Title

Retrieval and *in vitro* maturation of human oocytes from ovaries removed during surgery for endometrial carcinoma: a novel strategy for human oocyte research

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Concise and informative title

Human oocytes from surgically resected ovaries

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Structured Abstract

Purpose To collect human oocytes from ovaries removed as part of surgical treatment for endometrial carcinoma, and to induce *in vitro* maturation of those oocytes to obtain material for research on human ovarian aging.

Methods Design: Prospective clinical study. Setting: University Hospital. Patients: 8 patients, aged 35 - 44 years old with a preoperative diagnosis of Stage I endometrial cancer agreed to participate in this project. Interventions: Surgically-removed ovaries were punctured; oocytes were collected from follicular fluid and matured *in vitro*. Immunofluorescent detection of microtubules and DNA labeling were performed after *in vitro* maturation. Main Outcome Measures: Number of oocytes collected and their *in vitro* maturation stage.

Results 87 oocytes were collected, 11 of which had completed metaphase II. 75% of oocytes were collected from 3 patients in their aged 30s, while the remaining 25% were obtained from the 5 patients in their aged 40s. We could collect several stage of oocyte and the detection of the arrangement of oocyte microtubules and chromatin in several stages by fluorescence was possible.

Conclusion Material for research on human ovarian aging can be obtained from ovaries removed during surgery for endometrial cancer.

Key Words

Human oocytes, *in vitro* maturation, microtubules

Manuscript

Introduction

The axiom "Oocyte quality controls almost all of a couple's reproductive potential" is widely accepted in reproductive medicine. Oocyte quality begins to decrease after the age of 30 years, and drops rapidly after the age of 35 years [6, 7]. It was showed that the meiotic spindle in older woman is frequently abnormal [1], and the age-associated oocyte aneuploidy and meiotic spindle defects in mice were described [12]. However, to date, there are few reports investigating the association between aging and oocyte function. Studies on human oocytes are even fewer. Considering the number of women over the age of 40 years who wish to have children and are with infertility, it is clear that additional studies on the functional decline of oocyte quality associated with aging are urgently needed.

One reason for the paucity of relevant research to date is that it is difficult to develop animal models to simulate the aging of human oocytes, and it is difficult to obtain human oocytes for research purpose too. There are ethical problems as to whether we can use mature oocytes obtained through IVF for research, and the laws governing such use vary from country to country [9]. Additionally, because of the limited number of oocytes in women over their age of 30 years complicated the extension of oocytes research to the period preceding menopause.

In 2004, *Revel A* et al. reported human oocytes were obtained from an ovary that was resected during surgery for uterine endometrial cancer [11]. In their report, the ovary from a 43-year-old patient provided 17 oocytes after ovarian puncture, and 8 embryos were obtained by *in vitro* maturation (IVM). Uterine endometrial cancer occurs in women over a wide age range, including our target ages of 30 ~50. Therefore, we hypothesized that we could investigate age-related changes in oocyte function by using ovaries resected from patients in endometrial cancer. However, a thorough examination of oocytes obtained from an ovary resected at random during the menstrual cycle, including the growth stages of the oocytes, had not previously been performed. Here we report the collection of human oocytes removed during surgery for endometrial carcinoma, and the IVM of

those oocytes to obtain matured human oocytes for research. The organization of DNA and microtubules in the post-IVM oocytes was assessed by immunofluorescence microscopy.

Materials

8 patients under the age from 35 to 44 years with a preoperative diagnosis of Stage I endometrial cancer agreed to participate in this study. 3 of these patients were in their 30s (two patients aged of 35 years; case 1 and 8, one patient aged of 38 years; case 5) and 5 patients were in 40s (two patients aged of 41 years; case 3 and 4, wo patients aged of 42 years; case 6 and 7, one patient aged of 44 years; case 2). The menstrual cycle of them had been almost regular cycle before surgery.

The study was approved by the ethics committee of Tohoku University, and written informed consent was obtained before surgery.

Methods

After laparotomy under general anesthesia, surgeons confirmed that the patients had no macroscopic evidence of progressive cancer. After cytological sampling in the pelvic cavity, bilateral adnexae were resected, maintained in warm phosphate-buffered saline and immediately transported to the laboratory. We punctured macroscopically identifiable ovarian follicle-like structures with a 19-gauge needle and aspirated the follicular fluid (Fig. 1). We punctured each ovary about 15 times, and examined the collected oocytes under the microscope. Then, IVM was promoted using M199 medium (Gibco Labs, Grand Island, NY) containing 20% Systemic Serum Substitute (Irvine Scientific, Santa Ana, CA) and 75 mIU/mL recombinant follicle stimulating hormone (rFSH, Fertinome P, Serono, Tokyo, Japan) at 37°C in an atmosphere of 5% oxygen and 90% nitrogen for 24~48 hours. Cumulus cells were removed from oocytes after culture and the maturation stage of the oocytes was estimated. We fixed the oocytes that released a polar body after 24 hours of IVM, and fixed all remaining oocytes after 48 hours in IVM conditions. Fixation was carried out as previously described [3]. In brief, after removing the cumulus cells, oocytes were incubated in Buffer M (25% (v/v) glycerol, 50 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 1mM glycol ether diamine tetraacetic acid [EGTA], 50 mM imidazole hydrochloride and 1 mM 2-mercaptoethanol, pH

