PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **152**, 366-371 (1976)

A Simple Method for Preparing Suspensions of Luteal Cells[1] (39398)

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Methods for preparing suspensions of isolated cells have been developed for a number of tissues, including the adrenal (1-4), the anterior pituitary (5-11), the ovary (4, 12, 13), the pancreas (14), the testis (15), and the thyroid (16). The use of cell suspensions for biological studies offers several advantages including: (i) the physical and hormonal environment of the cell can be easily controlled and monitored; (ii) many biochemical parameters which can not be measured *in vivo* are more easily approached *in vitro;* and (iii) the ability to distribute cell suspensions homogeneously alleviates much of the variability associated with other *in vitro* systems. A variety of enzymatic procedures has been used to disrupt the tissue matrix and free the cells. The only method described for luteal tissue (12) employed a number of enzymes in a rather complex procedure. The following communication describes a simple and highly reproducible procedure for the dissociation of ovine corpora lutea into isolated cell suspensions.

Materials and Methods. Cells dissociation. Corpora lutea from ewes, superovulated as described by Sheridan *et al.* (17), were collected and placed immediately into iced Medium 199 (Grand Island Biological Company) containing 25 m*M* *N*-2-hydroxyethylpeperazine-*N'*-2-ethanesulfonic acid (HEPES, Sigma Chemical Company). Adherent connective tissue was removed from the corpus luteum and 0.5-mm slices were prepared with a Stadie-Riggs tissue slicer. The first and last slice of each corpus luteum was discarded to minimize capsular material. The slices were weighed and transferred to a spinner flask, and Medium 199 was added (5 ml/g tissue). The flask was placed in a water bath (32°), and the contents were stirred magnetically (100 rpm) for 10 min. The medium was then decanted, fresh medium with collagenase (2000 U/g tissue, Type I CLS, Worthington Biochemical Corporation) was added, and the incubation continued. At frequent intervals the medium was examined microscopically to evaluate the progress of the dissociation. When a few free, viable cells began to appear (30-40 min), the heavier material was allowed to settle and the supernatant was decanted and discarded. Fresh medium with enzyme was added and the dissociation was continued. When the slices were reduced to small clumps, the dissociation was hastened by repeatedly drawing these clumps into a pasteur pipet. The procedure was stopped when free cells predominated in the medium (2 hr). The contents of the flask were transferred and centrifuged (100 *g*, 5 min), the supernatant was discarded, and the cells were washed six times with fresh medium. The cells were resuspended and filtered through four layers of cheese cloth to eliminate large particles. This final preparation was evaluated for cell concentration and viability. If the suspension was not used immediately, it was stored at 4° with added antibiotics (100 U of penicillin G and 100 μg of streptomycin sulfate/ml, Difco Laboratories).

Viability of cells was estimated with a dye exclusion test using 0.2% trypan blue in 0.9% saline. Cells excluding the dye were considered viable (18). Cells were routinely counted in a standard hemocytometer and

[1] Supported in part by the Rockefeller Foundation and NIH Contract No. 69-2134.
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classified as small (av diam 9 μm) or large (av diam 28 μm). Each preparation was adjusted to a standard concentration. Approximately 30–40 × 10^3 viable large cells per ml were used to study progesterone synthesis.

Progesterone synthesis. Preparations of 30–40 × 10^3 viable large cells per ml (1–1.2 × 10^6 total cells) were dispensed in 0.4-ml aliquots to iced, round-bottom culture tubes (15 × 85 mm). NIH-LH-S16, NIH-prolactin-S8, and NIH-HCG-CR117 were added in 10 μl of phosphate (0.05 *M*) buffered saline (0.14 *M*) with 0.1% gelatin at pH 7.0 (PBS-gel). The tubes were incubated at 37° in a Dubnoff Metabolic Shaking Incubator up to 1 hr. The incubations were terminated by transferring the tubes to an 80° water bath for 20 min to kill the cells and denature any steroid enzyme systems. The samples were diluted with 8.6 ml of PBS-gel. Progesterone concentrations were determined by radioimmunoassay (19). In a preliminary experiment, 15 samples were extracted with glass-distilled chloroform and the progesterone in the extract was purified on silica gel-impregnated glass fiber sheets (Gelman Industries, Inc.) using 10 cyclohexane:1 ethyl acetate. Progesterone levels in aliquots of the media were identical to those obtained after extraction and chromatographic purification of the samples. Therefore, the incubation media were assayed directly for subsequent studies.

