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Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary

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Histological sections prepared from cortical parts of 25 bovine ovaries were used to study initiation of follicle growth *in vivo*. Small follicles were measured and characterized. Initiation of follicle growth consisted of two distinct consecutive phases. The first phase was characterized by transformation of granulosa cells from a flattened to a cuboidal shape and by their proliferation. In the second phase an increase in the number of granulosa cells was accompanied by a rapid increase in the size of the oocyte. Oocytes commenced growth when there were at least 40 granulosa cells in the largest cross-section (fourth generation of follicle cells). The oocyte diameter increased from 29.74 ± 0.30 μm (mean ± SEM) in primordial follicles to 92.90 ± 4.50 μm in small antral follicles. The zona pellucida first appeared as an island of periodic acid–Schiff positive material in small preantral follicles, but formed a complete ring around the oocyte when the late preantral stage was reached. Organ culture of ovarian cortical explants was used to study initiation of follicle growth *in vitro*. Within 2 days of culture most of the primordial follicles entered the growth phase: granulosa cells changed from a flattened to a cuboidal shape and entered S-phase as demonstrated by autoradiography after [^{3}H]thymidine incorporation. On day 2, 48.6% of follicles were labelled compared with 3% on day 0. Follicle growth started in the absence of gonadotrophins, in the serum-free medium, confirming the notion that gonadotrophins are not essential for this process. The culture system used here will be helpful in the study of the involvement of putative factor(s) in the initiation of follicle growth in large domestic animals.

Introduction

Initiation of follicle growth involves the passage of primordial follicles from the quiescent to growth phase and is characterized by three main events: change in shape of the granulosa cells from squamous to cuboidal, proliferation of granulosa cells and enlargement of the oocyte (reviewed by Hirshfield, 1991). In several species, changes in the granulosa cells precede oocyte growth. In mice and rats, the oocyte starts to grow when there are about 10 cuboidal granulosa cells in the largest cross-section of the follicle (Lintern-Moore and Moore, 1979; Andersen de Wolff-Exalto and Groen-Klevant, 1980). In humans and sheep, the oocyte enters the growth phase when it becomes surrounded by 15 cuboidal granulosa cells (Gougeon and Chainy, 1987; Cahill and Mauleon, 1981). Mhawi *et al.* (1991) reported that in newborn calves transition of flattened granulosa cells to cuboidal is followed by ultrastructural changes in the oocytes. However, the exact stage in which the oocyte enters the growth phase in the bovine ovary is unknown. In spite of accumulating information on follicle selection and dominance in cattle (reviewed by Fortune, 1994) very little is known about the initiation of bovine follicle growth and the early stages of folliculogenesis. Recently, several laboratories have attempted to culture preantral bovine follicles (Figueiredo *et al.*, 1994; Wandji *et al.*, 1996a; Nuttinck *et al.*, 1996), but with limited success as compared with mice (Spears *et al.*, 1994; Eppig and O'Brien, 1996). A better understanding of early folliculogenesis is needed to mimic follicle development *in vitro*.

The aim of the present study was (1) to characterize the sequence of events associated with the beginning of follicle growth in the bovine ovary, and (2) to establish and characterize culture conditions that will support the growth of bovine primordial follicles and allow the study of the effects of hormones and growth factors on the initiation of follicle growth.

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Materials and Methods

Sampling and processing

Bovine ovaries (Israeli Holstein) were collected at the local abattoir on several different occasions. Blocks of ovarian tissue containing cortex and antral follicles up to 4 mm in diameter from 25 ovaries were fixed in Bouin's solution, embedded in

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Table 1. Classification and characterization of small bovine follicles

Follicle	*n*	Layers of granulosa cells*	Number of granulosa cells* (range)	Follicle diameter (range, μm)	Oocyte diameter, μm (mean ± SEM)	Presence of zona pellucida	Clearly defined theca interna
Primordial (type 1)	105	1	< 10 (flattened)	< 40	29.74 ± 0.30^{a}	—	—
Primary (type 2)	93	1–1.5	10–40 (cuboidal)	40–80	31.12 ± 0.42^{a}	—	—
Small preantral (type 3)	17	2–3	41–100	81–130	49.48 ± 2.43^{b}	—	—
Large preantral (type 4)	13	4–6	101–250	131–250	68.61 ± 2.78^{c}	+	+
Small antral (type 5)	10	> 6	> 250	250–500	92.90 ± 4.50^{d}	++	++

