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Bovine cumulus expansion and corona–oocyte disconnection during culture *in vitro*

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Summary — Cumulus–oocyte complexes were aspirated from small antral follicles (3–5 mm in diameter) and divided into 2 groups: complexes in which a dark rim of corona cells were visible around the zona pellucida (group 1); and those in which the corona displayed the same density as the rest of the cumulus cell mass (group 2). Cumulus complexes of both groups were evaluated by aceto-orcein staining, scanning electron microscopy (SEM) and ^{3}H-uridine uptake during culture *in vitro*. Germinal vesicle breakdrown was initiated at 7 h of culture in group 1 while more than half of the oocytes in group 2 already displayed germinal vesicle breakdrown at the time of aspiration. However, whereas more than 80% of the oocytes in group 1 had reached metaphase II at 24 h of culture, almost half of the oocytes in group 2 were arrested in metaphase I after this time interval. As revealed by SEM the complexes of group 2 showed signs of expansion such as elongation of cumulus cells and presence of extracellular matrix already at the time of aspiration. In group 1 these features were noticed at 7–9 h of culture. A high level of metabolic coupling between corona cells and oocyte was maintained up to 9 h of culture in group 1 followed by a decrease to a constant low level at 13 h. In group 2 a high degree of coupling was maintained up to 7 h followed by a gradual decrease to a constant low level at 13 h. It is concluded that cumulus–oocyte complexes with a dark rim of corona cells as judged by stereomicroscopy mature at a higher rate and maintain unexpanded characteristics and efficient corona–oocyte coupling longer than complexes with even density of the cumulus mass. Consequently, the presence of a dark rim of corona cells may be used as a criterion for selection of oocytes for *in vitro* embryo production.

oocyte maturation *in vitro* / cellular coupling / cattle

Résumé — Expansion du cumulus oophorus bovin et déconnexion de l'ovocyte et de la corona pendant la culture *in vitro*. *Des ovocytes inclus dans leur cumulus oophorus ont été aspirés de petits follicules à antrum (3–5 mm de diamètre) et divisés en deux groupes : des complexes dans lesquels la corona radiata forme un anneau sombre autour de la zone pellucide (groupe 1) et des complexes dans lesquels la corona n'est pas plus opaque que le reste du cumulus (groupe 2). Des complexes des deux groupes ont été analysés par coloration acéto-orcéique, microscopie électronique à balayage (MEB) et incorporation d'uridine ^{3}H pendant la culture* in vitro. *La rupture de la vésicule germinative commence après 7 h de culture dans le groupe 1, tandis que la moitié des ovo-*

cytes du groupe 2 montrent déjà une rupture de la vésicule germinative au moment de l'aspiration. Cependant, tandis que plus de 80% des ovocytes dans le groupe 1 atteignent la métaphase II après 24 h de culture, presque la moitié des ovocytes dans le groupe 2 sont arrêtés en métaphase I après cet intervalle. Les complexes du groupe 2 observés par MEB montrent des signes d'expansion comme l'allongement des cellules du cumulus et la présence de matrice extracellulaire déjà au moment de l'aspiration. Dans le groupe 1, ces phénomènes se produisent après 7–9 h de culture. Un haut niveau de couplage métabolique entre les cellules de la corona et l'ovocyte se maintient jusqu'à 9 h de culture dans le groupe 1, suivi par une diminution à un bas niveau constant à 13 h. Dans le groupe 2, un fort couplage se maintient jusqu'à 7 h, suivi d'une décroissance graduelle jusqu'à un bas niveau constant à 13 h. Les complexes à corona sombre subissent une maturation plus rapide et maintiennent un état non expansé et un couplage efficace entre l'ovocyte et la corona plus longtemps que les complexes d'opacité homogène. En conclusion, la présence d'une corona sombre peut constituer un critère de sélection des ovocytes pour la fécondation in vitro.

maturation ovocytaire* in vitro / *couplage cellulaire* / *bovins

INTRODUCTION

Numerous studies have demonstrated that the cumulus and corona cells are linked to each other and the corona cells linked to the oocyte and extensive network of gap junctions (Anderson and Albertini, 1976; Moor *et al,* 1980; Motlick *et al,* 1986; Hyttel, 1987). The endogenous LH surge *in vivo* or the presence of gonadotropins under *in vitro* culture conditions initiate the process of cumulus expansion (Fléchon *et al,* 1986), which is associated with a loss of intercellular coupling between adjacent cumulus and corona cells and between the corona cells and the oocyte (Gilula *et al,* 1978; Szöllösi and Gérard, 1983; Hyttel *et al,* 1986a,b; Hyttel, 1987). In cattle a semiquantitative analysis of the gap junctions by transmission electron microscopy has demonstrated that the junctional contact is decreased during germinal vesicle breakdown up to 12 h of culture *in vitro*, and that gap junctions are no longer found at 18 h, when the oocyte has reached metaphase I (Hyttel, 1987).

