Scientific Research Publishing

International Journal of Clinical Medicine, 2018, 9, 377-399
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Ovarian Follicle Disaggregation to Assess Granulosa Cell Viability

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How to cite this paper: Asaduzzaman, M., Gonzalez, D.F. and Young, F. (2018) Ovarian Follicle Disaggregation to Assess Granulosa Cell Viability. *International Journal of Clinical Medicine*, **9**, 377-399.
https://doi.org/10.4236/ijcm.2018.95033

Received: March 26, 2018
Accepted: May 7, 2018
Published: May 10, 2018

Abstract

Background: Mammalian ovaries contain follicles containing an oocyte enclosed by layers of granulosa cells (GC). Follicle growth and oocyte maturation are largely dependent on GC numbers and viability, but there is no established, reliable method for assessing the number of viable GC within an isolated follicle. Methods: Centrifugation conditions and the Trypan Blue (TB) Exclusion assay were optimised for low cell densities compatible with the numbers of GC in follicles. Mouse ovarian follicles were disaggregated to produce a single cell suspension of GC which were examined by TB (n = 4), but also by crystal violet assay in a 96-well plate format after 24 h *in vitro* (n = 3). GC viability *in vitro* was characterised further by using enzyme-linked immunoassays to quantify GC production of anti-Mullerian hormone (AMH) and estrogen. Results: The centrifugation and low cell density TB protocol could accurately measure the viability of 78 GC in 10 µL, with an intra-assay coefficient of variation (CoV) 22%, and inter-assay CoV 7%. The best follicle disaggregation method (30 min 37˚C exposure to 2 mg/mL collagenase prior to 30 min exposure to 0.025% hyaluronidase) yielded (656 ± 87) GC per antral follicle of which 82% ± 5% were viable. Culturing 312 - 20,000 GC per well for 24 hours and assessing viability by crystal violet assay generated a linear correlation between OD value and viable GC number (R^2 = 0.98) and estrogen concentration per well (R^2 = 0.92). 20,000 GC per well produced 143 ± 16 pg/mL estrogen during 24 hours *in vitro*, but no detectable AMH. Conclusion: This is the first report describing the isolation of viable, estrogen-producing GC from murine follicles, and their subsequent culture. These procedures are transferrable to other species including humans and can be applied to screening the reproductive toxicity of pharmaceutical agents.

Keywords

Ovarian Follicle, Granulosa Cells, Viability, Collagenase, Hyaluronidase

1. Introduction

Follicles are isolated from ovarian tissue for many reasons [1]-[23] including *in vitro* maturation (IVM) to produce mature fertilizable oocytes [11] [20] [24] [25] [26] [27]. Isolated follicles are cultured *in vitro* in a number of different systems including 3D matrices [1] [24] [25] [28]-[36]. These methodologies often assess follicle growth *in vitro*, usually by measuring diameter, and occasionally by assessing the number and viability of granulosa cells (GC) within the follicle [21] [24] [25] [29] [31] [35] [36] [37] [38] [39]. However, there are few reports describing reliable and accurate methods for assessing the number of viable GC within an intact isolated follicle [39] [40] [41] [42], and the use of fluorescent markers of cell viability such as Live-Dead stain or CMXRos [4] [15] [16] [17] [19] [43] [44] to accurately quantify GC numbers requires careful optimization.

Follicles are the functional unit of ovary and are comprised of an oocyte surrounded by granulosa cells. The numbers of GC increase as the follicles grow and mature [45] [46] [47] [48]. Fixed haematoxylin and eosin-stained sections of murine ovaries were used to calculate the numbers of GC in primary (9 - 59), early to mid-secondary (60 - 185), late secondary to incipient antral (187 - 392) and antral (>395) follicles [42]. The basal laminae of murine follicles are composed of laminins (α1, β2 and γ1), collagens, particularly type IV, nidogen and perlecan [20] [49] [50] [51]. In addition to these, the extra cellular matrix (ECM) within the follicle also contains hyaluronic acid [49]. The molecular structure of a follicle suggests that a single cell suspension of GC might be obtained by digesting follicular basal lamina with collagenase IV, and the intra-follicular ECM with hyaluronidase.

The Trypan Blue (TB) Exclusion assay is based on the principle that viable cells have intact membranes that prevent the uptake of the dye, whereas ruptured and non-functional cell membranes are permeable to the dye and hence non-viable cells stain blue [15] [17] [52] [53] [54] [55]. After staining with Trypan Blue, cells are assessed using an objective lens light microscope and a haemocytometer [56]. The Trypan Blue Exclusion assay is an established and reliable direct estimation technique for the differentiation and enumeration of live and dead cells and was reported to be more reliable than Live-Dead stain for estimating the viability of fresh and thawed follicles [44].

Piccinini F, Tesei A, Arienti C and Bevilacqua A [54] found no difference in viability when cells retrieved from two dimensional (2D) or three dimensional (3D) culture systems were assessed by TB. Cell viability and density measures varied by 5% and 20% respectively, with percent error in estimating cell numbers higher for 3D systems (21%) than for 2D systems (17%) [54]. Other studies reported coefficients of variation (CoV) for estimating cell numbers using TB from 4.5% [57] to 6.9% [58]. It has been noted that the viability of cells decreases as the time after adding the dye increases, and also after cells have been harvested using trypsin [59] [60] [61]. The TB assay does not differentiate between

metabolically active and non-active cells [62].

