PORCINE GRANULOSA CELLS PRODUCE A PROGESTERONE SECRETION INHIBITORY ACTIVITY

R.Tz. Denkova, B. Nikolov, A. Russinova

Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Objective. To extend our previous observations of granulosa cell conditioned medium inhibition on basal and FSH-stimulated progesterone secretion by granulosa cells from large porcine antral follicles.

Methods. Granulosa cell conditioned media (SGCCM) were obtained from cultures of granulosa cells which were harvested from small porcine antral follicles. SGCCM were fractionated by filtration through an Amicon XM-50 and an PM-10 membrane.

Results. Two fractions – XM-50 filtrate (<50 kDa) and PM-10 retentate (10-50 kDa) – exhibited well expressed inhibitory effect on the progesterone secretion from large follicle granulosa cells *in vitro*. Such inhibitory effect was fully prevented by heat treatment, partially inactivated by trypsin digestion ($P<0.05$), but was resistant to several freeze-thaw cycles and storage at -20 or -70 °C for up to one year.

Conclusions. It is suggested that SGCCM contains some regulatory substance(s) capable of inhibiting progesterone secretion by cultured granulosa cells and that the molecular weight of such substance is presumably between 10-50 kDa.

Key words: Granulosa cells – Tissue culture – Progesterone secretion – Inhibitory substance

It is generally accepted that gonadotropins (FSH and LH) are essential for the gametogenic and steroidogenic processes in the mammalian ovary (Richards 1980). Recently it has become increasingly apparent that intraovarian regulatory mechanisms involving locally produced factors may also play a significant role in mediating follicular development and differentiation (Schomberg 1988; Cain et al. 1995; Zhou et al. 1997; Schotanus et al. 1997). It was reported that follicular fluids from ovaries of various species contain factor(s) which stimulates or inhibits the progesterone and estradiol secretion from granulosa cells (Ledwitz-Rigby 1986; Kigawa et al. 1986; Hillensjo et al. 1983; Sebokova and Kolena 1987). Granulosa cells collected from large porcine follicles possessed the capacity to produce and release progesterone secretion stimulatory activity under *in vitro* conditions (Sebokova et al. 1987).

In a preliminary study we have demonstrated that the conditioned media generated by primary cultures of granulosa cells (GCs) from small follicles (SGCCM) contain the nonsteroid regulator(s) capable of suppressing progesterone secretion by cultured granulosa cells isolated from large follicles (LGCs) (Denkova et al. 1993). The present study was designed to extend these findings in further detail. We report the progesterone inhibitory activities in different fractions of lyophilized small granulosa cell conditioned medium.

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); trypsin (from bovine pancre-

as 3.1 mg/ml and dialysis tubing with a pore size of 0.28 nm (molecular mass limit 10 kDa) from Serva (Heidelberg, Germany); thrombin (TH) and fibronectin (F) from bovine plasma, low density lipoprotein (LDL) from human plasma and insulin (I) from bovine pancreas were purchased from Sigma (St.Louis, USA). Ovine FSH (NIH-FSH-S_{11}) with a potency 1.15 NIH-FSH-S_1 units/mg and ovine LH (NIH– LH-S_{19}) with a potency of 1.01 NIH-LH-S_1 units/mg were obtained from Serono (Freiburg, Germany). FSH and LH were dissolved in sterile distilled water and stored at -20 °C in small aliquots of various concentrations. Vials of FSH and LH were defrosted immediately prior to addition to culture media, diluted with DMEM and added to the appropriate media to achieve final concentrations of 100 ng FSH/ml and 100 ng LH/ml. These were the minimal concentrations of FSH and LH required for maximal stimulation of steroid secretion. Other chemicals were of the highest purity commercially available.