Based on experiments in laboratory animals it has been suggested that interruption of junctional transfer of cumulus and corona generated oocyte maturation inhibitors triggers oocyte maturation as judged by germinal vesicle breakdown (Gilula *et al,* 1978; Dekel and Beers, 1980; Dekel *et al,* 1981). Other reports, also based upon laboratory animals (Heller and Schultz, 1980; Moor *et al,* 1980; Eppig, 1982; Eppig and Ward-Bailey, 1982; Salustri and Siracusa, 1983; Racowsky and Satterlie, 1985; Eppig and Downs, 1988) and swine (Motlik *et al,* 1986), however, indicate that small radiolabelled tracer molecules are continuously transported from the cumulus and corona cells to the oocyte for several hours after meiosis is resumed. Thus, at least in swine it is believed that disconnection of gap junctions within the cumulus investment rather than between corona cells and the oocyte is the actual trigger of oocyte maturation (Motlik *et al,* 1986).

In cattle only a single investigation of the corona–oocyte coupling using radiolabelled tracers has been reported (De Loos *et al,* 1991). This paper focused on the degree of coupling immediately after aspiration of cumulus–oocyte complexes from small antral follicles, and it was demonstrated that complexes with expanded heterogenously

clumped cumulus investment had a lower degree of coupling and developmental capacity following *in vitro* fertilization than oocytes with different types of more compact cumulus investment. No tracer studies, however, have been performed in order to elucidate the loss of corona–oocyte coupling during bovine oocyte maturation.

The objectives of the present study were to evaluate: 1), the nuclear oocyte maturation by aceto–orcein staining; 2), the process of cumulus expansion by scanning electron microscopy; and 3), the corona–oocyte coupling by tracer studies in 2 groups of oocytes classified according to the appearance of the cumulus investment at the stereo-microscopic level.

MATERIALS AND METHODS

Collection and culture of cumulus–oocyte complexes

Cumulus–oocyte complexes were collected from follicles (3–5 mm in diameter) from abattoir-recovered bovine ovaries. Complexes with compact multilayered cumulus investment were divided by stereomicroscopy into the following 2 groups: complexes in which the corona, *ie* the innermost couple of cell layers adjacent to the zona pellucida, appeared as a dark rim surrounding the zona (group 1; fig 1A), and complexes in which the corona had the same density as the rest of the cumulus investment (group 2; fig 1D). Complexes from both groups were cultured *in vitro* in 0.1 ml of medium under paraffin oil in an atmosphere of 5% CO_2, 10% O_2, and 85% N_2 at 38.5 °C. The culture medium was TC 199 (Sevac, Prague, Czechoslovakia) supplemented with 2.92 mM Hepes (Serva, Heidelberg, Germany), 50 iu per ml penicillin, 50 µg per ml streptomycin sulphate, and 20% heat treated estrous cow serum. After 0, 7, 9, 13 and 24 h of culture cumulus–oocyte complexes from each group were harvested for evaluation of nuclear oocyte maturation ($N = 1\ 812$), scanning electron microscopy (SEM, $N = 50$), and corona–oocyte coupling ($N = 1\ 000$).