In general practice, an accurate and precise TB estimation requires 0.4×10^6 to 1×10^6 cells per mL of cell suspension. Hence, suspensions with densities lower than 1×10^5 cells per mL cannot be assessed accurately using existing TB assay protocols [63] [64]. The number of granulosa cells in a single follicle, approximately 400 in an incipient antral follicle [42] [45] [46] is too low for accurate assessment using TB.

The aim of this study was to develop a technically simple and inexpensive method to disaggregate follicles and produce a single granulosa cell suspension which can be assessed in a modified Trypan Blue Exclusion assay. To do this, a preliminary study using human KGN, T47D and 184B5 cell lines was conducted to determine centrifugation conditions applicable to low cell densities, and to determine the limit of sensitivity of a standard TB Exclusion assay. Thereafter, the optimal centrifugation conditions were applied to the human granulosa KGN cell line to assess the reproducibility of the TB assay protocol. The resulting low cell-density TB Exclusion assay was then used to optimize follicle disaggregation methods before being applied to the characterization of granulosa cells obtained from disaggregated murine follicles. The TB evaluation was compared to follicle-derived GC that were cultured in a 96-well plate format before assessing their viability by using a crystal violet assay [65].

2. Materials and Methods

All the reagents used in this study were purchased from Sigma unless otherwise stated.

2.1. Cell Lines and Culture Media

184B5 (ATCC® CRL-8799™) and T47D (ATCC® HTB-133™) cell lines with passage numbers of 11 and 14 respectively were used for this experiment. The cells were cultured in RPMI with 10% foetal calf serum (FCS, DKSH, Melbourne, AUS) and 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) at 37˚C with 5% CO_2, and sub-cultured when 80% confluent. The KGN granulosa cell line derived from a human granulosa cell carcinoma [66] [67] was maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 1% insulin (5 ug/mL), transferrin (5 ug/mL) and selenium (5 ng/mL, ITS) and 10% FCS and 1% penicillin (10,000 units/mL) and streptomycin (10 mg/mL) and sub-cultured every 2 - 3 days as required using trypsin ethylene diamine tetra-acetic acid (EDTA) to detach cells.

2.2. Effect of Centrifugation on Cell Retrieval

A preliminary study used the KGN granulosa cell line to identify the optimal centrifugation conditions for maximizing the retrieval of viable cells. A low-density cell suspension containing 600 cells in 500 µL of media was distributed to 12 centrifuge tubes (1.5 mL) which were centrifuged at room tempera-

ture for 4, 5 or 6 minutes at 1306 g, 2040 g, 2938 g, or 4000 g. Supernatants were removed and the cells were re-suspended in 10 µL of media and 10 µL Trypan Blue. The numbers of viable and dead cells were determined in a Trypan Blue Exclusion assay. This experiment was repeated on four separate occasions (n = 4).

2.3. Trypan Blue Exclusion Assay Protocol

Cell suspensions were diluted 1:1 with Trypan Blue (2 mg/ml Trypan Blue in phosphate buffered saline; PBS) and 10 µL added to each side of a haemocytometer, *i.e.* onto each of two grids. Cells were viewed using an objective lens microscope at 10× magnification (Leica, Leitz Wetzlar, Germany). The numbers of unstained viable and blue-stained dead cells in all 9 large squares of the haemocytometer (Figure 1) were counted, and the average number of cells per large square determined. These were adjusted by the dilution factor and converted to reflect the number of cells in 10 µL cell suspension using the equations shown in Figure 1 [52] [68].

2.4. Accuracy of the Trypan Blue Assay

The ability of the Trypan Blue assay to accurately quantify viable cells was examined using 184B5 and T47D cell lines. The number of viable cells in each parent cell suspension was determined in the Trypan Blue Exclusion assay, and the volumes of parent cell suspension that contained 1250 (184B5), 930 (T47D) or 953 (T47D) viable cells were added to centrifuge tubes and the volume made

