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Hormonal requirements for the growth and differentiation of hamster preantral follicles in long-term culture

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Summary. Preantral follicles from pro-oestrous and oestrous hamsters were isolated enzymically (Stages 1–5) and by microdissection (Stage 6) and cultured for up to 168 h in the absence or presence of 100 ng ovine FSH or LH separately or combined or 1 or 10 μg progesterone or oestradiol-17β in serum-free defined medium and exposed to 1 μCi [^{3}H]thymidine for 24 h before termination. In the presence of insulin and hydrocortisone but not gonadotrophins, the morphology of follicles from pro-oestrous animals at Stages 1–4 (1–4 layers granulosa cells; no theca) were unaffected for up to 48 h whereas for Stages 5 (5–6 layers granulosa cells and developing theca) and 6 (7–8 layers granulosa cells and theca), atresia was prominent by 24 h. FSH significantly reduced the percentage of atretic follicles in Stages 1–5 throughout the culture period; but was effective only up to 96 h for Stage-6 follicles. LH was also effective, albeit to a lesser extent. FSH increased follicular labelling indexes during every 24-h labelling period and, during a pulse-chase period, follicular DNA content and granulosa cell numbers. FSH, but not LH, induced differentiation by 96 h of preantral follicles at Stage 6 into small antral stages (Stages 7–8). FSH and LH together induced almost the same effect as FSH alone. However, neither progesterone nor oestradiol had any significant long-term effects on DNA synthesis and oestradiol induced atresia beyond 24 h. Both FSH and LH induced follicular maturation *in vitro* as evident from increases in progesterone, androstenedione and oestradiol production. Follicles (Stages 1–4) collected from oestrous hamsters responded to FSH to a lesser extent than did those from pro-oestrous animals, possibly because of in-vivo exposure to periovulatory changes in gonadotrophins; however, an antrum formed in Stage-6 follicles by 72 h.

Keywords: preantral follicle; hamster; long-term culture; DNA synthesis; gonadotrophins

Introduction

The functions of follicular cells have been studied by incubating large preantral and antral follicles for brief periods (Hubbard & Greenwald, 1982) or by culturing granulosa cells as a monolayer in the presence of various factors (Orly, 1984; Baranao & Hammond, 1985; see review by Gore-Langton & Armstrong, 1988). However, disruption of the follicular microenvironment and in-vitro changes in cell shape (Ben-Rafael *et al.*, 1987) may lead to altered responsiveness of granulosa cells to endocrine factors (Hammond & English, 1987). Cellular maturation in parallel with follicular development from the primary to tertiary stages and its regulation by endocrine and paracrine factors remains mostly unexplored because it is impossible to monitor the growth of a given group of follicles *in vivo* since (1) folliculogenesis occurs as a continuum and (2) intact preantral follicles have not been cultured on a long-term basis. Using 2-h incubations, we have shown that follicular DNA synthesis in terms of [^{3}H]thymidine incorporation is critically influenced by FSH and LH (Roy & Greenwald, 1988); but in-vitro growth in terms of DNA synthesis and cell differentiation cannot be assessed by this system.

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Therefore, we have developed a long-term serum-free culture system which supports the growth and differentiation of intact hamster preantral follicles and provides a new dimension to studying the factors regulating folliculogenesis. Most of the study involved follicles from pro-oestrous hamsters because serum concentrations of FSH and LH were at their nadir and follicles were ready to respond to gonadotrophins (Roy & Greenwald, 1986a, b, 1988). For comparison, in the final experiment follicles from oestrous hamsters which had already been primed with endogenous gonadotrophins were used. This was done to determine whether hormonal pre-exposure would accelerate follicle growth *in vitro*.

Materials and Methods

Collagenase (Type I), DNase (bovine pancreas), crystalline bovine serum albumin (BSA) and hydrocortisone were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's MEM (DMEM) was obtained from Gibco (Grand Island, NJ, USA); ITS$^+$ (insulin, transferrin, selenium and BSA) from Collaborative Research (Bedford, MA, USA) and bacteriological agar was from Difco Laboratory (Detroit, MI, USA). [^{3}H]Thymidine (sp. act. 20 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL, USA). All other analytical grade chemicals were from local sources.

Follicle preparation. Adult, virgin golden hamsters (90–100 g, Harlan, Madison, WI, USA) which had exhibited 3 consecutive oestrous cycles were killed at 09:00 h on the day of pro-oestrus or oestrus. Preantral follicles at Stage 6 (7–8 layers of granulosa cells and developing theca) were dissected out and follicles in Stages 1–5 (Stages 1–4 = 1–4 layers of granulosa cells and no theca; Stage 5 = 5–6 layers granulosa cells with the appearance of thecal cells in follicles with 6 layers) were enzymically dissociated (Roy & Greenwald, 1985). After two sterile rinses in DMEM containing 15 mM-Hepes/0·12% $NaHCO_3$, 0·5% BSA, 10^3 units of penicillin G, 10 mg streptomycin and 35 μg fungizone (Hazelton, Lenexa, KS, USA) per ml of DMEM, follicles were collected in DMEM supplemented with 1% ITS$^+$ (final conc. 6·25 μg insulin, 6·25 μg transferrin, 6·25 μg selenium, 1·25 mg BSA and 5·35 μg linoleic acid per ml) and 40 ng hydrocortisone per ml DMEM (DMEM-complete).

