Human Ovarian Tissue Procurement and Processing for Ovarian Explant Cultures (Static and Fluidic conditions)

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ABSTRACT

The Cellular Senescence Network (SenNet) was recently established to map senescent cells in the human body. As part of this initiative, our goal is to profile senescent cells in the human ovary in vivo as well as ex vivo using a microphysiological platform called LATTICE. Human ovarian tissue is collected through the Northwestern University Reproductive Tissue Library. Tissue is collected from women undergoing bilateral salpingo-oophorectomies or hysterectomies with absence of ovarian neoplasia. With this model, we are generating a molecular signature of Doxorubicin-induced cellular senescence in static and fluidic culture which will then be mapped onto human ovaries across an aging series. To do this, human ovarian explants (cortex and medulla) are cultured in Doxorubicin (0.1 µg/ml) for 24 hours followed by up to 11 days of culture.

GUIDELINES

Guidelines for researchers working with human specimens

Researchers will adhere to all safety and training protocols required by the Northwestern Medicine/Northwestern University including but not limited to:

1. Biosafety Certification
2. Bloodborne Pathogens Certification
3. Working with Formaldehyde Certification
4. Collaborative Institutional Training Initiative (CITI program) certification

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MATERIALS

1. ORIGIO Handling IVF Medium (ORIGIO, #83100060)
2. Disposable Scalpel, Sterile, No. 10 and No. 22 (Fisher Scientific, 12-460-456)
3. 15 mL conical tubes (Fisher Scientific, 2610L35) for tissue collection.
4. Gibco™ DPBS, calcium, magnesium (Fisher Scientific, 14-190-250)
5. 60 mm Dish, Non-Treated, Corning™ Falcon™ Bacteriological Petri Dishes with Lid (Fisher Scientific 08-772-12 or equivalents) for processing
6. Stadie-riggs tissue slicer (discontinued)
7. Stadie-Riggs tissue slicer/microtome blades (Thomas Scientific 6727C18)
8. Stadie-Riggs tissue slicer blade handle (Thomas Scientific 6727C25)
9. Plastic Discs (Fisher Scientific, 1018001)
10. MEM Alpha (1X) + GlutaMAX (Thermo, 32561037)
11. Human serum albumin (Cooper Surgical Inc, ART-3003)
12. Fetuin (Sigma, F3385-25G)
13. Insulin-Transferrin-Sodium Selenite Supplement (Sigma-Aldrich, I1884-1VL)
14. Doxorubicin (Fisher Scientific, 22-521-0)
15. Millicell Cell Culture Insert, 12 mm, hydrophilic PTFE, 0.4 µm (Millipore Sigma, PICM01250)
16. Falcon® 24-Well Flat-Bottom Plate, Tissue Culture-Treated (Fisher Scientific, 353047)
17. Invitrogen™RNase-free Microfuge Tubes (ThermoFisher Scientific, AM12400)

SAFETY WARNINGS

Researchers will wear personal protective equipment (PPE) when working with human specimens which include gloves, mask, eye protection, and lab coat.

ETHICS STATEMENT

Human ovarian tissue procurement and processing for ovarian explant cultures adhere to the approved IRB protocol under NU (NU12G09) for the collection of human ovarian tissue through Northwestern Medicine.