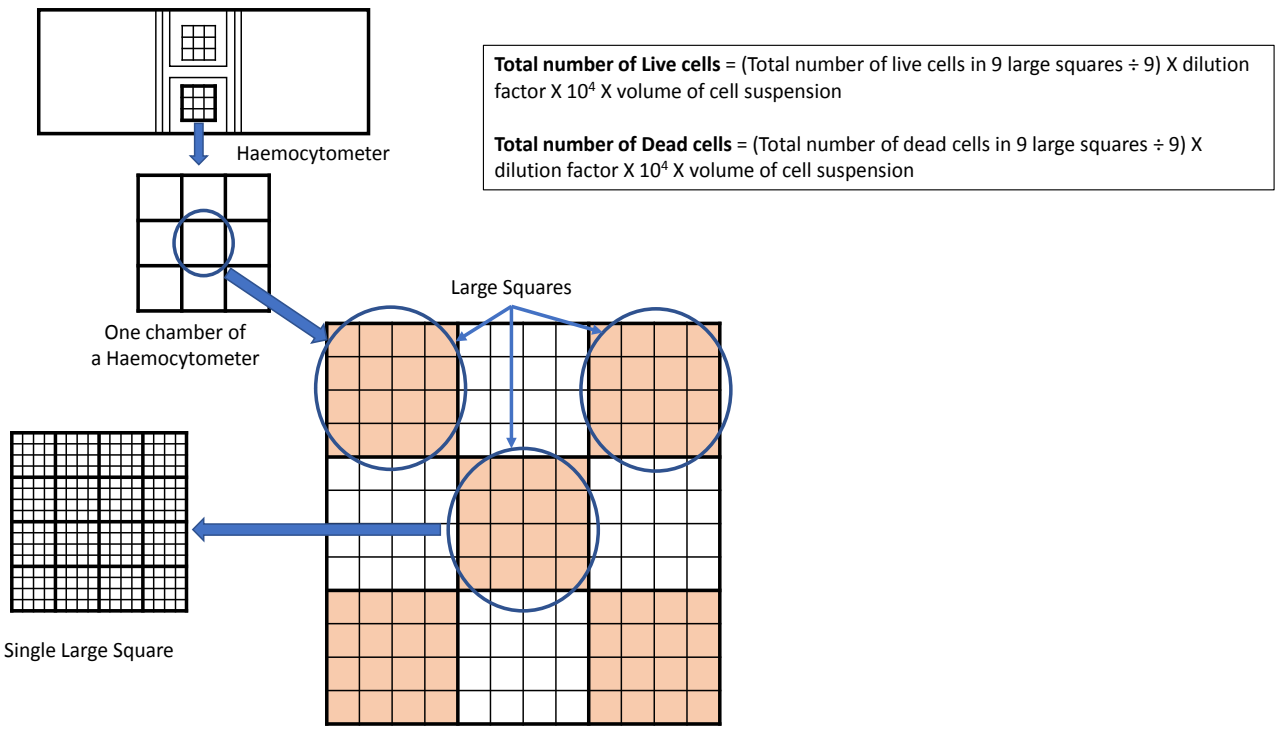


Figure 1. Haemocytometer chamber.

up to 100 µL with media. Four 1 in 2 dilutions were performed, and three separate dilution series were made from each parent cell suspension. Cells were centrifuged at 2040 g for 5 minutes and the supernatant removed before re-suspending the cells in 10 µL of media and 10 µL of Trypan Blue. The numbers of cells retrieved were determined in the Trypan Blue Exclusion assay.

2.5. Coefficient of Variation (CoV) of the Trypan Blue Assay

Three single cell suspensions of KGN granulosa cells containing 2500, 1250 and 156 cells per mL of media were produced on four separate occasions (n = 4). 500 µL of each cell suspension were added to 10 separate centrifuge tubes, so that they contained 1250, 625 or 78 KGN cells per tube. These were centrifuged at 2040 g for 5 minutes at room temperature and the supernatants removed before re-suspending the cells in 10 µL of media and 10 µL of Trypan Blue. The numbers of viable and dead cells were determined in a Trypan Blue Exclusion assay. Coefficients of variation (CoV) were calculated by dividing the standard deviation (SD) by the mean of viable cell numbers for each of the cell densities within each of the experimental replicates (intra-assay CoV) and between the experiments (inter-assay CoV, n = 4) and presented as a percentage; CoV (%) = (SD/mean) × 100 [69].

2.6. Mouse Ovary Collection

Female mice that were surplus to the College of Medicine and Public Health Animal Facility breeding colony needs were allocated to routine culling procedures. The Flinders Animal Welfare Committee approved our use of cull animals on condition that no requests were made for specific strains or ages. Swiss mice (n = 13) aged 6 - 8 weeks as calculated by their weight (20 - 25 grams) were used in these studies. Mice were killed by cervical dislocation and the ovaries were isolated by dissection. The ovaries and attached oviducts were placed in warm Hanks Balanced Salt Solution (HBSS) and transported to the laboratory at 37˚C. Whole ovaries were dissected free of attached tissues, blotted dry, cut in half and weighed.

2.7. Follicle Isolation from Ovarian Tissue

Follicles were isolated from ovarian tissue by disaggregation with 0.5 mL of 2 mg/mL collagenase IV (Worthington, 295 units/mg) in DMEM/F12 for 30 minutes at 37˚C in a humidified 5% CO_2 incubator. After incubation 100 µL FCS were added [70] [71] and each half ovary was mechanically disaggregated with 22 gauge needles for 5 minutes. All released follicles were collected and transferred to a 96-well plate containing DMEM/F12.

2.8. Allocation of Follicles into Size Cohorts

The diameters of follicles collected after ovarian tissue disaggregation were determined by using a camera (Scientific C-Mount Camera, 9MPX) mounted on a

dissection microscope to take a microphotograph of the well containing all the follicles from one half ovary. The well diameter was provided by the manufacturer and used to calibrate the Image J software. The area in pixels of each follicle in the well was determined by applying the circle tool to each follicle twice and calculating the average area. The area was used to calculate follicular diameter. The diameters of the follicles were used to allocate follicles to primordial, primary, early to mid-secondary, late secondary to incipient antral, or antral stage cohorts, according to the size ranges described by Griffin J, Emery BR, Huang I, Peterson CM and Carrell DT [42].

2.9. Follicle Disaggregation Using Hyaluronidase

Follicles were disaggregated using 50 µL hyaluronidase (1228 units/mg) at two concentrations (0.01% and 0.025%). A group of three secondary follicles from one mouse were exposed to 0.01% and another group of 3 secondary follicles from the same mouse were exposed to 0.025% hyaluronidase, for 2 hours at room temperature. 50 µL of FCS were added and the granulosa cells isolated from each group of 3 follicles were centrifuged at 2040 g for 5 minutes. The supernatants were discarded, and cells re-suspended in 20 µL DMEM/F12 with 10% FBS, and 20 µL Trypan Blue. This experiment was repeated using secondary follicles from two more mice (n = 3, a total of 18 secondary follicles).