Nuclear maturation

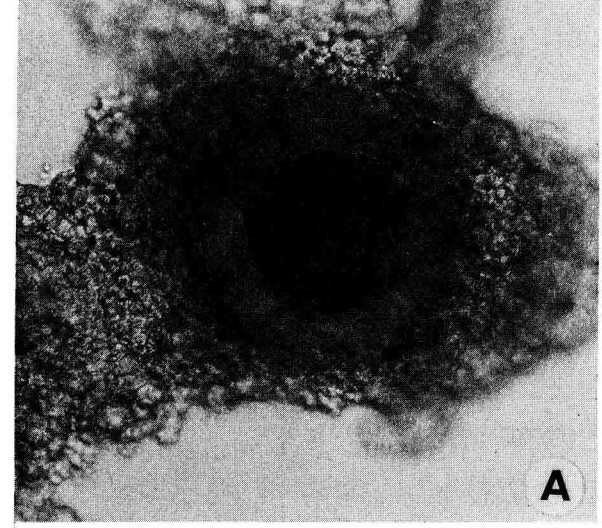
The nuclear oocyte maturation was examined in whole mount preparations by phase-contrast microscopy after fixation in acetic acid:methanol (1:3, V:V) and staining with 1% aceto–orcein.

Scanning electron microscopy

At each point of observation (0, 7, 9, 13 and 24 h) 5 cumulus–oocyte complexes from groups 1 and 2 were fixed in a mixture of 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer at 20 °C. Following 3 washes in fresh 0.1 M cacodylate buffer the complexes were post-fixed in 1.5% OsO_4 in 0.1 M cacodylate buffer. After 3 washes in redistilled water the complexes were gradually dehydrated in acetone, dried in a Polaron critical point dryer, and coated with a 20-nm thick layer of gold in a Polaron sputter coater (E 5100). Subsequently, the specimens were examined in a Jeol scanning electron microscope (JEM 100 CX II) equipped with a scanning adapter (ASID 4D) at 40 kV.

