Ultrastructure of in-vitro oocyte maturation in cattle

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Summary. Cumulus–oocyte complexes were collected from cows at an abattoir by aspiration from small (1–6 mm) antral follicles. After different periods of culture the complexes were processed for electron microscopy. Cumulus expansion occurred at 12–18 h of culture and concomitantly enlargement of cumulus cell projections in the perivitelline space was seen. At 48 h the innermost cumulus cells flattened and adhered tightly to the zona pellucida. In the oocyte the following changes occurred: at 0–3 h of culture the perivitelline space developed; at 3–12 h disconnection of the junctions between cumulus cell projections and the oolemma, and the concomitant breakdown of the nucleus was observed; at 12–18 h the mitochondria moved from a peripheral location to a more even spatial distribution and the Golgi complexes decreased in size; at ~18 h the smooth endoplasmic reticulum formed large aggregates surrounded by mitochondria; at 18–21 h the first polar body was abstricted; at 24–40 h the cortical granules spread; at 30–40 h the polar body degenerated in some specimens; at 40–48 h the perivitelline space decreased in size; and at 48 h one oocyte was in the process of fragmentation. It is concluded that nuclear and cytoplasmic oocyte maturation is simulated *in vitro*. However, certain deviations were noticed compared to in-vivo maturation.

Introduction

Oocytes liberated from their follicles can spontaneously resume meiosis and complete at least nuclear maturation when cultured. This has been described in a number of species including cattle (Edwards, 1965; Hunter, Lawson & Rowson, 1972; Shea, Latour, Bedirian & Baker, 1976; Fukui & Sakuma, 1980; for review see Fulka & Motlik, 1980). Attempts to fertilize such in-vitro matured oocytes *in vivo* by transfer to inseminated oviducts have revealed that the developmental capacity of the resulting zygotes is low (cattle: Hunter *et al.*, 1972; Trounson, Willadsen & Rowson, 1977; Newcomb, Christie & Rowson, 1978; sheep: Moor & Trounson, 1977; Staigmiller & Moor, 1984). Similar experiments in rabbits (Thibault & Gérard, 1973; Motlik & Fulka, 1974a) and pigs (Motlik & Fulka, 1974b) have demonstrated an inability to achieve male pronucleus formation possibly due to deviations in the course of cytoplasmic maturation.

In cattle, in-vitro fertilization of oocytes matured *in vivo* has given rise to live offspring (Brackett *et al.*, 1982; Lambert *et al.*, 1986) whereas in-vitro fertilization of oocytes matured *in vitro* has only resulted in development of early embryonic stages (Ball *et al.*, 1983; Leibfried-Rutledge, Critser, Eyestone, Northey & First, 1986), although one pregnancy has been obtained using oocytes matured in co-culture with granulosa cells (Critser, Leibfried-Rutledge, Eyestone, Northey & First, 1986).

Ultrastructural investigations have revealed certain aspects of cytoplasmic oocyte maturation *in vivo* in unstimulated (Fleming & Saacke, 1972; Kruij, Cran, van Beneden & Dieleman, 1983) and gonadotrophin-stimulated cattle (Hyttel, Callesen & Greve, 1986). However, only for human (Zamboni, Thompson & Moore Smith, 1972; Sathananthan, 1985) and mouse (Merchant &

Chang, 1971) oocytes have the ultrastructural features of oocyte maturation *in vitro* been compared with those of *in-vivo* maturation, revealing only minor differences.

The practical use of *in-vitro* fertilization in cattle relies upon achieving proper oocyte maturation *in vitro*. The objectives of the present study were to provide an ultrastructural description of the nuclear and cytoplasmic changes taking place in the cumulus–oocyte complex during maturation *in vitro*.

