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CULTURE OF BOVINE GRANULOSA CELLS IN A CHEMICALLY DEFINED SERUM-FREE MEDIUM: THE EFFECT OF INSULIN AND FIBRONECTIN ON THE RESPONSE TO FSH

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Summary—Granulosa cells from fully differentiated bovine follicles were cultured in serum-free medium for 4 days. At the end of culture, the number of viable cells was low (10–15% of cells plated on day one) and only progesterone secretion responded to FSH.

Insulin increased the number of viable cells at the end of culture (ED_{50} # 70 ng/ml) and stimulated progesterone secretion (ED_{50} # 50 ng/ml); the secretion of oestradiol-17β over basal value was evident only for concentrations of 1000 and 10,000 ng/ml.

FSH acted synergistically with insulin to modify steroid secretion. In the presence of 50 ng/ml of insulin, dose–response studies indicated that secretion of progesterone was maximal at 10 ng/ml of FSH and plateaud thereafter, while oestradiol output peaked at 2 ng/ml of FSH, decreasing at higher concentrations.

When cells were seeded in wells precoated with fibronectin, a comparison with cells cultured on plastic showed an increase (30–40%) in the number of viable cells at the end of culture and in oestradiol secretion but a decrease in progesterone output.

These results indicate that granulosa cells from large bovine follicles, cultured in a serum-free medium containing insulin, maintain their steroidogenic potency for at least 4 days. Moreover, they show that oestradiol and progesterone synthesis are differentially sensitive to FSH concentrations and that fibronectin increases oestradiol secretion in response to FSH.

INTRODUCTION

It has been clearly established that follicular growth depends on hypophyseal gonadotropins. More recent data have shown that the effects of these hormones are modulated by autocrine/paracrine factors. Evidence for this has been obtained from *in vitro* experiments using rat granulosa cells. However, it is increasingly apparent that species differences exist (for example see the recent review of Hutz [1] on the effect of oestrogens). Thus the regulation of granulosa cell function must be established for each species.

For cultured bovine granulosa cells, some data dealing with the control of their proliferation and their secretion of progesterone and oxytocine are available [2–8]. Most of these results were, however, obtained in the presence of serum which often has a more important effect than the agonist tested. Moreover, dose–response curves to establish optimal culture conditions were not performed and the secretion of oestradiol-17β, when studied, was not maintained.

The present report describes an investigation of culture conditions in a defined serum-free medium which allow bovine granulosa cells to remain responsive to FSH.

It has been observed that some responses of cultured granulosa cells may depend on the stage of development of the follicles (Rhesus monkey [9], Marmoset monkey [10], human [11], bovine [7], porcine [12]). The current investigation was restricted to granulosa cells obtained from fully differentiated follicles.

MATERIALS AND METHODS

Animals

Three-month-old Holstein × Friesian female calves were injected i.m. with 1500 IU PMSG (Intervet, Angers, France). Previous experiments [13] have shown that the occurrence of oestrus, the peak of oestradiol-17β concentration in peripheral blood and maximum follicular development are observed 110–130 h after this treatment. Therefore to obtain fully differentiated but not luteinized follicles as a

source of granulosa cells, the animals were killed 96 h after PMSG injection.

Cell culture

To collect granulosa cells, 8–15 mm diameter follicles were punctured and rinsed 10 times with INRA F_1 medium (IMV, L'Aigle, France) containing 2% heparine. The cell suspensions were pooled.

After centrifugation (300 *g*, 7 min) the supernatant was discarded. To improve dissociation the cells were treated according to Campbell [14]: 0.5 ml of 6.8 mM EGTA (SERVA) in Menezo B_2 medium without cholesterol (API System SA Montralieu-Vercieu, France) were added and the tubes incubated at 37°C for 15 min. After centrifugation the supernatant was discarded and the cells resuspended in Menezo B_2 medium. The viability, as assessed by trypan blue exclusion, was between 80 and 90%.

Cells (approx. 0.3×10^6/well) were plated in 48-well Costar plates in 0.5 ml Ham's F_{12} medium (Gibco) buffered with 20 mM Hepes buffer, pH 7.4, and supplemented with 1 mg/l human transferrin (Sigma), 100 μM ascorbic acid (Prolabo, Paris France), 20 mg/l gentamicin (Gentaline, Unicet Levallois Perret, France), 4 mg/l nystatin (Seromed) and 10^{-7} M Δ4-androstenedione (Steraloïds). Culture was performed at 37°C under a water-saturated gas phase of 95% air and 5% CO_2. Medium was collected and replaced every day.