Corona–oocyte coupling

At each point of observation (0, 7, 9, 13 and 24 h) 100 cumulus–oocyte complexes from groups 1 and 2 were sampled for coupling analysis. In each set of 100 complexes 50 were actively denuded from cumulus cells. All cumulus–oocyte complexes and oocytes were cultured for 1 h in the above-mentioned culture medium enriched with ^{3}H-uridine (UVVVR, Prague, Czechoslovakia; specific activity: 740 GBg per mmol) at a concentration of 10 µCi per ml (Eppig, 1982) under the above-mentioned conditions. After culture the rest of the cumulus–oocyte complexes were actively denuded from cumulus cells. Subsequently, 5 sets of 100 oocytes per observation point in each group were washed 5 times in phosphate-buffered saline at 4 °C, transferred to scintillation counting vials, dissolved in 1 N NaOH, and acidified with 1 N HCL. Following solubilization of the oocytes 6 ml of scintillation liquid scintillator was added. The intercellular coupling index was calculated using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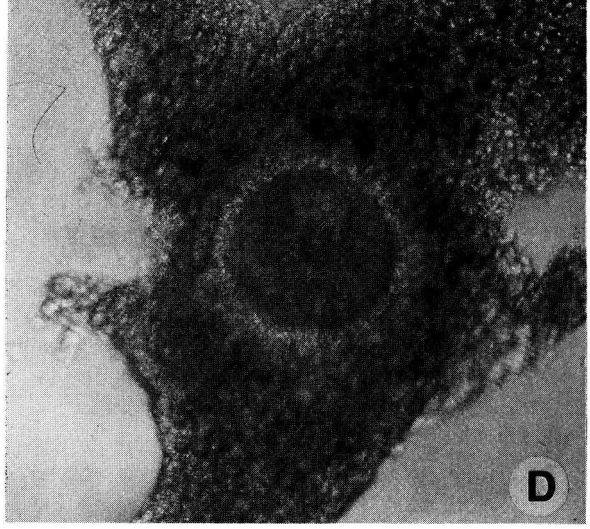
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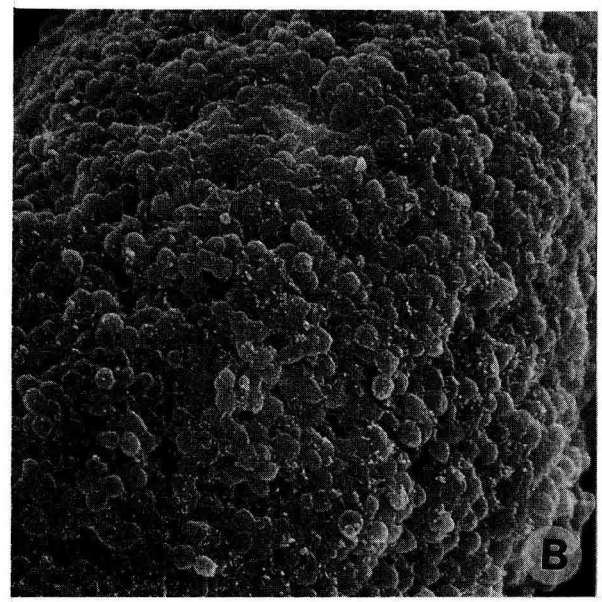
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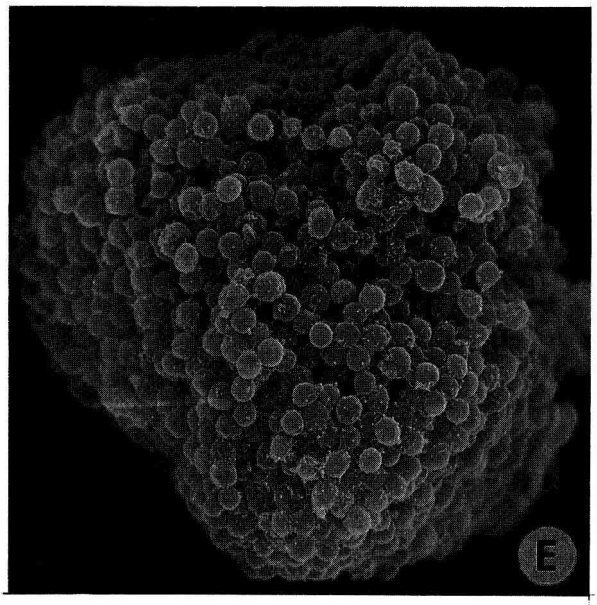
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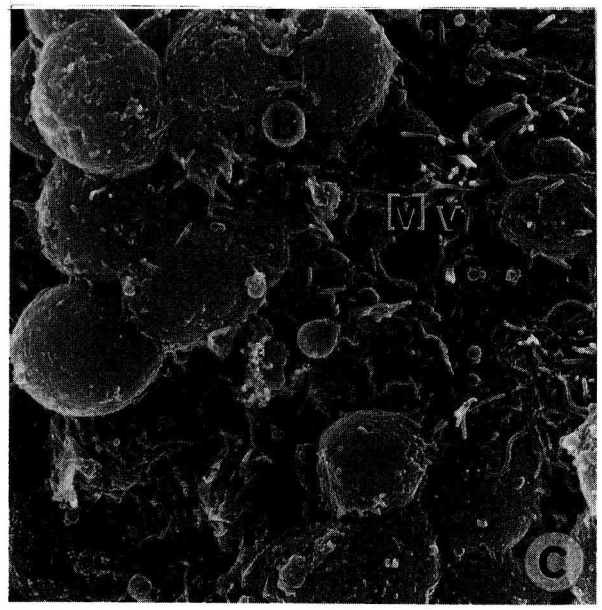
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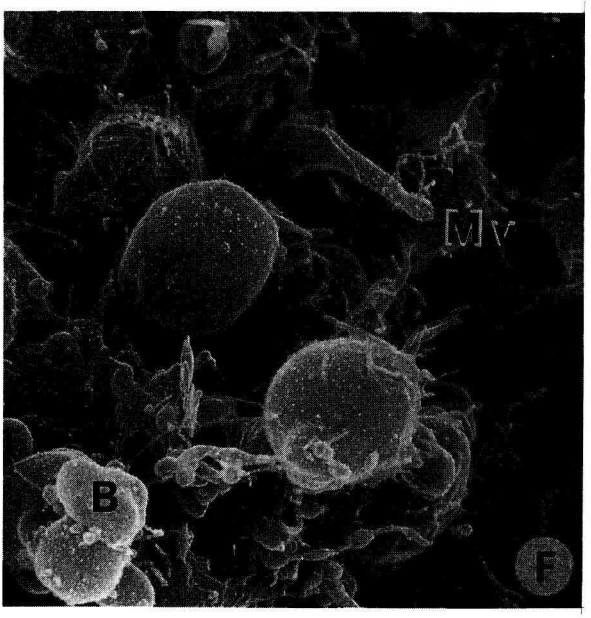
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the formula: Ci = $(A - B)/B$, where A is the amount of ^{3}H-uridine per set of 10 cumulus–oocyte complexes and B is the amount per set of 10 denuded oocytes (Heller *et al*, 1981). Differences in the coupling indexes groups 1 and 2 at each point of observation were tested by Student's *t*-test. Differences between sample point within each of groups 1 and 2 were treated by 1-way analysis using the Turkey multiple comparison test. Probability was determined by *U*-test after arcsin transformation $p_1 = x_1/n_1$.