Values with different superscripts are significantly different ($P < 0.05$).
n: number of non-atretic follicles examined.
*Largest cross-section of the follicle; the section where the nucleus of the oocyte is present.

paraffin wax and sectioned at 5 μm thickness. Sections were stained with either haematoxylin–eosin or periodic acid–Schiff (PAS) reagent.

Classification and measurement of follicles

Follicles were classified as described by Pedersen and Peters (1968) with modifications: (a) primordial follicles (type 1): oocyte surrounded by flattened granulosa cells; (b) transitory follicles (type 1+): oocyte surrounded by a mixture of flattened and cuboidal granulosa cells; (c) primary follicles (type 2): oocyte surrounded by one or one and a half layers of cuboidal granulosa cells; (d) small preantral follicles (type 3): two or three layers of granulosa cells; (e) large preantral follicles (type 4): four or more layers of granulosa cells; (f) small antral follicles (type 5): follicles with early antrum formation. Follicle and oocyte diameters were measured at right angles in the section where the nucleus of the oocyte was present (largest cross-section), and the mean diameters were then calculated. For each follicle, the numbers of flattened and cuboidal granulosa cells in the largest cross-section were estimated. Only non-atretic follicles (no pyknotic granulosa cells and no apparent sign of oocyte necrosis: eosinophilia of the cytoplasm, contraction of chromatin material) were considered in this study.

Culture of follicles

Ovaries were collected at the local abattoir in saline at about 30°C. Explants of ovarian cortex (1–2 mm^3) were prepared and cultured on floating lens paper (Topper *et al.*, 1975) at 38.5°C under an atmosphere of 5% CO_2. An average of 15 explants were cultured in one dish. Each experiment was repeated at least twice with two to three culture dishes for each treatment. The culture medium consisted of Minimum Essential Medium (α-modification) and was supplemented with Hepes (15 mmol l^{-1}), ITS (insulin, 5 μg ml^{-1}; transferrin, 5 μg ml^{-1}; selenium, 5 ng ml^{-1}), L-glutamine (20 mmol l^{-1}), sodium pyruvate (0.25 mmol l^{-1}), penicillin G (100 U ml^{-1}), streptomycin (10 mg ml^{-1}), neomycin (20 μg ml^{-1}) and 0.1% (w/v) BSA. All materials were purchased from Sigma Chemical Co., St Louis, MO. FSH (NIDDK oFSH-17, 100 ng ml^{-1}) was obtained from National Hormone and Pituitary program of NIDDK (Baltimore, MD) and was added to the culture medium as indicated. The biological potency of FSH was 20 × NIH-FSH-S1, as determined by a modification of the hCG Augmentation Bioassay of Steelman-Pohley. The medium was changed daily. The day of the beginning of culture was designated as day 0. After the incubation, explants were fixed in Bouin's solution, dehydrated and embedded in paraffin wax and serially sectioned at 5 μm. Between 30 and 50 sections were obtained from each explant and all non-atretic follicles were measured as described above.

Incorporation of [^{3}H]thymidine

[^{3}H]Thymidine (0.5 μCi ml^{-1}; Amersham Laboratories, Little Chalfont, Bucks; 25 Ci mmol l^{-1}) was added to the medium and explants were incubated for 4 h. Preliminary experiments showed linear [^{3}H]thymidine incorporation into explants over a period of 4 h. After the incubation, explants were washed in ice-cold PBS and fixed in Bouin's solution, dehydrated and embedded in paraffin wax and serially sectioned at 5 μm thickness. From each explant at least 50 sections were obtained. Sections were rehydrated, washed in 3% trichloroacetic acid and then washed in water, air-dried and covered with liquid photographic emulsion (Ilford K-5, Kodak, Rochester, NY). After 3 weeks of exposure, slides were developed (D19, Kodak), fixed and stained with haematoxylin. Slides from day 0, day 2 and day 4 were always processed together. All non-atretic follicles were examined in their largest cross-section. Granulosa cells with at least six grains (three times background) were considered labelled. Follicles with one or more labelled granulosa cells were considered labelled. The