Processing human ovarian tissue for ovarian explant cultures

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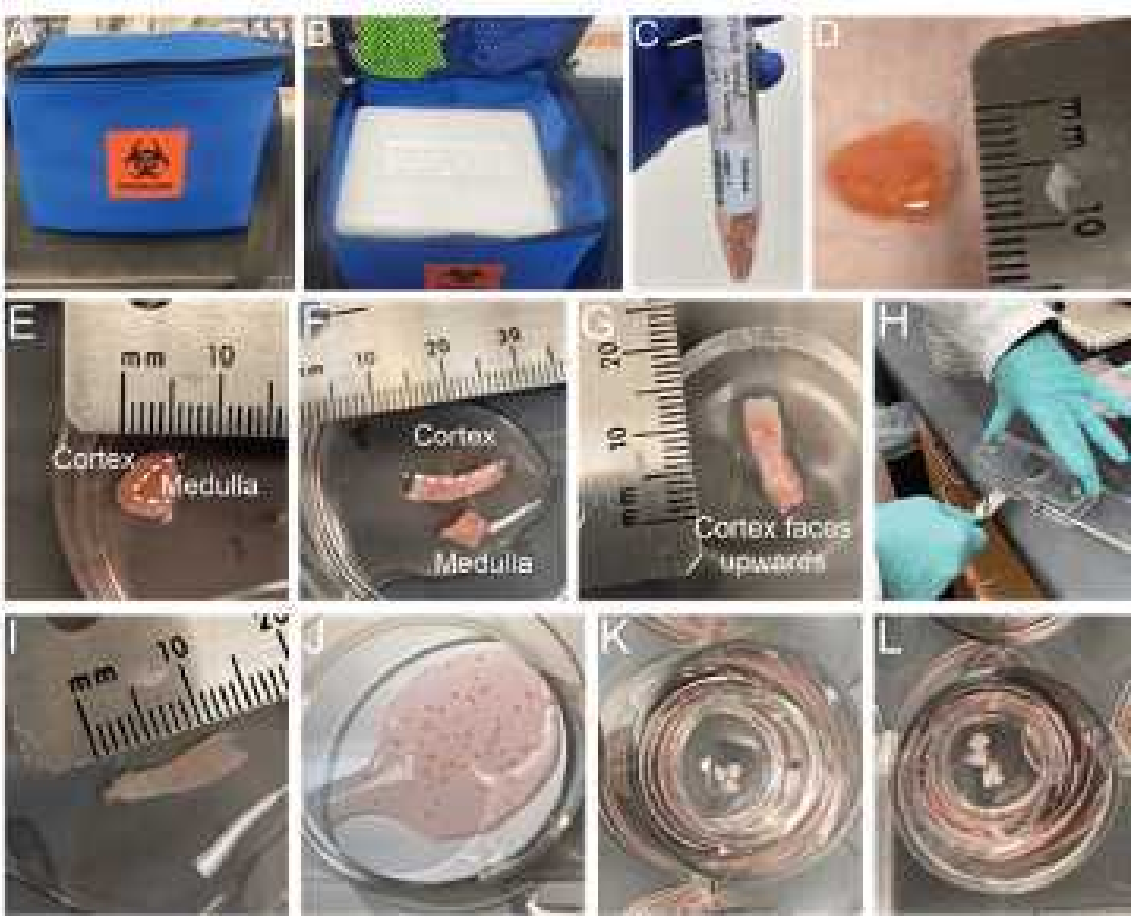

Fig. 1. Ovarian tissue pieces are collected for research through the IRB-approved Reproductive Tissue Library at Northwestern Medicine. Ovarian cross sections 3-5 mm in depth are brought to the lab for processing within 24 hours of surgery. All samples are transported in a blue biohazard labeled cooler bag (A) and on ice (2-4 °C) (B). Tissue pieces are transported and processed in ORIGIO Handling medium (C). Cross sections are measured on arrival (D) and should include both cortex and medulla tissue (E). A cut is made along the groove of the cortex leaving 2-3mm between the cut and the edge of the tissue piece, resulting in a long piece with cortex/medulla and a smaller piece of medulla(F). The strip containing the cortex and the medulla is placed cortex-side up on the Stadie-Riggs tissue slicer (G). A thin blade is used to make 0.5 mm slices through the entire piece of ovarian tissue (H). The resulting slices are measured (I). Individual slices are further sectioned into 1mm x 1mm squares (J) and pieces from the cortex (K) and medulla (L) are loaded onto cell culture inserts separately.

