Journal of Visualized Experiments

Fertility preservation in patients with severe ovarian dysfunction.

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE62098R1
Full Title:	Fertility preservation in patients with severe ovarian dysfunction.
Corresponding Author:	Kazuhiro Kawamura International University of Health and Welfare Narita, Chiba JAPAN
Corresponding Author's Institution:	International University of Health and Welfare
Corresponding Author E-Mail:	kazuhironanami@gmail.com
Order of Authors:	Yuta Kawagoe
	Kazuhiro Kawamura
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	

TITLE:

Fertility preservation in patients with severe ovarian dysfunction

AUTHORS AND AFFILIATIONS:

Yuta Kawagoe[1], Kazuhiro Kawamura[1]

[1]Advanced Reproduction Medicine Research Center, Department of Obstetrics and Gynecology, International University of Health and Welfare School of Medicine, Narita, Japan

Email address of co-authors
y-kawagoe@iuhw.ac.jp

Corresponding author:
kazuhironanami@gmail.com

KEYWORDS:

Drug-free IVA, fertility preservation, follicle growth, Hippo signaling, infertility, In-vitro activation, ovarian dysfunction

SUMMARY:

We present details of laboratory procedures for drug-free in vitro activation (IVA) of ovarian follicles for patients with severe ovarian dysfunction. This method could increase the number of retrievable oocytes per ovarian hyperstimulation and benefit fertility preservation for those patients.

ABSTRACT:

Ovarian function progressively declines during aging and in some pathophysiological conditions including karyotype abnormality, autoimmune diseases, chemo- and radiation-therapies, as well as ovarian surgeries. In unmarried women with severe ovarian dysfunction, fertility preservation is important for future pregnancies. Although oocyte cryopreservation is an established method for fertility preservation, these patients could only preserve a limited number of oocytes even after ovarian hyperstimulation, leading to repeated stimulations to ensure sufficient oocytes to guarantee future pregnancy. To solve this issue, we have recently developed a drug-free in vitro activation (IVA) procedure, which enable us to stimulate early stages of ovarian follicles to develop to the preantral follicle stage. These preantral follicles can respond to the unique protocol of gonadotropin stimulation, resulting in increased number of retrieved oocytes per ovarian stimulation for cryopreservation. The drug-free IVA comprised from the surgical approach and ovarian stimulation. We removed a part of cortex from one or both ovaries from patients under laparoscopic surgery. The ovarian cortical tissues were cut into small cubes to disrupt the Hippo signaling pathway and stimulate the development of early stage follicles. These cubes were grafted orthotropically into remaining ovaries as well as beneath the serosa of both Fallopian tubes. We have already published the surgical procedure of the drug-free IVA and the protocol of subsequent ovarian stimulation, but herein we present the details of laboratory methods required for drug-free IVA.

INTRODUCTION:

Ovarian function declines progressively during aging and some pathophysiological conditions including karyotype abnormality, autoimmune diseases, chemo- and radiation-therapies, and ovarian surgeries. Fertility preservation is one of the best options for unmarried women with severe ovarian dysfunction to preserve their potential for future pregnancy. For fertility preservation, currently two methods are available mostly in the onco-fertility field. Oocyte cryopreservation is a well-established procedure for fertility preservation and many successful cases have been reported[1,2]. On the other hand, ovarian tissue cryopreservation was also established for fertility preservation in cancer patients but it is still an experimental strategy[3,4]. In both methods, multiple numbers of mature oocytes are needed for pregnancy to occur. Generally, patients with premature ovarian insufficiency (POI), who become amenorrhea before 40 years of age, and middle-aged women with low ovarian reserve showed poor ovarian response (POR) to ovarian stimulation for yielding mature oocytes[5-7]. Furthermore, young patients with a low number of antral follicles also showed POR to the ovarian stimulation[7]. These patients have very limited number of retrievable oocytes even after proper ovarian hyperstimulation, thus requiring multiple expensive procedures to ensure a sufficient number of oocytes for pregnancy.

Oocyte donation followed by in vitro fertilization (IVF) with husband's sperm and embryo transfer (ET) is the only option for these POI and POR patients who have difficulties obtaining their own oocytes[8-10]. However, oocyte donation is complicated by ethical issues as well as autoimmune and pregnancy complications[11-14]. To solve these issues, the establishment of infertility treatment using patients' own oocytes is desired. For POI patients, we have developed the in vitro activation (IVA) approach to allow successful follicle growth and the generation of mature oocytes, leading a number of pregnancies and deliveries[15]. In IVA, we fragmented ovarian cortexes after removal of ovaries under laparoscopic surgery and cultured them for two days to activate follicles by Akt-stimulating drugs and then perform heterotopic grafting back into artificial pouches made beneath the serosa of Fallopian tubes under second laparoscopic surgery[15]. This procedure promoted growth of primordial, primary and secondary follicles after ovarian cortex fragmentation to promote Hippo signaling disruption[16], followed by two days culture with Akt signaling stimulators[17].