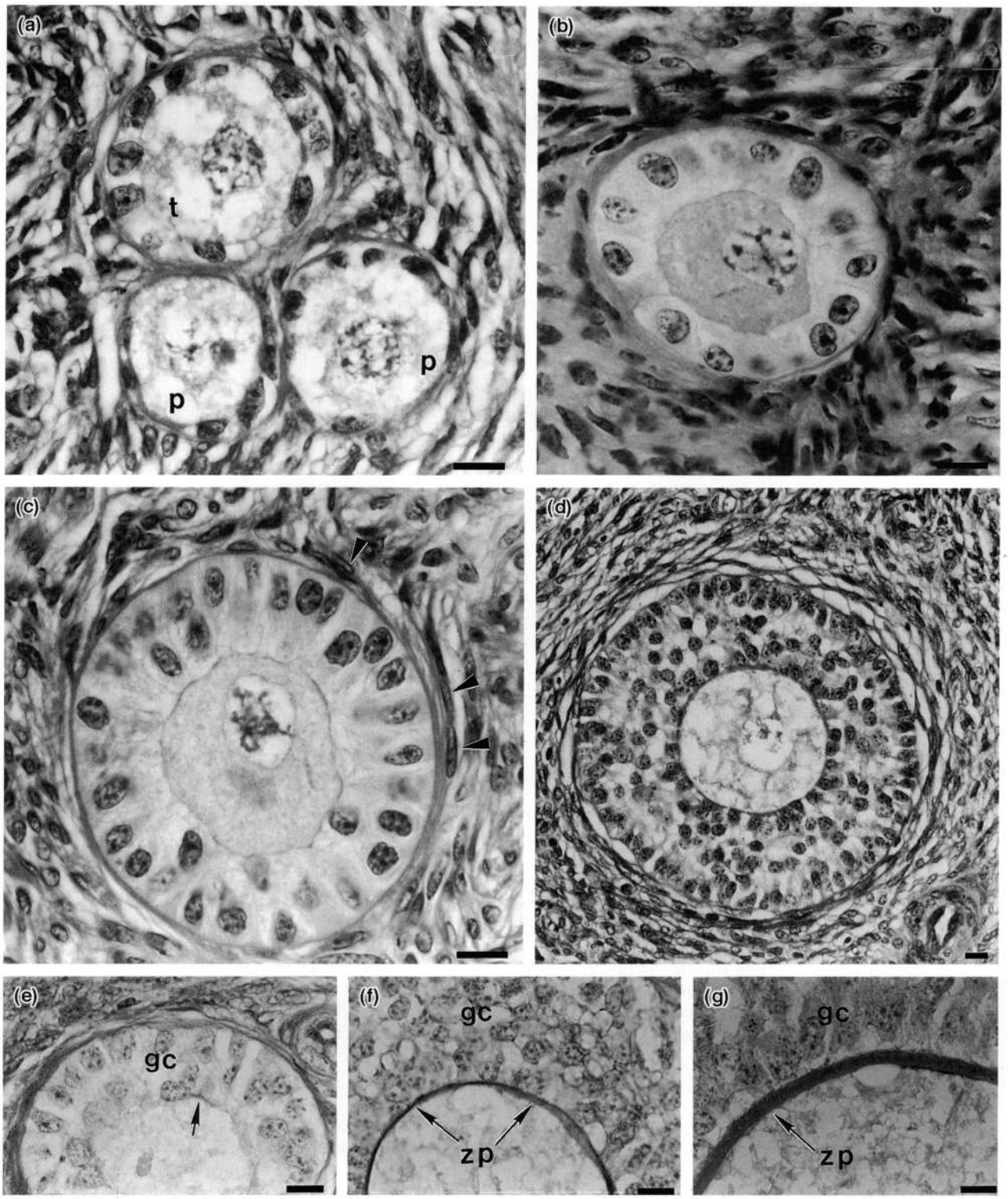

Fig. 1. Small follicles in the bovine ovary. (a) Two primordial follicles (type 1, p) with flattened granulosa cells, and transitory follicle (type 1 +, t) with a mixture of flattened and cuboidal cells. (b) Primary follicle (type 2): oocyte surrounded by a single layer of cuboidal cells. (c) Small preantral follicle (type 3): oocyte commences growth and is surrounded by two layers of granulosa cells. The beginning of theca formation can be recognized by presence of elongated cells (arrowheads) attached to the basement membrane. (d) Large preantral follicle (type 4): oocyte continues to grow and is surrounded by five layers of granulosa cells, and the theca interna is poorly defined. (e–g) Zona pellucida formation in bovine follicles; gc, granulosa cells. (e) Small preantral follicles with an island of periodic acid–Schiff (PAS) positive material (arrow). (f) Large preantral follicle