2 Ovarian tissue samples are collected from the Northwestern Pathology Department and placed immediately on ice following collection. The research coordinator will bring research specimens to the lab on ice (2-4 °C). Samples should be transported in a blue biohazard-labeled cooler bag (Fig.1A, B). Tissue should be kept at 2-4°C throughout all processing steps and should be processed within 24

hours of procurement. Ovarian tissue is kept in an ORIGIO Handling medium throughout the procurement and processing up until the transfer to culture growth media (Fig. 1C).

3 On arrival, ovarian tissue should be measured for length, width, and thickness (Fig. 1D). Also note the approximate anatomy of the cortex and the medulla (Fig. 1E). The cortex should be on the outermost edge surrounding the entire tissue piece and will have a glossy, somewhat translucent appearance. The medulla should be the innermost portion if the ovarian tissue slice is lying flat and will likely contain fibrotic-looking, white tissue, especially in postmenopausal ovaries.

4 Cut the ovarian tissue along the cortex and medulla region border, leaving approximately 2-3 mm between the edge of the piece and the cut surface (Fig. 1F). This cut should result in a piece containing both the cortex (the outer translucent-looking layer), and the medulla which will be softer and less rigid when held with forceps.

5 Place the cortex-containing portion flat on the center well of the Stadie-Riggs tissue slicer with the cortex side facing up (Fig. 1G).

6 Smoothly run the blade through the Stadie-Riggs tissue slicer (Fig. 1H). Avoid jagged movements as much as possible as it will result in uneven tissue slices. The first slice will yield one 500 µm slice of the ovarian cortex (Fig. 1I). The second slice can be discarded as this consists of both cortex and medulla tissue. The third slice is the ovarian medulla and can be treated like the first slice for culture preparations. Additional medulla slices and remaining tissue can also be fixed for histology.

7 Add 2-3 mL of ORIGIO Handling Medium, to a petri dish containing a tissue-slicing plastic disc (Fisher Scientific, 1018001) (Fig. 1J).

8 At room temperature under a dissection scope, manually cut the ovarian tissue slice into 1mm x 1mm squares in the droplet of ORIGO Handling Medium (Fig. 1J)

9 Once all ovarian tissue pieces (1 mm x 1 mm x 0.5 mm) are cut, submerge them in sufficient ORIGIO Handling medium to ensure they do not dry out during culture plate preparation.

10 Cut ovarian pieces can be transferred to equilibrated α-MEM-based media with supplements and then should be loaded onto cell culture inserts (Millipore Sigma, PICM01250) in groups of three separately as the cortex (Fig. 1K) and medulla (Fig. 1L). Follow the detailed procedure outlined for ovarian explant cultures to load tissues and maintain culture.

Human ovarian explant cultures

11 Growth media should be equilibrated for 4-6 hours before the tissue is received (Media contains MEM Alpha (1X) + GlutaMAX (Thermo, 32561037), 20 mIU ml−1 recombinant Follicle-Stimulating Hormone, 1 mg/mL fetuin, 1 µg/mL Insulin-Transferrin-Sodium Selenite supplement, and 30mg/10mL human serum albumin.

12 Before loading tissues into a standard 24-well plate add 400 µL of growth media to each well the tissue will be cultured. Any remaining wells should be filled with equal amounts of 1 x PBS. This can be done either at the time of culture if media is equilibrated in 60 mm dishes or ahead of time when equilibrating the media.

13 For the LATTICE plate, the donor wells should be loaded with 1.5 mL of media, each tissue well with 400 µL of media, and the acceptor wells should be kept empty. LATTICE allows for dynamic exchange of ~40µL of media every hour between all wells. The direction of flow and flow rate can be manipulated, but the flow is unidirectional for these cultures (Donor well -> Tissue A -> Tissue B -> Acceptor well; Fig. 2).