2.10. Follicle Disaggregation Using Combinations of Collagenase IV and Hyaluronidase

Three antral follicles from each half ovary from four mice (n = 4) were isolated (*i.e.* 48 follicles with mean diameter of 303 ± 65 µm from 8 ovaries). Four groups of three antral follicles from each mouse were exposed to collagenase IV (2 mg/mL) in a 96-well plate for 15, 30 or 45 minutes before adding 0.025% hyaluronidase. No hyaluronidase was added to the 4th group of follicles which were incubated in collagenase IV for 60 minutes. Follicles in all the treatment groups were incubated at 37˚C with one minute shaking every 15 minutes. After 60 minutes incubation, 50 µL of FCS were added and all the contents of each well were transferred to 1.5 mL centrifuge tubes. Each well was washed with 1X PBS and this was used to make the final volume in the centrifuge tubes up to 500 µL. The granulosa cells were centrifuged at 2040 g for 5 minutes. The supernatants were discarded, and the isolated cells were re-suspended in 10 µL of DMEM with 10% FCS. The numbers of viable cells were then determined in a Trypan Blue Exclusion assay as described previously.

2.11. Crystal Violet (CV) Assay Assessment of GC Viability

Granulosa cells were obtained from 250 - 265 disaggregated follicles from two mice on each of three separate occasions (n = 3). Aliquots from each of the three single GC suspensions were assessed in a Trypan Blue Exclusion assay to determine the numbers of viable cells. These primary-derived murine GC were added to sterile 96-well flat bottom plates in a final volume of 0.1 mL per well of

DMEM/F12 at densities of 0 to 20,000 cells per well, with each cell density in triplicate wells. The primary-derived murine GC were incubated at 37˚C with 5% CO_2 for 24 h to allow cell adherence [72]. The non-adherent, non-viable cells were removed by withdrawing supernatant from the wells. The supernatant was stored (−20˚C) for the later measurement of anti-Mullerian hormone (AMH) and estradiol by Enzyme-linked Immunoassay (EIA). The adherent viable cells in the wells were rinsed with sterile phosphate buffered saline (PBS) before adding 50 µL of 0.5% crystal violet in 50% methanol for 10 minutes, followed by the addition of 50 µL of 33% acetic acid and measurement of the absorbance at 570 nm with correction at 630 nm [65] [73].

2.12. Estradiol Enzyme Immunoassay (EIA)

The conditioned media were examined in a competitive Estradiol (E2) EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. The manufacturer reports a detection range from 6.6 to 4000 pg/mL, and an intra-assay coefficient of variation (CoV) of 7.8% to 18.8%. For this study, the estradiol standard was diluted in the DMEM/F12 cell culture medium to give concentrations that ranged from 6.6 to 4000 pg/mL. All supernatants from three separate primary-derived GC culture experiments were examined in one EIA, in which the standard curve R^2 value was 0.98.

2.13. Anti-Mullerian Hormone (AMH) Enzyme Immunoassay (EIA)

Conditioned media were examined in a two-immunological step sandwich type EIA (Immunotech, Marseille Cedex, France) that uses an anti-AMH monoclonal antibody for capturing AMH, and a biotinylated monoclonal antibody together with streptavidin-peroxidase for detecting bound AMH in the wells. The manufacturer reports a detection limit of 1 pg/mL, an intra-assay CoV of 12%, and an inter-assay CoV of 14.2%. For this study, the AMH standards were diluted in DMEM/F12 cell culture medium to give concentrations that ranged from 0 to 150 pM. All supernatants from three separate primary-derived GC culture experiments were examined in one EIA, in which the standard curve R^2 value was 0.99.

2.14. Statistical Analysis

The effect of centrifugation on viable cell retrieval was analysed by 2-way ANOVA with Bonferroni post-test. The expected numbers of T47D, 184B5 and KGN cells were compared to the numbers counted in the Trypan Blue Exclusion assay in a Chi Square analysis.

The numbers of GC obtained by follicle disaggregation using 0.01% or 0.05% hyaluronidase were compared in a Students Paired T-test. The numbers of GC obtained after follicle disaggregation using a combination of Collagenase IV and Hyaluronidase were compared to disaggregation using 0.05% hyaluronidase

alone by applying a two-way ANOVA with Bonferroni post-test. Statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Effect of Centrifugation on Viable Cell Retrieval

The centrifugal force significantly affected cell retrieval ($p = 0.0012$ overall effect in a 2-way ANOVA) but the duration of centrifugation had no effect ($p = 0.11$). Centrifugation at 2040 g for five minutes retrieved the highest number of viable cells (583 ± 14, $p > 0.05$) and the highest total number of cells 608 ± 14 ($p > 0.05$, Figure 2). Under these conditions, 96% of the cells were viable.

3.2. Accuracy of the Trypan Blue Assay

There was no significant difference between the numbers of cells calculated to be in a dilution series, and the number of cells counted directly in a Trypan Blue assay (Figure 3).

3.3. Coefficients of Variation of the Trypan Blue Exclusion Assay

The intra-assay co-efficient of variation decreased from 22.3% to 5.4% as the number of cells in the cell suspension increased, and so did the inter-assay CoV (Table 1).