RESULTS

Nuclear oocyte maturation

Before initiation of *in vitro* culture 92.5% of the oocytes in group 1 were at the germinal vesicle stage, while 53.6% of those in group 2 had initiated germinal vesicles breakdown (table I). At 7 h of culture 60% of the oocytes in group 1 were in the process of germinal vesicle breakdown, while 59.8% of the oocytes in group 1 were at metaphase I, while 28.9% and 54.8% of the oocytes from group 2 were at diakinesis or metaphase I, respectively. At 13 h the majority of the oocytes from both group 1 (92.3%) and 2 (83.2%) were at metaphase I or ana-telophase I. At 24 h 81.7% of the oocytes from group 1 had reached metaphase II, while only 27.8% of the oocytes from group 2 had done so due to the arrest of 42.0% and 13.7% at metaphase 1 and ana-telophase I, respectively.

Scanning electron microscopy

Before initiation of *in vitro* culture the cumulus investment of cumulus–oocyte complexes in group 1 appeared compact (fig 1B), and a close association between adjacent cumulus cells was clearly demonstrated (fig 1C). The cumulus cells were spherical and displayed some small microvilli and blebs. The cumulus investment of complexes in group 2 was more dispersed with a larger volume of intercellular space (fig 1E), and the individual cells were more elongated, made less contact with each other, and had longer microvilli and larger blebs than observed in group 1 (fig 1F). Moreover, in group 2 the cumulus cells were covered by intercellular matrix, giving the cells a fuzzy appearance.

At 7 h of culture the cumulus microvilli of cumulus–oocyte complexes in group 1 had undergone some degree of elongation, and initial covering of the cells by intercellular matrix was noticed. No morphological changes were observed in group 2. At 9 h (fig 2) the cumulus cells of complexes in group 1 had become more elongated

Fig 1. A. Light micrograph of a cumulus–oocyte complex with a dark rim of corona cells (group 1) before the onset of culture. x165. **B.** Scanning electron micrograph of a cumulus–oocyte complex in group 1 before the onset of culture. Note the compact appearance of the cumulus investment. x500. **C.** Scanning electron micrograph showing close contact between individual cumulus cells in a cumulus–oocyte complex in group 1 before the onset of culture. Note the moderate appearance of microvilli (Mv) and blebs (B). x3 000. **D.** Light micrograph of a cumulus–oocyte complex without a dark rim of corona cells (group 2) before the onset of culture. x165. **E.** Scanning electron micrograph of a cumulus–oocyte complex in group 2 before the onset of culture. Note the enlarged intercellular spaces. x500. **F.** Scanning electron micrograph showing the isolated cumulus cells in a cumulus–oocyte complex in group 2 before the onset of culture. Note the elongated microvilli (Mv), the large blebs (B) and the fuzzy appearance of the cells due to covering by extracellular matrix. x3 000.

Table I. Nuclear maturation of cumulus–oocyte complexes in groups 1 and 2 at different intervals of culture *in vitro*.

Group	Culture period	Oocytes (n)	Meiotic stages													
			GV		GVBD		ED/LD		MI		AI–TI		MII		DEG	
			n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
1		120	111	92.5	0	0									9	7.5
2	0	110	41	37.3	59	53.6									10	9.1
				++		++										
1		135	22	16.3	81	60.0	11	8.1	13	9.6	0	0			8	6.0
2	7	122	19	15.6	11	9.0	31	25.4	42	34.4	8	6.6			11	9.0
						++				++		++				
1		232					0	0	213	91.4					19	8.6
2	9	239					69	28.9	131	54.8					39	16.3
								++		++						++
1		260							169	65.0	71	27.3			20	7.7
2	13	173							75	43.3	69	39.9			29	16.8
										++						++
1		208							19	9.1	0	0	170	81.7	19	19.2
2	24	212							89	42.0	29	13.7	59	27.8	35	16.5
										++		++		++		

n = number of oocytes; GV = germinal vesicle; GVBD = germinal vesicle breakdown; ED = early diakinesis; LD = late diakinesis; MI = metaphase I; AI = anaphase I; TI = telophase I; MII = metaphase II; DEG = degenerated; + = $P < 0.05$, ++ = $P < 0.01$ (group 1 *vs* 2).

