CUMULUS CELL EXPANSION, ITS ROLE IN OOCYTE BIOLOGY AND PERSPECTIVES OF MEASUREMENT: A REVIEW*

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Cumulus expansion of the cumulus-oocyte complex is necessary for meiotic maturation and acquiring developmental competence. Cumulus expansion is based on extracellular matrix synthesis by cumulus cells. Hyaluronic acid is the most abundant component of this extracellular matrix. Cumulus expansion takes place during meiotic oocyte maturation under *in vivo* and *in vitro* conditions. Quantification and measurement of cumulus expansion intensity is one possible method of determining oocyte quality and optimizing conditions for *in vitro* cultivation. Currently, subjective methods of expanded area and more exact cumulus expansion measurement by hyaluronic acid assessment are available. Among the methods of hyaluronic acid measurement is the use of radioactively labelled synthesis precursors. Alternatively, immunological and analytical methods, including enzyme-linked immunosorbent assay (ELISA), spectrophotometry, and high-performance liquid chromatography (HPLC) in UV light, could be utilized. The high sensitivity of these methods could provide a precise analysis of cumulus expansion without the use of radioisotopes. Therefore, the aim of this review is to summarize and compare available approaches of cumulus expansion measurement, respecting special biological features of expanded cumuli, and to suggest possible solutions for exact cumulus expansion analysis.

oocyte; cumulus-oocyte complex; cumulus expansion; hyaluronic acid; spectrophotometry; high-performance liquid chromatography

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INTRODUCTION

Reproductive biotechnologies are a dynamically developing discipline of farm animal breeding. Their progress depends on a sufficient quantity of quality oocytes useful for various procedures, such as *in vitro* fertilization, cloning by somatic cell nuclear transfer and transgenesis. The quality of oocytes is determined by the completion of cytoplasmic maturation and the acquisition of developmental competence. In these processes, cell communication between oocyte and surrounding cumulus cells, and cumulus expansion are required (D e k e l et al., 1979; E p p i g , 1979).

The cumulus cells with adjacent extracellular matrix constitute cumulus, an essential component of cumulus-oocyte complexes (COCs). Cumulus surrounds the oocyte during growth and consecutive meiotic maturation of the oocyte, ovulation, fertilization, and early embryonic development. During meiotic maturation of the oocyte, cumulus cells change their morphology and metabolic activity. Thus, cumulus cells significantly influence oocyte maturation and

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developmental competence acquisition (Sutovsky et al., 1994; Procházka et al., 2000; Qian et al., 2003; Karja, 2008; Ju, Rui, 2012; Auclair et al., 2013). In addition to cross-talk between cumulus cells and oocyte, the cumulus cells are important in the synthesis of a large quantity of extracellular matrix occurring in the enlargement of COCs. This phenomenon is known as cumulus expansion. Cumulus expansion takes place in the follicle shortly before ovulation *in vivo,* as well as during meiotic maturation *in vitro* (Eppig, 1979; Dekel, Beers, 1980; Salustri et al., 1989; Chen et al., 1993).

Cumulus expansion is based on synthesis of glycosaminoglycan rich in hyaluronic acid (HA) into the extracellular space, where it plays a role as the structural component of expanded cumuli and signal molecule regulating oocyte maturation. A sufficient number of cumulus cell layers and adequate HA production followed by cumulus expansion are essential for successful oocyte maturation, fertilization, and early embryonic development (Chen et al., 1993; Tirone et al., 1993; Kimura et al., 2002; Han et al., 2006; Yokoo et al., 2010). Accordingly, the quality of cumuli and cumulus expansion are important markers of the COCs' quality necessary for reproductive biotechnologies (Han et al., 2006).

There are several methods of cumulus expansion measurement. Among these, non-invasive measurement by image analysis of expanded cumuli includes some disadvantages, such as the subjectivity of measurement and inability of three-dimensional structure inclusion. HA quantity measurement is the more exact invasive method of cumulus expansion quantification and measurement. Recently, HA measurement has been based on radioactive labelling of the precursor of HA synthesis (Eppig, 1980; Fagbohun, Downs, 1990; Daen et al., 1994; Nagyova et al., 1999). Alternatively, immunochemical and analytical methods are available for HA measurement (Kongtawelert, Ghosh, 1990; Volpi, 2000; Chen et al., 2005). The development of HA measurement could provide a more accurate study of cumulus expansion during oocyte maturation and qualified evaluation of the quality of COCs used for biotechnologies.

Chemistry of cumulus expansion

Cumulus cells and extracellular matrix synthesis

In the prenatal stage of ontogenesis, primordial oocytes are surrounded by one layer of follicular cells. Together with meiotic division of follicular cells and follicle growth, differentiation of the cells to mural granulosa cells and cumulus cells of cumulus oophorus occurs. Cumulus cells surround the oocyte and include corona radiata cells. Corona radiata mediates contact with the oocyte by cell connection type gap junction on corona radiata cell spurs permeating throughout the *zona pellucida* (Brower, Schultz, 1982; Wassarman, 1988; Morbeck et al., 1992). Individual oocytes and adjacent cumulus cells form cumulus–oocyte complexes (COCs).

