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Original article

Comparative analysis of the polypeptide pattern of cumulus cells during maturation of porcine cumulus oocyte complexes *in vivo* and *in vitro*

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Summary — The protein patterns of porcine cumuli oophori matured as intact cumulus oocyte complexes either *in vivo* or *in vitro* with or without FSH and LH for 46 h were investigated. In *in vivo*-matured cumuli oophori, a 53 kDa band disappeared after 24 h maturation, but reappeared at 46 h. Furthermore, the production of a polypeptide with a relative molecular mass of 44 000 ceased and the appearance of 2 other proteins with relative molecular masses of 38 000 and 28 000 was observed. In cumuli oophori matured *in vitro* with or without addition of FSH and LH the 53 kDa band ceased after a culture period of 12 h. This band was produced again after a culture period of 46 h. In contrast, the polypeptide with the relative molecular mass of 44 000 ceased only in cumuli oophori cultures supplemented with FSH and LH, and the 2 proteins of M_r 38 000 and 28 000 were detected only in the protein profiles of mature cumuli cultured with FSH and LH. It is concluded that the addition of FSH and LH to the culture medium is necessary for cumuli oophori to synthesize a protein pattern, which corresponds closely to that produced by cumuli oophori matured *in vivo.*

cumulus cell / polypeptide pattern / oocyte maturation

Résumé — Étude comparative des profils protéiques des cellules du cumulus oophorus pendant la maturation des complexes porcins cumulus–ovocyte *in vitro* ou *in vivo*. *L'étude porte sur les profils protéiques du cumulus oophorus de porcs maturé sous la forme de complexe intact cumulus–ovocyte, soit* in vivo *soit* in vitro *avec ou sans FSH et LH pendant 46 h. Pendant la maturation* in vivo, *une bande de 53 kDa disparaît après 24 h mais réapparaît à 46 h. En outre, la production d'un polypeptide d'une masse moléculaire relative de 44 000 cesse, simultanément apparaissent 2 autres protéines avec une masse moléculaire relative de 38 000 et de 28 000. Pour les cumulus oophorus maturés* in vitro *avec ou sans addition de FSH et de LH, la bande de 53 kDa cesse d'apparaître après une période de culture de 12 h. Cette bande est produite de nouveau après 46 h de culture. En revanche, le polypeptide d'une masse moléculaire relative de 44 000 ne disparaît que dans les cultures de cumu-*

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lus supplémentées avec FSH et LH; les 2 protéines de 38 000 et 28 000 de PMr ne sont aussi seulement décelées que dans le profil protéique des cumulus oophorus cultivés en présence de FSH et de LH. En conclusion, l'addition de FSH et de LH au milieu de culture est nécessaire pour les synthèses protéiques du cumulus oophorus correspondant étroitement à celles produites par le cumulus oophorus maturé in vivo.

cellules du cumulus oophorus / profils protéiques / maturation oocytaire

INTRODUCTION

Successful fertilization of *in vitro*-matured pig oocytes is limited by a high occurrence of polyspermy and, in the case of monospermic penetration, by the inability of the ooplasm to transform the sperm head into a male pronucleus. A further defect shown by embryos derived from *in vitro*-matured oocytes is represented by the failure of zygotes to progress to the blastocyst stage (Motlik and Fulka, 1974; Nagai *et al*, 1984; Toyoda *et al*, 1984; First *et al*, 1988; Parrish, 1991; Rath, 1991).

Recent experiments have shown that somatic support of the oocyte as well as supplementation of the culture medium with gonadotrophins have a beneficial effect on oocyte maturation *in vitro*. Denuded and/or only corona-enclosed ovine and porcine oocytes resume meiosis in culture but remain developmentally incompetent. In contrast, oocytes supported by cumulus and supplementary follicle cells or oocytes surrounded by the cumulus connected to the whole wall of the extroverted follicle undergo full maturation and subsequently normal embryonic development (Staigmiller and Moor, 1984; Mattioli *et al*, 1988a; 1989; Galeati *et al*, 1991). Moreover, the addition of LH and FSH to the culture medium facilitated and accelerated meiotic progression, and LH selectively improved cytoplasmatic maturation (Crosby *et al*, 1981; Mattioli *et al*, 1991). Gonadotrophins not only support oocyte maturation, but also induce major alterations in the protein profile of follicle and cumulus cells (Ball *et al*, 1985; Moor and Crosby, 1987).