Materials and Methods

Collection and culture of cumulus–oocyte complexes. Ovaries were obtained from cows within 30 min after slaughter at a local abattoir. Follicular contents were aspirated from visible antral follicles of about 1–6 mm in diameter. The aspirates were placed in a conical centrifuge tube containing 5 ml Hepes-buffered (20 mM) Ham's F-10 medium (Flow Laboratories, Irvine, U.K.) supplemented with 20% heat-treated fetal calf serum (Flow Laboratories), 0.4 mM-glutamine, 50 i.u. penicillin/ml and 50 µg streptomycin/ml (collection medium). After 30–40 min the sedimented cumulus–oocyte complexes were aspirated and transferred to fresh collection medium, and within 2–3 h the cumulus–oocyte complexes were brought to the laboratory in a Thermos flask at 30–37°C. Subsequently, they were washed in fresh collection medium and evaluated under a stereomicroscope. Only cumulus–oocyte complexes with a compact multilayered cumulus investment were included in the study (Fig. 1). The selected complexes were cultured in bicarbonate-buffered Ham's F-10 medium with the same supplementation as used in the collection medium plus 2 i.u. hCG/ml (Physex: Leo Pharmaceuticals, Ballerup, Denmark; 1 mg $\equiv$ 2252 i.u.) and 1 µg oestradiol benzoate/ml (Ovex: Leo Pharmaceuticals) (culture medium).

The start of culture was defined as the midpoint of the collection period. Culture was performed in 4-well multi-well dishes with 10–15 cumulus–oocyte complexes in each well containing 1 ml culture medium at 38°C in a 5% CO₂ in humidified air. Complexes were fixed after culture for 3, 6, 9, 12, 18, 21, 24, 30, 40 or 48 h.

To establish a 0 h control group, cumulus–oocyte complexes were evaluated and fixed immediately after aspiration ($n = 4$) or aspirates were stored for 2–3 h without medium at 0°C until evaluation and fixation of the complexes ($n = 7$).

Fig. 1. Uncultured cumulus–oocyte complex after fixation in glutaraldehyde. Note the tight multilayered cumulus investment. $\times 150$.

Fig. 2. Control cumulus–oocyte complex of type I with a rounded oocyte nucleus (arrow). $\times 300$.

Fig. 3. Type I cumulus–oocyte complex cultured for 3 h. Note the slightly flattened oocyte nucleus (arrow). $\times 300$.

Tissue preparation. Cumulus–oocyte complexes (0 h control group, $n = 11$; cultured, $n = 43$) were fixed in 3% glutaraldehyde in 0.1 M-phosphate buffer for 1 h at 4°C and post-fixed in 1% OsO_4 in 0.1 M-phosphate buffer for 1 h at 4°C. Subsequently, they were washed in distilled water and block-stained with 0.5% uranyl acetate, dehydrated and embedded in Epon following standard procedures, and finally serially sectioned in semithin and ultrathin sections. Ultrathin sections were prepared when an oocyte nucleus, condensed chromatin configurations, or a polar body appeared in the semithin sections and/or at the equatorial plane of the oocyte. The semithin sections were stained with toluidine blue while the ultrathin sections were stained with uranyl acetate and lead citrate and examined on a JEM-1200 EX (Jeol, Tokyo, Japan).

Results

Nuclear oocyte maturation

Based upon structural features the aspirated cumulus–oocyte complexes were divided into the following stages of meiosis: oocytes with an intact rounded nucleus (type I) (Figs 2 & 3), oocytes that had neither an intact rounded nucleus nor a polar body (type II) (Figs 4–6) and those that had a polar body (type III) (Figs 8 & 9). These features were related to the culture time as indicated in Table 1. Oocyte nucleus breakdown occurred after 3 to about 12 h of culture and the first polar body was abstricted in the period from 18 to 21 h. In some specimens at 40 and 48 h, however, polar bodies were either not seen or they were lightly stained and considered to be degenerate (Fig. 9).

Fig. 4. Detail of a type II cumulus–oocyte complex in which the oocyte nucleus is in the process of breaking down after 12 h of culture. Note the convoluted oocyte nucleus (ON) in which condensed chromatin (Chr) is seen. $\times 4400$. *Inset* showing the same cumulus–oocyte complex with the convoluted oocyte nucleus (arrow). $\times 300$.