Culture procedures. Ovaries were obtained from pigs, 4 months old or older, less than 20 min after sacrifice and were immediately placed on ice in a buffered salt solution containing penicillin (100 IU/ml), streptomycin (100µg/ml) and mycostatin (50 IU/ml). The antral follicles were removed from the ovary under sterile conditions and cleaned of adhering interstitial tissue. To exclude microscopically atretic follicles, those with a dull grey opaque appearance were discarded. Granulosa cells (GCs) from large follicles (LGCs) were isolated from follicles by the nonenzymatic needle puncture method described by Channing and Ledwitz-Rigby (1975). Viable cells (as determined by trypan blue dye exclusion) were seeded in tissue culture dishes at a density $1x10^6$ /ml and cultured at 37 °C under a water-saturated atmosphere of 95 % air an 5 % CO_2 in DMEM supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml fungizone and 5 % FCS for 48 h. For the next 48 h the cells were cultured in serum-free medium containing DMEM, 8µg/ml fibronectin (F), 1µg/ml insulin (I), 1 IU/ml thrombin (TH) and 10 µg/ml low density lipoprotein (LDL) (Buck and Schomberg 1987) with or without 50 % conditioned medium of GCs.

Conditioned media were obtained from 3-day cultures of GCs harvested from small follicles (SGCs). The SGCs (1.10^6/ml) were cultured as described above in DMEM and 5 % FCS for 24 h, followed by 48 h in DMEM without serum but supplemented with I+F+TH+LDL. The supernatants of the media were mixed with 10 % (wt/vol) activated charcoal, shaken at 4 °C for 15 min and centrifuged at 1000 g, 0 °C for 1 h to remove the steroid contained in them. This procedure was repeated 3 times. The remaining charcoal was removed from the fluid by filtration through 0.22 µ Milipore filters. Following the charcoal treatment conditioned medium was dialyzed against phosphate-buffered saline (PBS), 0.05 M, pH 7.4, for 48 h at 4 °C using dialysis tubing with a pore size of 0.28 nm and was concentrated by lyophilization. The media was reconstitted in 1/2, 1/5, 1/10 and 1/15 of the original volume of culture medium with no serum to give a 2-, 5-, 10– and 15-fold concentration (2x, 5x, 10x, 15x, respectively, giving 100, 250, 500, 750 µg/ml protein). The reconstituted conditioned medium (5x) was filtered through an Amicon XM-50 membrane (with a molecular weight exclusion limit of 50 KDa) to produce two fractions, XM-50 retentate (>50 KDa MW) and XM-50 filtrate (<50 KDa MW). XM-50 filtrates of SGCCM were further fractioned by Amicon PM-10 membrane (with of MW exclusion limit of 10 KDa) into two fractions – PM-10 retentate (>10KDa MW) and PM-10 filtrate (10 KDa MW). XM-50 filtrate of SGCCM was heated in a water batch at 60 °C for 30 min and treated with 500 µg trypsin.

Progesterone assay: The progesterone (P) level was estimated by means of RIA. The concentration of P in the media from cultured GCs was determined according to Kanchev et al. (1976) using rabbit antiserum (RD/4.10) at a dilution of 1:10000. The antiserum was prepared against 11a-hydroxy-progesterone succinyl-BSA. The sensitivity of the method was 10 pg per tube. The intra-assay coefficients of variations were 6.1 % and 10.2 %, respectively.

Statistical evaluation: The experimental data from replicate cultures are presented as mean ± SD. Comparable results were obtained in triplicate experiments. Statistical significance of differences was analyzed by Student's t-test.

Results

The effect of different concentrations of porcine SGCCM fractions on progesterone secretion by cul-