The fact that LH induces alterations in the protein synthesis of follicle cells, together with the observation that granulosa cells enhance the fertilizability of oocytes may indicate a causal relationship between the pattern of protein synthesis of the oocyte surrounding follicle cells and oocyte maturation *in vivo*. On the other hand, the inability of *in vitro*-cultured porcine and ovine oocytes to undergo normal fertilization and subsequent embryonic development could be related to the protein synthesis pattern of cumulus cells.

The aim of the present study was to compare the protein patterns of porcine cumulus cells matured as intact cumulus oocyte complexes either *in vivo* or *in vitro* with or without FSH and LH for various time intervals.

MATERIAL AND METHODS

In vivo *maturation*

Oocytes undergoing *in vivo* maturation were obtained from peripuberal (80–100 kg) gilts. Follicular growth and ovulation were induced by one im injection of 750 IE PMSG (Intergonan, Vemie, Germany) followed 72 h later by an im administration of 500 IE hCG (Ekluton, Vemie) (Hunter and Polge, 1966).

Ovaries were removed by ovariectomy 72 h after PMSG injection or 9, 21 and 43 h following the administration of hCG. Cumulus oocyte complexes were liberated by slitting the follicles and flushing the follicle content. They were placed in prewarmed (37°C) Dulbecco's phosphate-buffered saline (PBS, Serva, Heidelberg, Germany) supplemented with 1% heat-inactivated

fetal calf serum (FCS, Serva) until labelling with L-[^{35}S]methionine (500 µCi/ml; specific activity 1 280 µCi/mmol, Radiochemical Centre Amersham UK). One or 2 oocytes were separated each time, fixed in ethanol/ acetic acid (3:1 v/v) for 24 h, stained with 1% aceto-orcein and examined at 400 x using phase-contrast optics (Motlik and Fulka, 1976).

In vitro *maturation*

Ovaries were collected at a local abattoir from peripuberal gilts into 0.9% Na Cl solution (39.0°C) and immediately transferred to the laboratory. Cumulus oocyte complexes were released from follicles 3–5 mm in diameter by scraping the inner surface of the follicular wall with a needle and simultaneous flushing with prewarmed (37.0°C) PBS containing 1% heat-inactivated FCS. Cumulus oocyte complexes were examined under a stereo microscope (magnification 40 x). Only oocytes with a homogeneous ooplasm and surrounded by a compact cumulus oophorus (with or without an attached piece of parietal granulosa cells) were used in this study.

To determine the influence of oocyte nuclear maturation on the protein patterns of cumulus cells, the germinal vesicle of oocytes cultivated in medium with or without gonadotrophins for 21 h was visualized after centrifugation (9 980 *g* for 3 min) according to the method of Wall *et al* (1985). The presence or absence of the germinal vesicle was recorded for each oocyte prior to labelling.

Groups of 15–20 cumulus oocyte complexes were cultured in 2 ml bicarbonate-buffered TCM 199 with Earl's salt (TCM 199, Serva) supplemented with 10% heat-inactivated FCS either with or without 2.5 µg/ml FSH (NIH-FSH-B1, USDA-Reproduction Lab, Beltsville) and 5 µg LH (NIH-LH-B6). Culture of cumulus oocyte complexes was carried out at 39.0°C in a humidified atmosphere of 5%CO_2 in air.

Radiolabelling and electrophoresis

Cumulus oocyte complexes undergoing *in vivo* maturation were labelled immediately after isolation from the follicles (oocytes in GVI). Cumulus oocyte complexes matured *in vitro* were labelled 9 (GV I -IV), 21 (GVBD), and 43 h (M II) after initiation of culture (Motlik and Fulka, 1976). To determine the protein profile of cumulus oocyte complexes at the beginning of culture, cumulus oocyte complexes were labelled immediately after explanation from the follicles.

For L-[^{35}S]methionine labelling, the cumulus oocyte complexes were washed twice with labelling medium: Dulbecco's PBS, containing 0.036 mg/ml Na-pyruvate, 0.05336 mg/ml Na-lactate (50%), 0.99 mg/ml glucose and 1% dialyzed heat-inactivated FCS (Biochrom, FRG). Protein synthesis was allowed to proceed by incubating the cumulus oocyte complexes in labelling medium containing L-[^{35}S]methionine for 3 h at 39.0°C in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. Cumulus oocyte complexes matured *in vitro* without gonadotrophins were labelled in groups of 15 in 80 µl labelling medium under paraffin oil (paraffin oil light, Fisher, Germany). After maturation *in vivo* or *in vitro* with gonadotrophins the cumuli oophori were expanded and the cumulus oocyte complexes were labelled individually in 5 µl labelling medium supplemented with hyaluronic acid (0.025% w/v, Sigma, Germany).