Highly-purified FSH (pFSH prepared by Dr Y. Combarnous, INRA Nouzilly, France; batch C. Y. 1737 III; FSH activity 41 × NIH FSH P1, LH activity lower than 0.5%) and bovine insulin (Sigma) were added from the beginning of the culture and renewed daily. Within a culture, each dose was tested in 3 wells.

In some cases, fibronectin (fibronectin from bovine plasma, Sigma) was added to the wells (3 μg/well) and incubated for 30–60 min at 37°C before seeding [15].

The culture was stopped after 4 days. The medium was collected and stored at −20°C until assayed for oestradiol-17β and progesterone content, and the number of viable cells estimated.

Assay of oestradiol-17β and progesterone

Oestradiol-17β and progesterone were measured without extraction using previously described radioimmunoassays [16, 17]. The specificity of these methods was further checked by checking that dilution curves (dilutions corresponding to 50, 25, 12.5, 6.25 and 3.12 μl of medium) of 10 samples obtained in different culture conditions were parallel to the standard curves.

Each sample was measured in duplicate.

DNA assay

The number of cells plated 24 h after seeding and at the end of culture was estimated by measuring the amount of DNA in the wells. The assay was adapted from Sorger and Germinario [18] with the following modifications.

- We observed that both the DNA liberated during culture and the presence of dead cells lead to an overestimation of the number of viable cells (Table 1). Therefore, before the solubilization of the cells, dead cells and free DNA were eliminated by a trypsin/DNase treatment as suggested by Farookhi [19]: trypsin (20 μg/ml; Gibco) was added to the wells for 1.5 min at 37°C, inhibited by serum and followed by DNase (50 μg/ml; DNase I, type IV, Sigma) for 10 min at 37°C.
- In order to eliminate background due to inhomogeneity of samples, the extracts were sonicated for 5 s according to Brunk *et al.* [20].
- In view of range of DNA to be assayed, the concentration of 4′,6-diamidino-2-phenylindole (Dapi, Serva) was reduced to 20 ng/ml [21].

DNA was assayed using calf thymus DNA (Sigma) as standard and it was assumed that

Table 1. Validation of the DNA assay for the estimation of the number of viable cells at different times of culture

Duration of culture (h)	Methods			
	A	B	C	D
24	231.2 ± 3.6[1]	471.0 ± 4.6	342.2 ± 2.8	234.2 ± 1.4
72	202.1 ± 10.4	392.7 ± 12.8	288.1 ± 4.6	207.6 ± 4.4

[1]Number of cells $\times 10^{-3}$.

The number of viable cells was estimated after 24 or 72 h of culture by different methods: A—trypsinization (trypsin 0.1% in medium for 4–5 min at 37°C) and counting; B—DNA assay; C—DNA assay but the monolayer was treated with DNase before solubilizing the cells; D—DNA assay but dead cells and free DNA were removed by the trypsin/DNase treatment as described in Materials and methods. Data represent the mean ± SEM of 3 wells.

10 μg of DNA corresponded to 1.4×10^6 cells [22, 23].

Data analysis

Experimental data are presented as mean ± SEM. Data were subjected to one-way analysis of variance or to two-way analysis of variance to study interactions. The ED_{50} were calculated with the help of Graph PAD programm (ISI Software).

RESULTS

Effect of insulin on the response to FSH

When granulosa cells were seeded on plastic in the absence of insulin, less than 15% of cells attached 24 h after seeding were still present on D_4 of culture. A dose–response curve was observed for progesterone (Fig. 1) but secretion of oestradiol could not be detected (data not shown).

Insulin by itself increased in a dose-dependent manner both the number of viable cells on day 4 ($P < 0.001$, ED_{50} # 70 ng/ml, Fig. 2) and progesterone secretion ($P < 0.001$, ED_{50} # 50 ng/ml, Fig. 4). An effect of insulin on oestradiol secretion was noticed only at concentrations of 1000 and 10,000 ng/ml ($P < 0.01$).

FSH and insulin interacted to improve the maintenance of cell viability ($P < 0.001$, Fig. 3) with no differences among the doses of FSH tested. They also interacted to stimulate progesterone secretion ($P < 0.001$, Fig. 4). A similar interaction was also observed with reference to oestradiol secretion ($P < 0.001$). However, 1 ng/ml of FSH led to an increase, whereas 5 and 10 ng/ml of FSH decreased this secretion (Fig. 5).

