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Morphometric analysis of in vitro development of porcine immature granulosa cells stimulated by granulosa cell conditioned medium

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This paper examined whether ultrastructural changes characteristic of normal maturation or atresia of granulosa cells (GCs) accompany the steroidogenic alterations. Granulosa cells isolated from small porcine follicles (SGCs) unincubated or SGCs cultured in media supplemented with either serum or large follicle granulosa cell conditioned media (LGCCM) were studied. Under these conditions unincubated cells exhibited increase number of lysosomes, few osmiophilic lipid droplets and resembled atretic cells. In comparison serum cultured SGCs and specially LGCCM treated cells had increased osmiophilic lipid, mitochondria with vesicular cristae and smooth endoplasmic reticulum (SER) volume density. Progesterone secretion was stimulated in LGCCM treated cultures as compared to serum supplemented cultures. This suggests that LGCCM contains a factor(s) stimulating maturation while inhibiting ultrastructural correlates of follicular atresia.

Key words: ovary, granulosa, GC conditioned media, morphometry, progesterone.

It has been well demonstrated that porcine granulosa cell conditioned media (GCCM) possessed the capacity to produce and release a substance stimulating granulosa maturation (Š e b ö k o v a, K o l e n a, 1987). Conditioned media of granulosa cells obtained from small follicle (SGCCM) is at least equivalent to serum in supporting the steroidogenic activity. The effect of spent conditioned media obtained after 4-day culture of granulosa cells collected from small or large porcine follicles noted that the addition of GCCM increased progesterone (P) release in both systems studied. Higher amount of the active substance which stimulate progesterone secretion was released into conditioned media by granulosa cells from large follicles compared to small ones (Š e b ö k o v a, K o l e n a, 1987).

In the present study the effects of conditioned media, obtained from granulosa cells isolated from large follicles on the morphology of granulosa cells from small follicles were examined. Conditioned media of large follicle granulosa cells was used rather than spent media from small follicles because it contains greater stimulatory activity.

Materials and methods

Dulbecco's modified Eagle medium (DMEM) and tissue culture dishes were purchased from Flows Labs, UK; fetal calf serum (FCS) from Difco Labs, Detroit, Mich., USA. Human insulin (Pharmachim, Sofia) and human transferin (Sigma, St. Louis, Miss. USA) were gifts from Dr G. Miltchev. Other chemicals were of the highest purity commercially available.

Culture Procedures

Ovaries were obtained from pigs, 4-month-old or older, less than 20 min after slaughter and were immediately place on ice in a buffered salt solution containing 100 J. U./ml of penicillin, 100 µg/ml of streptomycin and 50 J. U./ml mycostatin. Granulosa cells from small follicles were isolated by the nonenzymatic needle puncture method described by Channing and Ledwitz Rigby (1975). Viable sells (as determined by trypan blue dye exclusion) were seeded in tissue culture dishes at a density 1.10^6 for 1 ml and cultured at 37 °C under a water-saturated atmosphere of 95 % air and 5 % CO_2 in DMEM supplemented with 50 J. U./ml penicillin, 50 µg/ml streptomycin, 2,5 µg/ml fungizone and 5 % FCS for 48 h. For the next 48 h granulosa cells were cultured in medium containing DMEM, supplemented with 25 % LGCCM, human insulin (J at 300mU/ml and human transferrin at 50 µg/ml.

Electron microscopy

At the end or culture period attached cells were scraped from the dishes with a rubber spatula and centrifuged at 600 xg. Unincubated freshly collected GCs and the pelleted cultured granulosa cells were embedded in gelly drop (30 % BSA + 50 % GA) and proceeded for electron microscopy.

Morphometric analysis

Morphometric analysis was performed on micrographs using the techniques of W e i b e l (1969). Fifteen micrographs were analyzed for each treatment group. Each cell constituent examined was outlined and the total area occupied by the specific organelle was recorded. Morphometric measurements were made, which involved stereological calculation of the volume density (the volume part occupied by a given compartment).