Simultaneously with follicle growth, *in vivo* oocyte growth takes place inside the follicle. On pig ovaries, only follicles with diameter >3 mm include fully grown oocytes capable of reinitiating and successful completing meiotic maturation (Marchal et al., 2002). Subsequently, with oocyte meiotic maturation within the follicle, cumulus cells synthesize structural components of extracellular matrix and the enlargement of COCs occurs. The COC extracellular matrix increases and formation during oocyte maturation has been described as mucification or cumulus expansion (Dekel et al., 1979; Eppig, 1979). Cumulus expansion occurs immediately before ovulation *in vivo*, as well as during maturation of COCs *in vitro*.

Cumulus expansion consists of the synthesis of the extracellular mass composed of proteoglycans. Proteoglycans are created by cumulus cell membrane proteins and glycosaminoglycans (GAGs). In animal tissues, hyaluronic acid (HA), chondroitin sulfate (ChS), keratan sulphate, and heparan sulfate are common GAGs (Murray Jr. et al., 2003). The HA is the most abundant compound in the extracellular matrix of expanded cumuli (Chen et al., 1990; Nakayama et al., 1996; Tirone et al., 1998).

Hyaluronic acid

Hyaluronic acid is an abundant biopolymer of the GAGs family. The β-(1-4)-glucuronic acid and β-(1-3)-N-acetylglucosamine are heterodimer components of HA (Murray Jr. et al., 2003). The heterodimer is generally repeated 10–10 000× and the final molecular weight of HA is usually 400 kDa in bovine eye vitreous, to 1400 kDa in poultry comb (Shiedlin et al., 2004). A large quantity of (-OH) groups are capable of binding many water molecules between HA chains (Murray Jr. et al., 2003). Sufficient quantity of HA synthesis precursors is a limiting factor of cumulus expansion. Their suboptimal quantity *in vitro* can create differences in cumulus expansion intensity between in vitro and in vivo conditions (Chen et al., 1990).

In many tissues, HA is extensively secreted into the extracellular matrix. Fibroblasts and other connective tissue cells are highly active in HA synthesis (Moscatelli, Rubin, 1974). Therefore, HA is ubiquitous polymer involved in joint structure and functions (Swann et al., 1974), HA is widespread in eye vitreous (Mizuno et al., 1991), umbilical cord (Lago et al., 2005), and embryonic tissues (Vabres, 2010). HA plays the role of lubricant responsible for tissue elasticity and compressibility (Swann et al., 1974). Moreover, HA participates in embryonic morphogenesis by allowing the migration of embryonic cells (Vabres, 2010). Hyaluronic acid is an impor-

tant component in wound healing (Prosdocimi, Bevilacqua, 2012). The HA abundance in an organism is the cause of releasing body fluids, such as pleural fluid (Jardillier, 1972), synovial fluid (Swann et al., 1974), urine, and blood (Morse, Nussbaum, 1967). The HA content in some fluids can be a useful marker of arthritis (Engstrom-Laurent, Hallgren, 1985) and liver cirrhosis (Nyberg et al., 1988). During *in vitro* cultivation of cells, HA is released into the surrounding environment such as culture medium (Johnsson et al., 1997; Chockalingam et al., 2004).

In addition, HA plays an important role in the reproductive physiology as a structural and signal molecule. HA participates in the regulation of oocyte meiotic maturation (Yokoo et al., 2010), cumulus expansion (Yokoo et al., 2003), ovulation and fertilisation of the oocyte (Chen et al., 1993), early embryonic development (Furnus et al., 2003), embryo nidation in uterine mucous membrane (Parikh et al., 2006), and foetal morphogenesis (Vabres, 2010). Moreover, the beneficial and protective effect of added HA on *in vitro* oocyte culture has been determined (Sato et al., 1987).

The HA synthesis is enzymatically catalyzed by hyaluronan-synthase (HAS) isoforms. In bovine and porcine COCs, HAS2 is necessary for cumulus expansion and its expression is regulated by gonadotropins (Kimura et al., 2002; Schoenfelder, Einspanier, 2003; Nagyova et al., 2012). The mRNA HAS3 was described in matured oocytes (Kimura et al., 2002), where HA is secreted into the perivitelline space between oolema and *zona pellucida* by the matured oocyte (Ueno et al., 2009).