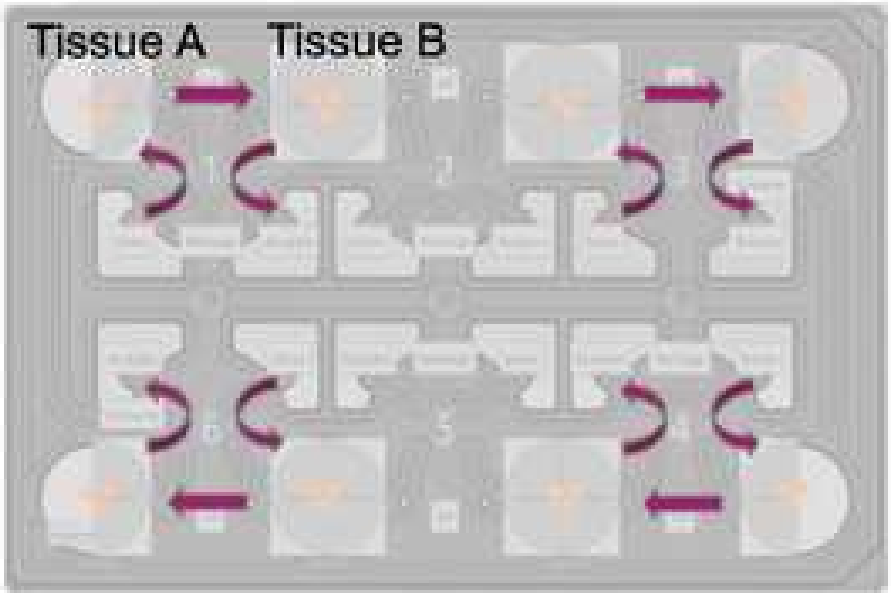

Fig. 2 Representative image of a LATTICE plate used in ovarian explant cultures. Red arrows represent the direction of media flow beginning from the Donor well -> Tissue A -> Tissue B -> Acceptor well.

14 Once 1 mm x 1 mm x 0.5 mm pieces of the ovarian cortex and medulla are cut, transfer to a new

60mm dish with ORIGIO handling media such that the tissue pieces are completely submerged/floating in media.

15 Cut the tip (approximately 1 cm) off of a 1000 µL pipette tip using a No. 10 scalpel.

16 Carefully load three pieces of tissue to each culture transwell (Millipore Sigma, PICM01250) as follows: sequentially take up some media (~100 µL) followed by a tissue piece with some media, and then take up an additional 100-200 µL of media into the pipette (Fig. 3A). Hover the pipette tip over the base of the transwell membrane and flick the edge so that the tissue pieces gather at the very bottom of the pipette tip in a bubble of media as seen in Fig. 3A. Gently touch the media/tissue bubble that bulges out of the tip (not the actual pipette tip as this could perforate the transwell and result in flooding of the tissues) to release a droplet with minimal media and tissue pieces in the center of the well.

