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CHRONOLOGICAL CHANGES OF BOVINE FOLLICULAR OOCYTE MATURATION IN VITRO*

By

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XU, K. P., T. GREVE, S. SMITH and P. HYTTEL: *Chronological changes of bovine follicular oocyte maturation in vitro.* Acta vet. scand. 1986, 27, 505—519. — Chronological changes of bovine follicular cumulus-oocyte-complexes were studied after in vitro maturation over a period of 48 h. According to their thickness and compactness of cumulus investments they were classified into 4 groups and cultured in enriched Ham's F-10 medium with or without human chorionic gonadotrophin (hCG) and estradiolbenzoate (EB) for 0, 6, 12, 18, 21, 24, 27, 30 and 48 h. Representative samples were taken at each time interval for evaluation of nuclear maturation stages, ooplasm quality and size of the perivitelline space (PVS). The results showed that oocyte nuclear breakdown (ONBD) required 6 to 12 h culture, and the peak of the first polar abstriction occurred at 24 h. The culture period required for ONBD and abstriction of the first polar body were related to the thickness and compactness of cumulus investments with and approximately 6 h delay in heavily compacted complexes. Ooplasm quality evaluation failed to show a clear trend, but the PVS increased in size from 0 h to 30 h and then retracted again from 30 to 48 h. The overall maturation rate in the presence of hCG and EB was 79.1 %, and a substantial proportion (68.8 %) of nude or partially covered oocytes reached metaphase II stage. In the presence of hCG and EB no block at either metaphase I or at anaphase-telophase I was observed. In the absence of hCG and EB the percentage of oocytes reaching metaphase II was much lower (48.6 %) in comparison with oocytes matured in the presence of these hormones (79.1 %). It was concluded a very high proportion of slaughterhouse oocytes could be matured in vitro and that the cumulus investments and addition of certain hormones affected the maturation rate.

nuclear maturation; hormones; cumulus; cattle.

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Most of mammalian oocytes including those of cattle mature in vitro when they are released from the follicular environments and placed in suitable culture media (*Edwards* 1965). The in vitro maturation events and the biochemical components involved in meiosis are poorly understood despite considerable research efforts in recent years (*Jagiello et al.* 1974, *Sato et al.* 1978, *Fulka et al.* 1981, *Süss & Wüthrich* 1985, *Motlik & Fulka* 1986, *Eppig & Schroeder* 1986).

The only reliable criteria for normal oocyte maturation are fertilization in vitro and subsequent development of the fertilized egg into live offspring (*Moor et al.* 1981, *Hensleigh & Hunter* 1985, *Leibfried-Rutledge et al.* 1985). However, the developmental capacity of most in vitro matured oocytes is low (*Ball et al.* 1984) and only recently has a pregnancy been established following in vitro fertilization of in vitro matured oocytes (*Critser et al.* 1986). Even in vivo matured oocytes have low survival rates following in vitro fertilization (*Greve et al.* 1984, *Lambert et al.* 1986).

There is still a need for more detailed studies of the requirements for and the conditions that allow proper in vitro maturation of bovine oocytes, and the aims of the present study are: (1) to establish a detailed chronology of the nuclear maturation of bovine oocytes cultured in vitro for different periods of time, and (2) to examine if certain factors such as hormones could influence this nuclear maturation process.

MATERIALS AND METHODS

Oocyte collection

Ovaries were obtained from cows within 30 min after slaughter at a local abattoir. Follicular contents from small visible antral follicles (approx. 1—6 mm in diameter) were aspirated and placed in a conical centrifuge tube containing 4 ml Hepes buffered Ham's F-10 (F-10), supplemented with 20 % heat treated foetal calf serum (FCS), 0.4 mmol/l glutamine, and 50 I.U. penicillin and 50 μg dihydrostreptomycin per ml. The tubes were kept at 30—38°C. After 30 to 40 min the sedimented oocytes with their cumulus investments were transferred to fresh medium of the same composition. To establish a zero hour control group follicular contents were stored without medium at 0°C until evaluation and fixation.