Fig. 5. Boxed area from Fig. 4 showing condensed chromatin (Chr) and disintegration of the nuclear envelope into vesicles (V). $\times 16\,800$.

Fig. 6. Detail of a type II cumulus-oocyte complex cultured for 18 h showing the zona pellucida (ZP) and condensed chromatin configurations (Chr) located peripherally in the ooplasm in a densely packed area. $\times 5000$. *Inset* showing the same cumulus-oocyte complex. Note the expanded cumulus investment and the condensed chromatin configurations (arrow). $\times 300$.

Fig. 7. Detail of a type III cumulus-oocyte complex cultured for 24 h showing the zona pellucida (ZP) which is traversed by a cumulus cell projection (CCP) with an enlargement in the perivitelline space (PvS). $\times 4800$. *Inset* showing the same cumulus-oocyte complex at a lower magnification at which the cumulus cell projection (arrow) exhibits similarities with a polar body. $\times 300$.

Cumulus morphology

All control cumulus-oocyte complexes had a multilayered cumulus investment in which the cells had well developed cisternae of rough endoplasmic reticulum and Golgi complexes. The cells adjacent to the zona pellucida had numerous projections which penetrated it and ended in invaginations of the oolemma. Junctions with a morphology similar to that of the zonula adhaerens and gap junctions were observed between these projections and the oolemma.

At 12 h of culture partial expansion of the cumulus cell investment was observed in some complexes, and at 18 h all complexes had completely expanded cumulus investments (Fig. 6). This cumulus morphology was maintained until 40 h of culture when decreased expansion was noticed in two complexes. At 48 h of culture the innermost cumulus cells flattened and adhered tightly to the zona pellucida (Fig. 11), and only one complex maintained partial expansion.

At 18 h of culture the oocyte of 2 complexes was degenerate although the expanded cumulus investment was unaltered. From 18 h of culture enlarged cumulus cell projections were frequently observed in the perivitelline space, some of a size similar to that of the first polar body (Fig. 7).

Oocyte morphology and cytoplasmic maturation

Control oocytes. The structure of the oocyte in the 0 h control complexes was similar to that described for oocytes aspirated before the occurrence of the LH peak *in vivo* (Hyttel *et al.*,

Table 1. Type of cumulus–oocyte complex relative to the period of culture

Culture period (h)	Type of cumulus–oocyte complex
Control complexes	I,I,I,I,I,I,I,I,I,I
3	I,I,I
6	I,II*, II*, II
9	I,I,II*
12	I,II*, II*, II
18	II, II, II, II, II, II, II, D,D
21	III, III, III
24	II, III, III, III, III
30	III, III, III, III, III
40	II, III, III†
48	II, II, III†, III‡

* Oocyte nucleus in the process of breaking down.
† Degenerated polar body.
‡ Oocyte in the process of fragmentation.
D, Degenerate oocyte.

Fig. 8. Type III cumulus–oocyte complex cultured for 30 h showing the first polar body (arrowhead) and condensed chromatin configurations (arrow) located peripherally in the ooplasm in a densely packed area. × 300.

Fig. 9. Type III cumulus–oocyte complex cultured for 40 h in which the first polar body (arrowhead) is degenerating. Again, condensed chromatin configurations are seen in the ooplasm (arrow). × 300.

Fig. 10. Detail of a type III cumulus–oocyte complex cultured for 24 h showing the zona pellucida (ZP) and cortical granules (CG) still located in a big cluster. × 17 000.

Fig. 11. Detail of a type III cumulus–oocyte complex cultured for 48 h showing cumulus cells (CC), zona pellucida (ZP) and in the ooplasm condensed chromatin configurations (Chr). $\times 3200$. *Inset* showing the same cumulus–oocyte complex at another section plane. Note the flat adhering cumulus investment. $\times 300$.

1986), i.e. absent or narrow perivitelline space, an eccentrically located nucleus, evenly distributed vesicles, peripheral mitochondrial clusters, well developed Golgi complexes and smooth endoplasmic reticulum (SER), large peripheral clusters of cortical granules and a close association between vesicles, lipid droplets and mitochondria throughout the SER.