After incubation cumulus oophorus cells were separated from the oocytes and from L-[^{35}S]methionine by washing twice with PBS by centrifugation at 9 980 *g* for 6 min. Individual cumuli oophori were placed in 25 µl SDS-sample buffer (Laemmli, 1970) and frozen at –70.0°C until electrophoresis. In order to complete lysis the cumuli were frozen and thawed 3 times. Two microlitres of each sample was used to determine the incorporation of radioactivity into TCA-precipitable material (Van Blerkom, 1978). Aliquots containing equal amount of radioactivity were heated for 1 min at 95.0°C and subjected to 1-dimensional 8–15% linear gradient SDS polyacrylamide slab gels (0.75 mm thick).

Electrophoresis was carried out according to Laemmli (1970) at 10 mA per gel. Gels were dried on a Biotec-Fischer-Geldryer under vacuum at 80.0°C and subsequently exposed to X-ray films (Hyperfilm-βmax, Amersham, UK) for 1–3 weeks.

A mixture of [^{14}C]-labelled proteins with a known molecular mass were run simultaneously as standards: myosin (200 kDa); phosphorylase b (92 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (21 kDa); and lysozyme (14.3 kDa).

Statistical analysis of data

In the protein pattern 4 bands (band A; 53 kDa; band B: 44 kDa; band C: 38 kDa; band D: 28 kDa) clearly displayed changes during maturation, and were selected for a closer examination. To obtain statistical data the appearance and disappearance of the 4 bands in each protein pattern were recorded as a function of the maturation periods and the presence or absence of FSH and LH in the culture medium. The data were analysed using least-square-mean procedure (LSQ) (Harvey, 1976) as a tool for calculating the percentage of cumuli oophori cells in the samples exhibiting the examined band, and qualified to 1 = not existent, 2 = faint, and 3 = existent. Furthermore, the influence of culture period, the presence or absence of the germinal vesicle after 21 h of culture, and the addition of FSH and LH to the culture medium were calculated by analysis of the variance for each band (Harvey, 1976).

RESULTS

Nuclear status

Among the 23 oocytes recovered at 72 h after PMSG injection or 9 and 21 h after hCG administration, 20 had an intact germinal vesicle (GV) whereas 3 had undergone germinal vesicle breakdown (GVBD). All of the 7 oocytes examined 43 h after hCG injection were in metaphase II.

Protein synthesis by cumuli oophori matured in vivo

The protein patterns of 170 cumuli oophori matured *in vivo* were analysed. Four protein bands showed major changes during the 43 h of maturation.

Representative examples of protein pattern synthesized by individual cumuli oophori isolated immediately before, and 9, 21, and 43 after hCG-injection are shown in figure 1. Band A disappeared within 21 h of maturation but reappeared 43 h after hCG injection. During the maturation period of 43 h the cumulus oophorus cells ceased the production of band B, and bands C and D began to appear.

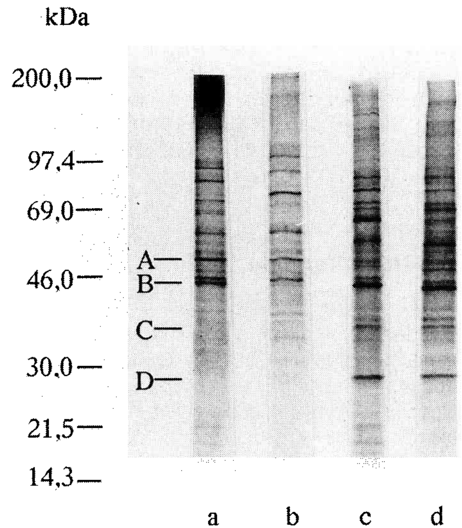

Fig 1. Autoradiodiagrams of L-[^{35}S] methionine-labelled polypeptides from individual cumuli oophori before initiation maturation (0 h) and 9, 21, and 43 h after hCG-injection (lanes a–d, respectively). Cumulus–oocyte complexes were removed from the ovaries immediately before (0 h) or 9, 21, or 43 h after hCG-administration and labelled for 3 h. Polypeptides were seperated on SDS polyacrylamide gels (8–15%). Changes in the synthesis of selected proteins during maturation (band A: 53 kDa; band B: 44 kDa; band C: 38 kDa; band D: 28kDa) are indicated by the lines.