3.4. Follicle Disaggregation Using Hyaluronidase

There was no significant difference in the number or viability of GC obtained

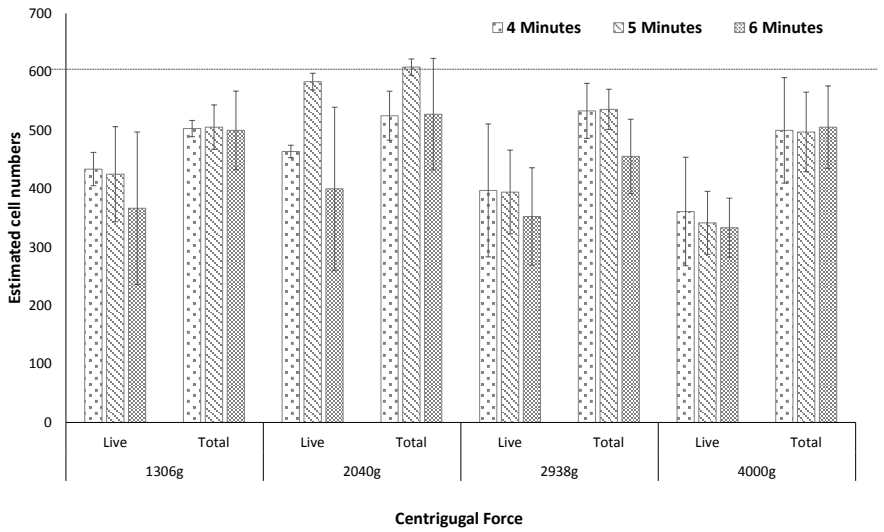
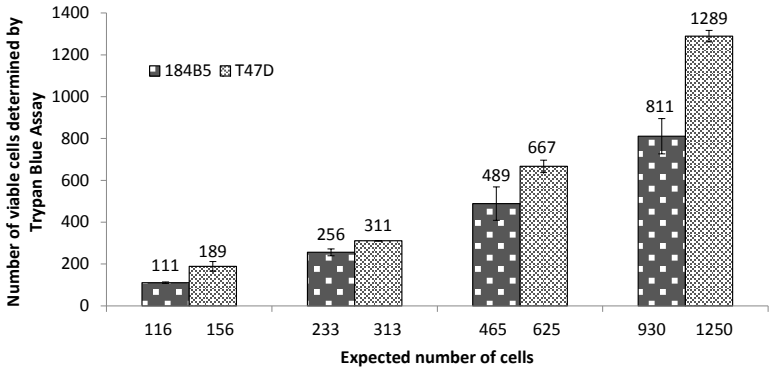
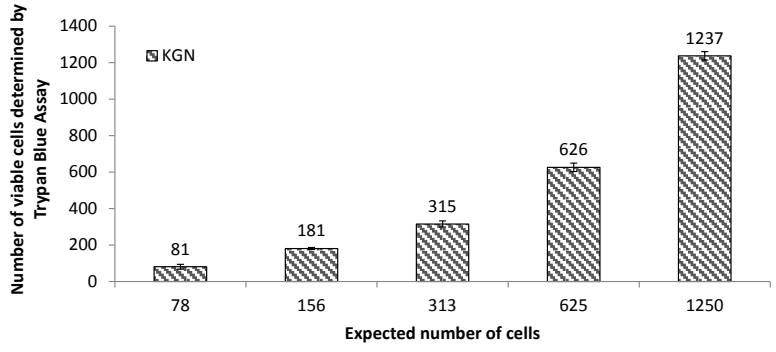


Figure 2. Effect of centrifugation on viable cell retrieval. KGN cells (600 cells in 500 µL of media) were centrifuged (1306 g, 2040 g, 2938 g and 4000 g) for 4, 5 or 6 minutes on four separate occasions (n = 4). Supernatants were removed and cells resuspended in 10 µL culture media and 10 µL of Trypan Blue before counting dead and viable cells using a haemocytometer and 10x objective lens microscope. Mean ± SD of cells in each cell suspension shown. Data were analysed by 2-way ANOVA with Bonferroni post-test, significance was assigned at $p < 0.05$.



(a)



(b)

Figure 3. Accuracy of the Trypan Blue Assay. Three parent cell suspensions of the human cancer cell lines; T47D, 184B5, (a) and KGN (b) were assessed using a standard Trypan Blue Exclusion assay. The volumes of cell suspension that contained 1250 viable 184B5 and KGN cells, or 930 viable T47D cells were made up to 100 µL, then subjected to four 1:2 dilutions. The numbers of cells calculated to be in each preparation were designated "Expected". The cells were centrifuged at 2040 g for 5 minutes, then re-suspended in 10 µL of media and 10 µL of Trypan Blue was added. Each stained cell preparation was assessed twice using a haemocytometer and 10× objective lens microscope, and the numbers of viable cells in the original 100 µL cell suspension determined. The experiment was repeated on three separate occasions (n = 3) and the mean ± SD shown. Data was analyzed with Chi Square test.

Table 1. Intra and inter assay Coefficients of Variation (CoV). 1250, 625 or 78 KGN cells per 500 µL of media, with each cell density examined in 10 separate centrifuge tubes, were centrifuged at 2040 g for 5 minutes. Supernatants were removed before re-suspending the cells in 10 µL of media and 10 µL of Trypan Blue. The experiment was repeated on four separate occasions (n = 4). The numbers of viable and dead cells were determined in a Trypan Blue Exclusion assay. CoV (%) = (SD/mean) × 100.