Whether insulin was tested alone or in the presence of FSH, the ED_{50} on the maintenance of cell viability and progesterone secretion was between 50 and 100 ng/ml.

Effect of fibronectin on the response to FSH in the presence of 50 ng/ml of insulin

It has frequently been demonstrated that at concentrations higher than 50 ng/ml the activity of insulin is due to interaction with IGF_1 receptors [24]. This dose was therefore selected in order to produce a "pure" insulin effect. The response of granulosa cells was further

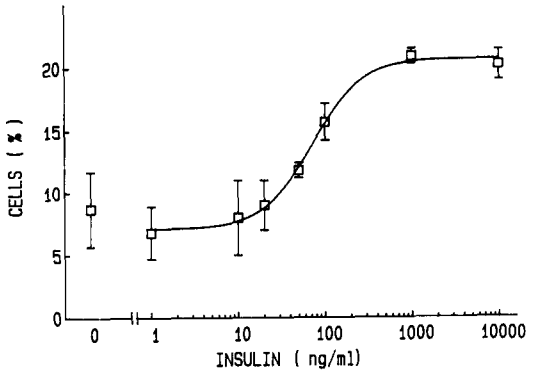

Fig. 2. Effect of treatment with insulin on the number of viable bovine granulosa cells. Cells were cultured as described in the legend of Fig. 1, with different amounts of insulin. The number of viable cells was estimated at the end of culture and expressed as a percentage of those present 24 h after seeding. Data represent the mean ± SEM of triplicate wells of 4 separate experiments.

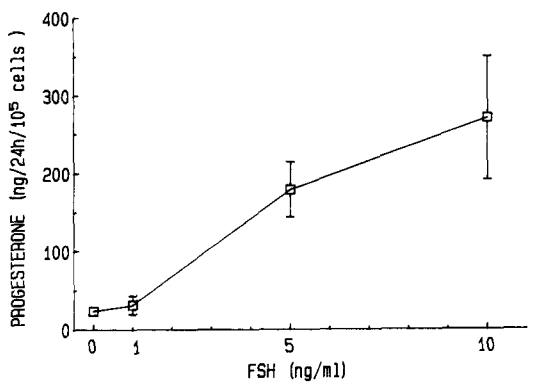

Fig. 1. Effect of treatment with FSH on progesterone secretion by bovine granulosa cells. The cells were cultured for 4 days in Ham's F_{12} medium supplemented with ascorbic acid (100 μM), transferrin (10 μg/ml), Δ4-androstenedione (10^{-7} M) and different amounts of FSH. The medium was changed every day. The secretion of progesterone during the last 24 h, corrected for the number of viable cells measured at the end of culture, is presented. Data represent the mean ± SEM of triplicate wells of a representative experiment repeated 4 times.

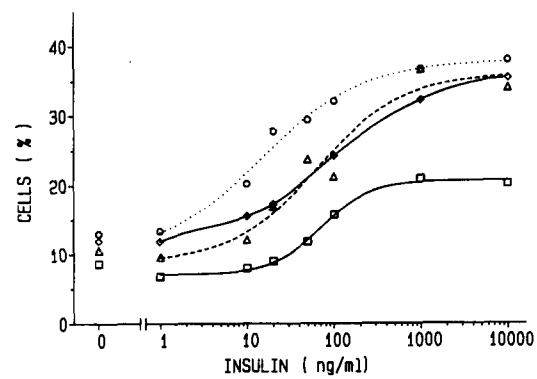

Fig. 3. Effect of treatment with insulin in the presence of different amounts of FSH on the number of viable granulosa cells. Cells were cultured as described in the legend of Fig. 1 with different amounts of insulin and 0 (□——□), 1 (△---△), 5 (◇——◇) or 10 (○·····○) ng/ml of FSH. The number of viable cells was estimated at the end of culture and expressed as a percentage of those present 24 h after seeding. Data represent the mean of triplicate wells of 4 separate experiments. SEM are not represented for clarity.