with zona pellucida (zp) forming complete thin ring around the oocyte (arrows). (g) Antral follicle with thick zona pellucida (arrow). (a–d) Haematoxylin and eosin staining; (e–g) PAS staining. Scale bars represent 10 μm.

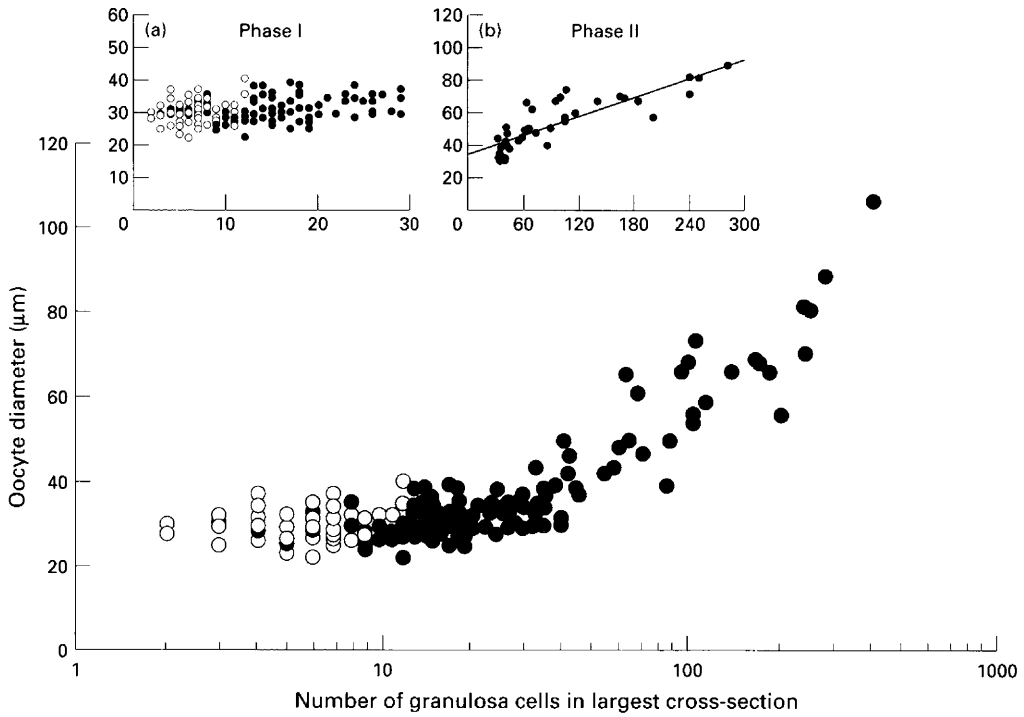

Fig. 2. Correlation between number of granulosa cells and oocyte diameter ($n = 240$) in bovine follicles. Two phases could be recognized: phase I (insert a), granulosa cells change shape from flattened (○) to cuboidal (●) and proliferate without increase in oocyte diameter; phase II (insert b), granulosa cells continue to proliferate and oocyte starts to grow, positive linear correlation between granulosa cell number and oocyte diameter ($y = 33.48 + 0.19\ x$, $R = 0.89$, $P < 0.001$, $n = 41$).

labelling index (LI) was calculated for labelled follicles as a percentage of labelled granulosa cells per total number of granulosa cells in the largest cross-section.