Oocyte evaluation and selection

Within 2 to 3 h of collection the oocytes were brought to the laboratory, washed 3 times in fresh medium and evaluated under a stereomicroscope. In Exp. I (a and b) pools of oocytes were assigned into 4 groups based upon their gross morphology and the integrity of the cumulus cells. The features and descriptions of different groups are shown on Plate 1, Figs. 1 to 5. No attempt was made to classify the ooplasm quality of groups I, II and III due to the thick layers of cumulus cells that made proper evaluation impossible. Oocytes with shrunk, vacuolated ooplasm or with other signs of degeneration were excluded from group IV. In Exp. II only healthy looking oocytes with more than 2—3 layers of cumulus were included.

Oocyte culture

The onset of culture time was defined as the mean time of the oocyte collection period. The medium used for oocyte culture was identical to the F-10 used for the collection with the exception, that it was bicarbonate buffered. In Exp. I_b no further supplementation was done while in Exp. I_a and Exp. II 2 I.U. hCG (Physex, Leo, Denmark) and 1 µg estradiolbenzoate (Ovex, Leo, Denmark) were added per ml. Culture was performed in 4 well multidishes, at 38°C/5 % CO_2 in humidified air for 0, 6, 12, 18, 24, 30 and 48 h (Exp. I_a and Exp. I_b), and 21, 24 and 27 h (Exp. II). For each group, 10 to 15 oocytes were placed in 1.0 ml medium which was filtered through a 0.22 µm milipore filter and equilibrated in 5 % CO_2 for at least 3 h prior to cultivation.

Oocyte evaluation before fixation

After various periods of culture, oocytes were sampled and the cumulus cells were removed with trypsin (original Trypsin-EDTA solution, GIBCO U.K.) along with repeated pipetting. The denuded oocytes were mounted on grease free slides and examined under a stereomicroscope. Oocytes were classified according to the size of the PVS as: absent, small, medium and large. Based upon the character of the ooplasm, three oocyte types could be defined. Those with finely granulated and evenly distributed organelles were regarded as type I, and those that were more coarsely granulated with unevenly distributed organelles and some vacuolation were designated as type II. Large

vacuoles and shrunken ooplasm were indicative of degeneration and these oocytes were classified as type III. Following this stereomicroscopic evaluation oocytes were fixed in methanol: acetic acid (3:1) for at least 48 h, and then stained with 1 % aceto-orcein for the final examination.

Oocyte evaluation after fixation

After fixation and staining, oocyte nuclear maturation was evaluated under interference phase contrast microscope (320 ×) to define the meiotic stages as follows.

Oocyte nucleus stage I (ON I): Oocytes had a distinct nuclear envelope, chromatin was only around the nucleolus, which may not always be present ,and/or in the appearence of a few orcein-positive zones.

Oocytes nucleus stage II (ONII): The nuclear envelope was still present but less distinct, the chromatin was distributed in separate, well stained clumps or the individual filamentous bivalents were distinguishable.

Diakinessis (D): The nuclear envelope was no longer visible, and the bivalents remained within the area of the oocyte nucleus.

Late diakinesis (LD): The oocyte nucleus area had totally disappeared and the bivalents were further condensed.

Metaphase I (MI): The chromosomes were maximally condensed, and sometimes present as a whole clump from an equatorial view.

Anaphase-Telophase I (A-T I): The chromosomes were under division or segregation, and the membrane of the first polar body might be present but the spindle was still not detached.

Metaphase II (M II): The contracted metaphase chromosomes and the first polar body were both present.

If none of the above mentioned nuclear features were identified in the preparation, the oocytes were classified as Non-Defined (ND).