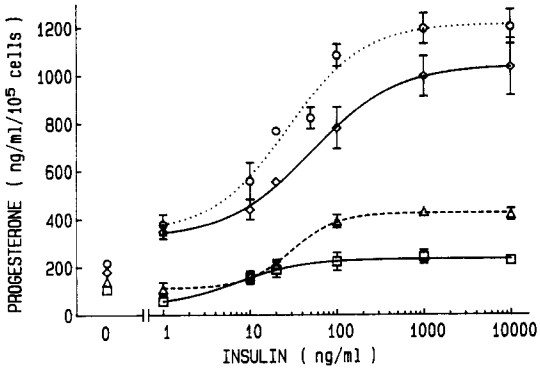

Fig. 4. Effect of treatment with insulin in the presence of different amounts of FSH on progesterone secretion by bovine granulosa cells. Cells were cultured as described in the legend of Fig. 1, with different amounts of insulin and 0 (□——□), 1 (△---△), 5 (◇——◇) or 10 (○·····○) ng/ml of FSH. The secretion of progesterone during the last 24 h corrected for the number of viable cells measured at the end of culture is presented. Data represent the mean ± SEM of triplicate wells of a representative experiment repeated 3 times.

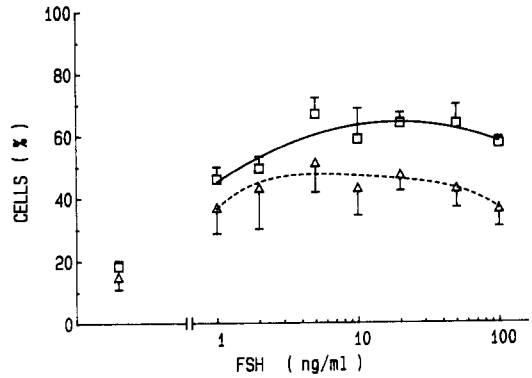

Fig. 6. Effect of treatment with FSH and fibronectin in the presence of 50 ng/ml of insulin on the number of viable bovine granulosa cells. Cells were seeded on plastic (△---△) or after treatment of wells with fibronectin (3 μg/cm²) (□——□) and cultured as described in the legend of Fig. 1 with 50 ng/ml of insulin and different amounts of FSH. The number of viable cells was estimated at the end of culture and expressed as a percentage of those present 24 h after seeding. Data represent the mean ± SEM of triplicate wells of 3 separate experiments.

characterized for a larger range of FSH concentrations (1–100 ng/ml) when cultured with or without fibronectin as an attachment factor.

In the presence of fibronectin, the number of cells attached after 24 h was slightly increased but the main effect was on the subsequent maintenance of viability ($P < 0.001$, Fig. 6). Whether the cells were cultured on plastic or on fibronectin, FSH increased the number of viable cells but no dose-related differences were observed. The secretion of progesterone increased with the dose of FSH, but the highest oestradiol secretion was observed with 2 ng/ml of FSH, dramatically decreasing thereafter (Fig. 7).

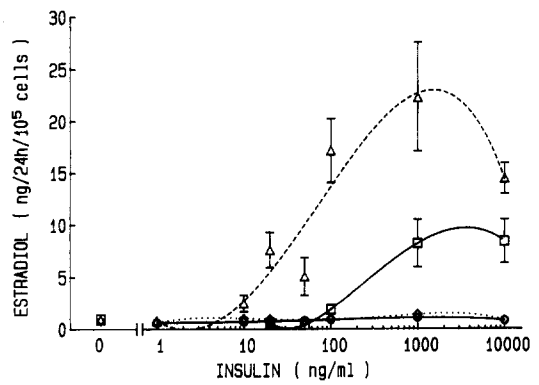

Fig. 5. Effect of treatment with insulin in the presence of different amounts of FSH on oestradiol secretion by bovine granulosa cells. Cells were cultured as described in the legend of Fig. 1, with different amounts of insulin and 0 (□——□), 1 (△---△), 5 (◇——◇) or 10 (○·····○) ng/ml of FSH. The secretion of oestradiol-17β during the last 24 h corrected for the number of viable cells measured at the end of culture is presented. Data represent the mean ± SEM of triplicate wells of a representative experiment repeated 3 times.

These patterns were similar in cells cultured with or without fibronectin, but in the presence of fibronectin the secretion of progesterone was decreased ($P < 0.001$), whereas that of

