Corpus luteum Cells Grown as Monolayers

Experiments in culturing corpus luteum tissue are few. Recently FAINSTAT[1] cultured whole rat ovaries, containing developed corpora lutea, for 3 days, and GOSPODAROWICZ[2] described a technique for obtaining lutein cells suspension for a rather short time in vitro.

However, cultures of isolated follicular granulosa or theca interna cells were successful[3,4], and this was the stimulus to undertake experiments to make corpus luteum cells grow as monolayers. Culture by this method could: 1. prolong the viability of the tissue in vitro, 2. make it possible to study it for a much longer period of time, as well as 3. its investigation free from the complex environment of the whole ovary.

In the present experiment, an attempt was made to adapt corpus luteum cells of pig and rat ovaries to tissue culture conditions.

Rat corpora lutea were dissected from the ovaries in early metestrus. Only corpora lutea forming from newly ovulated follicles were removed. Ovulation points were visible.

Porcine corpora lutea were dissected from ovaries in early luteal phase of the cycle. Corpora lutea from recently ovulated follicles were selected for the experiment. Ovulation points were closed but still visible and corpora lutea were pink and juicy.

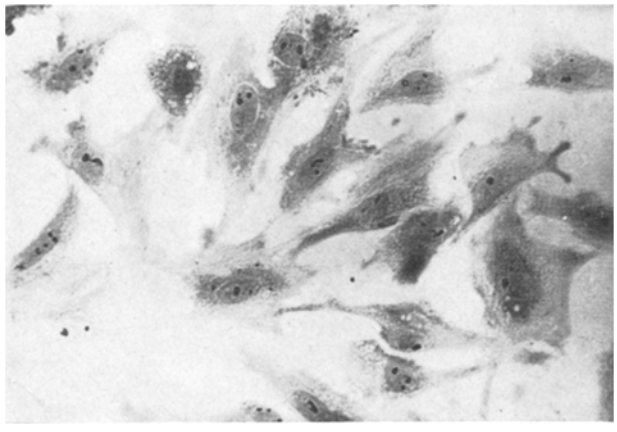

Fig. 1. Rat corpus luteum cells grown as monolayer, 4 days in culture. May-Grunwald-Giemsa stain (MG-G). ×81.

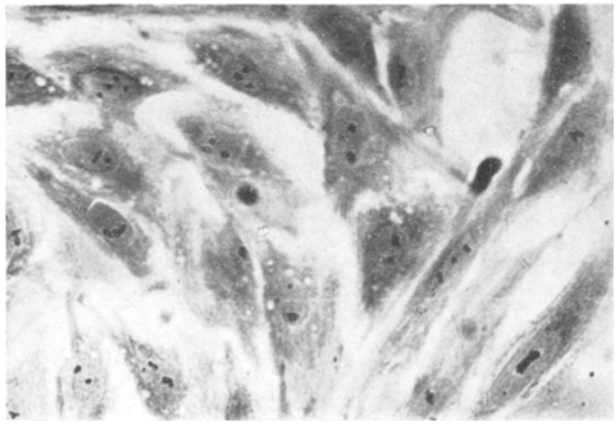

Fig. 2. Porcine corpus luteum cells monolayer, 6 days in culture, MG-G. stain. ×81.

Whole rat corpora lutea were minced mechanically, while with pig, first connective tissue theca was manually separated and discarded and then pure luteal tissue was dispersed using small, sharp scissors.

After mincing, tissue was trypsinized in 0.25% solution of trypsin[9] in PBS[9] twice for 10 min and then additionally for 15 min on the magnetic stirrer at 37 °C. Porcine corpora lutea with connective tissue theca removed were more easily dispersable by the enzyme digestion than rat ones. Next, trypsin was inhibited by the addition of cold calf serum[9]. Cell suspensions obtained, both of rat and pig corpora lutea, were rich in numerous large and viable cells which were morphologically homogenous. After centrifuging and washing, the cells were diluted with culture medium to obtain a suspension of ca. 800,000 cells per 1 ml of the medium, and then inoculated into the Leighton tubes, 1.5 ml into each.