Progesterone assay

At the end of the culture the media were centrifuged and supernatants were stored at -20 °C until progesterone was assayed. The concentration of progesterone in the media from cultured granulosa cells was determined by the method of K a n c h e v et al. (1976) using rabbit antiserum (RD/4.10) at a dilution of 1:10 000.

Statistical analysis

Statistical difference was determined by Student's t-test.

Results

Studies from our laboratory have shown that freshly collected granulosa cells (control) had a large oval shaped nucleus surrounded by a small amount of cytoplasm.

After 48 h incubation in 5 % fetal calf serum the granulosa cells had flattened, appeared healthy and were fibroblast-like, whereas those cultured in 25 % LGCCM were epithelioid (Fig. 1, *a* and *b*). After 4 days incubation nuclear: cytoplasmic ratio was significantly greater in the freshly collected cells than in any other group (5 %

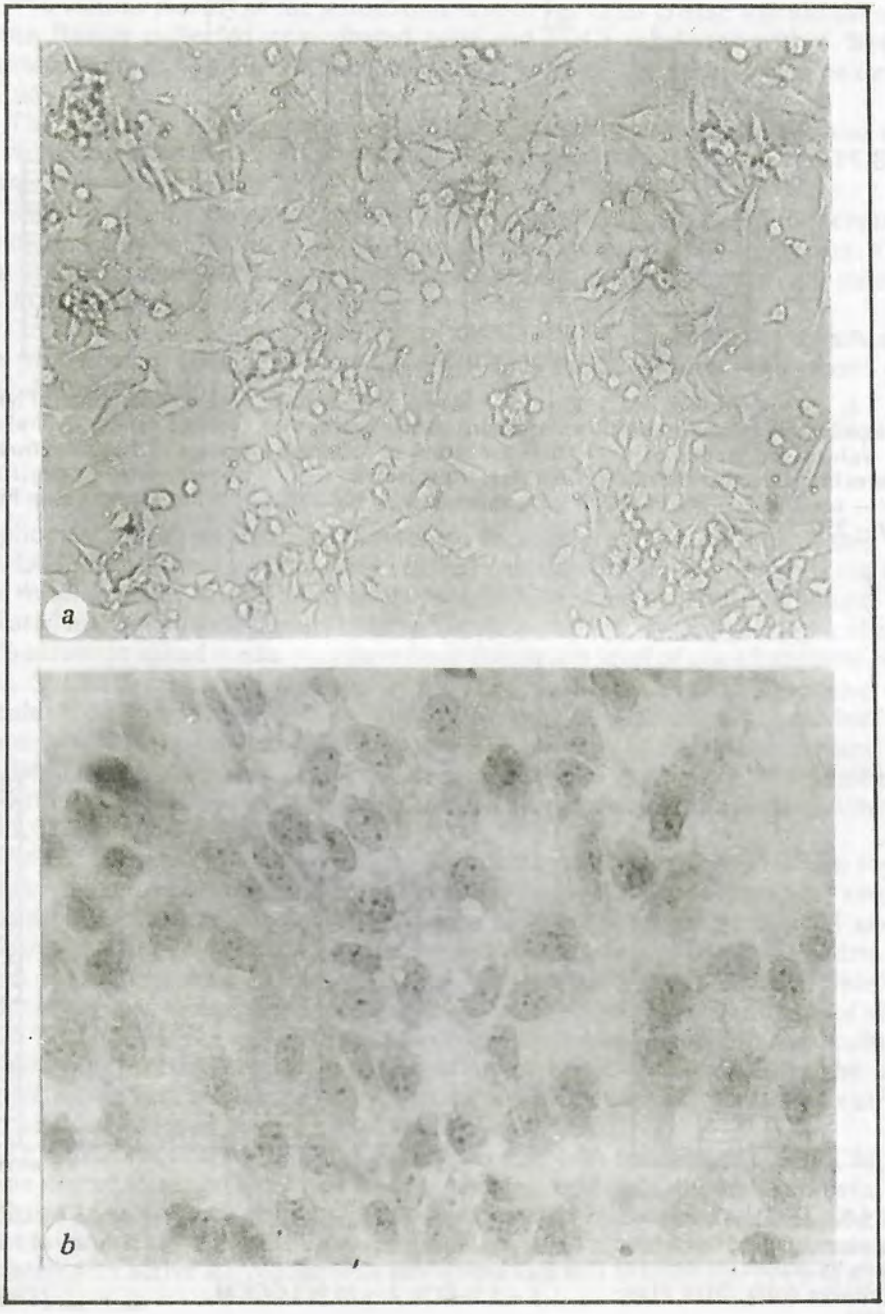

Fig. 1. Granulosa cells from small porcine follicles in 5 % FCS (fibroblast-like) (*a*) and 25 % LGCCM (epitheloid) (*b*)
a — 100×; *b* — 600×

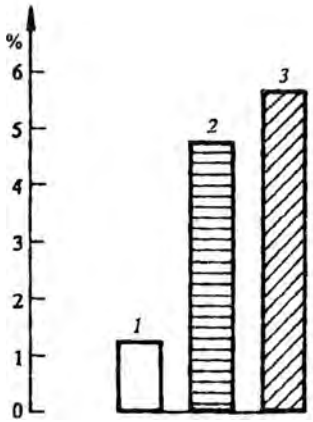

Fig. 2. Mean values of osmiophilic lipid volume density Vv. Values are means of 5 cultures from three experiments
1 — control; *2* — 5% FCS; *3* — 25% LGCCM

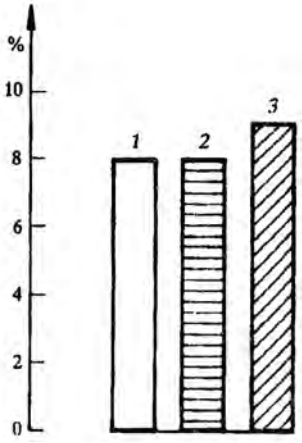

Fig. 3. Mean values of mitochondria volume density Vv. Values are means of 5 cultures from three experiments
Designations as in Fig. 2

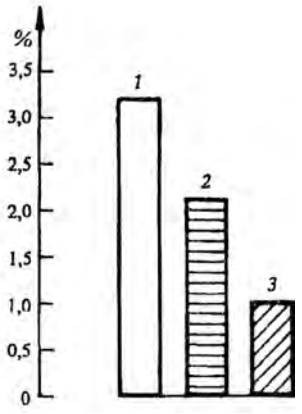

Fig. 4. Mean values of lysosome volume density Vv. Values are means of 5 cultures from three experiments
Designations as in Fig. 2

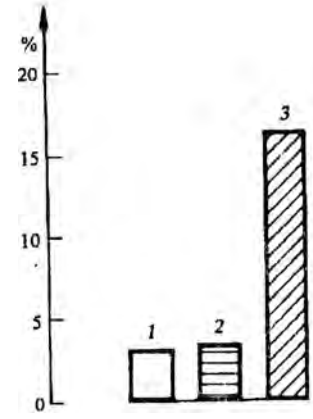

Fig. 5. Mean values of smooth endoplasmic reticulum volume density Vv. Values are means of 5 cultures from three experiments
Designations as in Fig. 2