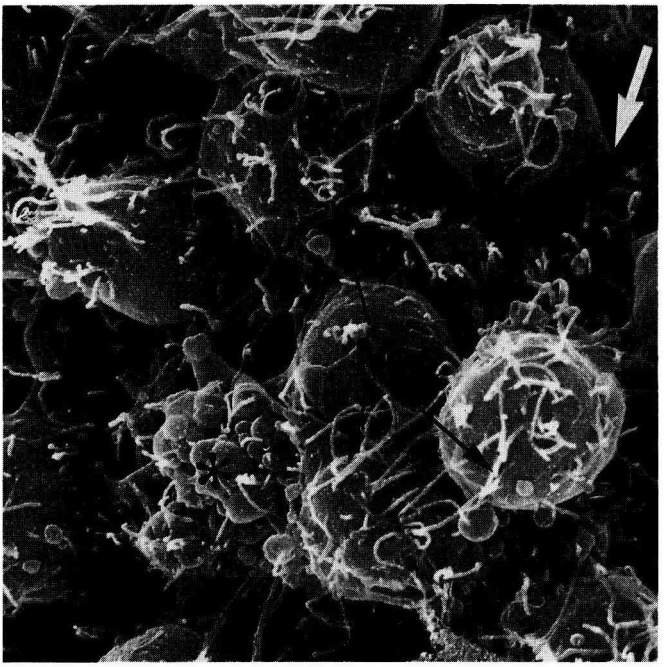

Fig 2. Scanning electron micrograph of a cumulus–oocyte complex in group 1 after 9 h of culture. Note the projections of elongated cumulus cells (arrow), blebs (*) and long microvilli (Mv). x7 200.

and more dissociated from each other, resulting in enlargement of the intercellular space. The cells showed more blebs and extracellular matrix covering longer microvilli than at any of the previous observation points. In complexes in group 2 the extracellular matrix was mainly located deeper in the cumulus investment. At 13 h the intercellular matrix of complexes in group 1 was also mainly observed in the deeper layers of the cumulus investment, while no morphological changes were noted in complexes in group 2. At 24 h the cumulus investment of complexes in both groups had large intercellular spaces, and the elongated cells displayed long microvilli and blebs, while the extracellular spaces, and the elongated cells displayed long microvilli and blebs, while the extracellular matrix was restricted to the deeper layers of the investment.

Corona–oocyte coupling

The tracer studies showed that a high degree of intercellular coupling was maintained in cumulus–oocyte complexes in group 1 up to 9 h of culture (fig 3). Between 9 and 13 h there was a marked decrease to a low level that was maintained up to 24 h. In complexes in group 2 the intercellular coupling was almost identical to

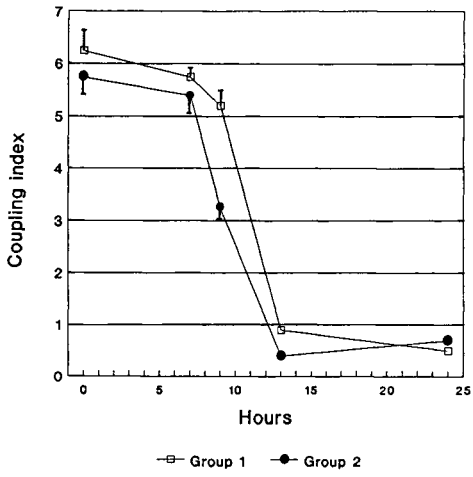

Fig 3. Coupling index in cumulus–oocyte complexes in groups 1 and 2 during culture *in vitro*. Vertical bars indicate SEM.

that observed in group 1 up to 7 h. Between 7 and 13 h there was a gradual decrease to a low level that was maintained up to 24 h.

DISCUSSION

It was clearly demonstrated by stereomicrocopy that it was possible to divide cumulus–oocyte complexes with compact multilayered cumulus investiment into 2 groups with different developmental capacities. Thus, complexes with a dark visible rim of corona cells (group 1) matured at a higher rate and maintained unexpanded characteristics and an efficient corona–oocyte coupling longer than complexes without a visible corona rim (group 2).