In contrast to severe POI cases, POR patients with decreased ovarian reserve have multiple secondary follicles. Because Hippo signaling disruption alone is effective in promoting secondary follicle growth[16], we recently demonstrated successful pregnancies and deliveries for POR patients using the drug-free IVA procedure involving cortical fragmentation and orthotopic grafting without treatment of the Akt stimulating drugs. Drug-free IVA stimulates early stage of ovarian follicles to develop to the preantral follicle stage after only one surgery and increased the number of retrieved oocytes for IVF-embryo transfer[15,18]. The drug-free IVA approach has several advantages as compared with our original IVA by 1) avoiding potential follicle loss during culture, 2) minimizing invasiveness and costs of the second surgery, 3) involving only short-term post-surgery bed rest and 4) potential of spontaneous pregnancy due to orthotopic grafting. We recently published a video article showing the surgical procedures of drug-free IVA[19] and detailed protocols of ovarian stimulation after the surgery[20]. Here, we present details of laboratory

methods required for drug-free IVA.

PROTOCOL:

Written informed consent was obtained from each POR patient with diminishing ovarian reserve who enrolled in the drug-free IVA treatment. This study was approved by ethical committee of International University of Health and Welfare (No. 17-S-21). Clinical trial was registered under number UMIN000034464 and carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

1. Ovarian cortex extraction

1.1 Remove part of ovarian cortex (10 x 10 mm, 2-3 mm thickness) from one or both ovaries under general anesthesia through laparoscopic surgery as previous described[19,20].

1.2 Place the collected ovarian cortexes in a sterile container containing 10 mL of modified HTF (mHTF) at 37 °C.

2. Ovarian cortex fragmentation

NOTE: Prior to dissection of the ovarian cortex, warm up mHTF to 37 °C. Maintain sterile conditions throughout the procedure. All tools for this procedure are listed in **Table 1**.

2.1 Place the collected ovarian cortexes in a plastic dish containing 5-10 mL of mHTF at 37 °C and place them on the heat plate to maintain the tissue at 37 °C (**Figure 2A**).

2.2 Take a picture of each cortex using a digital camera with patient's name before starting next procedure to link patient background data.

2.3 Place an individual cortex on a sterile gauze moistened with mHTF (**Figure 2B**).

NOTE: Apply additional mHTF with a disposable pipette on the gauze to prevent the surface of cortex being dried out during this procedure.

2.4 Remove residual medulla tissue where looks pink using a micro-scissors so that thickness of tissue reaches 1-2 mm (**Figure 2C and D**).

NOTE: To avoid the loss of follicles at early stage, do not prepare the cortical strips less than 1 mm in thickness, because the early-stage follicles are located within 1-2 mm from the surface of ovarian cortex[21].

2.5 Dissect a 1 mm x 1 mm x 5 mm of tissue piece from each ovarian cortical strip using a fine scalpel and subject it to histological analysis to determine the presence of residual follicles as previous described[18] (**Figure 2E**).

NOTE: The dissected tissues for histology are immersed in mHTF and keep at 4 °C until fixing (**Figure 2F**, see Step 4).

2.6 Cut the cortex into 1mm x 1 mm x 10 mm strips using a fine scalpel (**Figure 2G**).

NOTE: It is easier to cut by pressing down on the scalpel than by pulling on it.

2.7 Cut these tissue strips into 1 mm x 1 mm x 1 mm small cubes (**Figure 2H**).

NOTE: To avoid changes of osmotic pressure in the medium, add mHTF in moderation before tissue autografting.

3. Auto-grafting of ovarian cortical fragments

3.1 Open the package of the IVA cannula and put it on a sterile drape.

3.2 Rinse inside of the IVA cannula with mHTF by aspirating and discharging mHTF several times.

3.3 Aspirate 100-200 µL of mHTF to fill the tip of IVA cannula to avoid the cortical cubes from drying out during loading (**Figure 3A**).

3.4 Load the cortical cubes into the tip of IVA cannula using a fine tweezer (**Figure 3B-E**).

NOTE: The number of cortical cubes for loading depends on the grafting site. Generally, 20-30 cubes can be transplanted into an orthotropic remaining ovary, whereas 10-15 cubes can be transplanted into a pouch beneath the serosa of the Fallopian tube.

3.5 After loading the cortical cubes, hold the tip of the cannula containing the cortical cubes by the fingers until transferring the IVA cannula to a surgeon. This will avoid the cube temperature to decreases.

NOTE: The details of autografting procedure under laparoscopic surgery have been reported previously[19]. For grafting ovarian cortical cubes, a cannula-shaped device (IVA cannula) was developed by our collaborator, the Kitazato Corporation.

4. Histological analysis of ovarian cortex

NOTE: To count the number of residual follicles, perform histological analysis as previous described[15].

4.1 Mark the cortical strip surface with a tissue marking dye before sample fixation. This will indicate the side of cortex in strip for histology.

4.2 Fix the tissue strips overnight at 4 °C using Bouin's solution.

NOTE: To detect atretic follicles, we do not recommend using paraformaldehyde for fixing to avoid shrinkage of oocyte cytoplasm.

4.3 Dehydrate and embed the tissue strips in paraffin.

4.4 Serially section the paraffin block containing the tissue sample.

4.5 Stain each section using hematoxylin and eosin to visualize the follicles.

4.6 Count the follicles as previously described[17].

REPRESENTATIVE RESULTS:

In the first publication of the IVA approach[15], we transplanted the ovarian cortical cubes one by one into the grafting sites under laparoscopic surgery (**Figure 1A**). Because 100-150 ovarian cubes were used, it took 3-4 hours for tissue grafting under laparoscopic surgery. Also, some ovarian cubes were lost before grafting. Because the IVA cannula could transfer 20-30 cubes to a grafting site at one time (**Figure 1B**), we could shorten the operation time to 1-2 hours. Furthermore, the IVA cannula could eliminate the loss of ovarian cortical cubes during surgery.