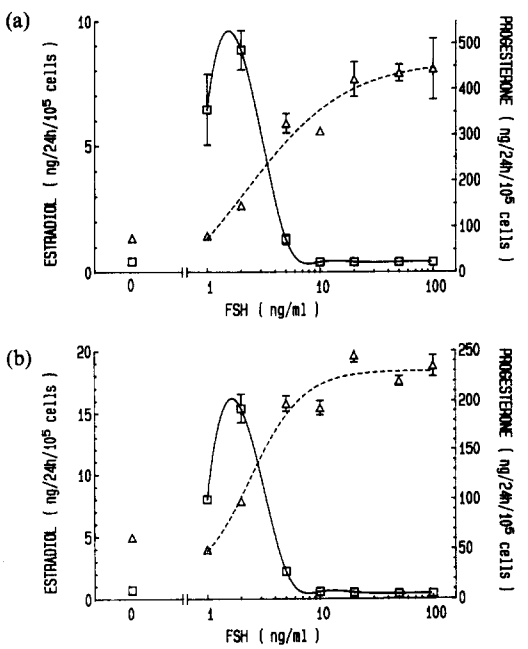

Fig. 7. Effect of treatment with FSH in the presence of 50 ng/ml of insulin on progesterone and oestradiol secretions by bovine granulosa cells seeded on plastic (a) or fibronectin (b). Cells were seeded on plastic or after treatment of wells with fibronectin (3 μg/cm²) and cultured as described in the legend of Fig. 1 with 50 ng/ml of insuln and different amounts of FSH. The secretion of progesterone (△-----△) and oestradiol (□——□) during the last 24 h corrected for the number of viable cells measured at the end of culture is presented. Data represent the mean ± SEM of triplicate wells of a representative experiment repeated 3 times.

oestradiol increased ($P < 0.001$) when compared with cells cultured on plastic.

Is the effect of fibronectin on steroid secretion related to its effect on the maintenance of cell viability?

As cell density is reported to affect steroid secretion of cultured granulosa cells [25, 26], we tested the hypothesis that the effect of fibronectin on steroid secretion in response to FSH was due to differences in the number of cells present at the end of culture.

Cells were seeded on plastic, and at the same, and at half the previous density on fibronectin in the presence of 50 ng/ml of insulin and 5 ng/ml of FSH. The results of two experiments reported in Table 2 show that the effect of cell density cannot account for all the differences in steroid secretion between cells cultured with or without fibronectin. Both oestradiol and progesterone secretions increased when cell density decreased for those cultured on fibronectin. However, at a similar density the presence of fibronectin increased oestradiol and decreased progesterone secretion on day 4 when compared with cells cultured on plastic.

Table 2. The effect of fibronectin and cell density on steroids secretion in response to FSH

	Cells cultured on plastic			Cells cultured on fibronectin					
				A			B		
	Cell number[1]	Progesterone[2]	Oestradiol[3]	Cell number[1]	Progesterone[2]	Oestradiol[3]	Cell number[1]	Progesterone[2]	Oestradiol[3]
Experiment 1	32.8 ± 1.7	537.2 ± 29.0	640.3 ± 78.1	66.1 ± 1.6	258.0 ± 14.2	759.2 ± 56.5	27.6 ± 0.9	312.3 ± 9.2	1655.2 ± 45.8
Experiment 2	55.8 ± 1.1	501.6 ± 20.8	452.5 ± 22.6	97.0 ± 2.9	236.0 ± 5.5	527.4 ± 10.3	43.2 ± 3.2	333.5 ± 8.8	939.5 ± 73.8

[1]($\times 10^{-5}$). [2](ng/24 h/10^5 cells). [3](pg/24 h/10^5 cells).
Cells were seeded on plastic and at the same (A) and at half (B) the previous density on fibronectin in the presence of 50 ng/ml of insulin and 5 ng/ml of FSH.
After 4 days both the number of cells and the steroid secretion were measured.
Data represent the mean ± SEM of 6 wells.
In experiment 1, the difference in oestradiol secretion by cells cultured on plastic and cells cultured at the same density on fibronectin is not significant; in experiment 2, this difference is significant with $P < 0.05$. Within an experiment, all other differences are significant with $P < 0.01$.

DISCUSSION

There are disadvantages to culturing cells in the presence of serum or on an extracellular matrix of unknown composition: the maintenance of cell viability is the result of an equilibrium between negative and positive effects of serum components (see for review [27] and [28]) and the effect of agonists can be masked (as for FSH [29, 30]) or amplified by unknown substance(s). For these reasons, we established our culture without serum and thus the observed effects are strictly a function of the substances added.

When granulosa cells obtained from fully differentiated follicles were stimulated with FSH alone, a dose–response curve for progesterone secretion was observed but the number of viable cells at the end of culture was low and no secretion of oestradiol-17β could be measured. Insulin alone improved the number of viable cells on day 4 and induced in a dose-dependent manner the secretion of progesterone as has previously been demonstrated for porcine granulosa cells [31, 32]. A limited effect on oestradiol secretion was noticed with the highest doses of insulin tested (1 and 10 μg/ml).