Table I presents the LSQ-mean analysis of the 4 selected protein bands of cumuli oophori isolated from the follicles 72 h after PMSG-injection (0 h). Band A was not detected in any of the analysed protein profiles 21 h after initiation of maturation. At the end of maturation (43 h) band A reappeared in all protein patterns of cumuli oophori. When the cumulus–oocyte complexes were labelled before hCG-injection,

Table I. LSQ mean of bands A, B, C, and D at 0, 9, 21, and 43 h after initiation of maturation *in vivo.*

Protein band	*Time (h) of maturation* in vivo			
	0	*9*	*21*	*43*
A (53 kDa)	100.0	65.9	0.0	100.0
B (44 kDa)	83.3	19.5	70.8	0.0
C (38 kDa)	15.3	100.0	85.3	100.0
D (28 kDa)	23.1	14.6	34.4	96.4
Samples *n*	39	41	48	42

the LSQ mean of the band B value reached 83.3; at 9 h this dropped to 19.5. The LSQ mean increase to 70.8 at 21 h and was not detectable at 43 h. Furthermore, during maturation *in vivo* the cumuli oophori began to produce bands C and D. Their LSQ mean increased during 43 h of maturation from 15.3 and 23.1 to 100.0 and 96.4, respectively.

Protein patterns of cumuli oophori matured in vitro

Representative protein profiles of cumuli oophori labelled immediately after isolation from the follicles and after maturation *in vitro* with or without FSH and LH for 9, 21, and 43 h are shown in figure 2. The patterns of

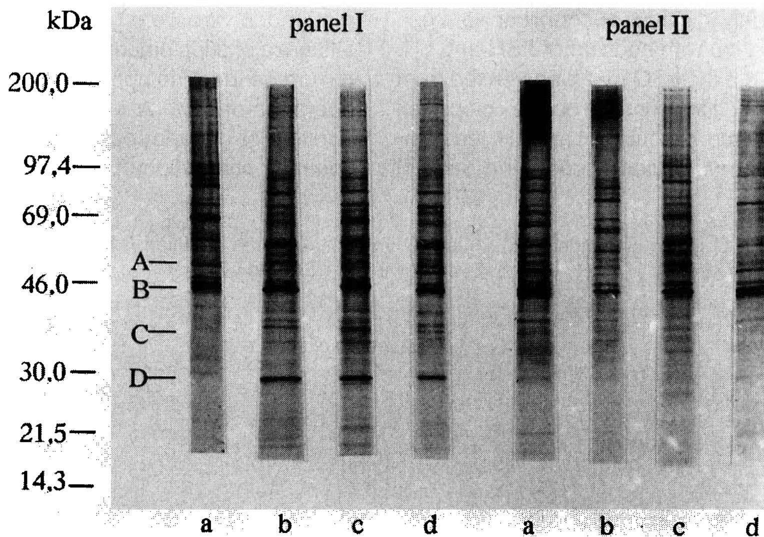

Fig 2. Autoradiograms of L-[^{35} S] methionine-labelled polypeptides from individual cumuli oophori labelled immediatedly after isolation fom the follicles (0 h) or 9, 21, or 43 h (lanes a–d, respectively) after start of culture either with (panel I) or without the addition (panel II) of FSH and LH. Polypeptides were separated on SDS polyacrylamide gels. The lines indicate the 4 bands examined during the culture period.

proteins synthesized by cumuli oophori cultured *in vitro* with FSH and LH resembled closely those observed when cumuli oophori were matured *in vivo.* Band A was present before initiation of maturation (0 h) and at the end of maturation (43 h). During the 43 h culture period production of band B ceased and the cells started to yield bands C and D. By contrast, the protein profiles of cumuli oophori cultured without FSH and LH differed substantially from the protein patterns of *in vivo*-matured cumuli oophori. Only the alterations of band A corresponded to the changes seen *in vivo.* These oocytes neither ceased the synthesis of band B nor started the production of bands C and D.