Cultured oocytes. From 0 to 3 h of culture the perivitelline space developed and it enlarged until 9 h to a size which was maintained until 40 h. During the initial enlargement of the perivitelline space the junctions between cumulus cell projections and the oolemma were maintained. At 48 h of culture all complexes had narrow perivitelline spaces (Fig. 11).

In 2 out of 3 oocytes the nucleus was flattened after 3 h of culture (Fig. 3). From 3 to about 12 h of culture the oocyte nucleus was broken down. An increasing undulation of the nuclear envelope was observed resulting in the formation of long slender projections in which the nuclear envelope doubled back on itself. Finally, the nuclear envelope was dissolved into vesicles (Figs 4 & 5). Concomitant with these events the chromatin condensed and an area of increased density appeared in the nucleus (Figs 4 & 5). In some specimens, the chromatin was associated with the inner membrane of the nuclear envelope during this process. During the condensation of the chromatin, microtubules with a haphazard orientation appeared in the nucleus. After completion of the oocyte nucleus breakdown, condensed chromatin configurations were observed at peripheral sites in a

Fig. 12. Detail of a cumulus-oocyte complex cultured for 48 h showing the zona pellucida (ZP) and part of the oocyte in the process of fragmentation as indicated by the indentation of the oolemma (arrow). In the oocyte mitochondria (M) and cortical granules (CG) are seen. $\times 13\,800$. *Inset* showing the same cumulus-oocyte complex. Note the flat adhering cumulus investment and the indentation of the oolemma (arrows). $\times 300$.

densely packed area of the ooplasm (Fig. 6). At this stage the microtubules formed a well-organized spindle. Concomitant with the oocyte nucleus breakdown the junctions between cumulus cell projections and the oolemma were disrupted. All type I complexes displayed junctions, but in the type II complexes in which the oocyte nucleus was in the process of breaking down some of the junctions were disrupted. All other type II and all type III complexes showed a complete lack of junctions.

From 12 to 18 h of culture the mitochondria moved from their peripheral location in the ooplasm to an even spatial distribution, and the Golgi complexes decreased in size. At about 18 h of culture the vesicles tended towards a more central location and the SER either decreased in size or, characteristically, formed large aggregates surrounded by mitochondria. This aggregation was especially pronounced at 48 h of culture.

From 18 to 21 h of culture the first polar body was abstricted. In many specimens the polar body was surrounded by cytoplasmic droplets, some of which contained cortical granules. Again, condensed chromatin configurations were observed at peripheral sites in a densely packed area of the ooplasm (Figs 8 & 9). At 24 and 30 h the cortical granules were dispersed from their location in large clusters to solitary positions forming a lining along the oolemma in 1 out of 5 and 2 out of 5 oocytes, respectively. In the remaining specimens the granules remained partly clustered (Fig. 10). At 40 and 48 h the cortical granules were dispersed in all oocytes.

At 48 h of culture one oocyte had a deep indentation from the oolemma and was in the process of fragmentation (Fig. 12). This oocyte had one set of condensed chromatin configurations peripherally in the ooplasm and still displayed a lining of cortical granules along the oolemma.

Discussion

The culture medium used in the present investigation has basically the same composition as that used by Newcomb *et al.* (1978) which, following in-vivo fertilization of in-vitro matured bovine oocytes, resulted in live offspring. Nuclear oocyte maturation apparently follows the same pattern *in vivo* and *in vitro*. In agreement with the findings of Sreenan (1970) and Motlik, Koefoed-Johnsen & Fulka (1978) in-vitro oocyte nucleus breakdown occurred from 3 to about 12 h of culture. *In vivo* this feature is reported as taking place from 4 to 8 h and 6 to 14 h after the LH peak in unstimulated (Kruip *et al.*, 1983) and gonadotrophin-stimulated (Callesen, Greve & Hyttel, 1986; Hyttel *et al.*, 1986) cattle, respectively. The abstriction of the first polar body occurred from 18 to 21 h *in vitro*, and is reported to take place about 20 h and 19 h after the LH peak in unstimulated (Kruip *et al.*, 1983) and gonadotrophin-stimulated (Callesen *et al.*, 1986; Hyttel *et al.*, 1986) cattle, respectively.