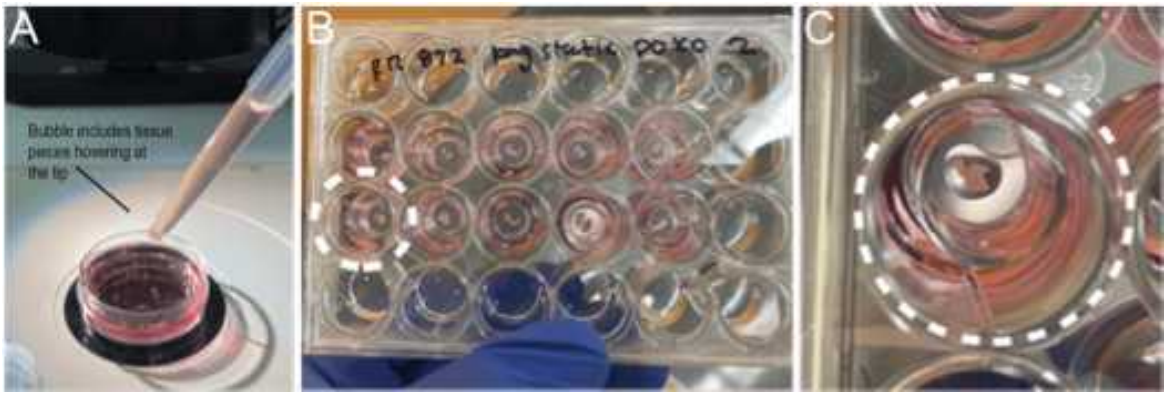

Fig. 3. Ovarian explant culture set up. Ovarian tissue pieces are loaded in groups of three using a cut 1000 µL pipette tip that has been filled with media and tissues (A). A fully prepared ovarian tissue plate has 400 µL of growth media per well and 400 µL of PBS in wells without tissue (B). Tissue should be resting on a membrane with a small droplet of growth media covering the tissue, but not spread to the walls of the insert, this is a representative well with 3 pieces of ovarian medulla tissue (C).

17 If necessary, remove any excess media using a 200 µL pipette making sure to never touch the transwell with the pipette tip as it may perforate the membrane.

18 After placing the ovarian pieces into the transwell the tissue should be resting in the center of the wells with a small droplet of media covering the tissues in each well of a 24-well plate (Fig. 3B-C). The droplet should not exceed 50 µl and should not extend more than 1-2 mm beyond the tissue edges.

19 Culture the tissues at 37°C with 5% CO2 (atmospheric O2 about 21%)for anywhere from one to eleven days.

20 For induced senescence with doxorubicin, doxorubicin is added to the culture media at a concentration of 0.01 µg/ml (Fisher Scientific, 22-521-0) for the first 24 hours of culture, regardless of the culture endpoint.

21 After 24 hours, remove the plate from the incubator and wash twice with standard growth media. Washes can be performed by lifting the transwell with curved forceps, make sure not to puncture the membrane when lifting. Then remove the full volume of media, ~400 µL. Replace with 400 µL of equilibrated growth media without doxorubicin. Repeat twice and then return to the incubator for the required time points. Ensure at this point that the droplet is intact, if the well has flooded, remove excess media with a 200 µL pipette without touching the surface of the membrane. If the tissue is dry add a small droplet of media, no more than 50 µL in volume.

22 For the static plate, half-volume changes of media should be performed every other day (200 µL) in each well that contains tissue. This can be done as detailed in step 10.

23 For the LATTICE plate, daily media changes/checks should be made as follows:

1. All media from the acceptor well should be removed and collected in pre-weighed tubes (for measurement of flow and other downstream analysis).
2. Donor wells should be replenished to full volume (1.5 mL)
3. Donor wells should be replenished to full volume (1.5 mL) in order to continue culture:
   i. Cotton balls under the used valves should be checked and replaced if necessary (In case of flooding or if the cotton balls are soaked).
   ii. Microfluidic channels should be checked to ensure they are empty.
   iii. The volume of culture media in the wells should be measured and media should either be removed or added to reach 400 µL.

24 When the culture period ends, remove the tissues from the inserts by carefully peeling away the mesh bottom with sterile forceps. With curved forceps, hold the edge of a transwell and with straight forceps, pierce the edge of the membrane so it breaks away from the plastic edge. Peel the remaining

membrane away from the edge.

25 Add the mesh membrane with the tissue pieces adhered to the center to a 1.5 mL microcentrifuge tube with 1 mL of Modified Davidson's Fixative Solution (Cat.#64133-50; Electron Microscopy Sciences, Hatfield, PA). If freezing the tissues, place the mesh bottom into a dish of PBS and then carefully move the tissue pieces to a dry 1.5 mL microcentrifuge tube using a similar technique used to load the tissues onto the wells: take up some PBS and tissue (100-200µL) into the cut tip of a 1000 µL pipette tip. Expel the contents into a dry 1.5 mL microcentrifuge tube and then remove excess PBS with a 200 µL pipette tip before placing them on dry ice. Store at -80°C.