Results. The first cells to appear free in the medium, other than erythrocytes, were small cells, possibly fibroblasts. These cells predominate in the final cell suspension and range from 5 to 13 μm (av 9 μm) in diameter. The first large cells to appear were not viable, but the proportion of free, viable, large cells gradually increased as the tissue was dissociated. The large cells ranged from 22 to 37 μm (av 28 μm) in diameter. The average yield of total cells from tissue obtained 11 days after superovulation (17) was 81.8 ± 9.1 × 10^6 cells/g tissue. There were 3.9 ± 0.3 × 10^6 large cells/g tissue. The micrograph in Fig. 1 shows a representative preparation after fixation and staining. The trypan blue dye exclusion test indicated that 75–85% of all cells were viable. Acridine orange gave comparable results.

The dissociated cells contained substantial quantities of progesterone at the beginning of the incubation. The addition of lu-

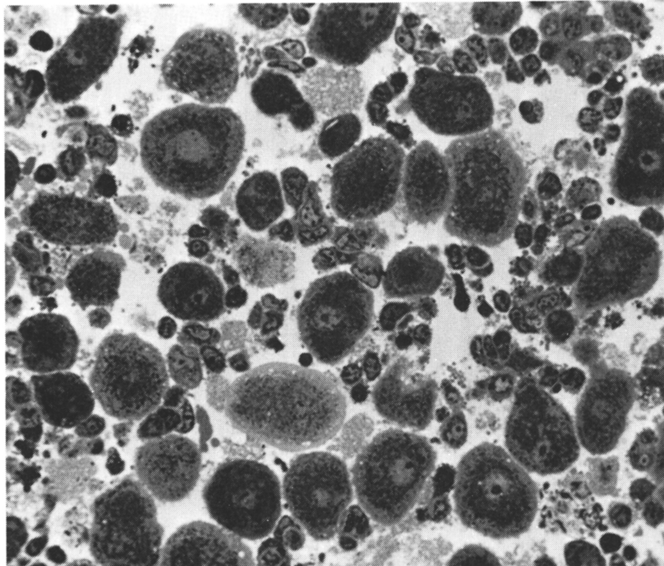

FIG. 1. Dissociated cells from corpora lutea of a superovulated ewe on Day 9 of the estrous cycle. The cells were fixed in a pellet with 4% glutaraldehyde, postfixed in osmium and embedded in epon. A 1-μm section was cut and stained with toluidine blue. (× 750).

teinizing hormone (LH) or human chorionic gonadotropin (HCG) to the cells stimulated a dramatic increase in progesterone output; however, the cells did not respond steroidogenically to prolactin (Table I). Increasing doses of LH produced a differential steroidogenic response which was maximal around 10 ng per incubated tube (Table II).

The initial progesterone content, the progesterone production with time, and steroidogenic response to LH all varied proportionately with cell number (Table III).

To determine if dissociated cells could be stored, paired samples of cells were incubated immediately or stored at 4° for 48 hr in Medium 199 or in 50% Medium 199 and 50% hypophysectomized sheep serum (LH free). The stored cells were washed and incubated as described above. Both groups of stored cells produced less progesterone than the fresh preparation and their steroidogenic response to LH was reduced although the percentage increase above unstimulated controls was similar to that of the fresh preparation (Table IV).

TABLE I. PROGESTERONE PRODUCTION BY LUTEAL CELLS AND THE EFFECT OF GONADOTROPINS ON PROGESTERONE PRODUCTION.

Treatment	Incubation (hr)	Progesterone production (ng)
None	0	237 ± 20
None	1	281 ± 17
LH (100 ng)	1	627 ± 37[a]
Prolactin (1 μg)	1	291 ± 11
None	0	105 ± 34
None	1	124 ± 10
HCG (25 ng)	1	231 ± 52[a]

Note: Values = Mean ± SD.
[a] Significantly different from respective 1-h control, $P < 0.01$.

TABLE II. PROGESTERONE PRODUCTION IN RESPONSE TO VARIOUS LEVELS OF LH.