Table II presents the LSQ mean of the 4 selected bands from cumuli oophori immediately after isolation from the follicles and after a culture period of 9, 21, or 43 h either with or without FSH and LH.

Independently of the addition of FSH and LH, band A disappeared and after a culture period of 43 h reappeared. Band B disappeared only in cumuli oophori that were cultured *in vitro* with addition of FSH and LH. Within 43 h the LSQ mean decreased from 92.6 to 3.0. If the cumulus oocyte complexes were cultured without FSH and LH, the number of cumuli oophori producing band B remained high. The LSQ-mean of bands C and D demonstrate that only cumuli oophori cultured in the presence of FSH and LH produced band C and D at the end of maturation. The LSQ-mean increased during the 43-h culture period from 0.0 and 2.1 to 95.7 and 91.5, respectively. The LSQ mean of band C and D from cumuli oophori matured without FSH and LH revealed that the proportion of cumuli oophori containing these 2 bands was low.

These results show that major changes in the protein profiles of cumuli oophori take place during *in vivo* maturation. Except for band A, the alterations in the protein patterns of cumuli oophori matured *in vitro* depend on the presence of FSH and LH in the culture medium.

Effect of FSH/LH and culture period on the protein profile

The protein patterns of 536 cumuli oophori matured *in vitro* were used to estimate the influence of supplemented FSH/LH and the culture period. The appearance and disappearance of band A were independent of addition of gonadotrophins ($p > 0.05$). By contrast, production of bands B, C, and D

Table II. LSQ mean of bands A, B, C, and D immediately after isolation from the follicle (0) and after a culture period of 9, 21, and 43 h with or without FSH and LH.

Protein band	*Duration of culture (h)*							
	0		*9*		*21*		*43*	
	FSH/LH	–	*FSH/LH*	–	*FSH/LH*	–	*FSH/LH*	–
A (53 kDa)	97.8	92.1	0.0	14.0	0.0	14.0	88.3	70.5
B (44 kDa)	92.6	76.3	43.7	47.5	3.3	40.4	3.0	72.1
C (38 kDa)	0.0	5.3	71.1	13.7	92.2	11.0	95.7	32.8
D (28 kDa)	2.1	0.0	59.1	0.0	95.6	6.1	91.4	18.0
Samples *n*	47	38	71	79	45	114	81	61

showed a significant difference ($p < 0.001$) between cumuli oophori which were matured with or without gonadotrophins. The protein profiles of cumuli oophori analysed immediately after isolation from the follicles and after a cultured period of 9, 21, and 43 h were also significantly different from each other ($p < 0.001$).

Effect of the nuclear status on the protein profile

Table III presents the LSQ mean of the 4 selected bands from cumuli oophori containing oocytes before and after GVBD at 21 h after initiation of culture.

The appearance of band C was influenced ($p = 0.001$) by the nuclear status of the oocytes. This band was more frequent in gels of cumuli oophori belonging to oocytes having undergone germinal vesicle breakdown. Furthermore a weak ($p < 0.05$) influence on the appearance of band A was noticed, and was more frequent in the presence of an intact germinal vesicle.

DISCUSSION

The present study demonstrates that marked changes occur in the protein pattern of cumuli oophori during maturation of porcine cumulus–oocyte complexes *in vivo* as well as in cumuli oophori matured *in vitro* in the presence of FSH and LH. The interpretation of the dynamic changes of polypeptides in the course of *in vivo* and *in vitro* maturation needs some caution.

Table III. LSQ mean of bands A, B, C, and D 21 h after initiation of culture.

Protein band	*G erminal vesicle of the ococyte*		
	Present	*Absent*	P-*value*
A (53 kDa)	13.7	6.3	0.017
B (44 kDa)	20.3	18.6	0.667
C (38 kDa)	30.5	41.8	0.001
D (28 kDa)	26.9	30.8	0.246
Samples *n*	114	122	

The absence of a band can be either explained by the total absence of synthesis or alternatively by a weak synthesis of unperceptible proteins. Using the present techniques it was not possible to differentiate between these 2 possibilities due to the complex protein profiles of cumulus cells. Therefore, we restricted the present study to 4 bands where clearly discernable changes were visually observed. It should be kept in mind that these polypeptides represent only a very small proportion of the total protein synthetic activity of the cumulus cells. After 1-dimensional electrophoresis each band consists of proteins with equal molecular masses, which might have perhaps different isoelectric points.

