluso JJ (2010): Progesterone receptor membrane component 1 expression and putative function in bovine oocyte maturation, fertilization, and early embryonic development. Reproduction 140, 663–672.

Montag M, Liebenthron J, Koster M (2011): Which morphological scoring system is relevant in human embryo development? Placenta 32, 252–256. doi:10.1016/j.placenta.2011.07.009.

Mrazek M, Fulka J (2003): Failure of oocytematuration: possible mechanisms for oocyte maturation arrest. Human Reproduction 18, 2249–2252.

Nemcova L, Machatkova M, Hanzalova K, Horakova J, Kanka J (2006): Gene expression in bovine embryos derived from oocytes with different developmental competence collected at the defined follicular developmental stage. Theriogenology 65, 1254–1264.

Nickson DA, Boyd JS, Eckersall PD, Ferguson JM, Harvey MJA, Renton JP (1993): Molecular biological methods for monitoring oocyte maturation and in vitro fertilization in bitches. Journal of Reproduction and Fertility 47, 231–240.

Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA (2004): Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 429, 154.

Otoi T, Yamamoto K, Horikita N, Tachikawa S, Suzuki T (1999): Relationship between dead cells and DNA fragmentation in bovine embryos produced in vitro and stored at 4 degrees C. Molecular Reproduction and Development 54, 342–347.

Otoi T, Murakami M, Fujii M, Tanaka M, Ooka A, Une S, Suzuki T (2000): Development of canine oocytes matured and fertilised in vitro. Veterinary Record 146, 52–53.

Pereira RM, Mesquita P, Batista M, Baptista MC, Barbas JP, Pimenta J, Santos IC, Marques MR, Vasques MI, Silva Pereira M, Santos Silva F, Oliveira Sousa MC, Fontes CM, Horta AE, Prates JA, Marques CC (2009): Doppel gene polymorphisms in Portuguese sheep breeds: insights on ram fertility. Animal Reproduction Science 114, 157–166.

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P (2009): Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reproductive Biology and Endocrinology 7, 40.

Royere D, Feuerstein P, Cadoret V, Puard V, Uzbekova S, Dalbies-Tran R, Teusan R, Houlgatte R, Labas V, Guerif F (2009):Non invasive assessment of embryo quality: proteomics, metabolomics and oocyte-cumulus dialogue. Gynecologie, Obstetrique and Fertilite 37, 917–920.

Santos P, Chaveiro A, Simoes N, Moreira da Silva F (2008): Bovine oocyte quality in relation to ultrastructural characteristics of zona pellucida, polyspermic penetration and developmental competence. **Reproduction in Domestical Animals** 43, 685–689.

Shimizu T, Krebs S, Bauersachs S, Blum H, Wolf E, Miyamoto A (2010): Actions and interactions of progesterone and estrogen on transcriptome profiles of the bovine endometrium. Physiological Genomics 42, 290–300.

Sikora J, Kempisty B, Jędrzejczak P, Jagodzinski PP (2006): Role of DNA damage in spermatozoa in assessment of ability of sperm to fertilization. Przeglad Lekarski 63, 800–802.

Silva DS, Rodriguez P, Galuppo A, Arruda NS, Rodrigues JL (2011): Selection of bovine oocytes by brilliant cresyl blue staining: effect on meiosis progression, organelle distribution and embryo development. Zygote 40, doi: 10.1017/S0967199411000487.

Singh R, Sinclair KD (2007): Metabolomics: approaches to assessing oocyte and embryo quality. Theriogenology 68, 56–62.

Sirisathien S, Brackett BG (2003): TUNEL analyses of bovine blastocysts after culture with EGF and IGF-I. **Molecular Reproduction and Development** 65, 51–56.

Song HJ, Kang EJ, Maeng GH, Ock SA, Lee SL, Yoo JG, Jeon BG, Rho GJ (2010): Influence of epidermal growth

factor supplementation during in vitro maturation on nuclear status and gene expression of canine oocytes. Research in Veterinary Science 50, doi: 10.1016/j.rwsc.2010.09.003.

Szczepanska P, Walczak R, Dziuban J, Jackowska M, Kempisty B, Jaskowski JM, Bargiel S (2009): Lab-on-chip quality classification of porcine/bovine oocytes. Procedia Chemistry 1, 341–344.

Szczepanska P, Walczak R, Dziuban J, Kempisty B, Jackowska M, Antosik P, Jaskowski J, Bargiel S (2010): Quality assessment of mammalian germ cells by using lab-chip microcytometer (in Polish). Elektronika 6, 93–96.

Tadros W, Lipshitz HD (2009): The maternal-to-zygotic transition: a play in two acts. Development 18, 3033–3042.

Tharasanit T, Colleoni S, Galli C, Colenbrander B, Stout TA (2009): Protective effects of the cumulus-corona radiata complex during vitrification of horse oocytes. Reproduction 137, 391–401.

Tkachenko OY, Delimitreva S, Isachenko E, Valle RR, Michelmann HW, Berenson A, Nayudu PL (2010): Epidermal growth factor effects on marmoset monkey (Callithrix jacchus) oocyte in vitro maturation, IVF and embryo development are altered by gonadotrophin concentration during oocyte maturation. Human Reproduction 25, 2047–2058.

Uhm SJ, Gupta MK, Yang JH, Chung HJ, Min TS, Lee HT (2010): Epidermal growth factor can be used in lieu of follicle-stimulating hormone for nuclear maturation of porcine oocytes in vitro. Theriogenology 73, 1024–1036.

Varga S, Diez C, Fernandez L, Alvarez J, Katchicualula A, Hidalgo CO, Tamargo C, Carbajo M (2011): Culture system and long-term storage of culture media in the in vitro production of bovine embryos. Acta Vetrinaria Hungarica 59, 129–139.

Vastenhouw NL, Zhang Y, Woods IG, Imam F, Regev A, Liu XS, Rinn J, Schier AF (2010): Chromatin signature of embryonic pluripotency is established during genome activation. Nature 464, 922–926.

Walczak R, Szczepanska P, Dziuban J, Kempisty B, Jackowska M, Antosik P, Jaskowski J, Chelmonska-Soyta A (2010): Lab-on-a-chip for developmental competence assessment of bovine oocytes. Elektronika 11, 30–33.

Wen X, Li D, Tozer AJ, Docherty SM, Iles RK (2010): Estradiol, progesterone, testosterone profiles in human follicular fluid and cultured granulosa cells from luteinized pre-ovulatory follicles. Reproductive Biology and Endocrinology 8, 117.

Willingham-Rocky LA, Hinrichs K, Westhusin ME, Kraemer DC (2003): Effects of stage of oestrous cycle and progesterone supplementation during culture on maturation of canine oocytes in vitro. Reproduction 126, 501–508.

Wongsrikeao P, Otoi T, Yamasaki H, Agung B, Taniguchi M, Naoi H, Shimizu R, Nagai T (2006): Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for in vitro production of embryos. Theriogenology 66, 366–372.

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