Follicle culture. Tissue culture plates (24 wells; Costar, Cambridge, MA, USA) were coated with 200 μl sterile 0·625% agar in DMEM at 40°C to prevent attachment of follicles to the plastic surface and the formation of a monolayer. Groups of follicles at each stage were transferred at random to 300 μl agar (0·625% in DMEM, 40°C) added to each well. There were 30 Stage-1, 20–25 each of Stages 2–5, and 6–10 Stage-6 follicles per well. After gelation, 1 ml DMEM-complete was added to each well. Follicles at each stage of development were distributed randomly into various groups and cultured at 37°C in 5% CO_2 in air in an humidified atmosphere for periods described in the experimental protocol.

Experiment 1. Follicles at Stages 1–6 were cultured for 96 or 168 h in (1) the absence (control) or presence of (2) 100 ng ovine FSH (NIH-17)/ml, (3) 100 ng ovine LH (NIH-25)/ml, (4) combined FSH and LH. Medium and hormones were replaced every 24 h; cultures were terminated at 24, 48, 72, 96, 120, 144 and 168 h after adding 1 μCi [^{3}H]thymidine/ml 24 h before termination. Follicles were fixed in Bouin's fixative and 7 μm thick sections were coated with NTB3 nuclear track emulsion (Kodak) and exposed for 7 days at 4°C, developed in Dektol and stained with Gill's haematoxylin. Labelled nuclei (only densely labelled ones) and the number of atretic follicles (a minimum of 3–5 pycnotic nuclei) were counted at ×250 magnification to determine the labelling index (LI) and the percentage of atretic follicles, respectively. Photographs of follicles cultured for up to 96 h are presented with the plane of focus emphasizing silver grains.

To determine whether there is an increase in follicular DNA content during the total period of in-vitro culture, follicles from pro-oestrous hamsters (Stages 1–6) were cultured in triplicate in the absence or presence of 100 ng FSH for 24 to 168 h as described above and the culture was terminated every 24 h. The follicles were sonicated in DNA assay buffer and DNA was estimated by fluorometry (Downs & Wilfinger, 1983; Roy & Greenwald, 1986a). The results were expressed as ng DNA per follicle.

Experiment 2. Follicles at Stages 1–6 were distributed randomly and treated as: (1) controls, (2) 100 ng FSH/ml, (3) 100 ng LH/ml or combined FSH and LH and cultured for 96 h. At the start of culture 1 μCi [^{3}H]thymidine was added to each group. The medium was changed every 24 h with full replacement of hormones and 2 μg unlabelled thymidine/ml to inhibit any possible incorporation of residual radioactivity remaining in the well. This dose of unlabelled thymidine blocked [^{3}H]thymidine incorporation without hampering follicular growth. The cultures were terminated at 24, 48, 72 and 96 h and follicles were processed for autoradiography as described above and labelled nuclei (both dense and light) were counted for indexing. We did not continue this experiment beyond 96 h because dilution of DNA-incorporated radionuclide introduced errors in counting.

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Experiment 3. Follicles were cultured for 96 h as (1) controls (0·1% ethanol); (2) 1 or 10 μg progesterone and (3) 1 or 10 μg oestradiol-17β. The cultures were terminated at 24, 48, 72 and 96 h after administration of 1 μCi [^{3}H]thymidine 24 h before termination. Follicles were processed for autoradiography to monitor the labelling index.

Experiment 4. To monitor steroidogenesis, follicles at Stages 5 (30 follicles/replicate) and 6 (6 follicles/replicate) from pro-oestrous hamsters were cultured for 120 h in the absence or presence of 100 ng FSH or LH separately or combined. Medium and hormones were replaced every 24 h and spent medium was assayed for progesterone, androstenedione and oestradiol-17β by RIAs as described by Terranova & Greenwald (1978).

Analyses. The results were analysed by two-way analysis of variance and Duncan's multiple range test using Biomedical Data Package Programs (BMDP, CA) and Student's *t* test with the level of significance at 5%.

Results

Experiment 1: role of gonadotrophins in maintaining follicular DNA synthesis and morphology

No apparent difference in follicular morphology between control and hormone treated follicles from pro-oestrous hamsters at Stages 1–4 was observed up to 48 h; however, the percentage of atretic follicles in the control group increased considerably by 72 h and for Stages 5 and 6, atresia was prominent (80%) by 24 h (Fig. 1) with 100% of Stage 6 follicles showing advanced atresia by 72 h (Figs 1 & 2). No attempt was made to classify the stage of atresia for small follicles because they contained few cells. For follicles at Stages 5 and 6, however, a clear zone of pycnosis around the oocyte was considered a sign of advanced atresia. In the presence of FSH or LH or combined treatment, a significant proportion ($\geq 80\%$) of follicles at Stages 1–5 remained free of pycnotic cells throughout the culture period but Stage-6 follicles showed early pycnosis by 96 h, regardless of hormonal treatment; none the less, overall follicular morphology was practically undisrupted compared to controls. Asynchrony in the 'S' phase was evident from the scattered distribution of densely labelled nuclei throughout the granulosa cell layer (Figs 2a & b). For Stage-6 follicles, granulosa cells showing active DNA synthesis were orientated mainly around the oocyte. DNA synthesis was also detected in the developing thecal layer. At the light microscopic level, oocytes of FSH-exposed follicles appeared normal with intact germinal vesicles, nucleoli, homogeneous ooplasm (which was eosin stainable) and discrete oolemmal boundaries. Both insulin and hydrocortisone were essential for follicle culture; lack of insulin resulted in follicular degeneration within 24 h for all stages regardless of hormonal treatment (data not shown). Therefore, these two hormones were essential components of the medium. Moreover, the addition of selenium as an acceptor of free radicals generated during cell metabolism improved culture conditions.