% CoV	78 cells	625 cells	1250 cells
Intra-assay	22.27	7.57	5.38
Inter-assay	6.97	4.14	1.57

from secondary follicles disaggregated with 0.01% or 0.025% hyaluronidase (**Figure 4**). The numbers of viable GC collected after disaggregation with 0.025% hyaluronidase however, were closer to those enumerated previously in H & E stained sections of mouse ovaries [42].

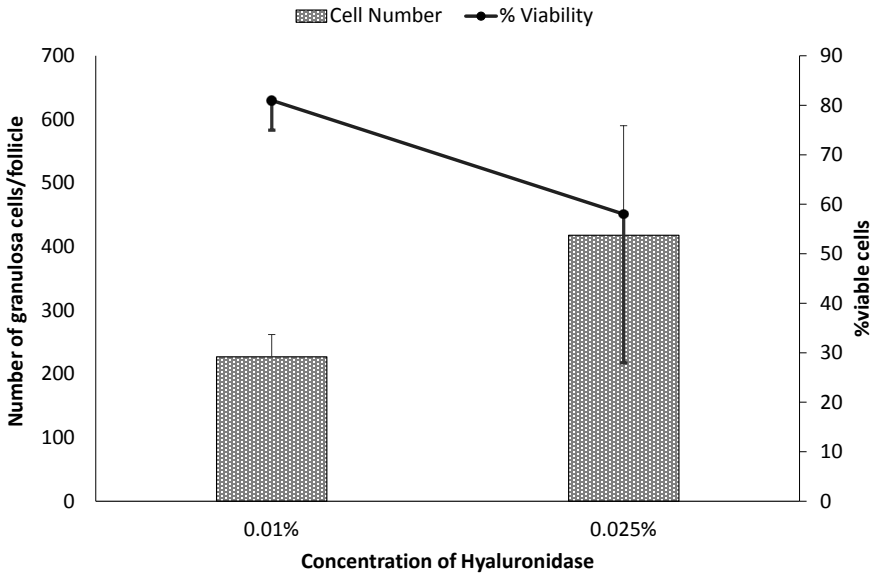


Figure 4. Follicle disaggregation using hyaluronidase. Groups of three secondary follicles were disaggregated with 0.01% or 0.025% hyaluronidase in DMEM/F12 for 2 h. The isolated cells were centrifuged at 2040 g for 5 minutes then re-suspended in 20 µL DMEM with 10% FBS before viable cell numbers were counted by Trypan Blue Exclusion assay. The experiment was repeated with 3 follicles for each hyaluronidase concentration from one mouse on three separate occasions (n = 3) and mean ± SD total cells and % viable cells per secondary follicle shown. Data were analysed using a students' T-test.

3.5. Follicle Disaggregation Using Combinations of Hyaluronidase and Collagenase IV

Antral follicles that were incubated with 2 mg/mL collagenase IV for 30 minutes then with 0.025% hyaluronidase for 30 minutes yielded the highest total number of granulosa cells per follicle (656 ± 87, $p < 0.05$) and the highest number of viable granulosa cells (542 ± 95, $p < 0.05$, **Figure 5**). The percent viability of granulosa cells was 82% ± 5% under these conditions, higher than when follicles were disaggregated with hyaluronidase alone (**Figure 4**). Follicular disaggregation using only collagenase (without hyaluronidase) resulted in isolation of the lowest number of GC (341 ± 69), and 76% of these were viable.

3.6. Primary-Derived Granulosa Cell Viability and Hormone Production *in Vitro*

Primary-derived GC obtained from disaggregated mouse follicles generated a linear correlation ($R^2 = 0.98$) (**Figure 6(a)**) between cell density per well and crystal violet absorbance (570 nm) after 24 h *in vitro* (**Figure 6**). Primary-derived granulosa cells did not produce any detectable AMH even at 20,000 viable GCs per well, but there was a density-dependent increase in basal estrogen (E2) production ($R^2 = 0.92$) (**Figure 6(b)**).

4. Discussion

Here we report a technically simple, highly reliable and inexpensive method for

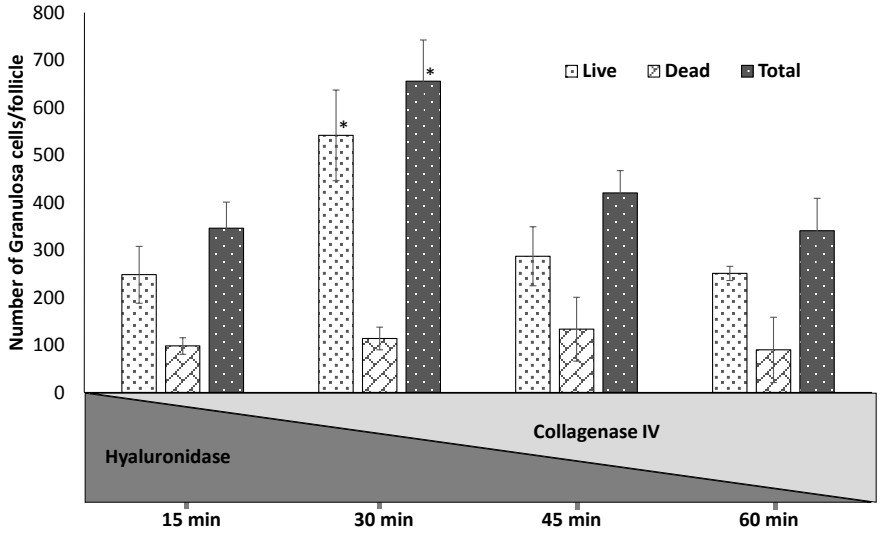
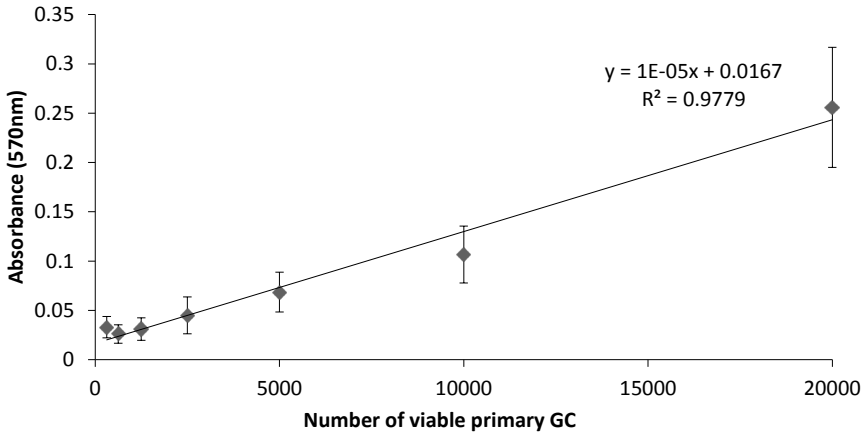


