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SHORT COMMUNICATION

HAMILTON, CANADA

A simplified method for preparing IVF granulosa cells for culture

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***Purpose*:** To validate an expedited method for the removal of erythrocytes when preparing IVF granulosa-luteal cells for culture.
***Methods*:** Granulosa cells were enriched for culturing from follicular aspirates by density gradient centrifugation and by hypo-osmotic lysis treatments.
***Results*:** Cells prepared by either method showed the same cell viability and produced progesterone in similar quantities.
***Conclusions*:** Using hypo-osmotic lysis to remove erythrocytes does not impair granulosa cell viability or steroidogenesis. It avoids multiple density gradient centrifugations and washings, and yields IVF granulosa cells ready for culture efficiently.

KEY WORDS: Cell culture; granulosa-luteal; hypotonic lysis; IVF cells.

INTRODUCTION

The advent of in vitro fertilization (IVF) treatment for infertility has made human granulosa-luteal cells increasingly available for endocrine researchers. In IVF protocols, the developed follicles are punctured under transvaginal ultrasound guidance and the contents are aspirated. The cumulus–oocyte complexes are then visualized and manually collected from the aspirated fluids. After the first follicle has been punctured, localized bleeding occurs, so that subsequent follicular aspirates contain some blood. Therefore, once follicular aspirates have been pooled, the majority of cells in these preparations are erythrocytes. These red blood cells (RBC) make it impossible to count the granulosa-luteal cells on a hemocytometer. Furthermore, they have a greater density than granulosa-luteal cells and settle to the bottom of culture dishes faster. If the culture surface is completely covered with these cells, the granulosa-luteal cells will not be able to attach. Therefore, it is necessary to remove these erythrocytes to maximize plating efficiency and obtain enriched cultures for study.

The methods in common use today employ the greater density of RBCs to separate them from the granulosa-luteal cells. Some techniques use repeated settlings under gravity of up to 20 min or longer and manually collect the granulosa cells from the upper layer after a portion of the erythrocytes had settled (1–3). A variation of this approach, and one that is more widely used, is density gradient centrifugation with colloidal silicas such as Percoll or Ficoll. This method varies slightly from lab to lab but in general it involves an initial 300–500 × *g* centrifugation for up to 15–20 min to concentrate the cells. The resuspended cell pellet is then gently layered over a 40–50% Percoll cushion (4–6) or similarly effective concentrations of Ficoll (7–9) or in some laboratories, a two-step 40 and 60% discontinuous Percoll gradient is used (10). This is followed by centrifugation at generally around 500 × *g* for 20 min. The cells then need to be carefully collected by aspiration from the colloidal silica/media interface, diluted with media and washed by a further centrifugation to remove the Percoll or Ficoll.

The above techniques, although efficient, are time consuming and require dexterity, practice and extra reagents. We sought to simplify the procedure based on the rapid lysis of erythrocytes by hypotonic solutions (11,12). Using this method, we can obtain follicular granulosa-luteal cells free of RBCs and ready for culture in less than half an hour.

MATERIALS AND METHODS

Cell Preparation

Follicular granulosa-luteal cells were obtained after oocyte retrieval from eight patients undergoing IVF treatment at the Hamilton Health Sciences Centre for Reproductive Care. Ovarian stimulation was accomplished using a long luteal protocol of GnRH agonist Lupron (Abbott Laboratories, Montreal, Que.) at 0.5 mg per day for 10–14 days and recombinant FSH, Gonal F (Serono Canada, Oakville, ON) followed by hCG, Profasi (Serono). The follicular aspirates from each patient were pooled in conical bottomed 50 mL polypropylene centrifuge tubes.

These were then centrifuged at $300 \times g$ for 5 min. At this speed, the resulting pellet shows no layering (as in a hematocrit) and is not firm enough to decant the tubes. Therefore, the supernatant was aspirated using a Pasteur pipette connected to a vacuum line and the remaining cell slurry was equally divided for granulosa-luteal cell enrichment via Percoll centrifugation and hypo-osmotic lysis.