Cumulus expansion and meiotic maturation

Endocrine and paracrine regulation of cumulus expansion

Endocrine regulation of cumulus expansion is based on the effect of gonadotropins, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), prostaglandin E2 (PGE2) secretion and steroidogenesis including progesterone production (Dekel et al., 1979; Eppig, 1981; Schuetz, Dubin, 1981; Phillips, Dekel, 1982). These hormones influence the metabolism of granulosa cells (Wassarman, 1988), which subsequently participate in the paracrine regulation of cumulus expansion within the follicle (Motlik et al., 1998a) (see Fig. 1.).

In paracrine regulation, the follicular fluid generated by secretion of follicular cells and substance infiltration from blood plasma during folliculogenesis, significantly contributes to cumulus expansion (Nakayama et al., 1996). Likewise, foetal bovine serum has a stimulatory effect on cumulus expansion and the chemical similarity to follicular fluid could be the explanation for this phenomenon (Eppig, 1980).

Production of the so-called cumulus expansion enabling factors (CEEFs) in granulosa and cumulus cells has been described (Motlik et al., 1998a; Prochazka et al., 1998). Whereas CEEF secre-

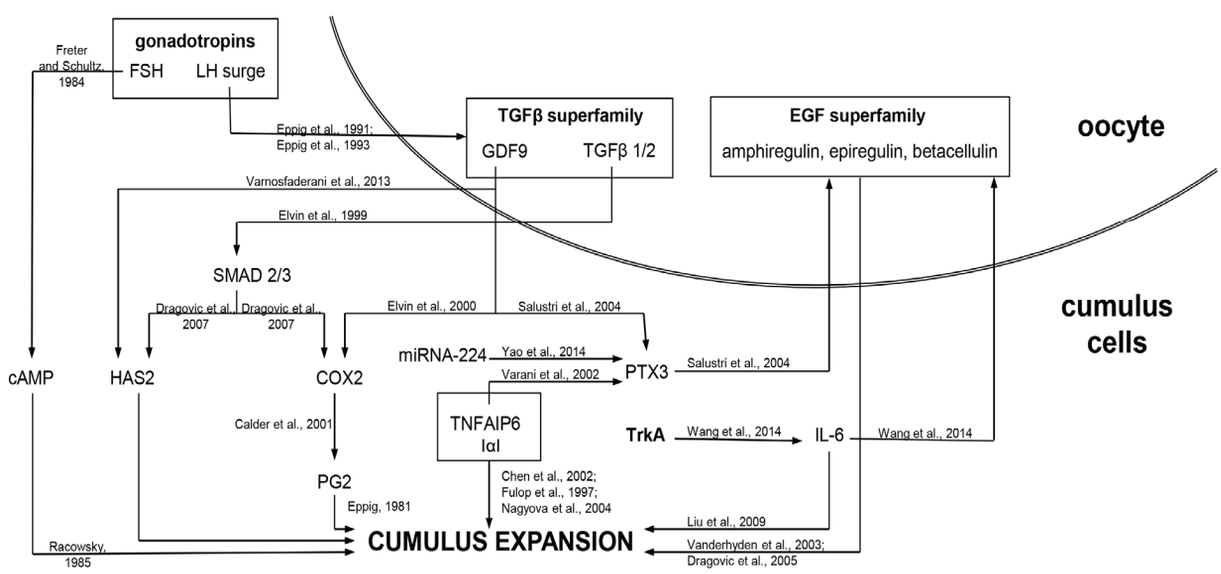

Fig. 1. Some of signalling pathways involved in cumulus expansion. Cross-talk between cumulus cells and oocyte leads to adequate hyaluronic acid production and cumulus expansion.

tion by granulosa cells of the fully grown follicle is strictly dependent on gonadotropin control, CEEF secretion by cumulus cells does not require hormonal action and paracrine signalling is sufficient (Zhang et al., 2008). Presumably, CEEF action is linked to the inability of the complete cumulus expansion of COCs with meiotic incompetent oocytes (Tirone et al., 1993). Although CEEF production by oocyte and cumulus cells of incompetent COCs is sufficient, the incompetent COCs lack the ability of reaction to paracrine regulation by CEEFs (Zhang et al., 2008). Many CEEFs have more exactly been defined as growth factors produced by cumulus cells as well as by oocyte.

The presence of growth factors in follicular fluid and positively modulating cumulus expansion are known as the epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) (Nagyova et al., 1999, 2012; Jezova et al., 2001; Nemcova et al., 2007). In addition to EGF and IGF-I, members of the transforming growth factor β (TGFβ) superfamily, including TGFβ1, TFGβ2, and growth differention factor 9 (GDF9), are required in gonadotropin-induced cumulus expansion (Vanderhyden et al., 2003; Dragovic et al., 2005). Subsequently, Drosophila mothers against decapentaplegic protein 2/3 (SMAD2/3) signal pathway is activated and the expression of important factors, such as HAS2 or PGE2 synthase, is triggered (Dragovic et al., 2007).