FIGURE AND TABLE LEGENDS:

Figure 1: The effectiveness of the IVA cannula. (**A**) Previous method with transplanting ovarian cortical cubes one by one using a forceps. (**B**) The current method with transplanting 20-30 cubes at one time using the IVA cannula.

Figure 2: Ovarian tissue dissection for drug-free IVA. (**A**) A piece of ovarian cortex is placed in a plastic dish containing mHTF at 37 °C. (**B**) To remove residual medulla tissues, the ovarian cortex is placed on a moistened gauze. (**C**) Medulla tissue is removed using a fine micro-scissor. (**D**) The thickness of cortex to be prepared is between 1 to 2 mm. (**E**) After removal of medulla tissue, a 1 mm x 1 mm x 5 mm of tissue piece from each ovarian cortical strip is dissected for histological analysis. (**F**) The dissected tissue is stored in 1.5 mL tube containing mHTF at 4 °C until fixing for histology. (**G** and **H**) The ovarian cortex is cut into 1 mm x 1 mm x 10 mm strips, and then further cut into 1 mm x 1 mm x 1 mm small cubes using a fine scalpel.

Figure 3: Auto-grafting of ovarian cortical fragments. (**A**) Before loading cubes, 100-200 µL of mHTF is aspirated to fill the tip of the IVA cannula to avoid the cortical cubes from drying out during loading (**B-E**) Loading ovarian cortical cubes into the tip of IVA cannula.

DISCUSSION:

In this manuscript, we showed a detailed laboratory protocol for drug-free IVA. The drug-free IVA is a new approach of infertility treatment for POR patient with diminishing ovarian reserve to promote secondary follicles growth, resulting in yielding more mature oocytes after ovarian

stimulation and increasing in successful pregnancy[20]. In 15 POR patients with diminishing ovarian reserve, this approach achieved one spontaneous pregnancy, in vitro fertilization-embryo transfer allowed four live births, together with one ongoing pregnancy[19].

For preparation of grafting ovarian cortex, medulla tissue was removed using a micro-scissors with 1-2 mm of thickness. Previously, we demonstrated that primordial, primary and majority of secondary follicles were located within 1 mm thickness from the surface of ovarian cortex in the patients with normal ovarian reserve[21]. In POI patients, we found that secondary follicles were located deeper than 1 mm but within 2 mm from the surface of ovarian cortex[21]. Antral follicles are known to locate in medulla tissues[21], but no follicle was found in medulla tissues in POI patients[21]. Although POR patients with diminishing ovarian reserve could have antral follicles in medulla tissue, antral follicles are difficult to survive after grafting due to lack of blood vessel communication[22]. Therefore, we determined the thickness of ovarian cortex to be 1-2 mm and the critical step within the protocol is not making the cortical strips < 1-2 mm to avoid loss of precious residual follicles. During preparation of cortical strips and cubes, we used mHTF, but one can use similar medium which include 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer.

In this procedure, we transplanted ovarian cortex after cutting it into 1 mm^3 cubes[15,20]. Due to the difficulty in handling such small fragments for grafting, previous grafting ovarian cubes one by one under the laparoscopic surgery took 3-4 hours with some cubes occasionally got lost during surgery. After development of the IVA cannula, we could shorten the operation time to 1-2 hours, resulting in minimizing the invasiveness of the surgery to allow the drug-free IVA becoming a single day-surgery. Moreover, we could eliminate the loss of ovarian cortical cubes during surgery. Because of limited number of follicles in ovarian cortex in POR patients with diminishing ovarian reserve, it is important to avoid loss of ovarian cortical cubes. If the IVA cannula is not available, you may try to find a similar cannula to be able to use under laparoscopic surgery.

The decline of ovarian function is progressively caused by aging and some pathophysiological conditions [23-25]. Thus, in unmarried women with severe ovarian dysfunction, fertility preservation is important to allow future pregnancy. For these patients, the methods of oocytes or ovarian tissue cryopreservation were reported[26-29]. Although oocyte cryopreservation is an established method for fertility preservation[1,2], these patients could have very limited number of retrievable oocytes even after ovarian hyperstimulation due to low ovarian reserve. Furthermore, in aging patients with ovarian dysfunction, high incidence of chromosome abnormalities was reported in ovulated oocytes[26,30]. This leads to the requirement of multiple expensive procedures to ensure sufficient number of oocytes to guarantee pregnancy. To solve these problems, we have developed the drug-free IVA approach for increasing the number of mature oocytes for oocyte retrieval. Because oocyte cryopreservation is no longer an experimental method[31,32], the drug-free IVA followed by oocyte cryopreservation is expected to be a promising method for fertility preservation in unmarried women with severe ovarian dysfunction. However, future clinical studies using the freeze-thawed oocytes obtained from the drug-free IVA will be required to determine the effectiveness of this approach for fertility preservation in unmarried women with

severe ovarian dysfunction.