Statistical analysis

Statistical differences between means were calculated using analysis of variance (ANOVA) with Duncan's multiple comparison test or Student's t test. Proportion of follicles of different types and follicles labelled with [^{3}H]thymidine in different experimental groups were compared using chi-squared analysis. Correlation between number of granulosa cells/largest cross-section and oocyte diameter was calculated using linear regression analysis. Differences of $P < 0.05$ were considered significant.

Results

Follicle growth in vivo

The relationship between number of granulosa cells, follicle diameter and oocyte diameter in different follicle types is shown (Table 1). Primordial follicles (type 1, Fig. 1a) contained an average (mean ± SEM) of 6.44 ± 0.21 flattened granulosa cells and had a diameter of 35.23 ± 0.37 μm. Transitory follicles (type 1 +, Fig. 1a) were follicles that entered the growth phase and contained a mixture of flattened and cuboidal cells. Primary follicles (type 2, Fig. 1b) were growing follicles and contained 19.93 ± 0.81 cuboidal granulosa cells that formed one or one and a half layers of cells around the oocyte. The diameter for primary follicles was 55.06 ± 1.22 μm. The analysis of the correlation between oocyte diameter and number of granulosa cells during early follicle growth showed two distinct consecutive phases (Fig. 2). Phase I was characterized by a change in the shape of the granulosa cells from flattened to cuboidal and an increase in their number without a significant increase in the oocyte diameter. In phase II, a linear and positive correlation between number of granulosa cells and oocyte diameter was found ($r = 0.89$, $P < 0.001$). The first significant change in oocyte diameter was observed in follicles with 41–60 granulosa cells in the largest cross-section and the oocyte continued to grow rapidly (Fig. 3). The oocyte diameter increased from 29.74 ± 0.30 μm in primordial follicles to 92.90 ± 4.50 μm in small antral follicles (type 5).

Zonae pellucidae first appeared as islands of PAS-positive material in type 3 follicles, but only in follicles of type 4 formed a complete ring around the oocytes (Fig. 1e, f). Theca interna cells could be recognized occasionally as elongated cells attached to the basement membrane in small preantral follicles (Fig. 1c), but a clear theca interna layer was formed in some of the large preantral and all small antral follicles. The beginning of an antrum formation was observed in follicles with at least 250 granulosa cells in the largest cross-section. These follicles had a clearly defined theca interna, and the oocyte was surrounded by a thick zona pellucida (Fig. 1g).

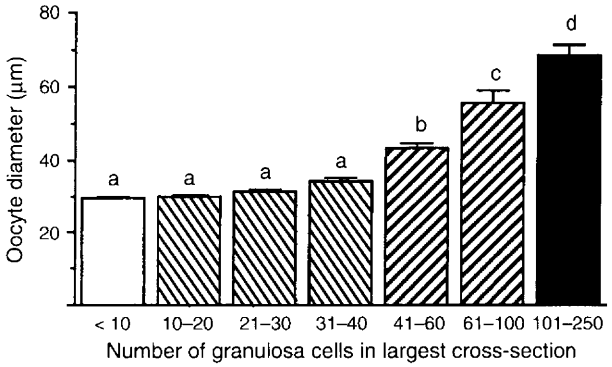

Fig. 3. Oocyte diameter in bovine small follicles with different numbers of granulosa cells. Primordial follicles (□), $n = 105$; primary follicles (▨), 10–20, $n = 55$; 21–30, $n = 24$; 31–40, $n = 14$; small preantral follicles (▨), 41–60, $n = 8$; 61–100, $n = 9$; and large preantral follicles (■), $n = 13$ follicles. Values are mean ± SEM. Columns with different superscripts are significantly different ($P < 0.05$).