Insulin enhanced the secretion of progesterone in response to FSH, as previously observed in other species [33–35], with an apparent ED_{50} of approx. 50 ng/ml.

It has, however, been reported that insulin has no effect (in porcine granulosa cells [35]) or amplifies (in human granulosa cells [11]) the activity of FSH on aromatase function. In bovine granulosa cells recovered from large follicles, our dose–response studies showed that both amplification and inhibition could be observed depending on the dose of FSH.

It is usually considered that the effects of doses of insulin higher than 50 ng/ml are mediated through IGF_1 receptors [24]. Hence, the effect of FSH was further characterized in the presence of 50 ng/ml of insulin in order to avoid responses to IGF_1 stimulation. In these conditions, the dose–response curve for progesterone plateaued at 20 ng/ml of FSH. The oestradiol response was unexpected: 2 ng/ml of FSH induced the largest response and for higher doses a dramatic fall of oestradiol secretion was observed. This observation could explain why Skinner and Osteen [7] could not maintain oestradiol secretion when stimulating bovine granulosa cells with 100 ng/ml of FSH.

The observation that oestradiol secretion decreases when that of progesterone increases, raised the question of the control of aromatase activity by steroids. Exogenous progesterone has been shown to inhibit the induction of aromatase activity in cultured rat granulosa cells [36, 37] but 10^{-5} M concentrations of steroids were necessary. More recently, Chan and Tan [38] have shown that when progesterone synthesis of porcine granulosa cells was decreased by aminoglutethimide, the FSH-induced aromatase activity was augmented. However, in the present work, the inverse relationship between oestradiol and progesterone secretion was dependent on the dose of FSH. In cows, according to the authors, the reported concentrations of FSH vary between 50 and 300 ng/ml in plasma [39–44] and between 0.5 and 125 ng/ml in follicular fluid [45–47]; differences in purity of standards cannot explain these discrepancies. Moreover, Schneyer *et al.* [48] suggest that FSH immunoactivity in follicular fluid, at least in pigs, is not pituitary FSH. Therefore, it is difficult to assess which of the concentrations of FSH tested *in vitro* mimics the degree of stimulation of granulosa cells *in vivo*.

Fibronectin was reported to improve granulosa cell attachment and the maintenance of cell viability in a defined medium [15, 49–51]. This effect was also seen in the present work. Orly *et al.* [29] reported that fibronectin was inhibitory to progesterone production by rat granulosa cells, whereas Morley *et al.* [50] found no differences in oestradiol and progesterone secretions of cells plated on plastic or on fibronectin.

In our experiments, the steroid response to FSH was dependent on whether the granulosa cells were seeded on plastic or on fibronectin but, again, the effects were different for progesterone and oestradiol secretion. Compared to cells plated on plastic, those plated on fibronectin secreted less progesterone, and more oestradiol. Therefore, as far as the steroidogenic response to FSH is concerned, seeding the cells on fibronectin or decreasing the dose of FSH had the same effect: the secretion of progesterone decreased, that of oestradiol increased.

Fibronectin modifies cell function after binding to a receptor of the integrin family (reviewed by Ruoslahti [52]). This receptor is connected with the cytoskeleton which has been reported to be involved in the transduction of signals evoked by FSH [53]. These authors have shown that fibronectin inhibits the action of FSH on the production of some cytoskeleton proteins associated with differentiation of granulosa cells. This could explain why, in our experiment, when cells were cultured on fibronectin, they responded as if they were stimulated by a lower amount of FSH.

Because fibronectin is secreted by granulosa cells under the control of FSH and insulin [54–56] and is one of the components of the extracellular matrix, the present results suggest that it is more important than just an attachment factor for *in vitro* studies. It may have a physiological function in the paracrine control of granulosa cells and follicles differentiation.

In conclusion, our data indicate that primary culture of bovine granulosa cells can be established and remain responsive to FSH under defined, serum-free conditions. The defined system will allow us to study the effects of growth promoters and differentiative agents on granulosa cell function in the absence of interference by serum components.

Our data also address the physiological significance of the inverse relationships between oestradiol and progesterone secretions when FSH concentrations increase.

The biological effects of fibronectin have been studied as far as cell adhesion and migration, organization and regulation of the cytoskeleton

elements are concerned. As reviewed by Amsterdam and Rotmensch [57], fibronectin and the other components of the extracellular matrix must be considered as possible factors in the regulation of granulosa cell differentiation.

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