Treatment (ng)	Incubation (hr)	Progesterone production (ng)	
		Expt. 1	Expt. 2
None	0	96 ± 25[a]	75 ± 7[a]
LH (0.1)	1	117 ± 8[ab]	110 ± 11[b]
LH (0.3)	1	126 ± 6[ab]	153 ± 8[c]
LH (1.0)	1	161 ± 13[ab]	197 ± 9[d]
LH (3.0)	1	204 ± 11[cd]	194 ± 7[d]
LH (10.0)	1	254 ± 9[d]	250 ± 25[e]
LH (30.0)	1	180 ± 46[c]	262 ± 25[eg]
LH (100.0)	1	188 ± 31[c]	297 ± 4[f]
LH (300.0)	1	207 ± 25[cd]	287 ± 44[fg]

Note: Means with different superscripts are significantly different ($P < 0.05$). Values = Mean ± SD.

Superovulated animals frequently had luteinized follicles and the cells from these follicles were dissociated to determine their steroidogenic capacity. Luteal tissue from the same animal was dissociated and incubated concurrently. There was no substantial difference in progesterone production (Table V) by these two cell preparations.

Luteal tissue from other species has been dissociated and the technique works well with bovine luteal tissue. However, the procedure is too harsh for use with rabbit or rat luteal tissue. It requires modification with respect to enzyme concentration and dissociation time before it can be used efficiently with tissue from these species.

Discussion. A variety of enzymes has been utilized to obtain suspensions of cells from endocrine tissues. The most extensive research regarding the dissociation of luteal tissue is that of Gospodarowicz and Gospodarowicz (12, 20) and Papaionanou and Gospodarowicz (21), who used a complex combination of enzymes and produced a pure population of large luteal cells. However, the population of cells produced is not representative of the cells which comprise the corpus luteum *in vivo*. This must have resulted from procedural damage to certain cell types which did not appear in the final preparation. The cells which remained may have also suffered enzymatic damage. Their cell preparations were responsive to LH simulation and appear to provide an excellent product for studying the interactions of gonadotropins and the large luteal cells.

The advantage of the procedure we have described is its simplicity and the fact that the population of cells obtained is typical of that found *in vivo*. Fifty-two percent of the volume of the ovine corpus luteum, during the midluteal phase of the cycle, is occupied by luteal cells (35% granulosa-lutein, 17%

TABLE III. EFFECTS OF CELL CONCENTRATION ON PROGESTERONE SYNTHESIS.

Treatment	Incubation (hr)	Progesterone production (ng)		
		60 K/ml	24 K/ml	12 K/ml
None	0	130 ± 12[a]	41 ± 4[b]	18 ± 3[b]
None	1	175 ± 23[c]	75 ± 5[d]	31 ± 5[b]
LH (1 μg)	1	656 ± 74[e]	261 ± 3[f]	83 ± 6[g]

Note: Means with different superscripts are significantly different ($P < 0.05$).

TABLE IV. EFFECTS OF CELL STORAGE ON PROGESTERONE PRODUCTION.

Treatment	Incubation (hr)	Progesterone production (ng)		
			After storage[2]	
		Original[1]	WHSS[3]	Medium 199[4]
None	0	110 ± 22[a]	26 ± 2[d]	90 ± 14[a]
None	1	228 ± 32[b]	102 ± 26[a]	121 ± 8[a]
LH (1 μg)	1	502 ± 3[c]	371 ± 51[e]	341 ± 35[e]

Note: Values = Mean ± SD. Means with different superscripts are significantly different ($P < 0.05$).
[1] Cell suspension used immediately after dissociation.
[2] Storage for 48 hr at 4°.
[3] Medium was 1/2 Medium 199 and 1/2 hypophysectomized sheep serum.
[4] Plain Medium 199.

TABLE V. PROGESTERONE PRODUCTION BY CELLS FROM LUTEINIZED FOLLICLES OR CORPORA LUTEA.

Treatment	Incubation (hr)	Progesterone production (ng)	
		Luteinized follicles	Corpora lutea
None	0	82 ± 7[a]	126 ± 8[a]
None	1	155 ± 9[b]	202 ± 19[b]
LH (2 μg)	1	656 ± 31[c]	558 ± 56[d]

Note: Values = Mean ± SD. Means with different superscripts are significantly different ($P < 0.05$).

theca-lutein). The remainder of the volume is connective tissue (35%) and blood vascular tissue (13%) (22). Our cell preparations average 4.8% large cells (av diam 28 μm) and 95.2% small cells (av diam 9 μm). Based on these figures, the large luteal cells in our preparations represent 59.7% of the cellular volume which approximates the *in vivo* condition. Under experimental conditions, where interaction between different cell types or their secretory products may influence the function of a particular population of cells, it is important that the dissociated cells be representative of the normal condition.

