Qualitative and quantitative structural changes during pig oocyte maturation

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Summary. Pig oocytes were examined at hourly intervals after stimulation with hCG. Meiosis was resumed between 20 and 30 h after hCG. This coincided with a decline in the number of mitochondria and evidence is presented which indicates that this was due to fusion. The number of lipid droplets increased and the volume fraction of large vesicles decreased. Both these organelles maintained a close spatial relationship with the endoplasmic reticulum (ER). Mitochondria were clustered at the periphery of the cell before hCG injection but dispersed with maturation. The volume occupied by large vesicles, 'protein bodies' and Golgi also decreased at the edge of the oocyte with the progression of maturation.

Introduction

The acquisition of the ability of oocytes to undergo normal fertilization and subsequent development involves a progression of nuclear and cytoplasmic events. While maturation of the nucleus has a central role, it has been demonstrated that resumption of meiosis can occur spontaneously in culture (Pincus & Enzmann, 1935; Edwards, 1965) without necessarily achieving competence for embryonic development (Thibault, Gerard & Menezo, 1976).

During maturation, major changes take place in protein synthesis (McGaughey & van Blerkom, 1977; Warnes, Moor & Johnson, 1977; Moor & Warnes, 1978; Crosby, Osborn & Moor, 1981; Moor, Osborn, Cran & Walters, 1981) and it has been proposed that such changes are essential for the continuation of cytoplasmic maturation (Thibault, 1977; Golbus & Stein, 1978; Moor, Hay, Dott & Cran, 1978). Structurally, the oocyte is a complex cell within which the organelles maintain specific spatial relationships. It has been demonstrated for the sheep (Cran, Moor & Hay, 1980) and cow (Kruip, Cran, van Beneden & Dieleman, 1983) that major structural rearrangements take place with maturation. Such changes are likely to be correlated with changes in protein synthesis and other, as yet, uninvestigated functions.

Information relating to structural changes during oocyte maturation in the pig is largely lacking, and there is little information of a quantitative nature for any species for the critical period after the LH surge. The present study examines changes in pig oocyte ultrastructure during maturation induced by hCG and provides quantitative information on such changes.

Materials and Methods

Collection of oocytes

Cross-bred gilts of the Large White and Landrace breeds, 8–10 months old, were injected intramuscularly with 1250 i.u. PMSG (Intervet Laboratories, Cambridge, U.K.) and 500 µg cloprostenol (Estrumate: ICI, Macclesfield, U.K.) on the 15th day of the oestrous cycle (onset of oestrus = Day 0) followed by 500 i.u. hCG (Intervet) 84 h after injection of PMSG. None of the

animals had shown spontaneous oestrus before injection of hCG. To collect oocytes at specific times during maturation, gilts were anaesthetized and ovariectomized at 0, 10, 20, 30 and 40 h after hCG. Those follicles that had undergone stimulation were punctured and the cumulus masses flushed in Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954) at 37°C. Ovulation occurs in the pig 40–42 h after hCG (Dziuk & Polge, 1962) and ovulated oocytes were collected 50 h after hCG by flushing the oviducts (Polge, 1982).

Electron microscopy

Fifty cumulus masses were processed for electron microscopy. After washing, the oocytes were fixed at room temperature in 4% glutaraldehyde in Dulbecco's saline (pH 7.2), post-fixed for 1 h in 1% OsO₄ in cacodylate buffer, block stained for 1 h in 1% uranyl acetate in maleate buffer (pH 5.2) and embedded in Epon. Thick sections (1 µm) were stained with 1% toluidine blue in 1% borax and thin sections with lead citrate.

Thick serial sections were cut through all the oocytes and at least 5 from each group were selected for ultrastructural examination. Thin sections were cut at at least two levels through each oocyte, one of which was through the nucleus and the other through the mid-point of the cell.

Quantitation

The entire visible area of the sections of the oocytes was photographed at a magnification of $\times 1600$ and montages made at a final magnification of $\times 3800$. In addition, extensive lengths of the perimeter were photographed at $\times 6300$ and montages made at $\times 15\,000$. A total length equivalent to the circumference of 2 entire oocytes was examined in this manner at each stage.