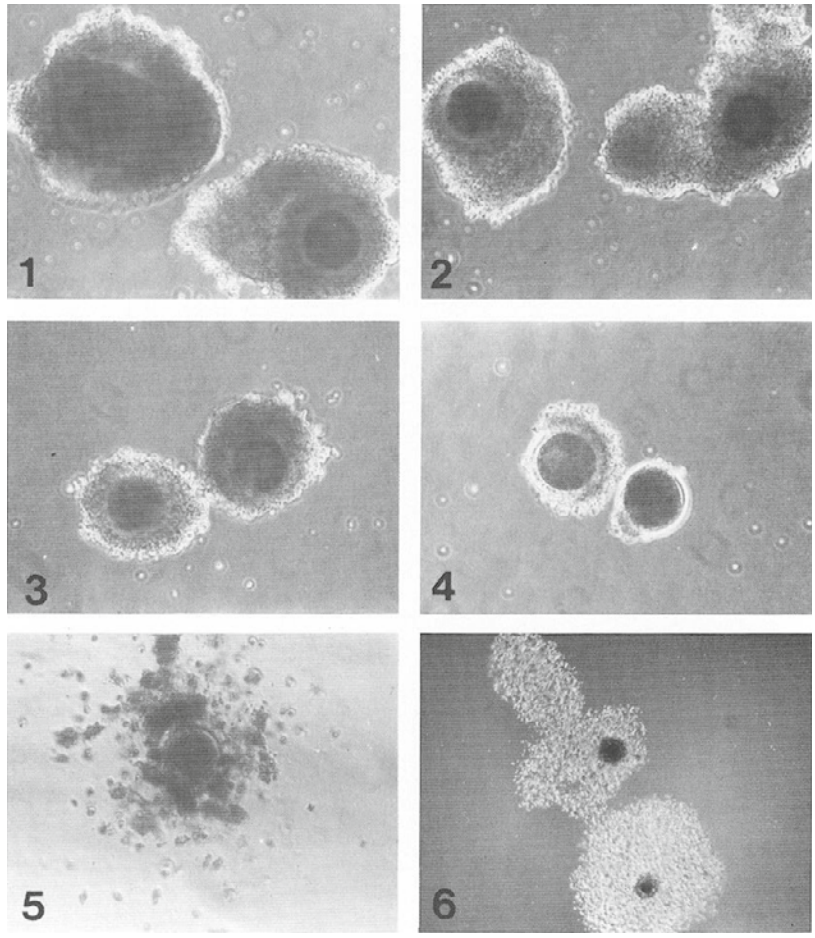

Plate 1. Morphologival appearance of cumulus-oocyte-complexes before and after culture.

Figure 1. (Group I): Oocytes with tight, completely compacted cumulus investments (70 ×).

Figure 2. (Group II): Oocytes with complete but not tightly compacted cumulus investments (70 ×).

Figure 3. (Group III): Oocytes with only a few layers of cumulus cells (70 ×).

Figure 4. (Group IV): Oocytes with only partial or none cumulus investments (70 ×).

Figure 5. Oocytes with degenerated cumulus investments. Note that cumulus cells are coalesced in scattered clumps. These oocytes were not included in the experiment (70 ×).

Figure 6. Typical morphological appearance of oocytes after culture for 30 h, showing expanded cumulus investments (20 ×).