In conclusion, we developed the drug-free IVA as a new approach of infertility treatment for POR patient with diminishing ovarian reserve and showed the detail laboratory protocol to be able to repeat our procedures. The methodological limitations of drug-free IVA are difficulty in prediction of residual follicle number before the surgery and also inability to evaluate the graft survival. The laboratory technique of drug-free IVA could become the basis of future application of ovarian tissue culture to obtain mature oocytes without grafting.

ACKNOWLEDGMENTS:

We thank Tatsuji Ihana, Sachiyo Kurimoto, Kazuko Takahasih, Yuki Yoshizawa, Maho Arashi, Kentaro Fujita, Erina Kudo, Yuka Kurimoto and Mayuko Wakatsuki for supporting the drug-free IVA procedure and Prof. Aaron J.W. Hsueh (Stanford University School of Medicine, Stanford, CA) for critical reading and editing of the manuscript. We also thank Rebecca Truman and Gregory Truman for inserting English narration. This study was supported by The Japan Society for the Promotion of Science (JSPS), Scientific Research B (19H03801), and Challenging Exploratory Research (18K19624).

DISCLOSURES:

The authors confirm that they have no conflict of interest.

REFERENCES:

1 Liang, T., Motan, T. Mature Oocyte Cryopreservation for Fertility Preservation. *Advances in Experimental Medicine and Biology.* **951**, 155-161 (2016).

2 Yoon, T. K. et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertility and Sterility.* **79** (6), 1323-1326 (2003).

3 Donnez, J. et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet.* **364** (9443), 1405-1410 (2004).

4 Meirow, D. et al. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. *New England Journal of Medicine.* **353** (3), 318-321 (2005).

5 De Vos, M., Devroey, P., Fauser, B. C. Primary ovarian insufficiency. *Lancet.* **376** (9744), 911-921 (2010).

6 Scott, R. T. et al. Follicle-stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome. *Fertility and Sterility.* **51** (4), 651-654 (1989).

7 Ferraretti, A. P. et al. ESHRE consensus on the definition of ‘poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria†. *Human Reproduction.* **26** (7), 1616-1624 (2011).

8 Huhtaniemi, I. et al. Advances in the Molecular Pathophysiology, Genetics, and Treatment of Primary Ovarian Insufficiency. *Trends in Endocrinology and Metabolism.* **29** (6), 400-419 (2018).

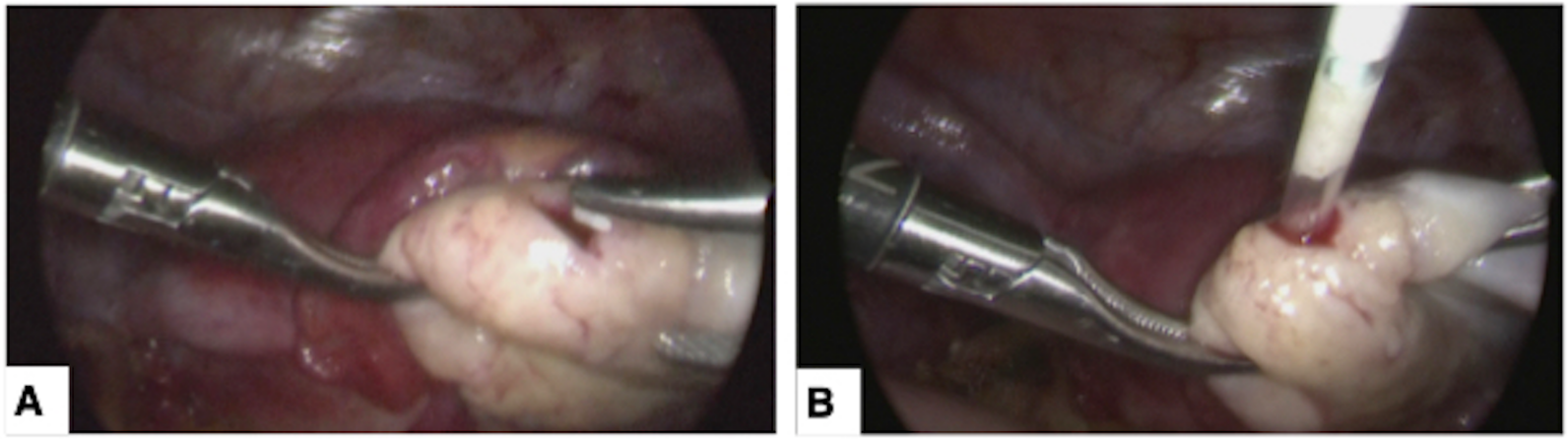
9 Męczekalski, B., Maciejewska-Jeske, M., Podfigurna, A. Reproduction in premature ovarian insufficiency patients - from latest studies to therapeutic approach. *Prz Menopauzalny.* **17** (3), 117-119 (2018).

