A simple and efficient method for preparation of isolated ovarian follicles for transmission electron microscopy

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Abstract

Objective A simple method for preparation of isolated ovarian follicles for transmission electron microscopy (TEM) using transwell inserts is described.

Materials and methods Pre-antral follicles were enzymatically isolated from mouse ovaries and cultured overnight on transwell insert polyester membranes. The following day, isolated ovarian follicles were processed for TEM by moving the transwell insert through successive wells containing the fixation and embedding reagents. After polymerization of the resin, the polyester membrane with the follicles embedded in the resin was disengaged from the transwell unit. The resin was sectioned. Semi-thin sections were stained with toluidine blue while ultra-thin sections were stained by uranyl acetate and examined by light microscopy and TEM, respectively.

Results Isolated ovarian follicles were easily processed in groups for TEM. Follicles were well embedded and there

appeared to be no loss of tissue during processing. The ultra-structure of processed isolated ovarian follicles was well preserved with little evidence of processing artifacts.

Conclusions In situ processing and preparation of isolated ovarian follicles by first allowing attachment on transwell insert membranes was shown to be a simple, rapid and effective method for TEM.

Keywords Isolated ovarian follicle · Ovary · Transmission electron microscopy · TEM · Transwell insert · Polyester membrane · Fertility preservation

Introduction

Cryopreservation of ovarian follicles, either isolated or in tissue pieces, is a promising fertility preservation method {reviewed in [1, 2]}. Follicles can be isolated from ovarian tissue by enzymatic digestion or mechanical dissection. The enzymatic technique is generally preferred for large mammals as well as humans, due to the dense ovarian stroma [3]. Observation of the ultra-structure of ovarian tissue/follicles by transmission electron microscopy (TEM) is a valuable tool for evaluation and comparison of follicular isolation and cryopreservation protocols [4]. Although there are numerous reports on the ultra-structure of ovarian tissue pieces [5–7], only a few have reported on the ultra-structure of isolated ovarian follicles [8, 9]. Moreover, the focus of these studies has not been the methodological aspects of preparing isolated follicles for ultra-structural examination using TEM

Processing a sample for TEM requires multiple steps of dehydration, infiltration and embedding. Thus TEM preparation of individual cells is usually challenging, as it is tedious to move cells one by one through different

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Capsule Use of transwell insert membranes as a vehicle for the preparation of isolated ovarian follicles for TEM is a very simple and easy technique.

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preparation steps. Traditional methods of TEM preparation include sample centrifugation [10] and pre-embedding in different matrices such as gelatin [11], agar [12], low melting point agarose [13], alginate [9] or bovine serum albumin/bis-acrylamide (BSA/BA) matrix [14].

Another method uses transparent porous cellulose tubes (200 μm inner diameter) through which cells are drawn by capillary action and then processed for TEM [15]. Alternatively, since the surface of all biological cells carry a negative charge, the cells can be adhered to poly-Lysine coated glass cover slips [16] or charged nylon membranes [17]. Individual cells can be also prepared for TEM in situ on supporting membranes like plastic [18, 19], cellulose esters [20], Teflon [21, 22] and polycarbonate [23].

In this study we report on an efficient and simple method for in situ preparation of isolated pre-antral follicles for light and TEM microscopy. The isolated ovarian follicles are cultured on a translucent polyester membrane in a transwell culture vessel. This culture system allows multiple follicles to be simultaneously cultured and retain polarity by growth on a membrane suspended between two fluid layers. Our TEM preparation technique is unique in that the follicles can be processed in their in situ growth configuration without disruption. Alternatively isolated follicles can be adhered to the membrane after overnight incubation. The application of this technique can be expanded to include any cell type that can attach to the polyester membrane.

Materials and methods

Animals and ovarian follicle isolation

Ovaries were harvested from C57BL6 X SJLs pups (14–16 days old) and placed in Leibovitz media (L15, Gibco, Carlsbad, CA) supplemented with 10% Synthetic serum substitute (SSS, Irvine, Santa Ana, CA). Ovarian tissue was enzymatically digested using 1 mg/ml collagenase Type I (132 u/mg, Worthington, Lakewood, NJ) at 37°C. Follicles were collected, rinsed free of enzyme and examined under a stereomicroscope. Intact pre-antral follicles with two or more layers of granulosa cells surrounding the oocyte were selected for this study.

Overnight in vitro culture of isolated pre-antral follicles

Isolated pre-antral follicles were cultured overnight in Minimal Essential Medium α (α -MEM, GIBCO, Carlsbad, CA) supplemented with 1% Nu-serum (BD Biosciences, San Jose, CA, USA), 100.5 mIU/ml follicle stimulating hormone (FSH, Follistim, Organon, Kirkland QC, Canada), 10 $\mu\text{g}/\text{ml}$ insulin, 5.5 $\mu\text{g}/\text{ml}$ transferrin and 0.67 $\mu\text{g}/\text{ml}$

selenium (ITS, GIBCO). Pre-antral follicles were group-cultured as close as possible on the central part of the transwell inserts (6 well plates, 24 mm in diameter and 0.4 μm pore size, Corning Incorporated Costar, Corning, NY). All cultures were performed at 37°C with 5% CO_2 in air.