For montages of entire oocytes the volume fractions (V_v) of mitochondria, lipid droplets and vesicles with a diameter $> 1\ \mu\text{m}$, termed 'large vesicles', were determined by point counting using a lattice in which the points were separated by 1 cm. The number of organelles per unit volume (N_v) was determined using the method of Weibel (Weibel & Gomez, 1962) in which $N_v = K/\beta \times (N_A)^{3/2}/(V_v)^{1/2}$ where K is a size distribution constant, β a shape constant and N_A the number of organelles per unit area. Since the coefficient of variation of the size frequency distribution was $< 25\%$, K was given a value of 1.05 (Williams, 1977). The value for β is dependent on the ratio of the axes of the organelles. These were 1.0, 1.53 and 1.44 for lipid droplets, mitochondria and large vesicles respectively ($n = 100$). These were accordingly given values of β of 1.382, 1.446 and 1.427, determined from the graph given by Weibel (1969). The cytoplasmic areas were traced, weighed and calculated from a standard.

Values of N_v were also determined for mitochondria present within $6\ \mu\text{m}$ of the oocyte plasma membrane. In addition, V_v values in this part of the cell for large vesicles, membranous constituents comprising endoplasmic reticulum (ER) and small vesicles, Golgi bodies, and 'protein' bodies were calculated. N_v was not determined for these organelles since they either did not consist of discrete entities or their dimensions were such that sections of entire organelles were frequently not included in micrographs taken at this magnification.

Results

Generalized structure

Apart from the nucleus the most prominent organelles in the pig oocyte were membrane-bound vesicles (large vesicles), lipid droplets and mitochondria. The membrane-bound vesicles which formed up to 15% of the oocyte volume measured $1.3 \pm 0.3\ \mu\text{m}$ (s.e.m.) ($n = 100$) in diameter. They were generally electron lucent (Pl. 1, Fig. 1) with occasional amorphous and membranous contents. A characteristic feature of such vesicles was an intimate relationship with the ER. This took the

Text-fig. 1. Volume fraction (V_v) and number/ μm^3 (N_v) of mitochondria (a & b), lipid droplets (c & d) and large vesicles (e & f) in whole pig oocytes after hCG.

form of a close apposition of smooth ER along part of the boundary of the vesicles (Pl. 1, Fig. 1). The lipid droplets which had an average diameter of $3.3 \pm 0.2 \mu\text{m}$ represented up to 20% of the cell volume. The structural appearance and dimensions of the lipid droplets varied with the stage of maturation (see below) and also maintained a close spatial relationship with the ER, which frequently formed an enclosing sheath (Pl. 1, Fig. 2). The mitochondria were similar to those observed in other domestic species, containing few cristae and frequently apposed vesicular or cisternal ER (Pl. 1, Fig. 3). Unlike the mitochondria of sheep and cattle oocytes (Senger & Saake, 1970; Fleming & Saake, 1972; Russe, 1975; Cran *et al.*, 1980), hooded forms were infrequent.

Golgi bodies were present in a peripheral position or adjacent to dictyate nuclei. Cortical granules were confined to the first $4 \mu\text{m}$ of cortical cytoplasm throughout maturation. Ribosomes were either in the form of rosettes or infrequently on rough ER and did not have any preferential distribution. An unusual structure formed by dilatation of rough ER (Szollosi & Hunter, 1973) was present throughout the cytoplasm (Pl. 1, Fig. 4). These took the form of vesicles ranging in diameter from 0.5 to $1.0 \mu\text{m}$. They frequently bore ribosomes on their external surface and for the sake of ease of description have been termed 'protein bodies' although the nature of their contents remains to be determined. The frequency of such ribosomes was less than in oocytes from unstimulated animals (Pl. 1, Fig. 5). In addition, protein bodies from stimulated animals were less electron dense than those from unstimulated pigs.

Quantitative changes

Whole oocyte. The volume fraction occupied by mitochondria remained constant throughout maturation (Text-fig. 1a) at about 0.06 (i.e. 6%). While the mean number was constant at $0.3/\mu\text{m}^3$

for the first 20 h after hCG there was a decline to $0.2/\mu\text{m}^3$ by 30 h, reaching $0.1/\mu\text{m}^3$ by 50 h (Text-fig. 1b).

There was considerable variation in the V/V value for lipid both within and between groups of oocytes (Text-fig. 1c) and there was no correlation for this measurement with time after hCG, the proportion of the cells occupied varying between 10 and 20%. As with the mitochondria, the number of lipid droplets remained constant for the first 20 h at about $0.007/\mu\text{m}^3$ (Text-fig. 1d). There was a rise thereafter such that by 30 h there were about 0.016 lipid droplets/ μm^3 .