The present study demonstrated a marked difference in nuclear status of oocytes in group 1 *versus* 2 at the start of culture. Oocytes in group 1 displayed an intact germinal vesicle in 92.5% of cases, in agreement with the findings of Motlik *et al* (1978), whereas Xu *et al* (1986) reported that 27.3% of oocytes with compact multilayered cumulus investment showed some degree of meiotic activation of the germinal vesicle. More than half the oocytes in group 2 in the present study were in the process of germinal vesicle breakdown. In accordance with this figure, Xu *et al* (1986) reported that 71.4% of oocytes with very sparse cumulus investment showed some degree of activation of the germinal vesicle. The discrepancies between the figures are probably due to differences in the subjective evaluation criteria employed. Oocytes in group 1 of the present study reached metaphase II at 24 h of culture in 81.7% of the cases, which is in accordance with the 75% reported for oocytes with compact cumulus investment by Xu *et al* (1986). Only 27.8% of the oocytes in group 2, however, reached metaphase II, which is considerably less than the 68.8% reported by Xu *et al* (1986) for oocytes with sparse cumulus investment. In the present study 42% of the oocytes in group 2 became arrested at metaphase I, while this was only the case for 12.5% of the oocytes with sparse cumulus investment according to Xu *et al* (1986). The reason for these discrepancies remains obscure.

From the surface morphology it was clear that the cumulus–oocyte complexes in group 2, in contrast to group 1, had achieved certain characteristics of expansion such as decreased contact between and elongation of the cumulus cells together with the presence of intercellular matrix already before the start of culture. In group 1 these features were not noted until 7–9 h of culture. The morphological features of expansion are in accordance with those reported previously in swine (Fléchon *et al*, 1986).

The possible relationship between the loss of intercellular coupling between the corona cells and the oocyte and the meiotic activation of the oocyte, *ie* whether the disconnection triggers resumption of meiosis has been the subject of much controversy (for review see Eppig and Downs, 1984). On the basis of experiments in laboratory animals such a causal relationship has been postulated (Dekel and Beers, 1980; Dekel *et al,* 1981; Bronsleager and Schultz, 1985), while other experiments in laboratory animals (Eppig, 1982; Eppig and Downs, 1984), man (Hyttel *et al,* 1986c), sheep (Moor *et al,* 19ℰ1) and swine (Gérard *et al,* 1979; Motlik *et al,* 1986) indicate that resumption of meiosis precedes intercellular uncoupling. Semiquantitative assessment of the frequency of gap junctions between the oocytes and the corona cells during *in vitro* culture of bovine cumulus–oocyte complexes has shown that this type of junction is lost at 12–18 h of culture, during which period the oocytes progress from germinal vesicle breakdown to metaphase I (Hyttel, 1987). In the present study the intercellular coupling in cumulus–oocyte complexes in group 1 was functionally maintained up to 9 h of culture in accordance with the previously-mentioned report. However, in the present material all oocytes had progressed to metaphase I at 9 h of culture, suggesting that a high degree of coupling is maintained up to and including this stage of oocyte maturation. The changes in surface morphology showed loss of the intimate contact between adjacent cumulus cells at 7–9 h of culture, *ie* before the decrease in corona–oocyte decoupling. These facts support the hypothesis based upon experiments in swine that resumption of meiosis is triggered by isolation of the cumulus–oocyte complex through disconnection in the cumulus investment rather than by isolation of the oocyte from the corona cells (Motlik *et al,* 1986). In group 2 a decrease in corona–oocyte coupling was already observed at 7-9 h of culture. It has been clearly demonstrated by Staigmiller and Moor (1984) that the corona-oocyte coupling during oocyte maturation is of great importance for the developmental capacity of the oocyte to undergo fertilization and embryonic development. Thus, in addition to a very low rate of nuclear maturation, oocytes in group 2 are expected to have a poor developmental capacity.

It is well recognized that oocytes collected from slaughterhouse material for *in vitro* production of embryos constitute extremely heterogeneous material. The results of the present investigation, however, indicate that the presence of a dark rim of corona cells may be used as a criterion for selection of oocytes with presumably the highest developmental capabilities.

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