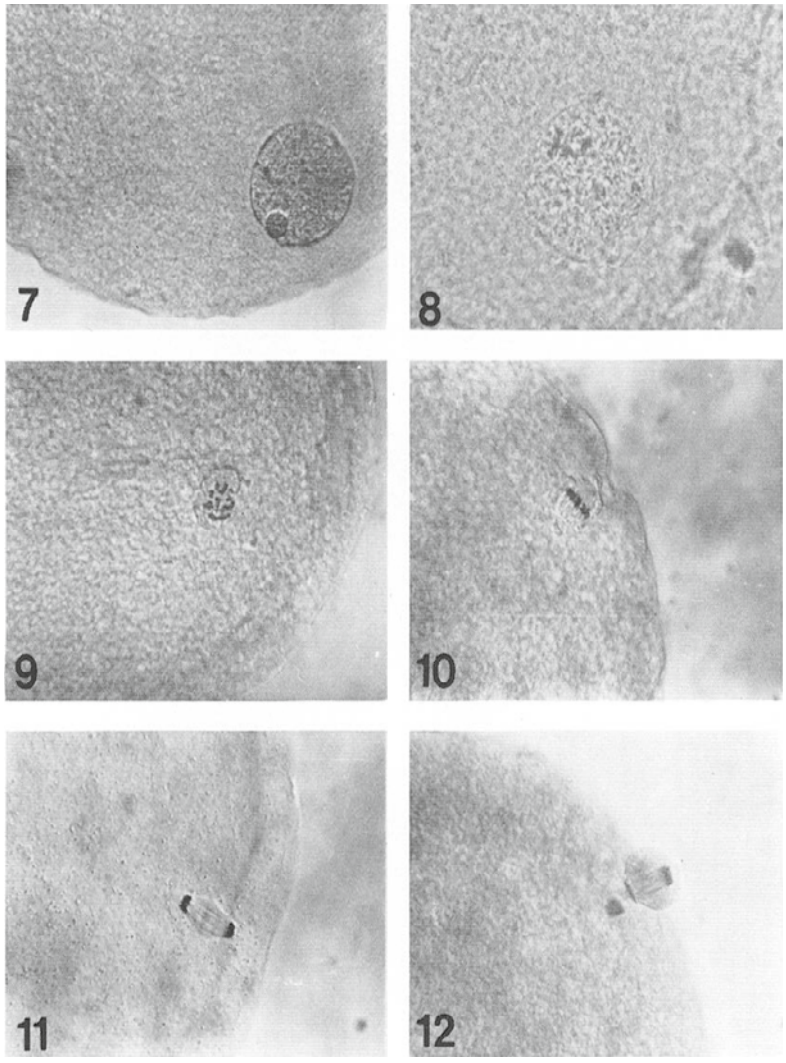

Plate 2. Representative examples of meiotic stages.

Figure 7. Oocyte nucleus stage I (ON I) (300 ×).

Figure 8. Oocyte nucleus stage II (ON II) (300 ×).

Figure 9. Diakinesis (D) (300 ×).

Figure 10. Metaphase I (M I) (300 ×).

Figure 11. Ana-Telophase I (A-T I) (300 ×).

Figure 12. Metaphase II with first polar body (M II) (300 ×).

RESULTS

Chronological changes of oocyte maturation

Photographic examples of the progressive meiotic stages are shown in Plate 2 (Figs. 7—12) and Table 1 depicts the distribution of meiotic stages over the culture periods, i.e. from 0 to 48 h ($n = 374$, Exp. I_a). The data in the table are based upon the identified oocytes, since the number of ND oocytes within each group were similiar, therefore, they were not included.

Table 1. Cytological changes of bovine oocytes cultured in vitro (Exp-I_a).

Culture period (h)	No. of identified oocytes	Meiotic stages (%)[1]						
		ON I	ON II	D	LD	M	A-T I	M II
0	75	69.3	25.3	4.0	1.3	0	0	0
6	35	8.6	22.9	60.0	8.6	0	0	0
12	39	5.1	2.6	28.2	53.8	7.7	2.6	0
18	64	1.6	1.6	4.7	26.6	15.6	25.0	25.0
24	67	0	0	0	6.0	7.5	7.5	79.1
30	54	1.9	0	5.6	5.6	14.8	1.9	70.4
48	40	0	0	0	0	70.0	0	30.0

[1] The meiotic stages are defined under Materials and Methods.

At the time of collection (0 h) the majority of oocytes (95 %) were at the oocyte nucleus (ON) stages. Approximately two thirds were at ON I (69.3 %), and one fourth (25.3 %) had shown some chromosome condensation which was classified as at the ON II. Only 5 % of the oocytes did not possess an ON.

At 6 h of culture, 69 % of the oocytes had undergone ONBD. At 12 h 92 % of oocytes showed ONBD, and most of them were at diakinesis or late diakinesis stage. The earliest of frist polar body abstriction was seen at 15 h. At 18 h 25 % of oocytes had extruded their first polar body, and the meiotic stages showed a most variable picture. The peak level (79 %) of polar body presentation occurred at 24 h. Following further culture, some of the polar bodies began to degenerate and disappear, as indicated by the declining percentage of M II after the peak level of 24 h. Only 30 % of oocytes had retained their first polar body at 48 h.

Table 2 shows the result of the Exp. II, i.e. the meiotic stages around the time of first polar body abstriction in 260 oocytes. At 21 h, approximately 20 % of oocytes were at A-T I, and

Table 2. Cytological changes of oocytes around the time of abstriction of the first polar body (Exp-II).

Culture periods (h)	No. of identified oocytes	Meiotic stages (%)[1]			
		Prometaphase[2]	M I	A-T I	M II
21	157	8.3	10.8	19.7	61.1
24	51	5.9	5.9	5.9	82.4
27	52	1.9	28.8	1.9	67.3

[1] The meiotic stages are defined under Materials and Methods.
[2] Prometaphase refers to ON I, ON II, D and LD.

following an additional 6 h culture only 2 % remained at this stage. The number of M II with polar body occurred 24 h, the same as in Exp. I_a (Table 1).