The volume fraction of large vesicles remained constant up to 30 h after hCG at about 0.15 (Text-fig. 1e). At 40 h this had declined to 0.075. There was considerable variation in vesicle number throughout oocyte maturation and no significant changes in this characteristic were detected (Text-fig. 1f).

Oocyte periphery. At 0 h there were $0.9/\mu\text{m}^3$ mitochondria present at the edge of the oocyte (Text-fig. 2) compared with a mean of $0.3/\mu\text{m}^3$ for the entire cell. This was followed by a decline which was greatest between 30 and 40 h such that, at 50 h, $0.1/\mu\text{m}^3$ were present, a value similar to that for the entire oocyte at this stage (Text-fig. 1b).

Text-fig. 2. N_v of mitochondria in the periphery of pig oocytes after treatment with hCG.

The volume fraction occupied by vesicles was less than for the entire oocyte ($0.1 \mu\text{m}^3$ compared with $0.15 \mu\text{m}^3$) and declined significantly by 30 h (Text-fig. 3a). There was considerable variation in the volume fraction occupied by membranous elements (ER and small vesicles) between oocytes taken at the same time after hCG. At 0 h a value of 0.14 ± 0.06 was obtained, while at 40 h this was

PLATE 1

Fig. 1. At 20 h after hCG. Membrane bound large vesicles (V) with flocculent (F) or membranous (M) contents. Three of the vesicles show a close relationship with ER (arrows). Multicisternal Golgi bodies (G) are also present. $\times 18\,000$.

Fig. 2. At 0 h. Lipid droplet with bounding layer of ER (ER). A few electron-lucent streaks (arrows) are present. $\times 18\,000$.

Fig. 3. At 20 h after hCG. Mitochondrial grouping at the cell periphery demonstrating the association with vesicular (V) and cisternal (C) ER. $\times 10\,000$.

Fig. 4. At 40 h after hCG. 'Protein body': a few ribosomes (R) are present on the bounding membrane. $\times 39\,000$.

Fig. 5. Oocyte from an unstimulated pig. 'Protein body' showing numerous attached ribosomes. $\times 25\,000$.

Fig. 6. At 40 h after hCG. A group of 3 mitochondria enclosed by ER (ER). The mitochondrial envelopes show marked protrusions (arrows). $\times 41\,000$.

Fig. 7. At 40 h after hCG. Cross-section through mitochondrial protrusions. Note the uniformity of the profiles. $\times 80\,000$.

Fig. 8. At 40 h after hCG. A mitochondrial grouping in which 3 are connected by narrow bridges (arrows). $\times 24\,000$.

Fig. 9. At 40 h after hCG. Two mitochondria connected by a narrow neck. $\times 38\,000$.

Fig. 10. At 40 h after hCG. Mitochondria with a markedly dilated envelope (arrow). It also bears protrusions of its envelope. $\times 61\,000$.

Text-fig. 3. Vv of large vesicles (a), ER and small vesicles (b), Golgi (c) and 'protein bodies' (d) in the periphery of pig oocytes after treatment with hCG.

Table 1. State of meiotic maturation of pig oocytes after treatment with hCG

Hours after hCG	No. of oocytes	Maturation stage
0	13	Germinal vesicle
10	7	Germinal vesicle
20	7	Germinal vesicle
30	8	Metaphase I-II
40	7	Metaphase II
50	8	Metaphase II

0.08 ± 0.03 . Although there was a downward trend this barely reached significance (Text-fig. 3b). The Golgi remained constant for the first 20 h (Text-fig. 3c) and the 'protein bodies' for the first 30 h (Text-fig. 3d). Thereafter, neither of these organelles was present within the oocyte periphery.

Qualitative changes

The nuclei of all oocytes up to 20 h after hCG were in the germinal vesicle stage. The mean nuclear diameter was $30 \mu\text{m}$. Since the pig oocyte has a diameter of $\sim 110 \mu\text{m}$, the germinal vesicle

PLATE 3

Fig. 11. At 50 h after hCG. Lipid droplets containing numerous electron-lucent streaks. The bounding ER is discontinuous. $\times 14\,000$.