During the first 24 h of FSH exposure, follicles from pro-oestrous hamsters at all stages showed marked increases in LI compared to controls (Fig. 2a); the degree of DNA synthesis varied for follicles at different stages of development. A significant increase in labelled nuclei was noticed by 24 h for Stage-6 follicles in which $\geq 80\%$ of cells were involved in DNA synthesis. By 96 h, follicular morphology in the control group had deteriorated considerably, especially for Stage-6 follicles in which the labelling index dropped markedly and granulosa cell boundaries were obscured (Fig. 2b). FSH prevented cellular pycnosis; labelled cells, although few in number, were still visible for follicles at Stages 1–3 and significantly prominent for Stages 4 and 5. Preantral follicles at Stage 6 showed the first signs of differentiation with the appearance of antral cavities (80–100% follicles developed antra; Fig. 2b). Although LH significantly increased labelled cells for Stages 5 and 6 (Fig. 2b), it failed to induce antrum formation in Stage-6 follicles.

Follicles from pro-oestrous hamsters at Stages 1–6 cultured for 24–168 h showed distinct labelling profiles (Fig. 3). In the absence of exogenous gonadotrophins, the LI for follicles at Stages 1 and 2 did not change appreciably during the first 48 h whereas for Stages 3 and 4 it declined steadily with the complete disappearance of labelled follicles by 96 h. For Stages 5 and 6, there were no healthy labelled follicles by 48 and 24 h, respectively. FSH, within 24 h, significantly ($P < 0{\cdot}05$) increased LI for Stages 1–6 and thereafter maintained it with ascending trends for most of the

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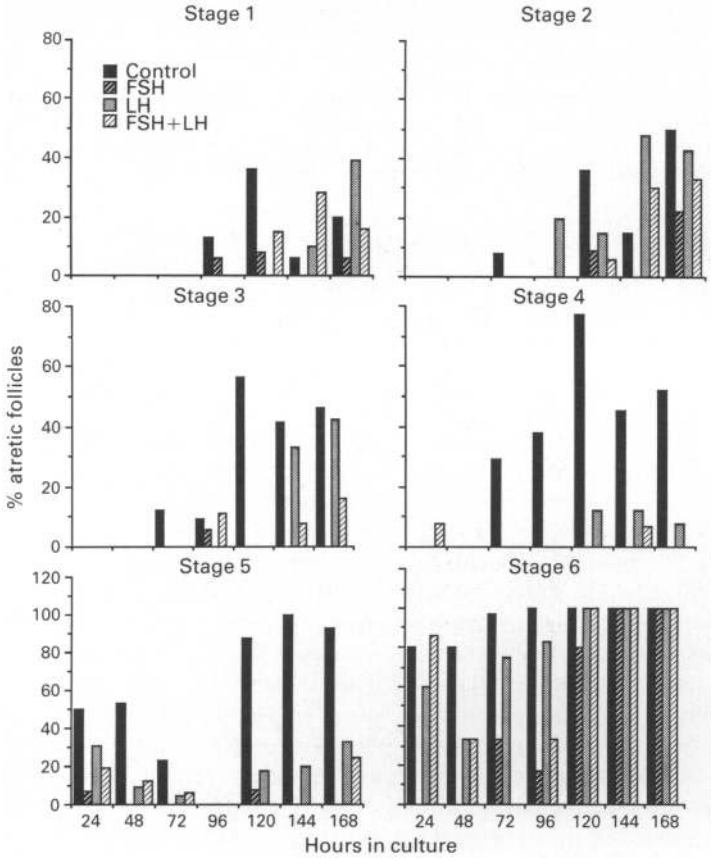

Fig. 1. Histogram showing the percentage of atresia in follicles from pro-oestrous hamsters, cultured *in vitro* for up to 168 h in the absence or presence of 100 ng FSH or LH.

stages except for Stage 6 in which a gradual decline was evident. LH stimulated LI for Stages 1–4 by 120–144 h and for Stages 5 and 6, by 24 and 72 h, respectively.

Since FSH markedly influenced LI *in vitro*, follicular DNA content was estimated in the absence or presence of only FSH (Fig. 4). In the absence of FSH, DNA content of follicles from pro-oestrous animals either remained at steady low levels or gradually declined, correlating therefore with the previous findings on atresia (Fig. 1). Within 24 h, FSH significantly increased DNA content for Stage 1–3 follicles while for Stages 4–6 a gradual increase was observed from 48 h. On the whole, compared to 0 h values, follicular DNA content in FSH-exposed follicles at Stages 1–3 and 5–6 almost doubled, while in Stage-4 follicles there was a significant rise by 96–120 h; control groups remained low. To determine whether increased follicular DNA content correlates with increments in cell number, in a separate experiment follicles of Stages 1–6 were cultured as described earlier in the absence or presence of 100 ng FSH for 5 days, fixed in Bouin's fixative, sectioned at 7 μm and stained with haematoxylin and eosin. The number of cells in sections showing the oocyte nucleoli of individual follicles at Stages 1–6 were counted and compared with follicles obtained at 09:00 h on pro-oestrus (Day 4–0 h; Table 1). In the absence of FSH, cell number in follicles at Stages 1 and 3–4 remained virtually unchanged, while for Stages 5–6 degenerative changes were prominent. In Stage-2 follicles, however, there was a marked increase. Again, FSH not only maintained a significant number of healthy follicles after 5 days of culture, but also markedly increased cell number in Stages 1–5; Stage-6 follicles possessed a small antrum and some cells were pycnotic.