Follicle growth in vitro

Freshly prepared explants contained mainly primordial follicles (72%), occasionally transitional or primary follicles (25%) and very few preantral follicles (3%). Within 2 days of culture many follicles started to grow: granulosa cells changed in shape from flattened to cuboidal and many of them incorporated [^{3}H]thymidine (Fig. 4). Only 10% of follicles were of the primordial type, while 86% were of the transitory or primary type (Table 2). The diameter of the follicles significantly increased from (mean ± SEM, µm) 37.94 ± 1.54 on day 0 to 50.83 ± 2.16 on day 2 of culture ($P < 0.05$, Table 2). Some follicles and oocytes remained viable for up to 4 days in culture, other oocytes degenerated or completely disappeared while granulosa cells looked healthy and continued to incorporate [^{3}H]thymidine. The diameter of the oocytes remained unchanged during the culture period. FSH had no effect on follicle or oocyte diameter (Table 2). Necrotic stromal cells were occasionally seen on day 2 of culture and were more common on day 4, mainly on the edge of the explants.

Incorporation of [^{3}H]thymidine

Only one follicle out of 33 had incorporated [^{3}H]thymidine on day 0 of culture (Table 3), while about half of the follicles were labelled on day 2, whether or not FSH was present in the medium. The labelling index (LI) increased about twofold on day 4 of culture compared with on day 2. The addition of FSH reduced the number of labelled follicles on day 4 but not on day 2 of culture, and had no effect on the LI of the follicles. Many stromal cells incorporated [^{3}H]thymidine on day 2 and day 4.

Discussion

The present study describes in detail the beginning of follicle growth in the bovine ovary. Our results show that follicle growth initiation can be divided into two distinct consecutive phases. The first phase is characterized by transformation of granulosa cells from a flattened to a cuboidal shape and by their

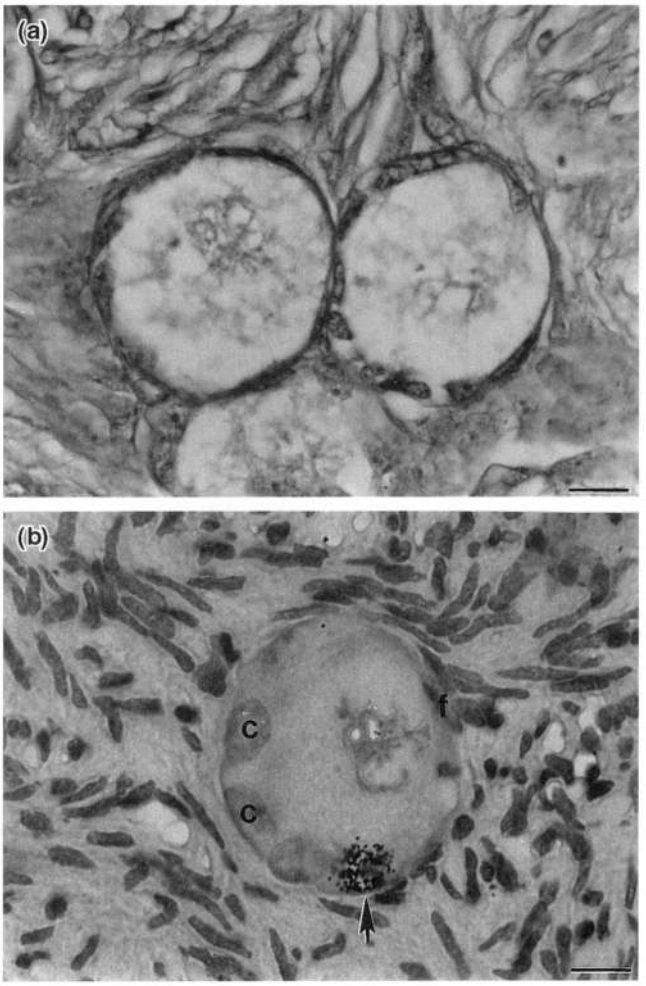

Fig. 4. Bovine primordial follicles *in vitro*. (a) Primordial follicles on day 0 of culture. (b) Transitory follicle on day 2 of culture: mixture of flattened (f) and cuboidal (c) granulosa cells and [^{3}H]thymidine incorporation into granulosa cell (arrow). Scale bars represent 10 µm.