Another paracrine factor regulating cumulus expansion is interleukin-6 (IL-6). The IL-6 binding to its soluble receptor in cells induces genes with an impact on cumulus expansion, throughout the key factors of oocyte maturation, such as mitogen activated protein kinases (MAPK) and Akt kinase (Liu et al., 2009). The role of IL-6 in cumulus expansion is modulated by tyrosine kinase receptor A (TrkA), which is required for gonadotropin-induced follicle development (Wang et al., 201).

Members of hyaluronic acid binding proteins (HABPs) have been indicated as substantial factors contributing to extracellular matrix formation in expanded cumuli. During cumulus expansion, inter-α-trypsin inhibitor (IαI) and tumour necrosis factor α-induced protein 6 (TNFAIP6) are responsible for extracellular matrix formation of expanded cumuli by the stabilization of HA chains (Chen et al., 1992; Fulop et al., 1997; Nagyova et al., 2004, 2012). In expanded cumuli formation, the proteolytical degradation of IαI throughout the ubiquitin-proteasomal system is substantially required (Yi et al., 2008). Cleavage of IαI to heavy chains HC1 and HC2 is necessary for HCs' binding to HA and for the stabilization of expanded cumuli (Nagyova et al., 2004). Thus, proteasome inhibition causes complete suppression of cumulus expansion in porcine COCs (Nagyova et al., 2012).

The interaction of TNFAIP6 with HA chains is essential for the further stabilization of expanded cumuli. This binding depends on TNFAIP6 interaction with pentraxin (PTX3), upregulated by GDF9 and produced during cumulus expansion into extracellular matrix (Varani et al., 2002; Salustri et al., 2004). Although the PTX3 does not influence HA production, it is necessary just as a further structural constituent of expanded cumuli (Salustri et al., 2004). In addition, endogenous small non-coding RNAs of protein importance (called microRNAs (miRNA)) are involved in expansion regulation. The cross-talk between *Ptx3* gene and miRNA-224 as negative regulator of cumulus expansion has been described (Yao et al., 2014).

Gasotransmitters, such as nitric oxide, hydrogen sulphide, and carbon monoxide (summarized by Smelcova, Tichovska, 2011) have been intensively studied factors of reproductive processes, including those in meiotic maturation and cumulus expansion. It has been observed that nitric oxide synthase (NOS) and nitric oxide presence as well as hydrogen sulphide in porcine COCs are important for successful oocyte maturation and cumulus expansion (Tao et al., 2005; Chmelikova et al., 2010; Amale et al., 2011; Nevoral et al., 2014). Some selected signal pathways involved in cumulus expansion regulation are shown in Fig. 1.

The concentration and proportion of the regulatory factors mentioned above are responsible for various effects of different weight fractions of follicular fluid and follicular fluid from different sizes of follicle on cumulus expansion intensity (Daen et al., 1994; Dostal, Pavlok, 1996; Qian et al., 2003). The possible reason for the different action of various sizes of follicle on cumulus expansion could be the decreasing concentration of inhibitory factors in the growing follicle (Yang et al., 1993; Dostal, Pavlok, 1996; Nandi et al., 2007). Simultaneously, the accumulation of steroid hormones (Dode, Graves, 2002) and the secretion of oocyte maturation stimulatory factors (Yoshida et al., 1992) as growth factors (Ding, Foxcroft, 1994) or amino acids (Hong et al., 2004) and electrolytes (Iwata et al., 2004) take place during follicle growth.

Role of cumulus expansion in regulation of oocyte maturation

Whereas the meiosis of dictyate fully grown oocyte in first meiotic arrest is reinitiated by hormonal stimulation in vivo, it is possible to evoke meiotic maturation by gonadotropins, serum proteins, and growth factors also under in vitro conditions (Singh et al., 1997; Uhm et al., 1998). Accordingly, germinal vesicle breakdown (GVBD) occurrs and oocyte maturation is completed by achievement of the second meiotic metaphase, where meiosis is spontaneously blocked. The mature oocyte after ovulation in vivo or in vitro cultivation is predetermined for fertilization

(Motlik, Fulka, 1976; Yanagimachi, 1988; Motlik, Kubelka, 1990).

Cumulus cells are necessary for meiotic and developmental competence acquisition during oocyte growth and maturation (Chesnel et al., 1994; Qian et al., 2003; Han et al., 2006; Auclair et al., 2013). The number of cumulus cell layers, cumuli quality, and cumulus expansion intensity are decisive in the success of oocyte maturation *in vitro* and required for viability of matured and fertilized oocytes (Procházka et al., 2000; Qian et al., 2003; Karja, 2008; Ju, Rui, 2012). Thus, only oocytes with an intact cover of cumulus cells in a sufficient number of layers are used for *in vitro* maturation.