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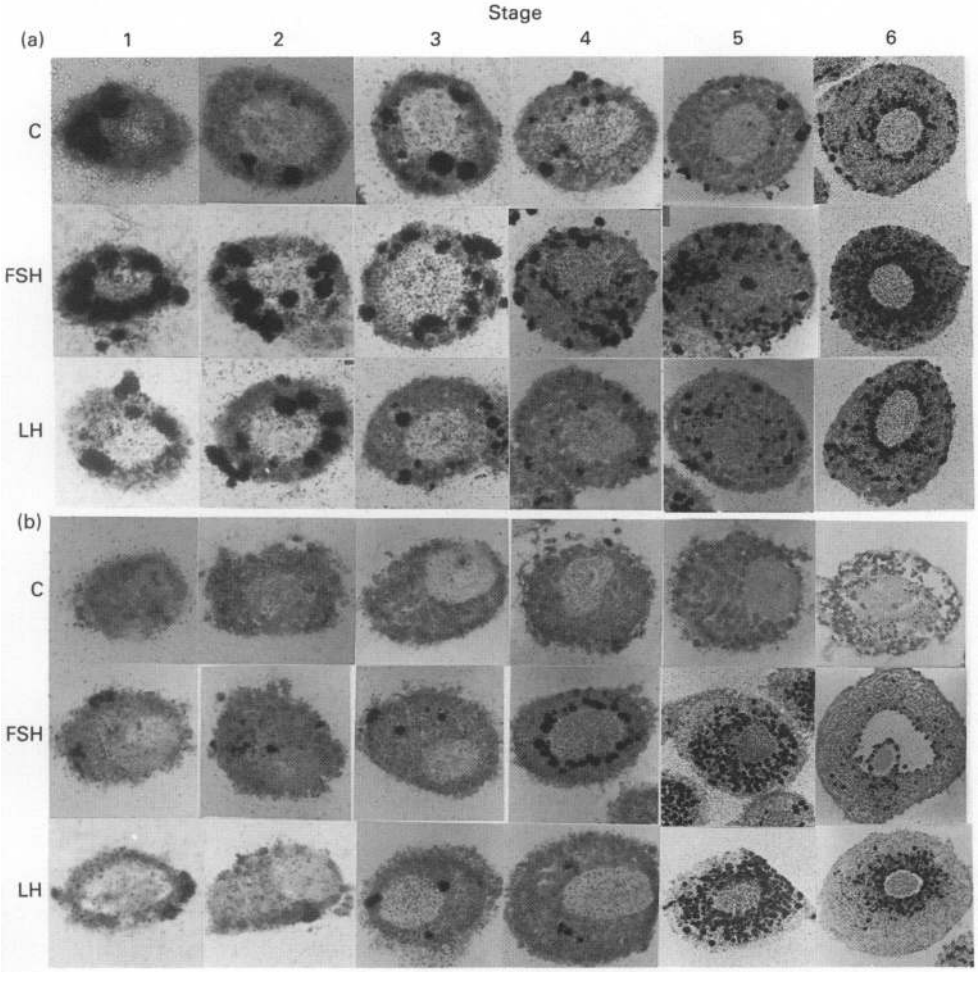

Fig. 2. Autoradiograms showing labelled pro-oestrous follicles at Stages 1–6 after (a) 24 h culture and (b) 96 h culture in the absence (C) or presence of 100 ng FSH or LH. In (a) note active DNA synthesis in follicular cells as evident from dense silver grain deposit on the nuclei. In (b) note the formation of an antral cavity in Stage-6 follicle after exposure to 100 ng FSH.

Experiment 2: pulse-chase with [^{3}H]thymidine to monitor follicular cell proliferation (Fig. 5)

When pulsed with [^{3}H]thymidine for 24 h, all stages of follicles from pro-oestrous hamsters showed dense grain deposits in granulosa cell nuclei. Because of the dilution of radioactivity incorporated into DNA with each cell division, labelled cells with at least ≥ 20 grains/nucleus were counted. As in Exp. 1, FSH significantly enhanced LI within 24 h for all stages and, thereafter, sustained a gradual ascent while the LI for hormone-deprived follicles either remained steady or declined during the 96 h of culture. LH, on the other hand, increased LI for all stages by the 24 h pulse period but thereafter maintained LI at low levels.

Experiment 3: role of steroids in in-vitro follicular growth

Progesterone (1 μg) enhanced LI only for Stages 1 and 3 by 24 h but showed no long-term effect; the higher dose was ineffective. Oestradiol (1 or 10 μg) was completely ineffective in influencing follicular LI and, moreover, induced follicular atresia by 24 h.