Preparation of isolated pre-antral follicles for TEM

All the TEM reagents were purchased from Electron Microscopy Sciences (Hatfield, PA).

Washing step

Sodium cacodylate (0.2M, PH 7.3) was used as the wash buffer in this study. The transwell insert with attached follicles was moved to a new 6 well plate for processing. Culture medium was removed and the outer and inner wells were quickly filled with cold (4°C) cacodylate wash buffer.

Fixation step

The transwell insert was then transferred into another well containing fixative solution (2.5% Glutaraldehyde+4% Paraformaldehyde in cacodylate buffer). The wash buffer in the inner well was immediately removed and replaced with fixative solution. The dish was refrigerated overnight at 4°C for fixation.

Post fixation step

The following day, the transwell was moved to a new well containing wash buffer. The fixative from the inner well was also removed and replaced with wash buffer. Washing was performed in 3 steps of 5 min each. Isolated pre-antral follicles were then post fixed with Osmium tetroxide (1%). The transwell insert was moved to a new well containing Osmium tetroxide. The wash buffer from the inner well was removed and replaced with the osmium tetroxide fixative. The plate with insert was incubated at 4°C. After 1 h, the transwell insert with attached follicles was transferred to a new well and once again washed three times for five minutes with cold cacodylate buffer.

Dehydration step

Dehydration of isolated pre-antral follicles was accomplished through successive transfer of the insert into wells containing increasing concentrations of ethanol. Dehydration steps were as follows: 1) 50% ethanol for 5 min at 4°C, 2) 70% ethanol for 5 min at 4°C, 3) 95% ethanol for 5 min at 4°C and 4) 100% ethanol for 10 min at room temperature, repeated three times.

Infiltration step

Following the third wash, the ethanol was replaced with a 1:1 solution of ethanol and embedding medium, (Embed-812 Resin kit [Embed-812+ DDSA+ NMA+ DMP-30] prepared as recommended by the vendor). The transwell insert was left covered overnight at room temperature to allow infiltration of the embedding agent. The next day, the 1:1 mixture was replaced with 100% embedding medium at room temperature.

Embedding step

After 4–6 h the polyester membrane was detached from the insert using a tuberculin syringe. A ~5 μ l droplet of pure embedding medium was placed on a rectangular piece of glass (5 mm thick, 2×10 cm). The transwell membrane with the follicles was placed on the drop of embedding solution with the follicles facing upwards. A gelatin capsule filled with pure embedding medium was then inverted on top of the membrane, with attached follicles. The glass plate with the membrane/follicles and gelatin capsule were placed in an oven at 60–70°C for 48–72 h. This assembly with the embedding capsule on top was then moved on to a hot plate, to facilitate the separation of the embedded sample and capsule from the glass holder. Semi-thin sections were cut and stained with 1% toluidine blue for examination with a light microscope. Ultra-thin sections were cut and stained with uranyl acetate and examined using a TEM (Philips CM12, FEI Company, Hillsboro, OR).

Results

Transwell inserts are commercially available in four membrane diameters; 6.5 mm (24 well plate), 12 mm (12 well plate), 24 mm (6 well plate) and 75 mm (100 mm dish). The polyester membranes, transfixed to the transwell inserts, are available in 3 pore sizes; 0.4 μ m, 0.8 μ m and 3 μ m. Transwell inserts have been used for in vitro culture and maturation of ovarian follicles [24–26]. The polyester membrane is fixed to the bottom of the insert, onto which ovarian follicles are cultured. Viable ovarian follicles usually attach well to the membrane after an overnight culture. Approximately 90–95% of the cultured follicles were found attached on the following day. Attached follicles were usually intact with a centrally located oocyte surrounded by tight layers of granulosa cells. Non-attached follicles were easily removed from the transwell insert prior to TEM processing using a glass drawn pipette.

Using the transwell inserts has greatly facilitated the processing of isolated follicles for TEM. It was very easy to sequentially move the whole insert with attached follicles

through the various reagents. The transwell membrane was efficient for follicle attachment and we experienced no follicle loss during the processing steps. After polymerization of the resin, the polyester membrane was easily peeled off the gelatin capsule. Embedded follicles were easily visualized within a single plane of the resin. This was especially helpful later on during sectioning.

Examination of the semi-thin sections stained with toluidine blue showed that the structure of ovarian pre-antral follicles was well preserved. Pre-antral follicles consisted of a central oocyte surrounded by few layers of granulosa cells. The basement membrane was intact and surrounded by layers of thecal (stromal) cells (Fig. 1).