During the 48 h of culture period, no apparent changes, such as vacuolization or uneven distribution of organelles, were observed in the ooplasm. However, there was a tendency towards a more finely granulated ooplasm, i.e. from type II ooplasm to type I ooplasm.

The size of the PVS changed during the culture period. At 0 h 85 % of oocytes had no visible PVS. As the culture proceeded the PVS became gradually enlarged, and reached a maximum size at 30 h. Subsequently, the PVS retracted or decreased in size.

Effects of cumulus investments on oocyte maturation

The meiotic stages of the oocytes grouped according the morphology of the cumulus investments are shown in Table 3. At 0 h only group III oocytes exhibited ONBD. By 6 h of culture a majority of group II (93.3 %) and group IV (85.7 %) oocytes had lost their ON, while approximately two thirds of group I and II oocytes retained theirs. At 12 h of culture none of group III and IV oocytes had ON, whereas one fourth of group I oocytes still possessed theirs (Fig. 13).

The time required for oocytes to reach the final nuclear maturation was also affected by cumulus investments. At 18 h, 40 % of group III oocytes had extruded their first polar bodies, and the peak level of first polar presentation appeared at 24 h. Group II and IV matured slower and exhibited a lower percentage of polar body formation than group III, but they reached the peak level at 24 h, i.e., at the same time as group III. Group I oocytes matured at the slowest rate and reached the peak level

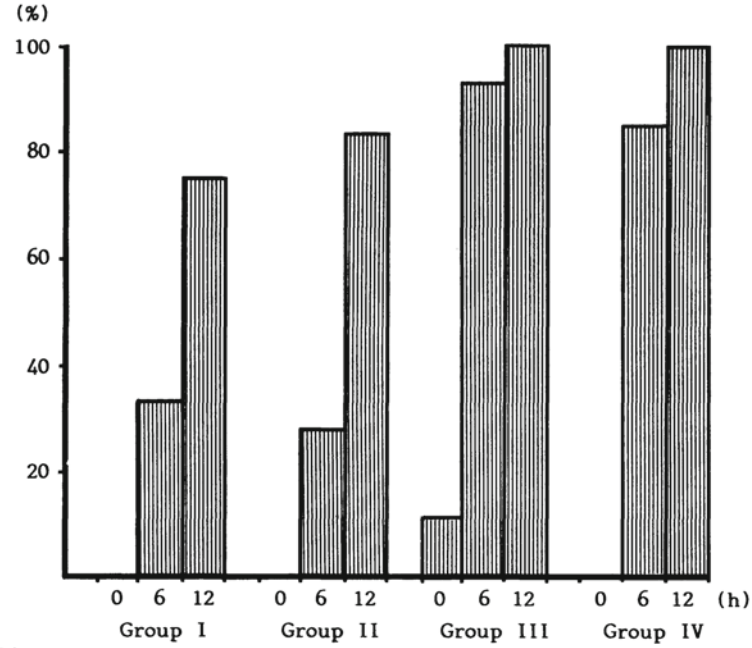

Figure 13. Time of oocyte nucleus breakdown (ONBD) of the 4 oocyte groups cultured in vitro.