10 Baker, V. Life plans and family-building options for women with primary ovarian

insufficiency. *Seminars in Reproductive Medicine.* **29** (4), 362-372 (2011).
11 Englert, Y., Govaerts, I. Oocyte donation: particular technical and ethical aspects. *Human Reproduction.* **13 Suppl 2**, 90-97 (1998).
12 Englert, Y., Rodesch, C., Laruelle, C., Govoerts, I. [Oocyte donation: ethical aspects related to the donor]. *Contraception, Fertilite, Sexualite (1992).* **25** (3), 251-257 (1997).
13 Storgaard, M. et al. Obstetric and neonatal complications in pregnancies conceived after oocyte donation: a systematic review and meta-analysis. *BJOG: An International Journal of Obstetrics and Gynaecology.* **124** (4), 561-572 (2017).
14 Storgaard, M., Malchau, S., Loft, A., Larsen, E., Pinborg, A. [Oocyte donation is associated with an increased risk of complications in the pregnant woman and the fetus]. *Ugeskrift for Laeger.* **179** (11) (2017).
15 Kawamura, K. et al. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. *Proceedings of the National Academy of Sciences.* **110** (43), 17474-17479 (2013).
16 Hsueh, A. J., Kawamura, K., Cheng, Y., Fauser, B. C. Intraovarian control of early folliculogenesis. *Endocrine Reviews.* **36** (1), 1-24 (2015).
17 Li, J. et al. Activation of dormant ovarian follicles to generate mature eggs. *Proceedings of the National Academy of Sciences of the United States of America.* **107** (22), 10280-10284 (2010).
18 Suzuki, N. et al. Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency. *Human Reproduction.* **30** (3), 608-615 (2015).
19 Tanaka, Y., Hsueh, A. J., Kawamura, K. Surgical approaches of drug-free in vitro activation and laparoscopic ovarian incision to treat patients with ovarian infertility. *Fertility and Sterility.* doi:10.1016/j.fertnstert.2020.07.029, (2020).
20 Kawamura, K., Ishizuka, B., Hsueh, A. J. W. Drug-free in-vitro activation of follicles for infertility treatment in poor ovarian response patients with decreased ovarian reserve. *Reproductive Biomedicine Online.* **40** (2), 245-253 (2020).
21 Haino, T. et al. Determination of Follicular Localization in Human Ovarian Cortex for Vitrification. *Journal of Adolescent and Young Adult Oncology.* **7** (1), 46-53 (2018).
22 Baird, D. T., Webb, R., Campbell, B. K., Harkness, L. M., Gosden, R. G. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196 C. *Endocrinology.* **140** (1), 462-471 (1999).
23 Qin, Y., Jiao, X., Simpson, J. L., Chen, Z. J. Genetics of primary ovarian insufficiency: new developments and opportunities. *Human Reproduction Update.* **21** (6), 787-808 (2015).
24 Domniz, N., Meirow, D. Premature ovarian insufficiency and autoimmune diseases. *Best Practice & Research: Clinical Obstetrics & Gynaecology.* **60**, 42-55 (2019).
25 Laven, J. S. Primary Ovarian Insufficiency. *Seminars in Reproductive Medicine.* **34** (4), 230-234 (2016).
26 Grynberg, M. et al. Fertility preservation in Turner syndrome. *Fertility and Sterility.* **105** (1), 13-19 (2016).
27 Tomao, F., Spinelli, G. P., Panici, P. B., Frati, L., Tomao, S. Ovarian function, reproduction and strategies for fertility preservation after breast cancer. *Critical Reviews in Oncology/Hematology.* **76** (1), 1-12 (2010).
28 Kim, S., Lee, Y., Lee, S., Kim, T. Ovarian tissue cryopreservation and transplantation in patients with cancer. *Obstetrics & Gynecology Science.* **61** (4), 431-442 (2018).

29 Donnez, J., Dolmans, M. M. Ovarian tissue freezing: current status. *Current Opinion in Obstetrics and Gynecology.* **27** (3), 222-230 (2015).
30 Wu, R. C., Kuo, P. L., Lin, S. J., Liu, C. H., Tzeng, C. C. X chromosome mosaicism in patients with recurrent abortion or premature ovarian failure. *Journal of the Formosan Medical Association.* **92** (11), 953-956 (1993).
31 De Munck, N., Vajta, G. Safety and efficiency of oocyte vitrification. *Cryobiology.* **78** 119-127 (2017).
32 Argyle, C. E., Harper, J. C., Davies, M. C. Oocyte cryopreservation: where are we now? *Human Reproduction Update.* **22** (4), 440-449 (2016).