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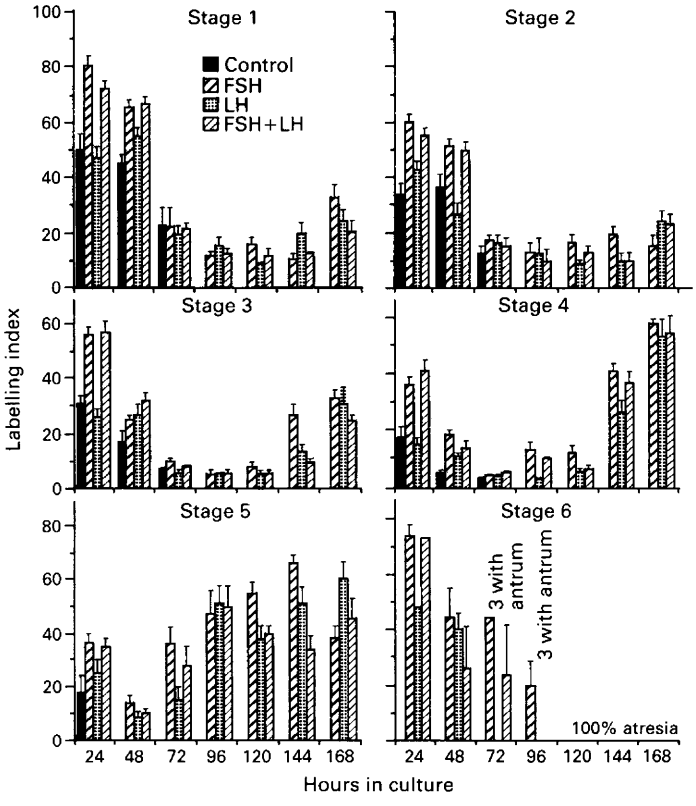

Fig. 3. Labelling indexes of pro-oestrous follicles at Stages 1–6 during 24–168 h culture in the absence or presence of 100 ng FSH or LH.

Experiment 4: steroidogenesis by large preantral follicles of pro-oestrous hamsters in the absence or presence of FSH or LH (Table 2)

In the absence of gonadotrophins for 24 h, Stage-5 and -6 follicles accumulated low basal amounts of hormones and the values declined significantly by 120 h, especially for Stage 6. When stimulated by 100 ng FSH, progesterone production by Stage-5 follicles increased significantly by 24 h and increased further by 120 h; for Stage-6 follicles progesterone production remained steady. However, follicular androstenedione and oestradiol production were significantly stimulated by FSH at 24 and 120 h. Because the culture medium was changed every 24 h, the accumulation of steroids reflects an active conversion of respective precursors rather than a release of stored products.

LH, within 24 h, stimulated progesterone production 4-fold for Stage-5 and 16-fold for Stage-6 follicles. Although LH significantly increased androstenedione production by Stage-5 follicles, the values were far below those of the FSH-treated group; Stage-6 follicles showed increased accumulation by 24 h and then dropped to lower levels by 120 h but were still at significantly higher levels than in controls. Accumulation of oestradiol also showed the same pattern as androstenedione. A combination of FSH and LH was also effective in stimulating follicular steroidogenesis but the pattern was similar to that for LH alone. Therefore, LH dampened the steroidogenic action of FSH.

For follicles from oestrous hamsters (Fig. 6), FSH increased LI for Stages 1 and 2 by 48 h and for Stages 3–6 by 24 h while controls showed a steady decline. However, at 24 h for Stages 1 and 2

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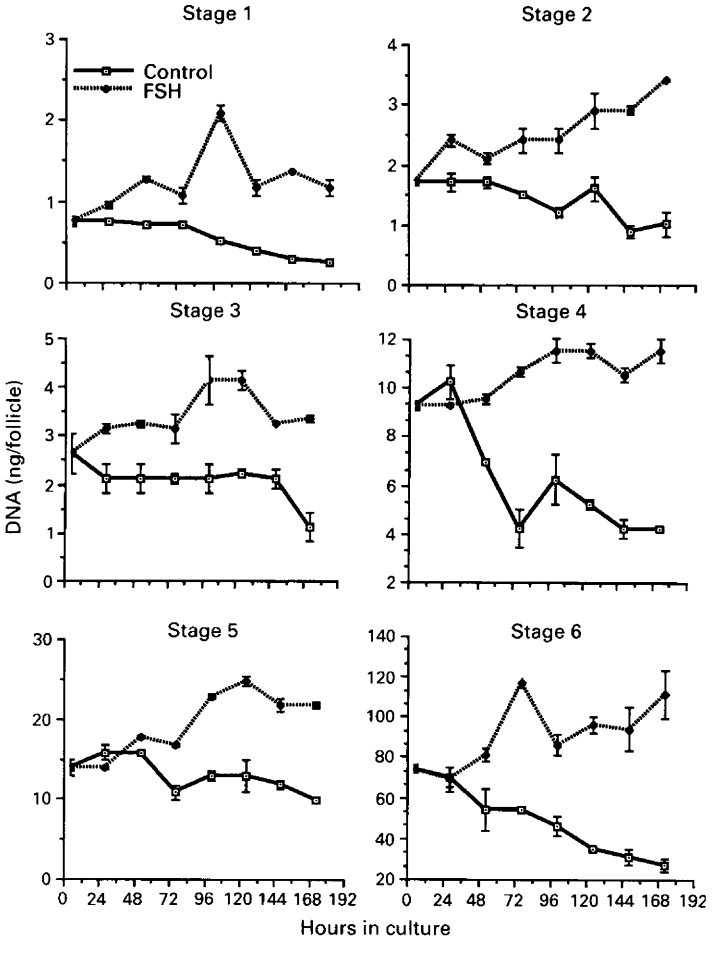

Fig. 4. Follicular DNA content during long-term culture of hamster follicles with or without 100 ng FSH.