For Percoll centrifugation, the cell slurry was diluted twofold with Hanks' balanced salt solution (HBSS) and then gently layered (via a hand held pipette with rubber bulb) on top of 5 mL of a 60% Percoll cushion in a 15 mL polystyrene centrifuge tube. The tubes were centrifuged at $500 \times g$ for 20 min and then the granulosa-luteal cell layer at the interface between the Percoll and HBSS was collected using a Pasteur pipette. These cells were then washed to remove Percoll by resuspending to 10 mL HBSS and centrifuging at $150 \times g$ for 3 min. The resulting cell pellets were resuspended in 0.5 mL of culture media and pooled for cell counting.

For the hypo-osmotic lysis technique, 0.5 mL of the cell slurry was pipetted into a 15 mL conical bottomed polystyrene centrifuge tube. To this was added 9.0 mL of sterile distilled water and the tube was capped and mixed. After 20 s, 1.0 mL of $10\times$ concentrated PBS, pH 7.4 was added and the tube was capped and mixed. The tubes were then centrifuged at $150 \times g$ for 3 min and then decanted by inverting the tubes. The cell pellet was resuspended in 0.5 mL of culture media. Aliquots from both preparation methods were counted for cell number and viability in 0.2% trypan blue on a hemocytometer.

Cell Culture and Assays

The cells from each protocol were plated in triplicate at 100,000 viable cells per well in 24-well Falcon culture dishes ($n = 8$). Culture medium consisted of Eagle's minimum essential medium with Earle's salts and was buffered with 2.5 g/L $NaHCO_3$ and 1.5 mM HEPES. The medium was further supplemented with 10% calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL Gentamicin and 1.25 μg/mL Fungizone (Gibco, Grand Island, NY). Cells were allowed to attach overnight and were then washed with two changes of HBSS to remove unattached cells and debris. Serum-free medium was then added. This was collected at 48 h and stored at -20°C until assayed for progesterone. After media collection, the wells were rinsed twice with HBSS and stored dry for the subsequent determination of protein content. The radioimmunoassay and protein determinations were as described previously (13).

RESULTS

Cell viability was assessed by trypan blue dye exclusion and was similar for granulosa-luteal cells prepared by either Percoll density gradient centrifugation or by the hypo-osmotic lysis technique ($64.0 \pm 6.4\%$ SD and $67.6 \pm 7.1\%$, respectively, $p = 0.37$). Likewise, there was no difference in progesterone production by cell cultures prepared by either method (12.9 ± 6.7 ng/μg protein SD and 14.0 ± 5.9 for Percoll and lysis, respectively, $p = 0.65$). This indicates to us that the simplified hypotonic lysis technique does not compromise the granulosa-luteal cells any more than does centrifugation on colloidal silica. If indeed the cells are comprised by these techniques, then they recover to the same extent after overnight plating and in vitro culture.

DISCUSSION

The first follicle that is aspirated in IVF procedures generally yields clear follicular fluid and is a rich source of luteinizing granulosa cells. To obtain more of these cells one needs to collect from the rest of the aspirated follicles, however due to the necessity of follicular puncture, these subsequent aspirates are mixed with blood, therefore necessitating the removal of RBCs. We have now adapted an uncomplicated lab technique, hypotonic lysis of erythrocytes, and shown that it is equally as effective as the currently used procedures for generating granulosa-luteal cell cultures.

The main advantages of our technique is that it is simple and can be done quickly and at a reduced cost. Furthermore, because there are fewer steps and manipulations involved, the lysis technique consistently results in a higher cell yield, usually close to 40% more cells. Additionally, the lysis technique yields a final preparation consisting mostly of single granulosa-luteal cells, whereas the Percoll technique contains many cell clusters. Having cells in association with adjoining cells makes it more difficult for counting and plating and has lead some (3,4) to add trypsinization and filtering steps to enrich for single granulosa-luteal cells.

Regardless of technique, one needs to remove any large (i.e. visible) blood clots from the follicular aspirates or the yield and purity will be compromised.

With our technique it is also important not to exceed the volume of 0.5 mL of cell slurry at the start of the lysis step. If one does this, not all of the RBCs will be removed and the resulting cultures will require more media changes to wash away this debris.

