J. Korean Fish. Soc. 29(6), 909~911, 1996 한수지, 29(6), 909~911, 1996

〈TECHNICAL NOTE〉

Optimum Concentration of the Granulosa Cells in Primary Culture to Study the Regulation of 17α-hydroxy, 20β-dihydroprogesterone Production in Rainbow Trout (*Oncorhynchus mykiss*) Ovary

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The aim of the present work was to determine the optimum concentration of the ovarian granulosa cells for *in vitro* regulation of 17α-hydroxy, 20β-dihydroprogesterone (MIS: maturation-inducing steroid) production in granulosa cells in the rainbow trout, *Oncorhynchus mykiss.* For this, we analyzed the influence of the cell numbers on the production of 17α-hydroxy, 20β-dihydroprogesterone (17α20βOHP) from 17α-hydroxyprogesterone (17α-OHP) in the presence of highly purified salmon gonadotropin (sGtH) for 48 hrs.

The techniques used for the preparation of granulosa cells has been initially suggested by Jalabert (personal communication) and described in detail elsewhere (Baek, 1990). In brief, the ovaries (subperipheral germinal vesicle stage reared at the fish farm of France) were cut into small clusters in TBSS (trout balanced salt solution, Jalabert and Fostier, 1984) and separated follicles were collected with fine forceps. Ovarian epithelia and theca folliculi were removed manually in SWIM S-77 medium (Gibco). The defolliculated oocytes were submitted to disperse in SWIM S-77 medium containing 2 mg/ml of collagenase at 12 ℃ for 4 hrs (method of Jalabert, unpublished data). Granulosa cells released from the oocytes were filtered through a fine nylon mesh, sedimented by centrifugation at 200×g for 7 min, and washed twice with SWIM S-77 medium. The cell pellet was resuspended in a medium consisted of SWIM S-77 with 1% bovine serum albumin (BSA) and placed in culture bottle in the presence of 20μg/ml DNase to reduce the extent of cell aggregation. It has been known that the dissociated granulosa cells showed a tendency of coalesce to form clusters. At the end of the culture period (12 hr), the cell pellet obtained by centrifugation was resuspended in SWIM S-77 with BSA. Cell number and viability were determined using a Thoma hemocytometer in the presence or absence of 0.2% erythrosin B. Granulosa cells (Fig. 1) were incubated at densities ranging from 6.25 to 200×10^3 viable cells per well (24-multiwell plates). It was performed in 1 ml of Leibovitz L-15 medium (Gibco) supplemented with 17α-OHP (50 ng/ml) and sGtH (200 ng/ml).

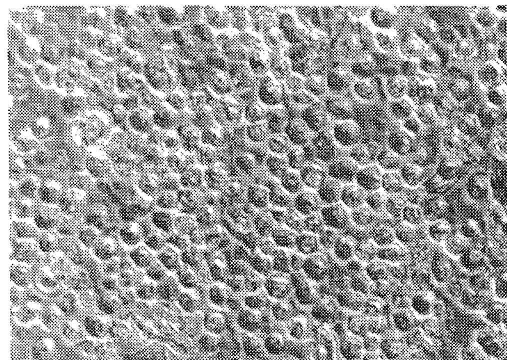

Fig. 1. Granulosa cells after enzymatic dissociation [collagenase (2 mg/ml)-DNase (20μg/ml), ×105]. Phase-contrast microscopy.

After 48 hr incubation, the amount of secreted 17α-20βOHP was estimated by radioimmunoassy as previously described (Fostier et al., 1981).

As shown in Fig. 2, produced 17α20βOHP was readily measured at 5×10^4 cells/ml density. With 1×10^5 cells/ml density, the concentration of 17α20βOHP in the wells was about 3 fold higher than that of 5×10^4 cells. However it showed a tendency of decreased production at 2×10^5 cells/ml density, and implied a saturation of metabolic capacity by high density of cells. In terms of cell numbers per milliliter of medium, routine seeding densities would be from 10^4 to 10^5 cells/ml or more, but as few as 10^3 or even 10^2 /cells/ml can be adequate under good conditions. With the latter low number of cells, there will be considerable lag phase (Wolf and Quimby, 1969). It can be concluded that 5×10^4 cells/ml (incubation well) was selected as an optimum density of cells to get a best responsiveness in subsequent experiments. 1×10^5 cells/ml was appeared to be excessive.

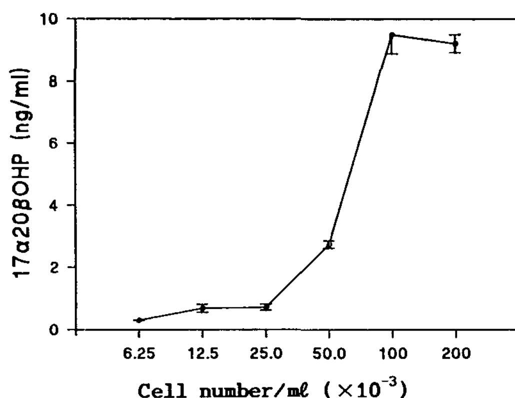

Fig. 2. 17α20βOHP production from the granulosa cells. Cells were incubated in the presence of sGtH (200 ng/ml) and 17α-OHP (50 ng/ml) for 48 hrs. Data are expressed as mean ± SEM of triplicate incubated wells.

High cell density may alter culture conditions because of accumulation of metabolic wastes, depletion of nutrients, changing of PH in the medium, or decreased oxygen tension may also directly alter the function of cells (Stocker and Rubin, 1967).

Several aspects of cell culture technique and concentration of used cells greatly influence to cell responsiveness in various experimental manipulations. The determined cell number in this experiment has been optimized for studying the regulation of 17α20βOHP production.

Acknowledgments

The author thanks Drs. B. Jalabert and A. Fostier for their helpful suggestions, and to Dr. B. Breton for generously supplying salmon gonadotropin.

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Received July 2, 1996
Accepted October 12, 1996