Figure 1



Figure

Click here to access/download;Figure;Figure 2.pdf

Figure 2

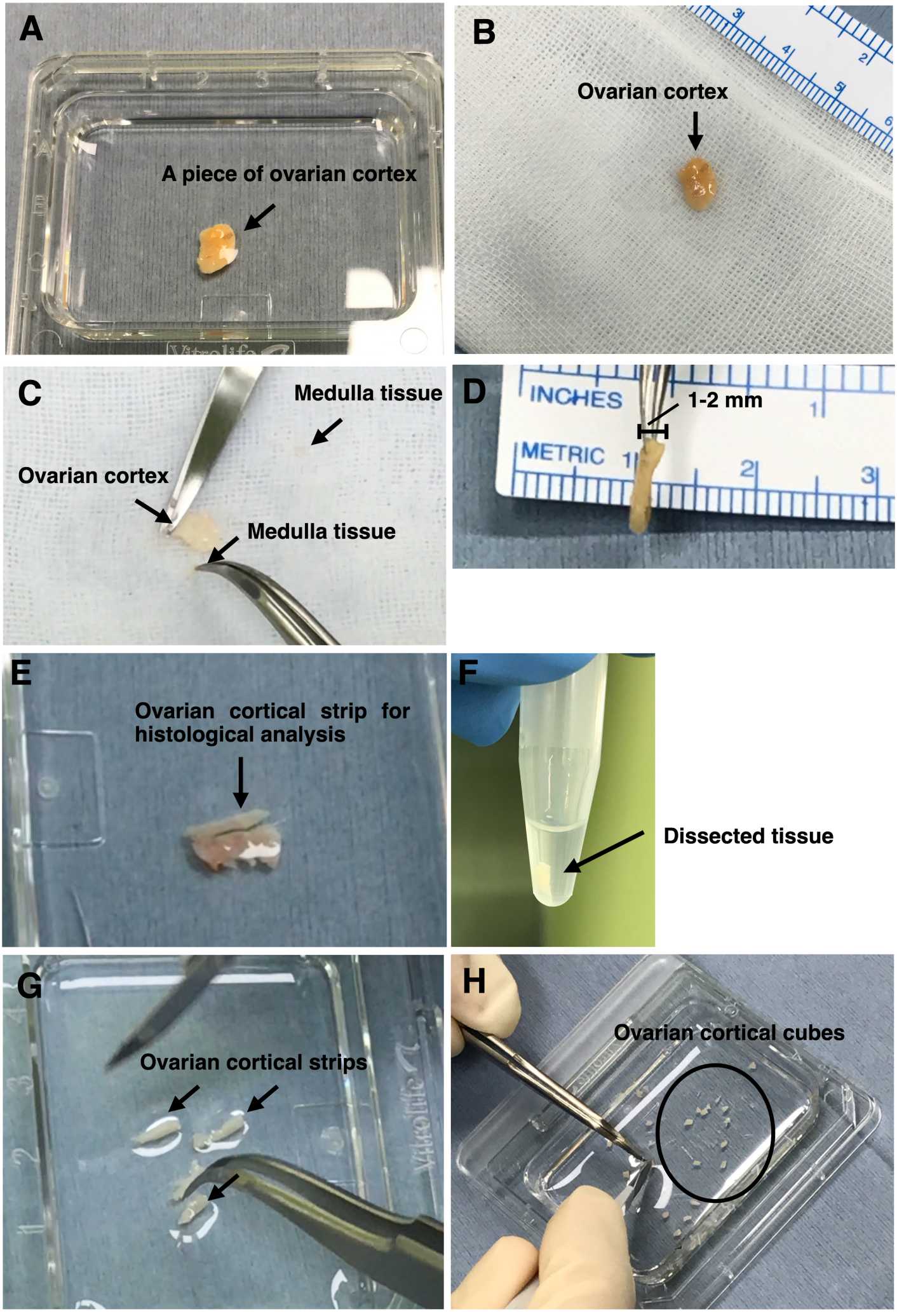


Figure 3

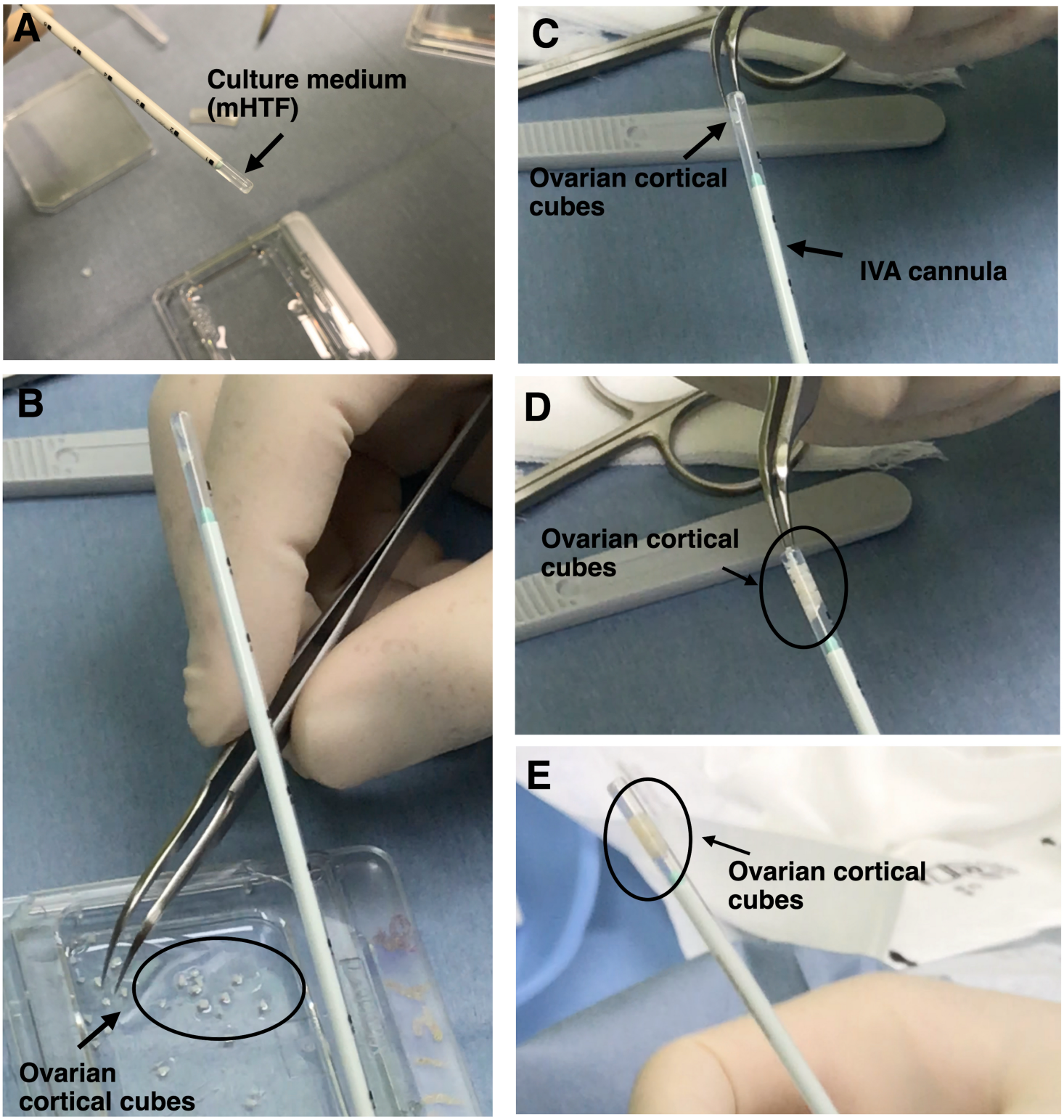


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials table.xlsx

Name of Material/ Equipment	Company	Catalog Number
4.5 onz specimen container	FALCON	354013
60mm dish	FALCON	351007
50 x 50 cm sterile drape	HOGY Medical	SR-823
Disposable pippete	FALCON	357575
Fine scissors, Curved	WPI	#14224-G
Hot plate	TOKAI HIT	TPiE-SP
Human Serum Albumin Solution	Irvine Scientific	9988
IVA cannule	KITAZATO	446030 IVA-6030E
KAI medical Disposable scalpel	WPI	#5 10-A
Micro scissors, Curved	WPI	#503364
Modified HTF Medium-HEPES	Irvine Scientific	90126
Sterile gauze	Osaki Medical	15004
Swiss Tweezers, Curved Tips	KAI	#504505

Comments/Description
Other products may also be suitable
Other products may also be suitable
Any type of sterile produsts may also be suitable
Other products may also be suitable
Although other products may also be suitable, we strongly recommend use this products
Use at operation room to maintain the temperature of dishes containing ovarian tissue before transplantation
Medium for handling ovarian tissue
Specific cannula for tissue autografting
Although other products may also be suitable, we strongly recommend use this products
Although other products may also be suitable, we strongly recommend use this products
Medium for handling ovarian tissue
Any type of sterile produsts may also be suitable
Although other products may also be suitable, we strongly recommend use this products

Editorial and production comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

-We have proofread the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points.

-We have changed the style of manuscript accordingly.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

- We have amended the protocol as suggested.

4. For the protocol steps please ensure you answer the “how” question, i.e., how is the step performed?

- We now amended the manuscript accordingly.

5. 2.2.: Do you take pictures under a microscope?

- Due to the large size of cortex, we took pictures by using a digital camera. We now clarified this point in the protocol.

6. 2.4: How do you visually identify the medulla?

- **We identified the medulla tissue based on the difference of color. Because of rich blood vessels in the medulla, the medulla looks pink, whereas the cortex looks white. We now added this information in the protocol.**