Figure 5. Collagenase IV and hyaluronidase disaggregation of murine follicles. Four groups of three antral follicles (348 ± 26 μm) were incubated in collagenase IV (2 mg/mL) for 15, 30, 45 and 60 minutes (shown on x-axis) before the addition of Hyaluronidase (0.025%) to the 15 - 45 minute groups. The isolated granulosa cells were assessed in a Trypan Blue Exclusion Assay. The experiment was repeated using follicles from four mice (n = 4). Data analysed by two-way ANOVA with Bonferroni post-test. Mean ± SD granulosa cells per follicle shown. $P < 0.05$ * compared to same cell category.

enumerating the number of viable and dead GC in an isolated ovarian follicle. To our knowledge, this is the first report of a straightforward process for disaggregating isolated follicles to produce a suspension of primary-derived GC from naturally cycling unstimulated adult mice, the *in vitro* culture of these GCs in a 96-well plate format, and their production of E2 and AMH.

The standard Trypan Blue protocol requires more than 1×10^5 cells per mL in the parent cell suspension, or a minimum of 100 cells counted on each grid [63] [64]. This limitation directed the development of our protocol. After allowing for dilution of a cell suspension with Trypan Blue dye, a minimum volume of 20 uL of cell suspension containing a minimum of 200 cells was required to allow the duplicate assessment of a GC preparation. Primary to mid-secondary follicles only contain 9 - 185 GC [42], therefore follicles were disaggregated in groups of three, to generate sufficient GC for an accurate assessment. The accuracy of this protocol is expected to decrease when applied to small primary follicles, which might only contain 9 GC, and we therefore recommend that the follicle disaggregation group size should be increased accordingly.

Previous reports describing the enumeration of granulosa cells in fixed ovarian sections [42] [45] [46] were comparable to the numbers of GC counted in this study after follicle disaggregation using 30 minute exposure to collagenase IV followed by 30 minute exposure to hyaluronidase. Under these conditions 82% of the GCs were viable. The loss of 18% viability may be attributed to the enzymes used to disaggregate the follicle [74], or to the disruption of the tight junctions between GC [28], or to mechanical sheer force during centrifugation.



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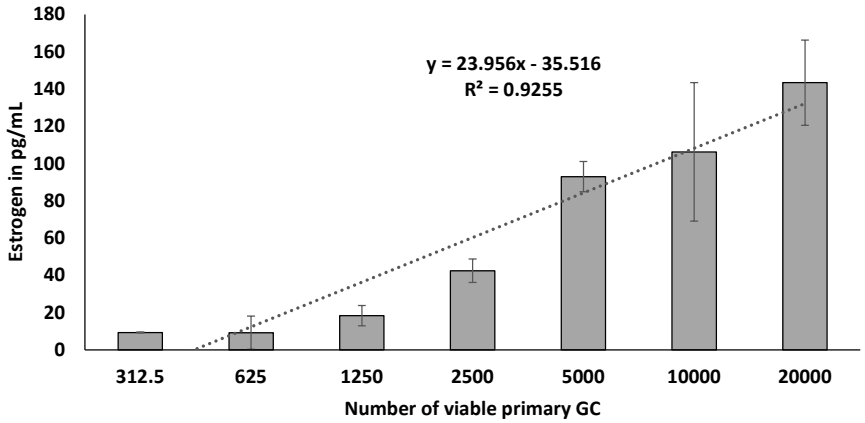

(b)

Figure 6. Estrogen produced by primary-derived granulosa cells. Primary-derived granulosa cells isolated from disaggregated murine follicles were cultured for 24 hours at 37˚C with 5% CO_2 at densities of 0 to 20,000 cells per well in a 96-well flat bottom plate on three separate occasions (n = 3). After 24 h, the numbers of viable cells in each well were determined in a crystal violet assay. Mean ± SD (n = 3) OD570 nm (with correction at 630 nm) was plotted against cell densities and fitted to a linear trendline (a). Conditioned media after 24 h culture were examined in a competitive estradiol immunoassay and E2 plotted against cell density (b).