Cumulus cells communicate with each other and with the oocyte by gap junctions (Gilula et al., 1978). Gap junctions facilitate the passage of molecules smaller than 1 kDa including molecules of second messengers, such as cyclic adenosine 3',5'-monophosphate (cAMP), cyclic guanosine 3',5'-monophosphate (cGMP), and Ca^{2+} ion, produced in cumulus cells. These messengers significantly inhibit key protein kinases of oocyte maturation – M-phase promoting factor (MPF) and the above mentioned MAPK, and thus GVBD and subsequent oocyte maturation (Moor et al., 1980; Dekel et al., 1988; Tornell et al., 1990; Caroll et al., 1994; Machaty et al., 1997). After the action of gonadotropin and growth factors, disruption and endocytosis of gap junction proteins and enlargement of expanding cumulus result in the prevention of cAMP/cGMP flow into oocyte (Dekel et al., 1981; Tornell et al., 1984; Chen et al., 1990; Tatemoto, Terada, 1998; Motlik et al., 1998b) (Fig. 2). Cumulus enlargement is based in particular on HA production by cumulus cells and its accumulation in the extracellular space (Chen et al., 1990, 1993; Nakayama et al., 1996; Tirone et al., 1998). In addition to mechanical action, HA has also been described as a signal molecule and ligand.

Widespread HA receptors participating in glycoprotein creation are proteins from the above-mentioned group of HABPs. As one of the HABP members, CD44 receptor, is localized in the cytoplasmic membrane of cumulus cells and oocyte (Kimura et al., 2002; Yokoo et al., 2002). Gonadotropin-induced CD44 receptor synthesis occurs intensively during oocyte maturation (Yokoo et al., 2002; Schoenfelder, Einspanier, 2003). Subsequently, the CD44 receptor becomes active by binding HA and takes part in communication between oocyte and cumulus cells (Kimura et al., 2002). Yokoo et al. (2007) described the necessity of CD44 for meiotic maturation of porcine oocytes. Active CD44 participates in phosphorylation of gap junction proteins, cell connection closing, and disruption of cAMP flow from cumulus cells to oocyte (Yokoo et al., 2010) (Fig. 2). The CD44 receptor activated by HA regulates cumulus expansion and participates in auto-acceleration and amplification

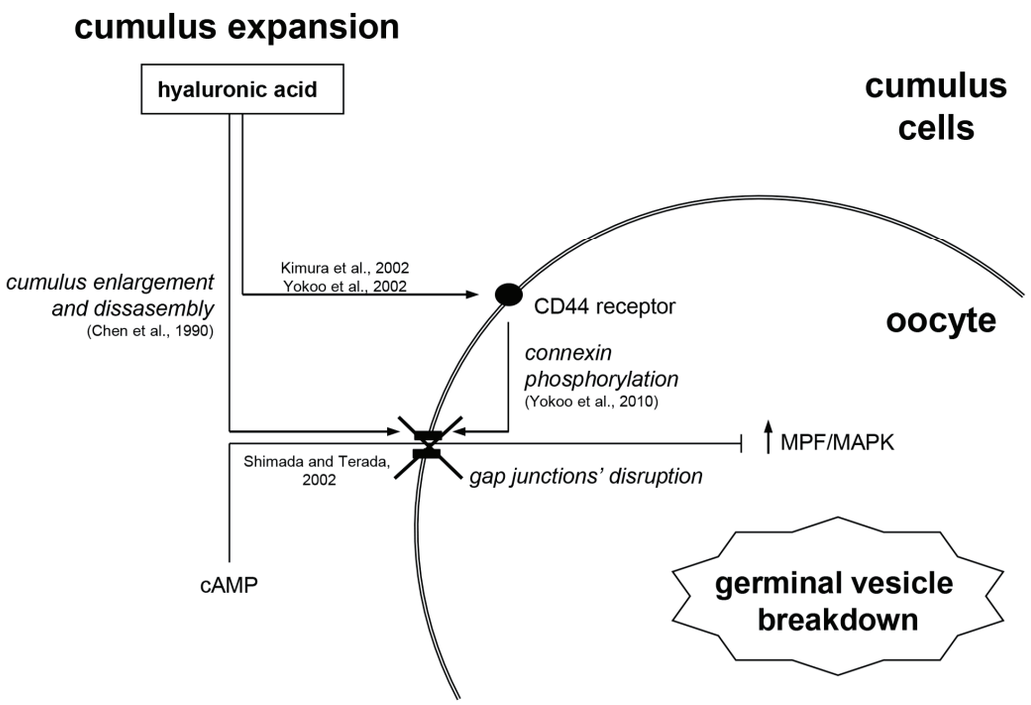

Fig. 2. Impact of cumulus expansion for trigger of germinal vesicle breakdown and re-initiation of oocyte maturation in pigs.