Table 1. Number of cells in cross-sections of hamster follicles before and after in-vitro culture for 5 days

	Stages of follicle					
Groups	1	2	3	4	5	6
Day 4–0 h†	12 ± 0·7 (11)	20 ± 0·9* (13)	40 ± 3 (11)	77 ± 3 (17)	110 ± 4 (15)	210 ± 3 (3)
Control	14 ± 0·8 (20)	31 ± 1 (22)	45 ± 2 (12)	70 ± 6 (5)	atretic	atretic
FSH-100 ng	27 ± 1* (15)	47 ± 3* (15)	81 ± 2* (15)	123 ± 5* (14)	233 ± 10* (10)	468‡ (1)

Values are mean ± s.e.m. for the no. of follicles indicated in parentheses.
*$P < 0·05$ compared with control value.
†Follicles collected from pro-oestrous hamsters and without culture.
‡Stage-6 follicles had formed antrum and some contained pycnotic cells; therefore, cells were not counted.

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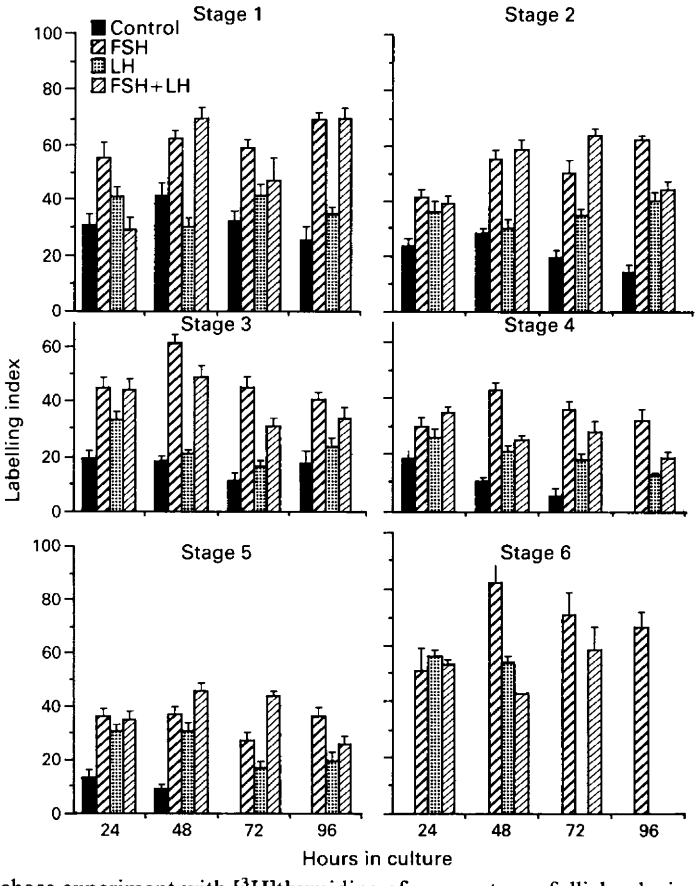

Fig. 5. Pulse-chase experiment with [^{3}H]thymidine of pro-oestrous follicles during 96 h culture in the absence or presence of 100 ng FSH or LH. Follicles were pulsed with radionuclide during the first 24 h and medium was then replaced every 24 h with DMEM containing 2 μg unlabelled thymidine.

Table 2. Production *in vitro* of progesterone, androstenedione and oestradiol-17β by follicles, in long-term culture, from pro-oestrous hamsters

Group	Time in culture (h)	Steroids (pg/follicle/24 h)					
		Progesterone		Androstenedione		Oestradiol	
		Stage 5	Stage 6	Stage 5	Stage 6	Stage 5	Stage 6
Control	24	30 ± 3	55 ± 10	2 ± 0·4	53 ± 6	1 ± 0·2	20 ± 3
	120	15 ± 1	14 ± 3	2 ± 0·4	1 ± 0·2	0·5 ± 0·06	3 ± 1
FSH	24	207 ± 0·1*	299 ± 58*	7 ± 2*	215 ± 31*	5 ± 1*	46 ± 5*
(100 ng/ml)	120	695 ± 40*	304 ± 36*	241 ± 44*	1885 ± 196*	1148 ± 160*	2036 ± 421*
LH	24	585 ± 69*	2203 ± 77*	8 ± 1*	770 ± 93*	2 ± 0·3*	15 ± 2*
(100 ng/ml)	120	2577 ± 475*	4771 ± 949*	34 ± 8*	116 ± 12*	219 ± 31*	196 ± 27*
FSH + LH (100+	24	936 ± 152*	1796 ± 147*	9 ± 1*	4981 ± 187*	2 ± 0·2*	50 ± 6*
100 ng/ml)	120	1453 ± 43*	4442 ± 197*	24 ± 4*	100 ± 9*	112 ± 14*	323 ± 10*

Values are mean ± s.e.m. for 4 replicates.
*$P < 0·05$ compared with the respective control.