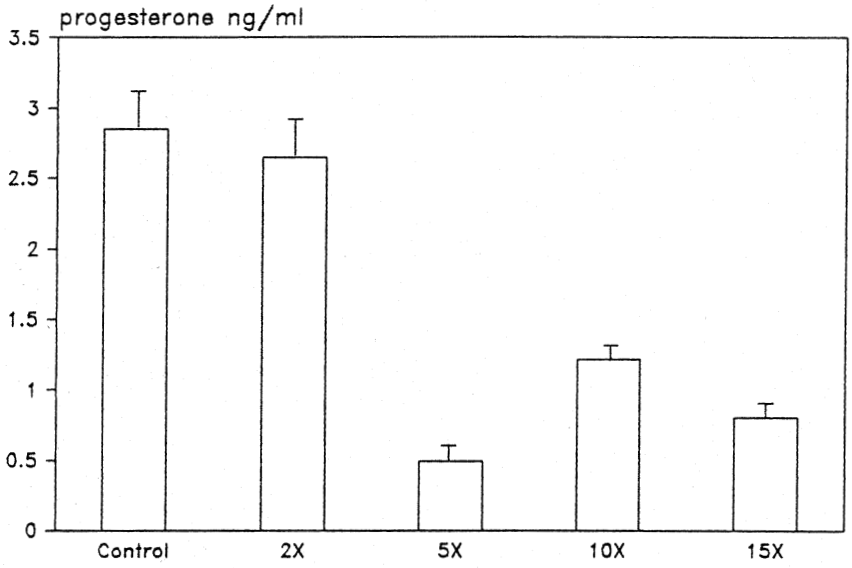

Fig. 1
Effect of various SGCCM concentrations on progesterone secretion by LGCs. The cells were cultured for 24 h in DMEM and 5 % FCS then for second 48 h in a serum free 50 % DMEM (with F+I+TH+LDL) and 50 % SGCCM from batches reconstituted to the concentrations of 2x, 5x, 10x or 15x. Each value is the mean ± S.E. of 15 cultures from three experiments.

tured GCs isolated from large follicles is shown in Fig. 1 (P<0.05); it is apparent that the concentration (5X) produced maximum inhibitory activity. For the characterization of active material granulosa cells harvested from large follicles were incubated for 3 days in the presence of the fractions which were obtained by filtration through an Amicon XM-50 or PM-10 membranes of 5X-concentrated SGCCM. During the period of GCs culture a very slight inhibitory effect of XM-50 retentate on progesterone secretion is visible (data not shown). The addition of XM-50 filtrates diminished significantly the P secretion (P<O.O01) (Fig. 2). As shown in the same figure, in the presence of PM-10 retentate (obtained by filtration through an Amicon PM-10 membrane of XM-50 filtrate) the progesterone secretion was also markedly decreased (P<0.001). PM-10 filtrates (<10 kDa) has faint effect on progesterone secretion by LGCs (data not shown). The two SGCCM fractions suppressed FSH-stimulated progesterone release (P<0.001) (Fig. 3). LH tested under the same conditions affected in a similar way the P secretion (P<0.001) (Fig. 4). Extraction with charcoal did not alter the inhibitory action of SGCCM (data not shown). In the group with heat treatment, progesterone secre-

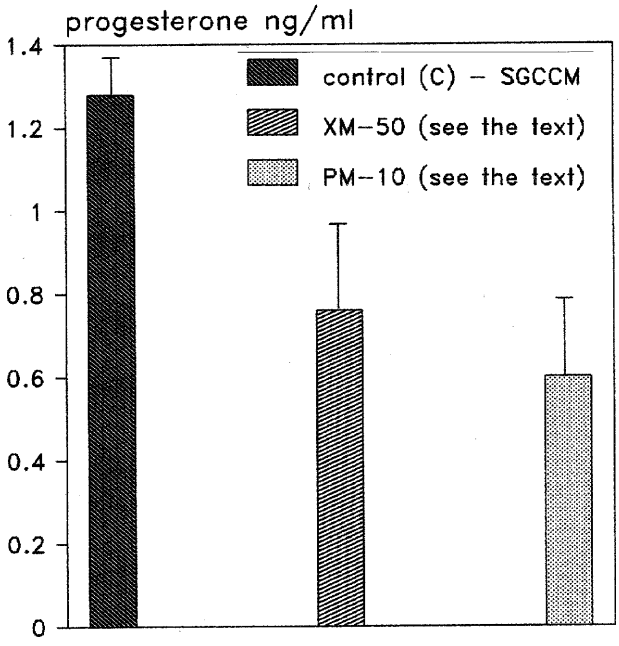

Fig. 2
Effect of 5x SGCCM fractions on progesterone secretion by LGCs. XM-50-filtrate (<50000 MW) – filtration of 5x SGCCM through an Amicon XM-50 membrane with a molecular weight exclusion limit of 50 KDa; PM-10 retentate (10000-50000 MW) – filtration of XM-50 through a PM-10 membrane with a molecular weight exclusion limit of 10 KDa. Each value is the mean ± S.E. of 15 cultures from three experiments.