7. 2.5: How do you dissect? Please include a citation for the determination of residual follicle via histological analysis.

- We dissected the cortical tissue using a fine scalpel. We now clarified this point in the protocol. We also included the citation as suggested.

8. 3.2: How do you rinse the inside of the cannula?

- We rinsed the inside of the IVA cannula by aspirating mHTF and then discharging it several times. We now added this information in the protocol.

9. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data. Previously published figures can be used in this case, if reprint permission is obtained.

- We have added new figure 1 showing the previous method with transplanting ovarian cortical cubes one by one using a forceps and the current method with transplanting 20-30 cubes at one time using the IVA cannula. Due to adding this new figure, we have changed the labelling of figure 1 and 2 to be figure 2 and 3, respectively.

10. The Representative Results section should be written in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.
- In this manuscript, we are showing the laboratory technique of IVA to allow physicians/embryogists could repeat our procedure for patients with ovarian dysfunction. Therefore, we represented the results related to laboratory technique of IVA.

11. If using figures from previously published literature, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
- We have not used figures from previously published literature.

12. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
- Thank you for your important suggestions. Following these suggestions, we have amended the discussion section.

13. Please do not abbreviate the journal titles in the references section.
- We have corrected them accordingly.

14. Please sort the materials table in alphabetical order.
- We have sorted the materials table accordingly.

Video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
- We have modified them accordingly.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.
- We have revised the narration accordingly.

3. Please ensure that the subheadings in the protocol section of the text and in the video are the same e.g., the first subheading in the text is ovarian tissue dissection and in the video - it is ovarian tissue fragmentation, etc.
- We have corrected the subheadings accordingly.

4. For the protocol section please have the narration in imperative tense as written in the text.

- We have revised the narration accordingly.