(Yokoo et al., 2003). However, CD44 activation is not strictly necessary for cumulus expansion (Yokoo et al., 2007). On the other hand, insufficient interaction of CD44 with HA during *in vitro* culture of COCs leads to unsuccessful oocyte maturation, fertilization, and early embryonic development (Yokoo et al., 2007). Suppression of CD44 and HA binding can be caused by glycosylation of CD44 extracellular domain by sialic acid (Bartolazzi et al., 1996). The other HA receptors are ion channels of *Xenopus laevis* oocytes (Fraser, 1997). Besides binding HABPs, HA can participate in meiotic maturation by regulation of ion flow and membrane potential (Fraser, 1997).

Hyaluronic acid, not only the most abundant factor produced by cumuli, is involved in the regulation of oocyte maturation. Expression of some candidate biomarkers in cumulus has been selected for non-direct oocyte quality evaluation (summarized by Assou et al., 2010). Among them in cumuli, inducible nitric oxide synthase (iNOS) and heme oxygenase 1 (HO-1), factors positively correlated with cell stress, are considered as a negative marker of developmental competence acquired during oocyte maturation (Bergandi et al., 2014). On the other hand, PTX3, angiogenin (ANG) and regulator of G-protein signalling 2 (RGS2) are highly expressed in oocytes with significantly better developmental competence (Feuerstein et al., 2012). Expression of the mentioned GDF9, HAS2, SMAD2/3, cyclooxygenase 2 (COX2), PTX3, and TNFAIP6 in cumulus cells is linked with improved embryonic development through adequate cumulus expansion (Elvin et al., 1999, 2000; Pangas et al., 2002) (Fig. 1). There is possibly a method of utilizing an expression profile of defined developmental competence biomarkers as an additional tool coupled with cumulus expansion measurement.

Significance of oocyte in cumulus expansion regulation

The oocyte participates in the regulation of cumulus expansion (Dekel, Beers, 1980; Salustri et al., 1989). Only the fully grown oocyte is capable of adequate stimulation of cumulus expansion (Tirone et al., 1993). During oocyte maturation, the oocyte produces the above-mentioned CEEFs (Eppig et al., 1993). Oocyte activity in paracrine factor secretion, including CEEFs, decreases during meiotic maturation, especially after metaphase I achievement (Nagyova et al., 2000). The role of the oocyte in regulation of cumulus expansion differs, depending on animal species (Vanderhyden, 1993). While CEEFs' production by the oocyte is necessary for cumulus expansion for mice COCs (Eppig et al., 1993), cumulus expansion of porcine COCs is independent of CEEF secretion by the oocyte (Prochazka et al., 1998). Oocyte removal by oocytectomy and *in vitro* cultivation of oocytectomized complexes have demonstrated the cumulus cell ability of CEEF production compensating for the removed oocyte (Prochazka et al., 1991; Motlik et al., 1998a). Nevertheless, cumulus expansion can be slightly inhibited in porcine oocytectomised complexes (Nakayama et al., 1996; Kimura et al., 2002). The oocyte presence influences the extracellular matrix composition of expanded cumuli: the oocyte determines the intensity of HA synthesis, but does not involve ChS quantity (Nakayama et al., 1996).

Cumulus expansion and oocyte developmental competence

Cumulus expansion and sufficient GAG synthesis by COC in the follicle are necessary for ovulation and the increased probability of enlarged COC interception by the infundibulum (Chen et al., 1993). Expanded cumuli of ovulated COCs play an important role in polyspermy prevention by selective barrier establishment. Moreover, cumulus expansion and HA synthesis are essential for sperm selection during fertilization (Tesařík, Kopecný, 1986). Only fully capacitated sperm with complete enzymatic equipment, including hyaluronidase from sperm acrosome digesting HA polymers, are capable of passing through expanded cumuli (Dunbar et al., 1976).

Another polyspermy prevention is the enlargement of the perivitelline space between oocyte and *zona pellucida* immediately after sperm penetration. The HA synthesis by oocyte and water molecule binding by HA is the reason for perivitelline space enlargement (Talbot, Dandekar, 2003). It was found that HA production by the oocyte without the need for cumulus cell synthesis causes perivitelline space enlargement. The inhibition of GAGs' synthesis in oocytes increases polyspermy incidence as response to the HA quantity decrease in the perivitelline space (Flechon et al., 2003; Ueno et al., 2009).

Cumulus expansion and HA synthesis in COCs during oocyte maturation are an important condition for the fertilization capability and developmental competence acquisition by the oocyte (Han et al., 2006). Initial cumuli quality and size expressed by a number of cumulus cell layers are in positive correlation with the developmental competence of GV-oocytes. Although a smaller number of cumulus cell layers is not strictly limiting for oocyte maturation, it unfavourably influences early embryonic development, in comparison with a high quality cumuli cover of oocytes (Schoevers et al., 2007). It is possible to compensate for the insufficient cumuli of matured oocytes by co-culture with intact COCs during *in vitro* maturation (Luciano et al., 2005), or with disperged oviductal epithelial cells during zygote culture (Qian et al., 2005).