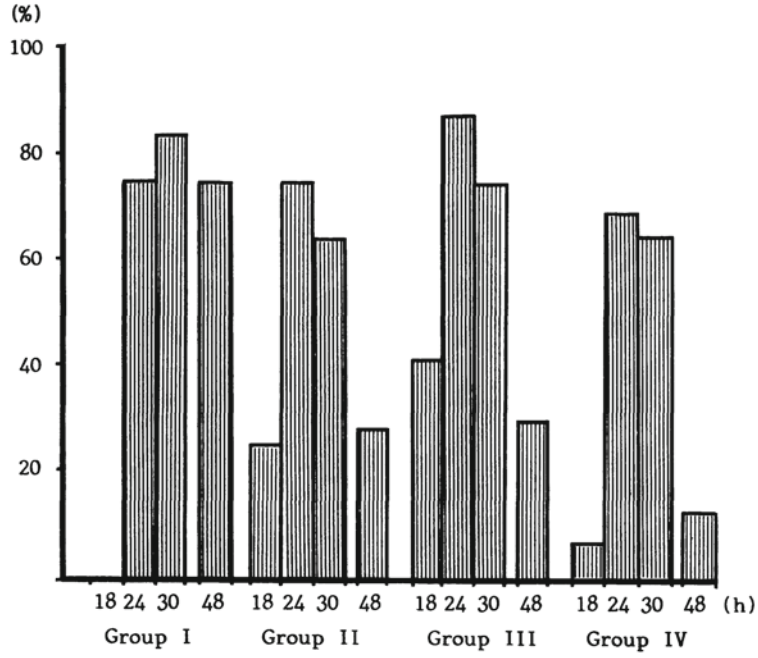

Figure 14. Time of first polar body appearance of the 4 groups of oocytes cultured in vitro.

Table 3. Cytological features of the 4 cumulus groups of oocytes cultured in vitro (Exp-I_a).

Cumulus group	Culture period (h)	No of oocytes evaluated	Meiotic stages (%)[1]						
			ON I	ON II	D	LD	M I	A-T I	M II
I	0	11	72.7	27.3	0	0	0	0	0
	6	6	16.7	50.0	33.3	0	0	0	0
	12	8	12.5	12.5	50.0	25.0	0	0	0
	18	6	0	0	16.7	33.3	50.0	0	0
	24	12	0	0	0	0	25.0	0	75.0
	30	6	0	0	0	0	16.7	0	83.3
	48	4	0	0	0	0	25.0	0	75.0
II	0	22	86.4	13.6	0	0	0	0	0
	6	7	28.6	42.9	28.6	0	0	0	0
	12	6	16.7	0	16.7	66.7	0	0	0
	18	16	0	0	12.5	25.0	12.5	25.0	25.0
	24	8	0	0	0	12.5	0	12.5	75.0
	30	11	0	0	0	9.1	27.3	0	63.6
	48	11	0	0	0	0	72.7	0	27.3
III	0	35	65.7	22.9	8.6	2.9	0	0	0
	6	15	0	6.7	80.0	13.3	0	0	0
	12	15	0	0	13.3	86.7	0	0	0
	18	27	0	0	0	25.9	3.7	29.6	40.7
	24	31	0	0	0	6.5	0	6.5	87.1
	30	23	0	0	4.3	4.3	13.0	4.3	73.9
	48	17	0	0	0	0	70.6	0	29.4
IV	0	7	28.6	71.4	0	0	0	0	0
	6	7	0	14.3	71.4	14.3	0	0	0
	12	10	0	0	40.0	20.0	30.0	10.0	0
	18	15	6.7	6.7	0	26.7	26.7	26.7	6.7
	24	16	0	0	0	6.3	12.5	12.5	68.8
	30	14	7.1	0	14.3	7.1	7.1	0	64.3
	48	8	0	0	0	0	87.5	0	12.5

[1] The meiotic stages are defined under Materials and Methods.

of polar body presentation at 30 h, approximately 6 h later than any of the other groups (Fig. 14). The peak level of first polar body occurrence was 83.3 %, 75.0 %, 87.1 % and 68.8 % for groups I, II, III and IV, respectively.

Effects of hormones on oocyte maturation

In the absence of hormones in the culture medium (Exp. I_b), oocyte maturation did proceede, as shown in the Table 4. At 18

Table 4. Cytological features of oocytes cultured in the medium with (Exp-I_a) or without (Exp-I_b) hormones.

Type of medium	Culture period (h)	No. of oocytes evaluated	Meiotic stages (%)[1]			
			Prometaphase[2]	M I	A-T I	M II
with hormones	18	64	34.5	15.6	25.0	25.0
	24	67	6.0	7.5	7.5	79.1
	30	54	13.1	14.8	1.9	70.4
without hormones	18	40	32.5	12.5	27.5	27.5
	24	37	29.7	5.4	16.2	48.6
	30	33	51.5	12.1	3.0	33.3

[1] The meiotic stages are defined under Materials and Methods.
[2] Prometaphase refers to ON I, ON II, D and LD.

h, 27.5 % of oocytes reached M II stage, which was similar to the proportion achieved in the hormone-containing medium (Exp. I_a, 25 %). At 24 h, however, the percentage was much lower in the hormone-free medium (48.6 %) as compared with the hormone-containing medium (79.1%). A similar decline in the percentage of M II was observed at 30 h (from 48.6 % to 33.3 %) under hormone-free conditions.