The ultra-thin sections examined by TEM further confirmed that the ultra-structure of isolated pre-antral follicles was well maintained throughout the processing (Fig. 2). Artifacts typically associated with sample preparation technique were not evident. The ultra-structure of the oocyte contained within the follicle was well preserved with a central nucleus surrounded by a nuclear membrane (Fig. 2a & b). Abundant mitochondria and endoplasmic reticula were shown to be scattered throughout the ooplasm (Fig. 2b & c). Numerous finger-like processes (microvilli) were shown projecting into the perivitelline space (Fig. 2c). Cortical granules were seen scattered beneath the oocyte membrane (Fig. 2c).

The surrounding granulosa cells appeared polyhedral in shape and arranged in concentric layers around the oocyte (Fig. 2a). Each granulosa cell consisted of a large irregular nucleus surrounded by a nuclear membrane (Fig. 2a). The cytoplasm was populated with numerous organelles (Fig. 2d). The junctions between adjacent granulosa cells were well preserved (Fig. 2a & d).

Fig. 1 A semi-thin section stained by toluidine blue showing an intact isolated ovarian pre-antral follicle. O: oocyte, ZP: zona pellucida, GC: granulosa cell, TC: thecal cells

Fig. 2 a–d Electron-micrographs of an isolated ovarian pre-antral follicle prepared on the transwell insert membrane: **a** Part of an oocyte (O), its zona pellucida (ZP) and surrounding granulosa cells (GC). Note the contacts between the oocyte and granulosa cells (astrix *). **b** The oocyte nucleolus (NU) and nucleus (N) within the nuclear membrane. Note the ooplasm (OP) with its abundant organells. **c** Part of an oocyte ooplasm (OP) and surrounding zona pellucida (ZP). Note the abundant mitochondria (M) and endoplasmic reticulum (arrow head) within the ooplasm. Also note the finger like processes (micro-villi, MV) projecting into the perivitelline space. Some cortical granules (arrow) were seen beneath the oocyte plasma membrane. **d** A higher magnification of the inset shown in (a). It shows 2 adjacent granulosa cells. Note the granulosa cell nucleus (GCN) and the abundant mitochondria (M) populating the cytoplasm. Note the junction between the 2 granulosa cells (astrix)

Discussion

In this study, we described a very simple and efficient technique for in situ processing of large numbers of isolated ovarian follicles for TEM using the transwell insert polyester membrane. Isolated ovarian follicles remained well attached to the membrane and were not lost during the various processing steps for TEM.

This method has the advantage of providing in situ processing without the need to handle or manipulate the ovarian follicles individually. Moreover it allows for the efficient and simultaneous preparation of a large number of follicles. Cultured follicles can be processed for TEM at any time point during the culture period. As the follicles grow and develop antral cavities, they become more fragile, and would be difficult to remove and process, without causing any injury to them. Thus the in situ processing described here would reduce the risk of any mechanical injury caused by follicular manipulation. The transwell insert with the attached membrane can be easily moved from well to well, unlike the use of a membrane alone which can float about in different reagents and flip over.

Preparation of individual cells for TEM usually involves centrifugation of the sample at high speed to form a pellet. Centrifugation of isolated ovarian follicles may cause follicular injury and detachment of oocytes from the surrounding granulosa cells. Moreover it can result in some degree of sample loss. The method presented here avoids these dangers.

Our method does not rely on matrices, which pose several problems. Pre-embedding of individual cells in different matrices (gelatin, agarose, agar and BSA/BA) adds an extra step during the processing of the sample for TEM. Moreover, some of these matrices (gelatin and agarose) are fragile and lack translucency and consistency in sample preparation [14]. Embedding follicles in matrices is also not ideal for simultaneous processing of large numbers of follicles, since they often become embedded at different levels within the matrix, making examination more difficult and necessitating ultra-thin sectioning of the entire block.

Although the use of cellulose capillary tubes for TEM processing [15] of individual cells is effective, it requires expensive high pressure freezing apparatuses [17]. Supporting

membranes such as Teflon [21, 22] and polycarbonate [23] have been used for preparation of individual cells for TEM, but not for isolated ovarian follicles. Polycarbonate membranes require staining for cell visualization. In contrast, the polyester membrane in the transwell insert is translucent, allowing excellent cell visualization under phase contrast microscopy without the need for staining. The transwell insert with polyester membrane is a well documented system for in vitro culture and maturation of ovarian follicles [24–26]. The major limitation with this technique is that the original three-dimensional structure of the ovarian follicles is lost by attachment to the transwell membrane. Further investigation is needed to develop techniques that maintain the follicle three-dimensional architecture during processing for TEM.

In summary, this paper provides a detailed description of a simple, rapid and reproducible technique for preparation of isolated ovarian follicles for TEM. The advantage of this technique is its ability to process isolated follicles in situ. All of the TEM processing steps can be performed on the membrane, including the embedding and sectioning, thus maintaining the growth architecture of cultured isolated follicles. To our knowledge, this is the first study to describe technical aspects of follicle preparation for TEM.

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