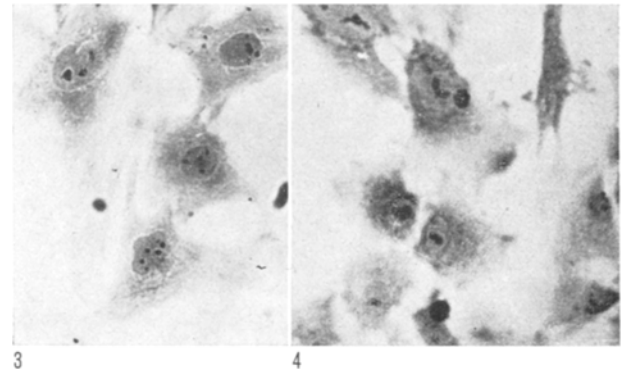

Fig. 3. Porcine corpus luteum cells 5 days in culture. MG.-G. stain. ×81.

Fig. 4. Porcine follicular theca interna cells, 5 days in culture. MG-G stain. ×81.

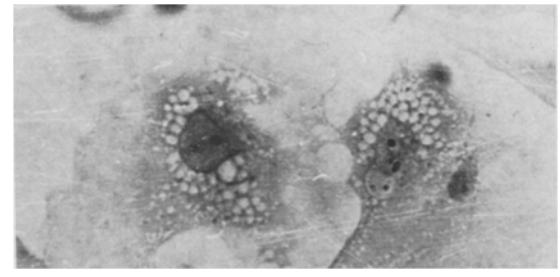

Fig. 5. 8 days culture of porcine corpus luteum cells note numerous vacuoles. MG-G stain. ×125.

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Medium 199[9] enriched by 15% of calf serum was used. Cells were cultured at 37 °C. Medium was changed every 3rd day. Each time it was newly oxygenated. Cultures were continued for 8 days. Slides with monolayers were stained according to the May-Grunwald-Giemsa method to judge their viability.

Beginning from the 4th day of culture, parallel monolayers were submitted to a histochemical test for Δ^5-3β-OH steroid dehydrogenase activity[5,6]. This enzyme participates in the transformation of pregnenolone into progesterone and was observed in corpora lutea in vivo[6,7]. Its presence within cultured cells provided information about their possible hormonal activity.

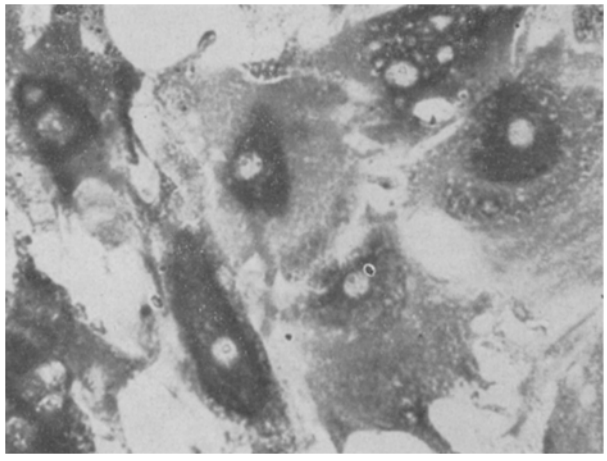

Fig. 6. Distinct Δ^5-3β-OH steroid dehydrogenase activity within porcine luteal cells on the 4th day in culture. ×125.