DISCUSSION

The time sequence of bovine oocyte nuclear maturation in vitro has previously been studied by *Hunter et al.* (1972), *Jagiello et al.* (1974), *Motlik et al.* (1978), *Süss & Wüthrich* (1985) and others. Most papers have placed their emphasis either on the early or the late meiotic stages. The present experiment provided a camplete chronology of bovine follicular oocyte maturation in vitro. It should be mentioned that the culture medium used in this study is comparable to thta used by *Newcomb et al.* (1978) who succeeded in obtaining two calves from follicular oocytes matured in vitro and fertilized in vivo.

In agreement with *Hunter et al.* (1972) we found that the majority of oocytes fixed shortly after aspiration possessed an intact oocytes nucleus (ON). The time required for ONBD lasted from less than 6 h up till more than 12 h, which is in agreement with *Jagiello et al.* (1974), but some what longer than those found by *Motlik et al.* (1978). A possible explanation for this descrepancy may be the definition of the onset of culture.

Oocytes examined at 12 h were mainly in diakinesis stages with a few in Metaphase I and Ana-Telophase I stages. First polar body abstriction was observed at 15 h in agreement with *Süss & Wüthrich* (1985), and then reached a peak level at 24 h. Reports in the literature give a wide range of the peak period for Metaphase II occurrence, from 20 h (*Fulka* 1981), 21 h (*Jagiello et al.* 1974), 20—24 h (*Süss & Wüthrich* 1985), 24—30 h (*Sato* 1978) to 31 h (*Edwards* 1965). Some of those difference are inherent to the culture conditions and methods of observation. The percentage of nuclear maturation was reportedly in the order of 60 % to 85 %. Less than 6 h was required for the nuclear change from M I to M II to occur in the study, which is about 4 h less than found by *Sato et al.* (1978).

Contrary to *Edwards* (1965) and *Hunter et al.* (1972) the present study failed to reveal any block at the Metaphase I or Anaphase I stages. Thus, the requirement for substantiating at least a proper nuclear maturation was fulfilled. This includes the Mg^{++} concentration, which is of particular interest, since *Liebfried & First* (1979b) showed that absence of Mg^{++} had a deleterious effect upon the completion of frist meiotic division.

It may under certain conditions be impossible to distinguish Metaphase II chromosomes by the whole mounting technique (*Trounson et al.* 1977), unless combined with the evidence of the first polar body as it was done in this study. However, when this polar body degenerates and disappears ,the Metaphase II chromosomes could be classified as Metaphase I, and this may explain why the percentage of Metaphase II showed a declining pattern after the peak.

In order to interprete the outcome of in vitro oocyte maturation, a better understanding of the heterogeneous developmental competence among the oocytes is required. *Fukui & Sakuma* (1980) found that the presence of cumulus cells was the most important factor for in vitro maturation of oocytes. *Dahlhausen et al.* (1981) found that oocytes devoid of thier cumulus cells did not mature in vitro. In the present study, however, 68.8 % of group IV oocytes (nude or partially covered oocytes) completed their first meiotic division, which is rather high in comparison with 44 % from similar oocytes reported by *Leibfried & First* (1979a). The reason why these denuded oocytes still have the ability to mature in vitro could be that they still possess cumulus cell projections embedded in the zona pellucida (*Hyttel et al.*

1986b), from which cAMP may be transferred to the oocytes (as reviewed by *Moor et al.* 1983, *Eppig & Downs* 1984). Whether these matured nude and/or partially nude oocytes have further developmental competence as in the mouse (*Schroeder & Eppig* 1984) remains to be investigated in order to make full use of these oocytes. Previous results from sheep, however, showed that oocytes matured in the absence of all associated follicle cells did not acquire the capacity for subsequent embryonic development (*Crosby et al.* 1981, *Staigmiller & Moor* 1984).