Fig. 12. At 40 h after hCG. Cisternal ER (C) is close to the oolemma and vesicular ER (V) is also present. $\times 46\,000$.

Fig. 13. At 20 h after hCG. Degenerating ends of somatic cell processes (P). No junctional contact is evident. $\times 16\,000$.

represents ~2% of the total volume. At 30 h the nuclei were in metaphase I or telophase I while those at 40 and 50 h were in metaphase II (Table 1).

At 0 h, as was also indicated by the quantitative data, the mitochondria, which had a mean diameter of $0.41 \pm 0.01 \mu\text{m}$, were aggregated towards the edge of the oocyte. With maturation, these and the large vesicles dispersed, resulting in, at 40 h and 50 h, a cortical region which was relatively organelle free. At this stage the mitochondria measured $0.62 \pm 0.02 \mu\text{m}$. Up to 30 h the mitochondria tended to occur in small groups with associated ER (Pl. 1, Fig. 3). From 30 h they became progressively associated with sheet-like cisternae of the type adjacent to lipid droplets and large vesicles (Pl. 2, Fig. 6). In addition, the cisternae were frequently related to groups of 2 or 3 mitochondria (Pl. 2, Figs 6 & 8). From 30 h marked finger-like blebbing of the mitochondrial outer chamber was observed (Pl. 2, Figs 6, 7 & 8). In addition, the mitochondria were also often linked by narrow bridges (Pl. 2, Figs 8 & 9). Generally only two mitochondria as seen in thin section were involved in the association, but occasionally three or more were linked (Pl. 2, Fig. 8). At 40 h and 50 h after hCG mitochondria with markedly dilated outer chambers were also present (Pl. 2, Fig. 10).

The lipid droplets at 0 to 20 h had a generally uniformly grey image and measured $3.4 \pm 0.2 \mu\text{m}$. At 30 h some contained many electron-lucent streaks. This form increased in frequency with time such that by 50 h after hCG virtually all the lipid was of this form (Pl. 3, Fig. 11). Lipid droplets in such oocytes had a mean diameter of $3.0 \pm 0.2 \mu\text{m}$. In addition, while at 0 h the droplets were generally totally enclosed by ER, at 40 and 50 h the association was more fragmentary (Pl. 3, Fig. 11).

Up to 20 h Golgi comprising several cisternal layers were present close to the plasma membrane and the nucleus and occasionally in deeper regions of the cell (Pl. 1, Fig. 1). After 20 h this form of Golgi was no longer observed. However, at this time extensive lengths of ER running parallel to the plasma membrane were present at the periphery of the oocytes (Pl. 3, Fig. 12). Occasional dilatations of such ER were associated with small vesicles and cortical granules.

Processes from somatic cells were in contact with the oolemma by intermediate junctions up to 20 h after hCG. However, at this time swollen remnants of processes lacking junctional contact were also present (Pl. 3, Fig. 13). After this time no junctional contact was evident.

Discussion

The morphological changes in the pig oocyte during maturation may be broadly divided into two phases; a phase before about 20 h after hCG and the subsequent period up to ovulation. In the first phase the nucleus remained in the germinal vesicle stage while in the second, nuclear maturation took place. During the first phase both the volume fraction and the number of mitochondria, lipid droplets and large vesicles remained constant. In the second phase, there was an abrupt change in these characteristics. Superimposed upon these changes were redistributive events and qualitative changes of organelles.

In the cow (Kruip *et al.*, 1983) and sheep (Cran *et al.*, 1980; Moor, Polge & Willadsen, 1980) morphological and functional events may be broadly related to germinal vesicle breakdown (GVBD). In these two species the time leading up to GVBD is about 6 and 9 h respectively. This phase has been termed the "inductive" period (Moor *et al.*, 1980) and it has been demonstrated that during this period the oocyte requires follicle cell support (Moor *et al.*, 1980). In the pig junctions between follicle cell processes and the oocyte were present up to 20–30 h after hCG. After GVBD in the sheep, there was a period of some 12 h, the "synthetic" period during which follicle cell support was not required (Moor *et al.*, 1980). In the pig the "inductive" phase appears to be particularly prolonged. Fertilization, however, can be achieved some 40 h after the LH surge (Hancock, 1959). The "synthetic" period in the pig may therefore be of a duration similar to that in the sheep and cow.