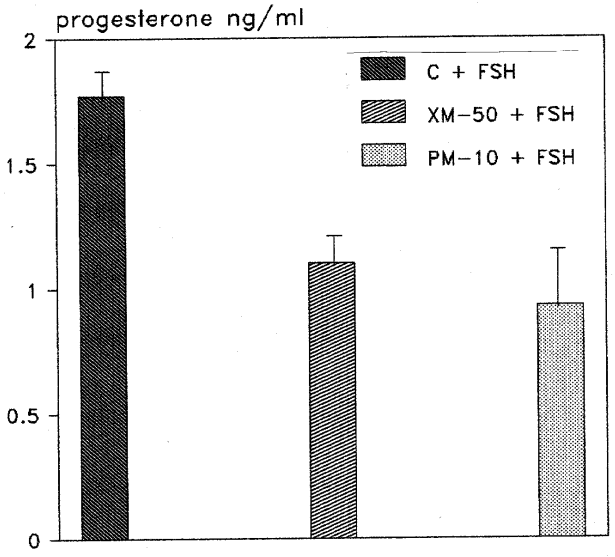

Fig. 3
Effect of 5x SGCCM fractions (XM-50 and PM-10) on progesterone secretion by LGCs after FSH-stimulation. Each value is the mean ± S.E. of 12 cultures from three experiments.

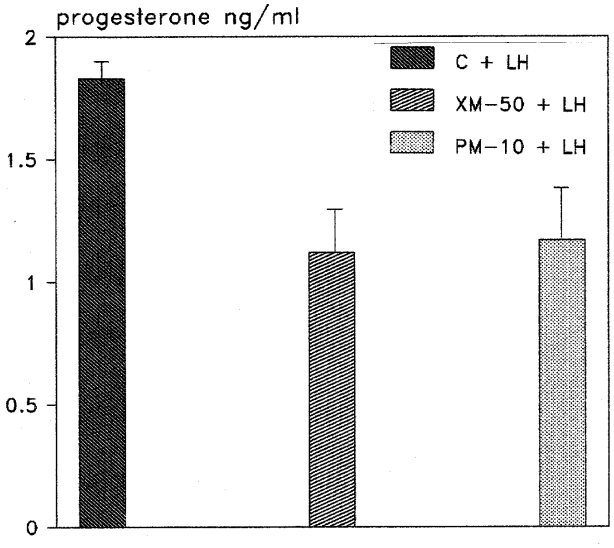

Fig. 4
Effect of 5X SGCCM fractions (XM-50 and PM-10) on progesterone secretion by LGCs after LH-stimulation. Each value is the mean ± S.E. of 12 cultures of three experiments.

tion did not differ from the control. The inhibitory effect by fractions (10-50 kDa MW) was partially inactivated by trypsin digestion ($P<0.05$) (Tab. 1), but survived several freeze-thaw cycles and the storage at -20 or -70 °C for up to one year.

Discussion

In the present study we reported the progesterone inhibitory activities of some fractions of 5X concentrated small granulosa cell conditioned medium on progesterone secretion. Suchc inhibitory activity was detected in the filtrate (<50 kDa MW) of SGCCM obtained by filtration through a XM-50 membrane and the fraction (PM-10 retentate) obtained by filtration through a PM-10 membrane of the XM-10 filtrate of SGCCM. In the presence of the two fractions either basal and gonadotropin (FSH and LH) stimulated progesterone secretion was suppressed. In agreement with our data Kigawa et al. 1986 observed a protein like substance from XM-50 filtrate of porcine follicular fluid which inhibited the progesterone secretion of GCs. In contrast, however, an inhibitory activity was also detected in a low molecular weight fraction (<1000 MW) obtained by PM-10 membrane filtration and Sephadex G-25 column chromatography of a pool of small follicle follicular fluid (Hillensjo et al. 1980). Our results may suggest that the inhibitory activity in the fractions is probably a heat-stable and trypsin sensitive molecule, non lipid in nature with MW possibly between 10-50 kDa. It is tempting to speculate that this locally produced progesterone inhibitory substance may play an important autocrine role in modulating the GCs steroidogenesis.