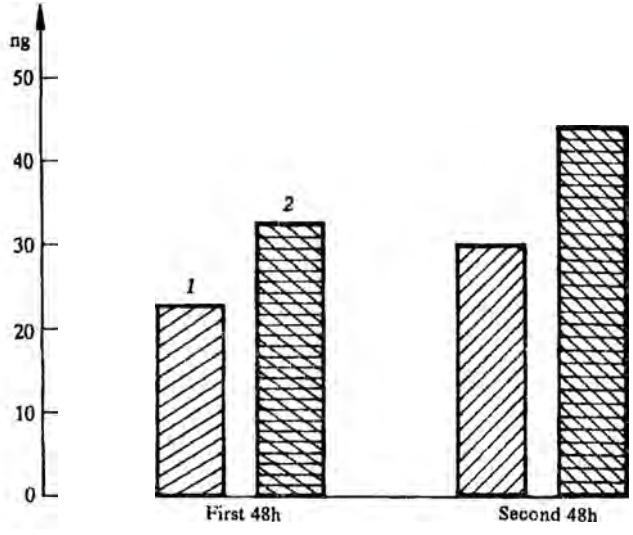

Fig. 6. Effect of FCS and LGCCM on P secretion by GCs from small follicles (ng/ml per 1.10^6 cells) Values are means of 8 cultures from 3 experiments
1 — 5 % FCS; *2* — 25 % LGCCM

FCS or 25 % LGCCM) and the number of microvilli increased in the case of 25 % LGCCM treatment (data not shown).

Lipid volume density was significantly higher after 4 days incubation in 25 % LGCCM in comparison with other treatment (5 % FCS) or in freshly collected unincubated granulosa cells ($p<0,001$) (Fig. 2).

The volume density of the mitochondria with vesicular cristae was almost equal in both freshly collected unincubated cells and SGCs incubated with 5 % FCS. Treatment with 25 % LGCCM increased slightly the mitochondrial volume density ($p<0,05$) (Fig. 3).

The lysosomal volume density was highest in freshly collected granulosa cells. The volume density decreased after incubation of granulosa cells with 5 % FCS and declined considerably after 25 % LGCCM treatment ($p<0,001$) (Fig. 4).

Smooth endoplasmic reticulum (SER) volume density appeared to increase in cells incubated with 5 % FCS, but the increase was not statistically significant. There was a dramatic increase in the volume density of SER in the granulosa cells cultured with 25 % LGCCM ($p<0,001$) (Fig. 5).

Granulosa cells incubated in 25 % LGCCM for 4 days secreted significantly more progesterone than cells cultured in 5 % FCS specially during the second 48 h of incubation ($p<0,001$) (Fig. 6).

Discussion

Morphometric analysis identified differences between granulosa cells incubated with 25 % LGCCM, serum or unincubated cells. Four-day-incubation of granulosa cells from small follicles in 5 % FCS or 25 % LGCCM alter their ultrastructure. The decreased nuclear volume density observed in the present study after 4 days incubation in 5 % serum or spend media may have been due to low level of gonadotropins in the media. Such a decrease was observed by Mc L e a n et al. (1986) after incubation with follicular fluid from large follicles. The appearance of microvilli on granulosa cell surface has been correlated with an increased number of LH receptors and LH stimulable adenylyl cyclase (J a r r i et al., 1994). An increase in the number of microvilli has been associated with granulosa cells maturation after incubation with 25 % LGCCM (W a d a, 1993).

One of the marked changes in granulosa cell morphology noted in this report was the change of osmiophilic lipid droplets, lysosomes, mitochondria, smooth endoplasmic reticulum content of granulosa cells cultured with 5 % FCS and in SGCs treated with 25 % LGCCM. The highest volume density of mitochondria was seen in culture treated with 25 % LGCCM. The most striking increase in lipid and smooth endoplasmic reticulum volume density was observed after treatment of SGCs with 25 % LGCCM. The lysosome volume density decreases when SGCs were cultured with serum and reaches minimal values after treatment with LGCCM. The large autophagocytic vacuoles seen in unincubated cells have been associated with atresia and resembled in vivo atretic cells (E l f o n t et al., 1992).

An important observation of our data was that cells incubated in LGCCM and in some degree in serum exhibited fewer signs of atresia than unincubated cells.

Progesterone secretion by SGCs was stimulated by serum and LGCCM to a greater extent on the 4th day than on the first 2 days and correspond to characteristics associated with active steroidogenesis and granulosa cell maturation (S t e w a r t et al., 1982).

In conclusion data presented in this paper demonstrated that spent media fron granulosa cells of large follicles alter small follicle granulosa cell morphology enhancing granulosa cell maturation as well as steroidogenesis.

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