The early embryonic development success depends on a sufficient quantity of HA and HA synthesis precursor (Chen et al., 1990). The HA presence in culture medium for early embryos is able to improve embryo development to blastocyst stage (Kano et

al., 1998; Miyoshi et al., 1999). In addition, HA binding to the CD44 receptor expressed in the early bovine embryo (Furnus et al., 2003) enhances early embryonic development (Toyokawa et al., 2005).

The cumulus cell presence significantly influences the first mitotic division of the zygote, blastocyst stage achievement, and viability of embryos (Ju, Rui, 2012). Cumulus expansion intensity is positively linked to the success of embryonic development to 2–4 blastomers and blastocyst (Qian et al., 2003). Thus, cumulus expansion can be used as a developmental competence marker, useful for *in vitro* matured oocyte selection for *in vitro* fertilization.

In addition to polyspermy suppression and early embryonic development enhancement, cumulus expansion prevents an extrauterine conception by enhanced interception by the infundibulum (Chen et al., 1993). Owing to cumulus expansion physical features, secretion of growth factors and cytokines by uterus mucous membrane important for adhesion within the uterus, expanded cumuli of early embryos prevent a nidation in the oviduct (Parikh et al., 2006).

With respect to the importance of cumulus expansion, the measurement of cumulus expansion intensity is necessary for the evaluation of *in vitro* matured oocyte suitability for *in vitro* fertilization, cloning, and transgenesis.

Methods of cumulus expansion measurement

Visual evaluation of cumulus expansion and measurement of cumulus area.

The original method of cumulus expansion measurement is a subjective classification into groups by cumulus expansion intensity (Fagbohun, Downs, 1990). The result of the evaluation is a COC frequency in groups. Daen et al. (1994) described a quantifying method of cumulus expansion evaluation. On taken photographs, the area occupied by COCs is measured using a ruler and calculated using the formula: area (mm^2) = length (mm) × width (mm) × 0.7854. Currently, photographs can be processed using image analysis software and the area is calculated by a programmed algorithm. Both methods of classification and image analysis are non-invasive and continuous data collection during *in vitro* maturation is possible. Subjectivity and lack of the three-dimensional structure record are disadvantages of these methods. For these reasons, classification and image analysis cannot be considered exact and relevant methods for cumulus expansion measurement.

Measurement of GAGs content

The quantity of GAGs in expanded COCs can be used as a reliable marker of cumulus expansion. The HA, as the most abundant GAG, is suitable for detection and indication of cumulus expansion intensity. For HA measurement, the use of radioactively labelled precursors of synthesis is possible (Eppig, 1980). The [3H]glucosamine (100 μCi ml^{-1}) as HA precursor, or [35S]sulfate (60 μCi ml^{-1}) as ChS precursor, are used as components of culture medium. After HA synthesis from labelled precursors by cultured COCs, cumulus cells of expanded cumuli are proteolyzed and followed by HA extraction to solution. Subsequently, HA is enzymatically degraded by hyaluronidase, alternatively by chondroitinase ABC for ChS chains (Salustri et al., 1990). The HA content is deduced by radioactive signal emitted by the labelled HA precursor (Solursh, 1976). Disadvantages of the method are the inability of repeating cumulus expansion evaluation during culture of the same COCs and the manipulation with radioactive material.

Perspectives of cumulus expansion measurement

Apart from radio-labelled precursor usage, there are analytical methods for measurement of the degraded products of HA. Among the methods worth considering are spectrophotometry and high-performance liquid chromatography (HPLC) in ultraviolet (UV) spectra (Rehakova et al., 1994; Volpi, 2000).