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in control groups, LI was much higher than in the follicles from pro-oestrous hamsters at corresponding stages. For Stages 5 and 6, FSH significantly increased LI by 96 h and antrum formation was observed in Stage-6 follicles by 72 h, 1 day earlier than in the follicles of pro-oestrous animals. Although LH maintained follicular viability for all stages, it was unable to induce antrum formation. FSH and LH together increased LI for Stages 1–5; however, for Stage 6 LI decreased considerably.

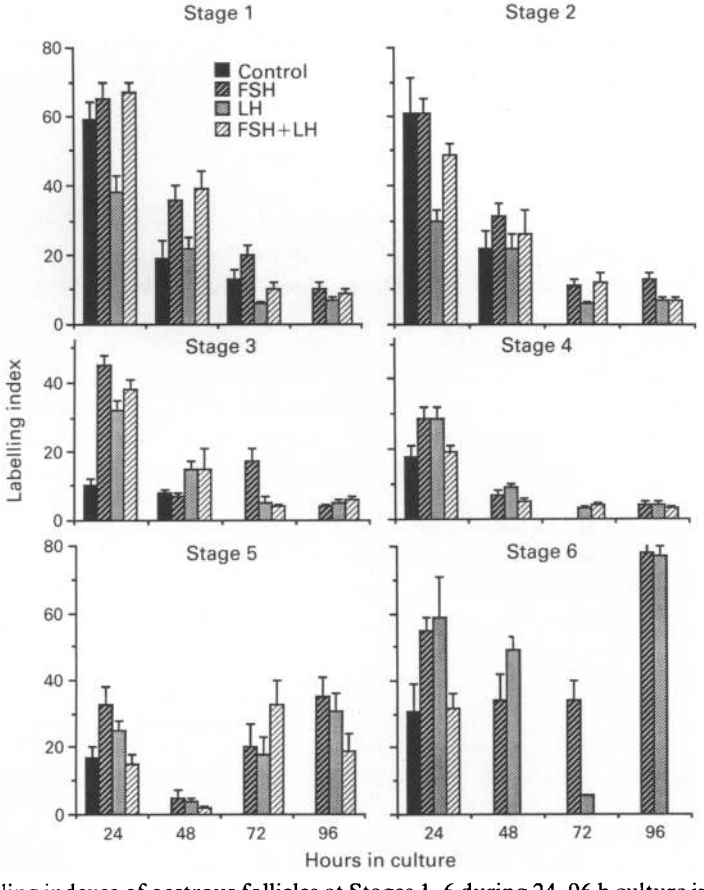

Fig. 6. Labelling indexes of oestrous follicles at Stages 1–6 during 24–96 h culture in the absence or presence of 100 ng FSH.

Discussion

The results of the present studies provide evidence for an efficient long-term culture system (4–7 days) which supports the growth and differentiation of hamster preantral follicles. The presence of insulin in the culture is vital from the beginning to support follicular viability. However, in our system, insulin alone is insufficient to maintain healthy follicles (control, Fig. 4), suggesting the requirement for other factors for follicular survival.

In the hamster, FSH causes follicular recruitment *in vivo* (Kim & Greenwald, 1984) and DNA synthesis in preantral follicles both *in vivo* (Roy & Greenwald, 1986a, b) and *in vitro* (Roy & Greenwald, 1988). The results of granulosa cell culture need to be interpreted with caution since

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extensive intercellular communication by gap junctions between granulosa cells as observed *in vivo* (Albertini & Anderson, 1974) is virtually destroyed during harvesting of granulosa cells which may induce membrane perturbations resulting in premature activation of intracellular signals for differentiation and alterations in cell functions, and reappearance of gap junctions *in vitro* (Amsterdam *et al.*, 1981) may not represent their counterparts *in vivo*.

Differentiation of Stage-6 preantral follicles to early antral stages (Stages 7–8) indicates the efficiency of the culture system to maintain large preantral follicles for at least 4 days. The failure to maintain Stage-6 follicles beyond 96 h could be due to several factors: (1) Stage-6 follicles are the immediate preantral pool to be recruited within 4 days to the next set of ovulatory follicles and so culturing them beyond 96 h may exceed their normal lifespan resulting in follicular atresia; (2) the complex cellular maturational processes accompanying growth may no longer be supported by the simple defined medium; or (3) because granulosa cells in multilayered follicles depend on diffusible products from thecal vascularity, growth *in vitro* may ultimately lead to cell death due to inaccessibility of nutrients.