5. Please do not include the step number in the video, these do not match with the protocol text. Only chapter subheadings are enough (e.g., ovarian tissue dissection, auto grafting of ovarian cortical fragments, histological analysis of ovarian cortex) to match the text in the video.
- We have corrected the subheadings accordingly.

6. The audio levels are too loud. Please reduce the overall volume to 50% of what it currently is. (Or adjust by approximately -6 dB).
- We have decreased the audio levels accordingly.

7. 02:48: The graphics of the ovary and ovarian cortical tissue are very close to the edge of the screen. Consider moving the graphics a little further from the edge to give a small margin around the important information.
- We have modified the graphics accordingly.

8. 04:04: There is a series of breathy sounds here that could be cut out with some audio editing.
- We are sorry about it. Now, we removed the breathy sounds.

9. 05:10: The word "at" is trimmed a little too closely here and sounds cut-off at the beginning. Try adjusting the in-point for this audio clip back a nudge to capture the beginning of the 'a' sound.
- We have revised it accordingly.

10. 7:44, 10:18: Please include footage to go with the narration.
- We have modified the footage accordingly.

11. Please make representative results and conclusions as separate chapters in the video. Please ensure that the representative result includes a table or a figure to show the efficacy of the technique presented. Figures and tables can be reprinted from previously published literature after obtaining the reprint permission.
- We now prepared the representative results and conclusions as separate chapters in the video.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The laboratory procedures for drug-free IVA procedure to be performed in patients with severely diminished ovarian reserve has been described in the current manuscript.

Major Concerns:
None.

Minor Concerns:
None.

- We thank the reviewer for positive comments.

Reviewer #2:
Manuscript Summary:
JoVE62098
The authors developed the drug free method for treatment of women with ovarian insufficiency not due to cancer. This was done by cutting the ovaries to small pieces and IVA by the hypo stimuli signal. The paper describes the lab methods of this procedure. The paper is interesting for people in this field especially the embryologist in the lab.

Major Concerns:
My only problem is that I could not see the Figures in the reviewer draft I received. I think this paper can be easily accepted if the figures are visible. Maybe the journal can send me the figures seperatly.

Minor Concerns:
None

Comments on video:
I recommend publication

- We thank the reviewer for positive comments.

Reviewer #3:
Manuscript Summary:
In this manuscript, Kagawoe & Kawamura describe the protocol applied for the drug-free IVA ovarian stimulation in poor ovarian response patients. While the protocol is written in detail, there are some few points that need to be improved.
- We thank the reviewer for positive comments.

Minor Concerns:

- Replace "removal of part" (L105) by "remove part".

- We have corrected it.

- How thick should be this 10x10 mm strip (L105)?
- **The thickness of strip is 2-3 mm. We now included this information in the protocol.**

- Replace "stripes" (L113, L118) by “strips".
- Replace "stipe" (L116) by “strip”.

- Thank you for the detail check-up. Instead of strip or strips, “cortex” is more appropriate word and thus we modified them as “cortex or cortexes”.

- Replace "stripe" (L126) by “strip".
- Replace "is depending" (L146) by “depends".
- **- We have corrected these typos.**
-
- L150-152: Hold the cannula containing the cortical cubes by the fingers until transferring the IVA cannula to a surgeon. This will avoid the cube temperature to decrease.

- L158-159: To count the number of residual follicles, histological analysis should be performed as previously described15.
- L160-161: Mark the cortical strip surface with a tissue marking dye before sample
- **We now amended these suggested parts of text accordingly.**

- L161: What do the authors mean by "strip orientation”?
- **It means the cortex on one side and the medulla on the other side. We now modified the text to clarify it.**

- L162: Fix the tissue strips overnight at 4°C using Bouin's solution.
- L165-167:

4.3 Dehydrate and embed the tissue strips in paraffin.
4.4 Serially section the paraffin block containing the tissue sample.
4.5 Stain each section using hematoxylin and eosin to visualize the follicles?
4.6 Count the follicles as previously described17.

- Replace "DOR" (L216) by “POR”.
- **We now amended the sentence as instructed. For staining, we used hemetoxylin and eosin.**

- Discussion: please add the number of babies born using this procedure in POR patients.
- **According to the suggestions, we added the number of babies born using drug-free IVA in POR-DOR patients.**
-

Dr. Kyle Jewhurst,
Science editor, JoVE
Dr. Federica Franciosi,
Guest editor, JoVE

Re: JoVE62098

Dear Drs. Jewhurst and Franciosi:

We greatly appreciate the thorough review by the referees and editor on our video manuscript, entitled "Fertility preservation in patients with severe ovarian dysfunction."

Following their suggestions, we have amended the protocol, results and discussion. We have also added a new figure (Figure 1) and revised the video.

We believe that we have fully addressed the comments of the reviewers and the supervising editor. Our point by point responses to their comments are addressed below and all changes are highlighted with yellow in the revised manuscript.

We hope that the revised version of our manuscript now meets the standards required for possible publication in JoVE.

Sincerely yours,

Kazuhiro Kawamura, M.D., Ph.D.,

Department of Obstetrics and Gynecology, International University of Health and Welfare Graduate School Medicine, Narita, Chiba, 286-8686, Japan
Tel.: +81-90-2252-7169
E-mail: kazuhironanami@gmail.com