Table 1
Effect of SGCCM (PM-10 retentate) treated with heat and trypsin on progesterone secretion by LGCs

	Progesterone (ng per 100 cells per 24 h)
Control	2.95 ± 0.82
PM-10 retentate	1.60 ± 0.90
with heat treatment	1.58 ± 0.52
with trypsin treatment	2.25 ± 0.06[1]

[1] – $P<0.05$ vs. control

PM-10 retentate was treated by heat (60°C for 30 min) and trypsin (Kigawa et al. 1986). Each value is the mean ± S.E. of four estimations.

Acknowledgments

This work was supported by the grants from the National Found of "Scientific Research"-B-519/95 and K-530/95. The authors thank Prof. L. Kanchev for the RIA estimation of progesterone.

References

CAIN L, CHATTERJEE S, COLLINS TY: In vitro folliculogenesis of rat preantral follicles. Endocrinology **136**, 3369-3377, 1995

DENKOVA RT, IVANOV IG, KANCHEV LN: Porcine granulosa gell gonditioned gedia as autocrine regulator of progesterone secretion. Reprod Fertil Dev **5**, 95-102, 1993

HILLENSJO T, POMERANTZ SH, SCHWARZ-KRIPNER A, ANDERSON LD, CHANNING CP: Inhibition of cumulus cell progesterone secretion by low molecular weight fractions of porcine follicular fluid which also inhibit oocytes maturation. Endocrinology **106**, 584-591, 1980

HILLENSJO T, CHARI S, NILSON L, HANBERG L, DAUME E, STRUM G: Inhibition of progesterone secretion in cultured human granulosa cells by a low molecular weight fraction of human follicular fluid. J Clin Endocrinol Metab **56**, 835– 838, 1983

KANCHEV LN, DOBSON H, WARD WR, FITZPATRICK RJ: Concentration of steroids in bovine peripheral plasma during the oestrus cycle and the effect of betamethasone treatment. J Reprod Fert **48**, 341-345, 1976

KIGAWA T, OGAWA T, MATSUOKA R, KOIKE S: Inhibitory effects on the porcine follicular fluid on estradiol and progesterone secretion by cultured rat granulosa cells. Endocrinol Japon **33**, 221-231, 1986

LEDWITZ-RIGBY F: Local regulation of granulosa cell maturation: J Steroid Biochem **27**, 1-2, 385-391, 1986

RICHARDS JS: Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev **60**, 51-59, 1980

SCHOMBERG DW: Regulation of follicle development by gonadotropins and growth factors. In: The Primate Ovary (Ed. R.L. Stouffer) pp 25-33, Plenum Press, New York, 1988

SCHOTANUS K, HAGE WJ, VANDERSTICHELE H, VANDENHURK R: Effect of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. Theriogenology **48**, 3, 471-483, 1997

SEBOKOVA E, KOLENA J: Effect of follicular fluid on the maturation of porcine granulosa cells: stability of luteinization stimulator. Endocrinol Exper **21**, 2, 103-113, 1987

SEBOKOVA E, DANISOVA A, KOLENA J: Granulosa cells in culture are able to produce luteinization stimulatory factor. Endocrinol Exper **21**, 1, 3-12, 1987

ZHOU J, KUMAR TR, MATZUK MM, BONDY C: Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. Mol Endocrinol **11**, 13, 1924-1933, 1997

Corresponding author: R.Denkova, M.D.
Bulgarian Academy of Sciences
Institute of Experimental Morphology
and Anthropology
G. Bonchev str. B-25
1113 Sofia, Bulgaria

Accepted: January 15, 1999