proliferation. In the second phase an increase in the number of granulosa cells is accompanied by a rapid increase in the size of the oocyte. Similarly, in other species, changes in the granulosa cells precede growth of the oocyte. In mice and rats, the oocyte starts to grow when there are about 10 granulosa cells in the largest cross-section (Lintern-Moore and Moore, 1979; Andersen de Wolff-Exalto and Groen-Klevant, 1980). For sheep and humans the critical point is about 15 cells (Cahill and Mauleon, 1981; Gougeon and Chainy, 1987). The present study shows that in the bovine ovary oocytes start to grow later, and the critical point is about 40 granulosa cells. According to our results, the bovine primordial follicle contains approximately six granulosa cells on its largest cross-section (first generation of follicle cells). Thus oocyte growth is initiated during the fourth generation of follicle cells, compared with the second or third generation in rodents and humans, respectively.

Oocyte growth is accompanied by the formation of a zona pellucida. In mice and humans, the zona pellucida already forms a complete ring around the oocyte in the primary follicles (Himelstein-Braw *et al.*, 1976; Oakberg, 1979). In the bovine

Table 2. Effect of culture and FSH (100 ng ml^{-1}) on development of bovine follicles *in vitro*

Day of culture (*n*)	FSH	Primordial follicles (% of total)	Primary or transitory follicles (% of total)	Preantral follicles (% of total)	Follicle diameter (mean ± SEM, μm)	Oocyte diameter (mean ± SEM, μm)	Number of granulosa cells*
0 (69)	—	50[a] (72.0)	17[a] (25.6)	2 (2.9)	37.94 ± 1.54[a]	28.50 ± 0.49[a]	9.81 ± 1.48[a]
2 (58)	—	6[b] (10.3)	50[b] (86.2)	2 (3.4)	50.83 ± 2.16[b]	27.91 ± 0.52[a]	11.47 ± 1.22[a]
2 (57)	+	3[b] (5.3)	53[b] (93.0)	1 (1.7)	50.77 ± 2.67[b]	27.91 ± 0.53[a]	10.84 ± 1.85[a]

Within columns, values with different superscripts are significantly different ($P < 0.05$).
n: number of non-atretic follicles examined.
*Largest cross-section of the follicle.

Table 3. Incorporation of [^{3}H]thymidine into granulosa cells of bovine follicles *in vitro*

Day of culture	FSH (100 ng ml^{-1})	Number of labelled follicles/total number of follicles (%)	Labelling index (mean ± SEM)
0	—	1/33[a] (3.0)	3.03[a]
2	—	17/35[b] (48.6)	20.52 ± 3.18[b]
2	+	18/36[b] (50.0)	26.11 ± 3.36[b]
4	—	24/42[b] (57.1)	39.02 ± 4.54[c]
4	+	16/42[c] (38.1)	47.68 ± 6.84[c]

Labelling index has been calculated for labelled follicles: percent of labelled granulosa cells/total number of granulosa cells in the largest cross-section.
Within columns, values with different superscripts are significantly different ($P < 0.05$).

ovary, however, this occurs much later, when the follicle reaches the late preantral stage. Collagenase treatment has been used successfully for isolation of mouse and human preantral follicles (Eppig and Schroeder, 1989; Roy and Treacy, 1993; Spears *et al.*, 1994; Eppig and O'Brien, 1996). When a similar digestion protocol was used for isolation of bovine preantral follicles, it resulted in the death of most of the oocytes (Figueiredo *et al.*, 1993; Wandji *et al.*, 1996a; R. Braw-Tal and S. Yossefi, unpublished). The high sensitivity of bovine oocytes to the collagenase treatment may be due to the absence of a complete zona pellucida in early preantral follicles.