Rabahi *et al* (1991) examined 3 major peaks of the newly synthesized proteins of bovine granulosa and cumulus cells, with molecular masses of 76, 56, and 30 kDa before and after the LH surge. In contrast to the granulosa cells exhibiting a changing pattern of cytosolic proteins, neither the cytosolic nor the secreted proteins of the cumulus cells altered significantly. In the present study the analysis of a large number of proteins patterns of individual porcine cumuli oophori revealed significant differences in the intensity of bands A, B, C, and D depending on *in vivo* or *in vitro* maturation, as well as on the duration of the maturation period. The start of appearance of bands C (38 kDa) and D (28 kDa) during maturation is consistent with previous data in other mammalian species. Moor and Crosby (1987) investigated the hormonal regulations and biochemical characteristics of polypeptides secreted by isolated Graafian follicles of sheep. Within 15 h of the LH

surge, the secretion of M_r 46 000–60 000 polypeptides had ceased and were replaced by a M_r 30 000 secretion product. The experiments of Landefeld *et al* (1979) in granulosa cells of rats also showed the synthesis of 2 specific proteins 30 min after treatment with hCG. Furthermore, 4 additional new proteins appeared 3 h after hCG-injection. The molecular mass of these proteins ranged from 23 000–40 000 Da (Landefeld *et al*, 1979). These data are in close agreement with the present observations and may reflect common changes in protein synthetic activity of cumulus cells in the course of preovulatory maturation. Despite apparent identical maturation conditions in the *in vitro* experiments, not all protein patterns showed the typical *in vivo* alterations. The absence of these changes in protein patterns may be attributable to the inability of these cumuli oophori to respond to hormonal stimuli. This, in turn, may reflect the individuality of cumulus–oocyte complexes. It is possible that the non-responsive cumulus oophori are those which did not undergo complete maturation.

The maturation-dependent protein synthesis during maturation raises the question on the nature and precise role of these proteins. Because of the ability of follicle cells to influence the quality of maturation, the changes in protein synthesis might be relevant for oocyte maturation (Moor and Crosby, 1987; Mattioli *et al*, 1988a, b). Analysis of the relationship between protein synthesis of cumulus cells and stage of oocyte maturation revealed that only the synthesis of band C is related to the stage of maturation. This conclusion is based on the observation that band C appears predominantly in the protein pattern of cumulus cells from cumulus–oocyte complexes containing an oocyte after GVBD as compared with that of cumuli oophori from complexes with an oocyte in the GV stage (table III).

In the protein pattern of ovine oocytes, as well as cumulus cells, a band with a molecular mass of 45 kDa was identified as actin. This band was greatly reduced after removal of the cumulus cells (Osborn and Moor, 1982). It has been postulated that this actin belongs to granulosa cell processes that remain within the zona pellucida as a consequence of oocyte isolation procedures (Canipari *et al*, 1988). During *in vivo* maturation intercellular coupling persisted up to 32 h after hCG-injection. However, the functional coupling between cumulus cells and the oocyte *in vitro* was maintained for only 16 h (Motlik *et al*, 1986). Because of the apparent molecular mass of our band B, and since the disintegration of intercellular coupling correlates with the time of disappearance of band B in the protein pattern, we speculate that it may be actin.

Cumulus expansion and mucification of the cumulus oophorus in porcine cumulus oocyte complexes by FSH and LH has been reported (Mattioli *et al*, 1991). Our data support this observation since only cumuli oophori matured *in vivo* or *in vitro* with FSH and LH showed expansion and mucification. Cumuli oophori cultured without FSH and LH remained compact and dense. Therefore, LH- and FSH-dependent changes in protein synthesis may be related to expansion and mucification of the cumulus oophorus.

Whether the altered proteins have no regulative function but might be products of an activated metabolism during expansion and mucification needs further investigation.

In conclusion, during *in vivo* maturation of porcine cumuli oophori apparent alterations in the protein synthesis pattern take place. During *in vitro* culture only the protein patterns of cumuli oophori matured with FSH and LH correspond to the profile of protein synthesized by cumuli oophori during *in vivo* maturation. Thus, addition of FSH and LH to the culture medium seems to be necessary

to enable cumuli oophori to produce an *in vivo*-like protein pattern.

Further investigations of the protein synthesis of cumuli ooophori may help in understanding the importance of somatic support for the oocyte to undergo complete maturation.

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