The marked increases in LI by 24 h for follicles at Stages 1–6 after FSH exposure support our earlier in-vivo findings during the periovulatory gonadotrophin surges (Roy & Greenwald, 1986a). The labelling pattern for follicles of pro-oestrous animals at different stages during the 168-h culture period indicates that the rates of growth of developing follicles depend on their maturational status at the time they are cultured. Although Stage-6 follicles have 100-fold more DNA than do Stage-1 follicles, no such massive increase in follicular DNA occurred in any stage during the 7-day culture period. Chiras & Greenwald (1977) have demonstrated that, *in vivo*, follicles with 2–3 layers of granulosa cells (Stages 2–3) take 8 days to acquire 4–5 layers of cells (Stages 4–5) while another 8 days is required to form a follicle with 7–8 layers of cells (Stage 6). Therefore, it takes 16 days for Stage-2 follicles to become Stage-6 follicles *in vivo*. Considering the use of serum-free defined medium, a slower rate of cell division *in vitro* and different rates of cell proliferation in different stages of follicles, it cannot be expected that Stage-1 follicles will transform into Stage 6 within 7 days *in vitro*. The inability of LH to increase follicular labelling indexes by 24 h for follicles smaller than Stage 5 supports our previous findings (Roy & Greenwald, 1986b, 1988). Follicles at Stages 1–4 do not possess any LH binding sites or LH-responsive adenylate cyclase which appear from Stage 5 and onwards (Roy *et al.*, 1987; Roy & Greenwald, 1988). LH-induced increases in LI for Stages 3 and 4 by 48 h could be due to the acquisition of LH receptors during in-vitro growth by insulin action on granulosa cells. Insulin has been shown to induce LH receptors in rat granulosa cells in culture (Davoren *et al.*, 1986). Although, in short-term (2 h) incubation, LH counteracted FSH-induced [^{3}H]thymidine incorporation by Stage-5 and -6 follicles (Roy & Greenwald, 1988), no such effect was discernible in the present study. The antagonistic effect may be limited to the preovulatory surge period or to a brief duration of in-vitro exposure, but thereafter the tonic levels induce follicular recruitment and maturation.

The low, steady labelling indexes for follicles in the control group for Stages 1–3 during the pulse-chase period indicates a cessation in cell proliferation; a sharp drop by 72 h for Stages 4 and 5 along with increased atresia may reflect the loss of radionuclide by DNA fragmentation. The characteristic increases in LI by 24 and 48 h due to FSH for most of the stages strongly suggests that cells which were actively synthesizing DNA by 24 h underwent mitosis. The decrease in LI from the 48 h value may reflect the proliferation of unlabelled cells during the chase period, thereby also affecting the percentage of labelled cells. The results of follicular DNA content and cell number support this view. The in-vitro increase in follicular DNA within 24 h for Stages 1–3 in the presence of FSH correlates well with the increase in [^{3}H]thymidine incorporation (Roy & Greenwald, 1986a, 1988) and LI, and provides convincing evidence that FSH stimulates follicular DNA synthesis and cell proliferation in small preantral follicles. The slow ascent in follicular DNA content following FSH exposure can be ascribed to asynchrony in cell division whereby only a few new cells are recruited every 24 h which may not add enough DNA to double the total content (considering 12 pg DNA per hamster granulosa cell and the maximum sensitivity of the assay is 10 ng/tube: Roy

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& Greenwald, 1986a). However, when the values between 0 and 168 h of culture are compared, a clear-cut rise in follicular DNA is evident by 96–120 h. This is further substantiated by the rise in the number of follicular cells during the 120-h culture period. Beyond 96 h the rise in DNA content for Stage 6 when granulosa cells undergo considerable degeneration may indicate the proliferation of thecal cells as well as the remainder of the viable granulosa cells. Incorporation of radioactivity by Stage-6 follicles at 144 h was seen in autoradiograms (data not shown).

The steady high LI for LH-exposed follicles at Stages 5 and 6 for up to 48 h during the chase period suggests that LH has a different influence on the cell cycle than does FSH. Increased [^{3}H]thymidine incorporation by Stages 5 and 6 incubated with LH has been previously observed (Roy & Greenwald, 1988). However, increased LI for Stage-5 follicles by 48 h and for Stage 6 by 72 h by combined FSH and LH suggests that both hormones are necessary for normal functioning of these stages. The mandatory requirements for both FSH and LH for the development of antral and preovulatory follicles in the hamster have already been demonstrated (Moore & Greenwald, 1974; Kim & Greenwald, 1984).

Oestradiol does not influence folliculogenesis *in vivo* either in the hamster (Kim *et al.*, 1984) or in the monkey (Koering *et al.*, 1986). The present in-vitro studies also demonstrate that oestradiol has no stimulatory effect on follicular DNA synthesis; however, slight stimulation by progesterone was evident.

Significant increases in steroidogenesis by follicles at Stages 5 and 6 after exposure to FSH suggest that the follicles are functionally active even after 120 h culture. Furthermore, the rise in androstenedione and oestradiol production by Stage 6 and oestradiol by Stage 5 after 120 h provides further evidence for FSH-induced follicular maturation *in vitro*; however, LH mainly affected progesterone production. Increases in progesterone, androstenedione and oestradiol accumulation by hamster follicles at Stages 5 and 6 during short-term (2 h) exposure to FSH and LH *in vitro* has been previously reported (Roy & Greenwald, 1987). Luteinization of granulosa cells is associated with a shift in steroidogenesis towards progesterone (Channing, 1970). In the present study, LH causes thecal hyperplasia and differentiation (data not shown) leading to a gradual transformation of follicular cells to luteal-like cells with maximal progesterone production.

Although follicles of oestrous animals showed almost the same trend in DNA synthesis in response to gonadotrophins, the failure of those at Stages 1 and 2 to respond to FSH by 24 h could be due to refractoriness induced by the endogenous gonadotrophin surges. This was evident from the higher LI observed for follicles from oestrous compared to pro-oestrous hamsters and from our previous results (Roy & Greenwald, 1986a). The faster rate of differentiation of follicles of oestrous animals due to FSH and LH priming is substantiated by the onset of antrum formation in Stage-6 follicles by 72 h.

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