The fact that the maturational speed among four oocytes groups is different deserves special attention. It was shown that ONBD as well as first polarbody abstriction occurred approximately 6 h earlier in oocytes with few surrounding cumulus cells than in such with many cumulus cells (Figs. 13 and 14). It remains unclear whether this could be an indication that the different groups might come from different size of follicles and, thus, be at the different development stages (*Dahlhausen et al.* 1981, *Motlik et al.* 1984), or it could be an indication of a faster and easier removal of maturational inhibitor(s) (*Tsafriri & Channing* 1975, *Moor et al.* 1983) from less covered (group II and IV) and slightly expanded (group II) oocytes.

Monitoring the ooplasm quality indicated that there was no pronounced degenerative change taking place during the culture period. On the contrary, the proportion of oocytes with finely granulated ooplasm (type I) increased, which is comparable to in vivo conditions (*Greve et al.* 1984) and may reflect organelle redistribution that takes place during cytoplasmic oocyte maturation in vivo (*Hyttel et al* 1986a) as well as in vitro (*Hyttel et al.* 1986b).

It is generally accepted that the preovulatory LH surge triggers the resumption of meiosis and that steroids are important for full maturation and subsequent embryonic development in vivo (*Kruip et al.* 1983, *Dieleman et al.* 1983, *Callesen et al.* 1986). Furthermore, both gonadotrophins and estradiol-17β are required for intrafollicular oocyte maturation in vitro (*Moor et al.* 1984). However, contradictory results regarding the role of gonadotrophins in oocyte maturation in vitro have been reported by *Bae & Foote* (1975) and *Flemming et al.* (1985), who found that LH did not affect oocyte maturation in vitro at least of rabbits and pigs, respectively. Our results showed that in the absence of hCG and estradiol benzoate the percentage of oocytes complet-

ing the first meiotic division declined significantly, from 79 % to 49 %. Another striking finding was that oocytes cultured without hormones showed little cumulus expansion, a feature which is believed to be an important maturational character *(Hensleigh & Hunter* 1985) (Fig. 6).

In conclusion, this study showed that a large proportion of follicular oocytes aspirated after slaughter can be matured in vitro under proper experimental conditions, and that the time sequence of events was affected by the character of the cumulus investments and the presence of hormones in the culture medium. To test the further developmental capacity, contemporary experiments at this laboratory have shown that oocytes matured under these in vitro conditions can be penetrated by bovine sperm in vitro and that the zygotes will develop to at least the pronuclear stages.

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SAMMENDRAG

Kronologiske ændringer i bovine follikulære oocyter modnet in vitro.

Oocytmodningens kronologiske forløb blev studeret efter in vitro modning af slagtehusoocyter. Deres cumulus oophorus, perivitelline rum størrelse samt cytogenetiske karakteristika blev undersøgt umid-

delbart efter aspiration (kontroloocyter) og efter dyrkning i henholdsvis 6, 12, 18, 21, 24, 27, 30 og 48 timer i Ham's F-10 medium eller Ham's F-10 medium tilsat humant chorion-gonadotropin (hCG) og østradiolbenzoat (EB). Resultaterne viste, at oocytkernen blev nedbrudt (ONBD) inden for de første 6—12 timer, og at hovedparten af oocyterne afsnørede deres første pollegeme (PB1) efter 24 timers dyrkning in vitro. Begge disse processers tidsmæssige forløb var påvirket af cumuluscellelagenes antal og tæthed, således at oocyter omgivet af mange lag, tætsluttende cumulusceller brugte længere tid både til ONBD og til udstødelse af PB1.

Oocytmodningen var ligeledes under indflydelse af dyrkningsmediets hormonindhold, idet Ham's F-10 med hCG og EB resulterede i en højere modningsrate (79,1 %) and Ham's F-10 uden hormoner (48,6 %), bl. a. fordi en stor procentdel af oocyterne tilhørende denne sidste gruppe ikke nåede metafase II stadiet, men standsede ved metafase I og anafase-telofase I.

Nærværende undersøgelse har vist, at en meget høj procentdel af slagtehusoocyter kan modnes in vitro, at tilsætning af hormoner samt cumuluscellelagenes antal og tæthed påvirker det kronologiske forløb af denne proces.

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