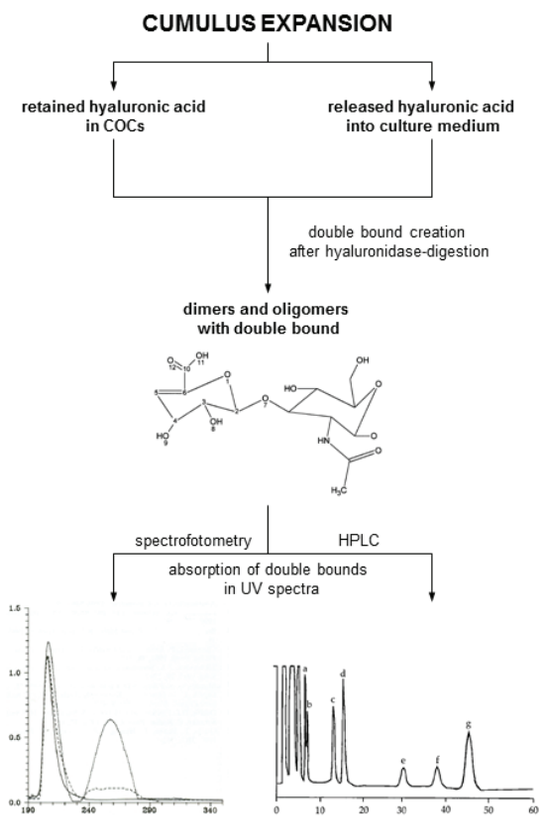

Fig. 3. The possible approach to hyaluronic acid measurement and quantification of cumulus expansion (adjusted by Alkrad et al., 2003; Rehakova et al., 1994; Stern and Jedrzeias, 2006; Volpi, 2000).

For analysis of HA degradation products, precise sample preparation consisting of expanded COCs is necessary. The preparation includes especially the washing out of culture medium components and the complete transfer of washed cumuli into tubes for analysis. In addition to COCs' preparation, aliquots of culture media can be removed and analyzed for HA release by *in vitro* cultured COCs into the surrounding space. Subsequently, HA degradation is based on total enzymatic HA digestion by hyaluronidases. Only digesting HA chains by lyases without hydrolysis by water molecule enable double-bound creation in heterodimer of the glucuronic acid and N-acetylglucosamine absorbing the light in UV spectra (Alkrad et al., 2003; Stern, Jedrzeias, 2006). The amount of double bounds determining UV light absorbance is measurable by spectrophotometry and/or HPLC (Volpi, 2000; Chen et al., 2005) (Fig. 3).

Adequate sample preparation and sensitivity of the afore-mentioned methods would enable precise analysis of HA content retained in COCs, as well as of the HA released into the culture medium. Just the analysis of the HA released into the culture medium could be a potent mode for non-invasive evaluation of cumulus expansion during *in vitro* COCs' cultivation.

Currently, spectrophotometry and HPLC methods are not routinely established for quantification and evaluation of cumulus expansion. The efficiency of HA measurements during cumulus expansion depends on further studies.

CONCLUSION

The cumulus expansion of COCs is based on GAG synthesis, especially HA (Dekel, 1979; Eppig, 1979). HA acts as a structural component and signal molecule during oocyte maturation and early embryonic development. Thus, cumulus expansion intensity is positively correlated with meiotic and developmental competence (Furnus et al., 2003; Yokoo et al., 2003, 2010; Han et al., 2006). The evaluation of cumulus expansion can be a marker of quality COCs used for *in vitro* fertilization. HA measurement is the method of cumuli quality and cumulus expansion evaluation as a marker of oocyte maturation success and oocyte developmental competence.

There are only a few methods for the measurement of cumulus expansion intensity, either by the expanded cumuli area, or indirectly by HA quantity measurement. Recent methods used for cumulus expansion evaluation contend with problems such as subjectivity and manipulation with radioactive material.

Analytical methods of HA content measurement in UV spectra, i.e. spectrophotometry and HPLC, are prospects for real sample preparation, analysis of HA content retained in COCs and released into culture medium during cultivation, and thus for the sensitive determination of cumulus expansion.

Further testing experiments are necessary for the establishment of a suitable method of HA measurement as exact evaluation of cumulus expansion in routine practice.

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LIST OF ABBREVIATIONS:

ANG = angiogenin, cAMP = cyclic adenosine 3',5'-monophosphate, cGMP = cyclic guanosine 3',5'-monophosphate, CEEF = cumulus expansion enabling factor, ChS = chondroitin sulfate, COCs = cumulus-oocyte complexes, COX2 = cyclooxygenase 2, GAGs = glycosaminoglycans, GDF9 = growth differention factor 9, GV = germinal vesicle, GVBD = germinal vesicle breakdown, EGF = epidermal growth factor, ELISA = enzyme-linked immunosorbent assay, FSH = follicle stimulating hormone, HA = hyaluronic acid, HABP = hyaluronic acid binding protein, HAS = hyaluronan-synthase, HC = HA heavy chain, HO-1 = heme oxygenase 1, HPLC = high-performance liquid chromatography, IGF-I = insulin-like growth factor I, IαI = inter-α-trypsin inhibitor, IL-6 = interleukin-6, iNOS = inducible nitric oxide synthase, LH = luteinizing hormone, MAPK = mitogen activated protein kinases, miRNA = microRNA, MPF = M-phase/maturation promoting factor, PGE2 = prostaglandin E2, PTX3 = pentraxin, RGS2 = regulator of G-protein signalling 2, SMAD2/3 = Drosophila mothers against decapentaplegic protein 2/3, TGFβ = transforming growth factor β superfamily, TNFAIP6 = tumour necrosis factor α-induced protein 6, TrkA = tyrosine kinase receptor A

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