Both rat and pig corpus luteum cells adapted well to tissue culture conditions, adhesed to the glass early and showed mitotic activity during the culture time. Cells showed typical luteal morphology with no tendency to fibroblastic growth (Figures 1–2). These cells in pig were very similar to separately cultured theca interna cells of the follicle (Figures 3–4). Large and strongly vacuolized cells appeared in 7–8 days cultures (Figure 5). Such cells showed distinct dehydrogenase activity. This would suggest their participation in hormonal synthesis, or aging of cells, if 20α-OH steroid dehydrogenase enzyme would show its activity[8]. Further experiments will decide wheter it is true or not. Cultured corpus luteum cells showed distinct $\Delta^5$3β–OH steroid dehydrogenase activity during investigation time, especially well visible in 4 days culture (Figure 6). The histochemical reaction was both granular and diffuse.

The experiments discussed above are preliminary and further studies are being continued.

Zusammenfassung. Die Fähigkeit der Corpus-luteum-Zellen als Monolayers zu wachsen wurde untersucht. Die von Schwein und Ratte entnommenen Gelbkörper wurden zerkleinert und mit einer 0,25%igen Trypsinlösung in eine Suspension übergeführt. Die Kulturen wurden bis zum 8. Tag beobachtet. Die wachsenden Luteinzellen zeigen die typische Form und auch die ausgeprägte Aktivität der Δ^5-3β-OH Steroiddehydrogenase.

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Reconstitution of *Cancer pagurus* Hemocyanin with Copper (II) and Hydroxylamine

Hemocyanin is a non-heme, copper-containing, respiratory protein found in the hemolymph of arthropoda and mollusca. The essential copper cannot be removed from hemocyanin by EDTA or ion-exchange resins. Copper-free hemocyanin can only be prepared without any denaturation of the protein by cyanide treatment[1]. A partial reconstitution of hemocyanin has been achieved by CuC_2^{-1}, Cu_2O and copper (I) amine complex[2]. A practically quantitative reconstitution of functional hemocyanin with copper (I) acetonitrile complex has been reported formerly from these laboratories[3]. In the present communication, a much more simple and quicker method of fixation of copper with copper (II) and hydroxylamine is described.

Copper-free *Cancer pagurus* hemocyanin is prepared by adding dropwise a solution of IM potassium cyanide in 0.01 *M* hydroxylamine hydrochloride, pH 8.2 (adjusted with acetic acid) to 2% fresh hemocyanin in 0.01 *M* calcium acetate until the blue protein colour becomes yellowish. This solution is dialyzed for 4 days against 0.05 *M* potassium cyanide in 0.01 *M* calcium acetate and 0.01 *M* hydroxylamine hydrochloride, pH 8.2 (adjusted with acetic acid) at 4 °C. Cyanide is removed by dialysis against acetate buffer (pH 5.7, ionic strength 0.1).

The reconstitution of *Cancer pagurus* hemocyanin is studied through the changes in absorbancy at 335 nm in Beckman DU Spectrophotometer. For the reconstitution, a 2% apohemocyanin solution is treated at pH 5.7, acetate buffer, ionic strength 0.1, for 5 h in presence of nitrogen with 2 equivalents of copper (II) and 50 equivalents of hydroxylamine hydrochloride per mole of copper in native hemocyanin. The reconstituted hemocyanin is dialyzed against EDTA, M/40 in acetate buffer, pH 5.7, ionic strenght 0.1 and afterwards against the acetate buffer alone.

The absorption coefficient, K_a at 335 nm for a protein concentration 1 g/l and a path length of 1 cm, corrected for the absorbance of the protein due to light scattering, is determined. It amounts to 0.231, 0.030, 0.203 for fresh, apo and reconstituted hemocyanin respectively corresponding to 86% reconstitution. Total copper is determined photometrically with 2,9-dimethyl-1,10-phenanthroline at 454 nm in glacial acetic acid in the presence of 0.1% hydroxylamine hydrochloride. The fresh, apo and reconstituted hemocyanins have copper contents of 0.165, 0.001, 0.141% respectively, corresponding to 85% reconstitution.

The reconstituted hemocyanin appears to be identical with the native hemocyanin e.g. the oxygenation-deoxygenation cycles, optical absorption spectra. Copper

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