To dissociate the ovine corpus luteum, various enzymes were tried, including crude trypsin, purified trypsin, subtilisin, hyaluronidase, crude collagenase, and purified collagenase. The best results were obtained with crude collagenase which was twice as effective as its highly purified form with respect to dissociation time. This indicates that other proteolytic enzymes in the preparation were beneficial. Various concentrations of enzymes were tested, but the most extensive trials were made with crude collagenase (300–4000 U per g of tissue). The addition of fresh collagenase at frequent intervals did not alter dissociation time or cell yield.

Krebs ringer, bicarbonate buffer plus glucose (KRBG) with one-half the normal Ca^{2+} failed to maintain the cells through the dissociation and incubation procedures. Fortification of KRBG with 4% bovine serum albumin (BSA) (12) produced satisfactory results; however, there are disadvantages to the use of BSA. BSA contains a small, but variable, amount of LH which would confound any studies of progesterone production. Four lots of commercial BSA were assayed for LH by radioimmunoassay and found to contain <2, <2, 8.3, and 14.5 pg/mg of BSA. BSA and other common cell culture additives, such as fetal calf serum (FCS), also protect the cells from stains such

as trypan blue and result in erroneously high viability ratings. Using BSA or FCS, the estimated viability of cells was 95%, whereas without BSA or FCS, viabilities were reduced to the normal range. When cell preparations with viabilities as low as 65% were resuspended in medium containing BSA or FCS, they had viability ratings of 90–95%. Tennant (18) showed that serum protected cells against the toxic effects of trypan blue and that cells rendered metabolically inert by heat treatment produced excellent viability ratings in the presence of serum. For these reasons, defined media such as Dulbecco's Modified Eagles and Medium 199 were selected.

Cell suspensions, prepared as described, synthesize progesterone and are stimulated to increase progesterone output in response to either LH or HCG, indicating the presence of functional luteal cells. Cell preparations vary with respect to steroidogenic capabilities, possibly due to animal-to-animal variation. Sayers *et al.* (14) felt that similar variability in adrenal cell suspensions may have resulted from heterogeneous cell populations. Total cell counts would fail to reflect these differences; however, in the present study cell counts were standardized on the basis of large luteal cells.

Increasing doses of LH elicited a graded response in progesterone production. This system could serve as a bioassay for LH or HCG. The concentration of LH (10^{-8} *M*) at which maximum progesterone production was reached compares favorably with that found by Gospodarowicz (12) (10^{-7} *M*) for maximum progesterone synthesis and Kammerman (23) (10^{-8} *M*) for maximum binding of HCG. When the cell concentration was varied, the progesterone synthesis and the response to LH varied proportionately, indicating that the medium was adequately supplying the requirements of the cells during incubation. Cells stored at 4° for 48 hr after dissociation maintained viability and responded steroidogenically. The steroidogenic response, however, was not so great after 48 hr as when the preparation was fresh. This decrement could signify cell deterioration or a gradual loss of differentiated function as a result of culturing techniques.

Procedures for separating dissociated luteal cells into the cell populations described in this report are presently being developed. Centrifugation on both isopycnic and isokinetic gradients composed of sucrose, Ficol, or Metrizamide have been employed with limited success. The most promising method appears to be the separation of cell types on a Sepharose 2B-300 column. Using this procedure, it has been possible to obtain functional small lutein cells devoid of contamination by large cells.

Summary. A simple and highly reproducible method was developed for the dissociation of ovine luteal tissue into isolated cell suspensions using a crude collagenase preparation in Medium 199. The procedure required less than 3 hr. The mean yield of cells was $81.8 \pm 9.1 \times 10^6$ cells/g tissue. The resulting cell preparation synthesized progesterone and responded to the addition of LH or HCG, but not prolactin, with increased progesterone production. The quantity of progesterone produced was related to the level of LH added. The cells were still steroidogenically capable and responsive to LH after storage for 48 hr at 4°.

The PMSG, LH, HCG, and prolactin used were supplied by the National Institutes of Health. The sheep were synchronized with the aid of Sil Estrus implants, a gift of Abbott Laboratories, North Chicago, Illinois. Ovulation was induced with Gonamone, a gift of Fort Dodge Laboratories, Fort Dodge, Iowa.

The authors acknowledge the technical assistance of Bobbie Livingston, Wayne Hopper, and Vaughn Cook.

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Received December 10, 1975. P.S.E.B.M. 1976, Vol. 152.