The timing of the onset of meiosis is similar to that reported by others (Spalding, Berry & Moffit, 1955; Hunter & Polge, 1966; Ainsworth, Tsang, Downey, Marcus & Armstrong, 1980) and corresponds with a marked decline in follicular fluid oestrogen levels and also with cumulus expansion (Ainsworth *et al.*, 1980). While there is little evidence for the induction of meiosis through hormone stimulated steroidogenesis (Foote & Thibault, 1969; Tsafiriri, Lindner, Zor & Lamprecht, 1972; Lieberman *et al.*, 1976) it is clear that initial high levels of oestrogen are necessary for the cytoplasmic maturation required for subsequent embryonic development (Moor & Trounson, 1977; Moor, 1978; Moor *et al.*, 1980). The nature of the signals passing between the two cell types is still obscure. However, the rapid change in oocyte metabolism evidenced by the alteration in organelles together with the onset of meiosis would suggest the presence of a strict control mechanism.

During maturation there was a decline in mitochondrial number such that at 50 h after hCG there was only one third the number at 0 h. Concomitantly, there was no change in the volume fraction indicating an increase in mitochondrial size, and direct measurements showed that over this period there was a $> 300\%$ increase in individual mean mitochondrial volume. There are two processes which might explain the loss of mitochondria; degeneration or fusion. While the observed dilatation of the mitochondrial envelope may be an indication that some loss of mitochondria by degeneration was taking place, few markedly degenerate organelles were observed. It seems unlikely, therefore, that the reduction in number may be accounted for by this means. After GVBD small groups of mitochondria become enveloped in cisternae of ER and also developed numerous extensions. In addition dumb-bell-shaped configurations were observed. In view of the numerical data, it is unlikely that these represent fission. It is rather more likely that they were indicative of fusion. The physiological significance of these changes is not clear. Dekel, Hultborn, Hillensjo, Hamberger & Kraicer (1976) demonstrated a decline in oxygen consumption during maturation of mouse cumulus-oocyte complexes. However, the majority of oxygen consumption may be accounted for by that of the somatic cells, although Magnusson, Hillensjo, Tsafiriri, Hultborn & Ahren (1977) have indicated that the maturation of denuded oocytes elicits an increase in oxygen consumption which is correlated with the onset of meiosis. The present observation may therefore be a structural manifestation of this change in metabolism.

Before hCG the number of mitochondria in the oocyte periphery was some 3-fold greater than the average for the entire cell. Following hCG this declined progressively, eventually reaching levels found elsewhere in the cytoplasm. Congregation of mitochondria close to the plasma membrane of non-matured oocytes is a common feature (Sotelo & Porter, 1959; Hadek, 1969; Fleming & Saake, 1972; Cran *et al.*, 1980; Kruij *et al.*, 1983) and may represent an economical position for processing molecules entering the cell. The movement to the interior may represent a loss of dependency of the follicular environment and appears to precede loss of intercellular coupling.

A close inter-organelle relationship, particularly between mitochondria and the ER is a common feature in mammalian oocytes (Fleming & Saake, 1972; Cran *et al.*, 1980; Kruij *et al.*, 1983). The relationship between the ER and lipid and the large vesicles may, however, be unique to the pig. Since the ER is a site of protein manufacture and transport, such an association may be indicative of a phospho-protein content within the droplets. However, the significance of such associations, the change in structure of the lipid droplets with oocyte maturation and the potential for the ER to exert a controlling influence on product utilization and intracellular transport must await the appropriate biochemical evidence.

As in the cow oocyte (Kruij *et al.*, 1983), Golgi bodies were infrequent after GVBD. However, cortical granule numbers (Cran & Cheng, 1985), as in other species, increase during late maturation (Zamboni, 1970; Sathananthan & Trounson, 1982). Such an increase does not appear to be due to centrifugal migration (Cran & Cheng, 1985). The Golgi has been shown to be the source of cortical granules in several species (Szollosi, 1967; Baca & Zamboni, 1967; Zamboni, 1970; Kang, 1974; Kang & Anderson, 1975; Selman & Anderson, 1975). In the pig, however, continued production of

granules could not be from multi-layer Golgi bodies. At 30–40 h after hCG extensive lengths of ER were present lying parallel to the plasma membrane. This was often associated with numerous small vesicles. While definitive evidence is lacking, this may represent a specialized form of Golgi involved in cortical granule formation.

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