The cytoplasmic oocyte maturation *in vitro*, as indicated by the ultrastructural changes, basically followed the same pattern as under in-vivo conditions and the biological implications of this sequence of events have been discussed in detail previously (Kruip *et al.*, 1983; Hyttel *et al.*, 1986). However, a certain variation and delay in the time of cortical granule spread was noticed *in vitro* compared to *in vivo*, in which the spread occurs around 19 h after the LH peak in unstimulated cattle (Kruip *et al.*, 1983) and from 21 to 22 h after the peak in gonadotrophin-stimulated cattle (Hyttel *et al.*, 1986). The cortical granule spread is regarded as being essential for the proper course of oocyte maturation (Sathananthan & Trounson, 1982), and the release of the granules during gamete fusion is thought to act in the block against polyspermia (Austin, 1956; Szollosi, 1967). Deviations in the cortical granule spread might therefore decrease the likelihood of subsequent normal fertilization of the oocyte. However, contemporary in-vitro fertilization experiments using in-vitro matured oocytes clearly show that the frequency of polyspermia is low (K. P. Xu, unpublished data).

Major rearrangements of mitochondria and vesicles of the oocyte taking place between 12 and 18 h of culture are also reported as occurring *in vivo* around 19 and 15 h after the LH peak in unstimulated (Kruip *et al.*, 1983) and gonadotrophin-stimulated (Hyttel *et al.*, 1986) cattle, respectively. In both types of animals these rearrangements coincide with a shift from oestradiol dominance to progesterone dominance in the follicular fluid (Dieleman, Kruip, Fontijne, de Jong & van der Weyden, 1983; Callesen *et al.*, 1986). One could speculate that this shift might be the inducing factor of the organelle rearrangements *in vivo* and the existence of a similar steroidogenic shift in the present culture system due to cumulus cell activity cannot be excluded but needs further elucidation.

The clustering of the SER, described as one of the later characteristics of in-vitro oocyte maturation, is also observed *in vivo* (Hyttel *et al.*, 1986). Kruip *et al.* (1983) suggested the existence of metabolic units in which lipid droplets, vesicles and mitochondria are combined through the SER. Thus, the clustering of the latter organelle may indicate a decrease in oocyte metabolism to a lower level which is maintained throughout the culture period.

From 18 h of culture enlarged cumulus cell projections were frequently observed in the perivitelline space, a finding which has also been reported *in vivo*, but at a much lower frequency (Hyttel *et al.*, 1986). Light microscopically, the largest of these projections may be misinterpreted as polar bodies in unstained preparations and therefore erroneously suggest the completion of nuclear oocyte maturation. Correspondingly, K. P. Xu & W. A. King (unpublished data) have observed metaphase I in oocytes which appeared to have abstricted the first polar body.

At 40 and 48 h of culture the first polar body had degenerated and disappeared in some

complexes and the perivitelline space decreased in size. Also, the degree of cumulus expansion decreased, resulting in the formation of a flat cell layer tightly adherent to the zona pellucida which probably deprives the oocyte of its ability to be fertilized as spermatozoa are phagocytosed by these cumulus cells (Pijnenborg, Gordts, Ongkowidjojo & Brosens, 1985; cattle: P. Hyttel, unpublished data). Finally, one oocyte was in the process of fragmentation. Altogether, these changes suggest that long culture periods result in a certain overmaturation of the cumulus–oocyte complex, rendering it unsuitable for in-vitro fertilization purposes.

In conclusion, both nuclear oocyte maturation and the sequence of structural events which is part of the cytoplasmic oocyte maturation are simulated *in vitro*. However, certain deviations which may be causally involved in the low capability of fertilization and further embryonic development inherent to in-vitro matured oocytes were noticed.

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