In working with granulosa-luteal cell cultures, one must contend with a modest amount of cell contamination, as absolutely pure preparations are unobtainable (9). Granulosa-luteal cell cultures prepared by either of the methods discussed contain a small percentage of other nucleated cells such as leukocytes and resident ovarian macrophages. Although density gradient centrifugation with Percoll or Ficoll does not remove leukocytes from the follicular cells (4,9) it has however become the accepted norm or the standard preparation in this area of research. Loukides *et al.* (14) have shown that resident macrophages and monocytes comprise 5–15% of follicular tissue cells. This proportion remains constant in the corpus luteum, so that maintaining some of these cells in the cultures would actually be more reflective of the luteal environment. Similarly, Beckmann *et al.* (9) have shown that adding back extra lymphocytes to granulosa-luteal cell cultures does not alter progesterone production by the cells.

Another cell type in the preparations prior to plating are squamous epithelial cells arising from the transvaginal aspiration. These cells do not attach in culture and are removed when the cultures are washed.

In conclusion, our hypo-osmotic lysis technique expedites the removal of RBCs from follicular aspirates and yields granulosa-luteal cell preparations of equal quality to that of centrifugation over colloidal silica but with a reduction in time and cost.

REFERENCES

1. Bar-Ami S: Increasing progesterone secretion and 3β-hydroxysteroid dehydrogenase activity of human cumulus cells and granulosa-lutein cells concurrent with successful fertilization of the corresponding oocyte. J Steroid Biochem Molec Biol 1994;51:299–305
2. Lin Y, Kahn JA, Hillensjo T: Is there a difference in the function of granulosa-luteal cells in patients undergoing in-vitro fertilization either with gonadotrophin-releasing hormone agonist or gonadotrophin-releasing hormone antagonist? Hum Reprod 1999;14:885–888
3. Figenschau Y, Sundsfjord JA, Yousef MI, Fuskevag OM, Sveinbjörnsson B, Bertheussen K: A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells. Hum Reprod 1997;12:523–531
4. Wang LJ, Brännström M, Pascoe V, Norman RJ: Cellular composition of primary cultures of human granulosa-lutein cells and the effect of cytokines on cell proliferation. Reprod Fertil Dev 1995;7:21–26
5. VandeVoort CA, Overstreet JW, Lasley BL, Stewart DR: Effects of progesterone receptor blockers on human granulosa-luteal cell culture secretion of progesterone, estradiol and relaxin. Biol Reprod 2000;62:200–205
6. Fuji A, Harada T, Yamauchi N, Iwabe T, Yoshihiro N, Yanase T, Nawata H, Terakawa N: Interleukin-8 gene and protein expression are up-regulated by interleukin-1β in normal human ovarian cells and a granulosa cell tumor line. Fertil Steril 2003;79:151–157
7. Nakamura Y, Tamura H, Takayama H, Kato H: Increased endogenous level of melatonin in preovulatory human follicles does not directly influence progesterone production. Fertil Steril 2003;80:1012–1016
8. Park D-W, Cho T, Kim MR, Kim YA, Min CK, Hwang KJ: ATP-induced apoptosis of human granulosa luteal cells cultured in vitro. Fertil Steril 2003;80:993–1002
9. Beckmann MW, Polacek D, Seung L, Schreiber JR: Human ovarian granulosa cell culture: Determination of blood cell contamination and evaluation of possible culture purification steps. Fertil Steril 1991;56:881–887
10. Guet P, Royère D, Paris A, Driancourt MA: Aromatase activity of human granulosa cells in vitro: Effects of gonadotrophins and follicular fluid. Hum Reprod 1999;14:1182–1189
11. Westwood A: Rapid micromethod for the preparation of leucocyte-free haemolysates for the determination of pyruvate kinase and other erythrocyte enzymes. Ann Clin Biochem 1975;12:263–268
12. Hansel TT, Pound JD, Thompson RA: Isolation of eosinophils from human blood. J Immunol Methods 1990;127:153–164
13. Lobb DK, Soliman SR, Daya S, Younglai EV: Steroidogenesis in luteinized granulosa cell cultures varies with follicular priming regimen. Hum Reprod 1998;13:2064–2067
14. Loukides JA, Randall AL, Edwards R, Honig J, Visintin I, Polan ML: Human follicular fluids contain tissue macrophages. J Clin Endocrinol Metab 1990;71:1363–1367

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