The present results demonstrate that bovine primordial follicles can enter the growth phase *in vitro*. Within 2 days of culture most granulosa cells had changed shape from flattened to cuboidal and entered S-phase as demonstrated by autoradiography following [^{3}H]thymidine incorporation. Follicle diameter increased by 13 μm and numbers of granulosa cells by about 20%. Although follicle growth could be initiated in culture, it was limited to granulosa cells, while oocytes had not commenced growth. According to our findings *in vivo* (see above) bovine follicles should have at least 40 granulosa cells in their largest cross-section before the oocyte enters the growth phase. In our culture system degenerative changes in the cultured tissue appeared before this step could be reached. Improvement of culture conditions would most likely allow oocyte growth to occur.

Several studies have shown that gonadotrophic hormones are not essential for the initiation of follicle growth in mice (Eshkol *et al.*, 1970; Ryle, 1972; Peters *et al.*, 1973a), rats (Nakano *et al.*, 1975; Hirschfield, 1985; Cain *et al.*, 1995) and sheep (McNatty *et al.*, 1990). Our study *in vitro* supports previous findings and extends them to the bovine ovary. Primordial follicles entered the growth phase when cultured in the absence of gonadotrophins and serum. Ninety percent of the follicles were growing on day 2 of culture, compared with 28% at the beginning of the experiment. Incorporation of [^{3}H]thymidine indicated that about half of the follicles entered S-phase on day 2 and day 4 of culture and twice as many granulosa cells per follicle were labelled on day 4. Granulosa cells of isolated human and bovine preantral follicles cultured under similar conditions also incorporated [^{3}H]thymidine, as reported by Roy and Treacy (1993), and Wandji *et al.* (1996a), respectively. In addition, Wang and Greenwald (1993) reported that preantral follicles in hypophysectomized mice incorporated [^{3}H]thymidine *in vivo* and Wandji *et al.* (1996b) reported that primordial follicles from fetal calves can survive and initiate growth *in vitro* in serum-free conditions. Gonadotrophins are apparently neither essential for follicles to enter the growth phase, nor for granulosa cells to proliferate. However FSH, although not essential, exerts an effect on small follicles, and absence of FSH results in abnormal folliculogenesis in the mouse ovary (Lunenfeld *et al.*, 1976; Wang and Greenwald, 1993), while addition of FSH into culture of isolated bovine preantral follicles increases proliferation of granulosa cells and follicle survival (Hulshof *et al.*, 1995; Wandji *et al.*, 1996a). In the present study, the presence of FSH did not affect the proportion of follicles that entered the growth phase, but slightly increased the labelling index of granulosa cells of growing follicles. Binding sites for FSH are present at very early stages of bovine folliculogenesis (Wandji *et al.*, 1992). Whether the effect of FSH on bovine granulosa cells is direct or

is mediated through locally produced growth factors such as basic fibroblast growth factor (bFGF), a potent mitogen of granulosa cells *in vitro* (Gospodarowicz *et al.*, 1977; Wandji *et al.*, 1996a), remains to be established. bFGF is secreted by bovine granulosa cells, ovarian surface epithelial cells and stromal tissue (Neufeld *et al.*, 1987; Gospodarowicz *et al.*, 1989). The higher proliferation rate of granulosa cells in our cultures, as compared with that reported for isolated follicles by Wandji *et al.* (1996a) may be due either to the effect of bFGF, most likely present in the cultured explants, or to other epithelial/stromal factors, often found in close proximity to growing small follicles (Bukovsky *et al.*, 1995).

The mechanism of primordial follicle recruitment into the pool of growing follicles remains among the major unsolved problems of biology of the ovary. The presence of an intraovarian inhibitor that prevents follicle growth initiation has been suggested by Peters *et al.* (1973b). In the present study, primordial follicles entered the growth phase within 2 days of culture and without any apparent stimulus, and this may imply the existence of a such an inhibitor within the ovary. This culture system will prove useful to the study of the involvement of putative factor(s) in the initiation of follicle growth in large domestic animals.

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