However, others have reported that approximately 80% of granulosa cells in intact follicles were viable when assessed by whole follicle Live-Dead staining [4] [15] [16] [17] [32], which suggests that our follicle collection and disaggregation procedure had no adverse effects on the viability of the GC.

The Trypan Blue Exclusion assay is notorious for low accuracy, poor reproducibility and high variation between counts of the same preparation of cells, thought to be caused by non-specific binding of the TB dye to cellular artifacts [54]. Our optimized TB assay has coefficients of variation similar to, or lower than those reported previously [54] [57] [58]. In the present study, when preparations containing 625 granulosa cells were counted on four separate occasions, the intra-assay CoV of 7.6%, and the inter-assay CoV was 4%. Since the GC were isolated from groups of three follicles, this meant that when there was an average

of 209 GCs per follicle (equivalent to a late secondary stage follicle [42], the CoVs for the TB assay were of the same order of magnitude as those reported by the manufacturers of commercially available EIA kits, and the CoVs for cell numbers corresponding to groups of three small primary follicles were within the ranges previously found acceptable for other cell-based assays [75]. From this we conclude that we developed an assay protocol with relatively high precision and accuracy.

Although there are numerous reports of primary-derived human granulosa cells being cultured *in vitro* [76]-[82] and many instances in which GC obtained from the follicles of larger mammals have been cultured [83] [84] [85] [86] [87], as far as we know this is the first report describing the *in vitro* culture of GC obtained from the follicles of naturally cycling adult mice in a 96-well format. Cell viability assays that use the high throughput 96-well plate formats benefit from the application of control standard plots, in which known cell densities are related to optical density values [88] [89] [90]. Previously granulosa cells have been reported as having a doubling rate of 46.4 hours [67], hence we considered it likely that the murine GC would not proliferate in the first 24 h, but would only adhere to the floor of the wells [82]. Since this is the first study to examine primary-derived murine GC *in vitro*, we established baseline control conditions, which can be referred to in future studies to examine the effects of gonadotrophins such as follicle stimulating hormone (FSH), and other reproductive parameters, during longer culture periods.

Murine follicles cultured *in vitro* increase E2 production when stimulated by Follicle Stimulating Hormone (FSH) [33] [91] [92], and Adriaens I, Cortvrindt R and Smitz J [92] found that intact follicles containing approximately 20,000 GC produced the equivalent of 125 pg/mL E2 every 48 h. We found that 20,000 murine GC in a monolayer *in vitro* produced 143 pg/mL E2 in the first 24-h of culture. The difference may be because only 80% of the GC may have been viable in the follicles *in vitro*, whereas it was more likely that all the primary-derived GC were viable in the monolayer. On the other hand, the GC monolayer did not produce any detectable AMH, even though Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, Themmen AP and Visser JA [93] reported data suggesting that a follicle containing approximately 100 GC may produce 1.3 pM AMH *in vivo*. Since the analytical sensitivity of the AMH kit was 1 pM, we were therefore surprised that no AMH was produced by 20,000 GC *in vitro*. There are two key differences between our *in vitro* system and the *in vivo* situation; the *in vitro* system did not include FSH and hence the GC were not stimulated to proliferate [94] [95], and follicle disaggregation detaches GC from other GCs and the oocyte which interfered with the intercellular communication essential for the production of AMH [28] [32] [96] [97].

5. Conclusion

Although the Trypan Blue Exclusion assay is the standard laboratory workhorse

for assessing cell viability, it has not previously been applicable to suspensions with low cell densities. This study presents a straightforward and simple method to assess the viability of suspensions with cell densities as low as 7800 cells per mL (less than 10 cells/μL). There are few reports of follicles isolated from ovarian tissue being disaggregated to produce a single cell suspension [19] [98], but the ability to obtain GC from pools of follicles of defined size cohorts will allow examination of reproductive parameters that are affected by follicular development, such as AMH regulation or sensitivity to chemotherapeutics.

Acknowledgements

We appreciate the financial support awarded to us by the publicly owned company Flinders Fertility, which supported some of this research with a grant managed by the University. The company had no oversight or influence on the research or results.

Ethics Approval and Consent to Participate

The Flinders Animal Welfare Committee approved our use of cull animals on condition that no requests were made for specific strains or ages.

Consent for Publication

Not Applicable.

Availability of Data and Material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interests

I confirm that none of the authors have any conflict of interests in the manuscript.

Funding

Not Applicable.

Authors' Contributions

Mohammad Asaduzzaman: made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data and production of the manuscript.

Daniela Figueroa Gonzalez: contributed to the acquisition of data, analysis and interpretation of data.

Dr. Fiona Young: contributed to the conception, experimental design, analysis and interpretation of data, and production of the manuscript.

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List of Abbreviations

2D:	Two Dimensional
3D:	Three Dimensional
AMH:	Anti-Mullerian Hormone
ANOVA:	Analysis of Variance
CoV:	Coefficients of Variation
DMEM:	Dulbecco's Modified Eagle's Medium
E2:	Estradiol
ECM:	Extra Cellular Matrix
EDTA:	Ethylene Diamine Tetra-Acetic Acid
EIA:	Enzyme-Linked Immunoassay
FCS:	Foetal Calf Serum
FSH:	Follicle Stimulating Hormone
GC:	Granulosa Cell
HBSS:	Hanks Balanced Salt Solution
ITS:	Insulin, Transferrin and Selenium
IVM:	*In vitro* Maturation
PBS:	Phosphate Buffered Saline
SD:	Standard Deviation
TB:	Trypan Blue