6.8) at 37°C for 30 min. Then oocytes were placed on glass coverslips and fixed with - 20°C methanol. Immunofluorescence staining to detect microtubules and DNA visualization with Hoechst 33342 were performed as previously described [10]. To put it simply, microtubules were labeled with a mixture of monoclonal antibody againstβ-tubulin (clone 2-28-33; diluted 1: 100; Sigma) and acetylated α-tubulin (clone 6-11-B1; diluted 1: 100; Sigma). Primary antibodies were detected by fluorescein-conjugated goat immunoglobulin G (IgG; diluted 1: 40; Zymed, San Francisco, CA, USA). DNA was detected after labeling with 10 mg/mL of Hoechst 33342.

Results

The number of oocytes collected from each patient is presented in Table 1. In total we obtained 87 oocytes from 8 patients. We obtained 65 oocytes (an average of 21.7 per patient) from the 3 women in their 30s. By contrast, we obtained only 23 oocytes (an average of 4.4 per patient) from the 5 women in their 40s. Of the 87 oocytes, only 11 oocytes (all from women aged 35 years) reached metaphase II (MII) after IVM. By contrast, a significant number of oocytes arrested maturation even after IVM. With respect to the developmental stages observed, the most common phenotype was ‘‘unclassifiable’’, applying to 31 oocytes. The status of rest oocytes were germinal vesicle (GV) or germinal vesicle break down (GVBD) of 23 oocytes, and metaphase I (MI) of 22 oocytes. The results of oocyte response to IVM are summarized in Table 1.

Nuclear staining by Hoechst 33342 and immunofluorescent detection of microtubules after IVM revealed the spindle apparatus of oocytes in various nuclear phases. We observed characteristic microtubule and chromatin formation of the oocytes that reached MII after IVM, and also noted the oocytes that were arrested before arriving at the MII stage after 48 hours of IVM (Fig. 2). Also, we had observed the various malformation of the meiotic spindle in MII oocytes obtained from 2 patients by immunofluorescence staining (Fig. 3). There were oocytes showing the misalignment of chromosome and the loss of arrangement of microtubule that comprise the meiotic spindle.

Discussion

Ethical issues constitute an obstacle to obtaining oocytes for human research. In this respect, the

bilateral salpingo-oophorectomy as standard treatment [5] for patients with uterine endometrial cancer represents an opportunity to solve ethical problems and obtain usable material for research without impacting the pathological diagnosis. In humans, meiotic chromosome segregation errors, a major cause of aneuploidy, increase dramatically as women age [4, 13]. A common age-related problem during the maturation of human oocytes is poor sister chromatid cohesion during meiosis I and meiosis II [14]. It is known manifestation of some cohesion protein, for example; Rec 8 and Shugoshins, which control meiotic chromosome segregation decrease in oocytes of aged mouse [2, 8]. On the other hand, there are no reports to date about the aged change of manifestation of those cohesion proteins in human oocytes. One reason is that it is difficult to obtain human oocytes as research material. We could obtain oocytes in several stages of several ages in this study. It would be useful for these researches. To our knowledge, this is the first report of immunofluorescent detection of microtubules after IVM of human oocytes from ovaries resected in patients with endometrial carcinoma. The number of collected oocytes in 40s was clearly smaller than that of 30s in this our study. And, we could not obtain oocytes in metaphase II in 40s. Wiser et al reported IVM was a procedure best suited to patients younger than 40 years of age [15]. The number of oocytes and total MII oocytes were significantly lower in their study of IVM in women of different ages. Unfortunately, we did not examine their hormone status: for example, luteinizing hormone, follicle stimulating hormone, anti-mullerian hormone around the perioperative period. We have to study about effect of IVM itself with setting control group of development of oocyte in vivo in future to consider contributing factor besides aging.

We conclude that material for research on human ovarian aging can be obtained from ovaries removed from patients during surgery for endometrial cancer. We think that this represents a useful and ethical method to advance research on age-associated changes in oocyte function.

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References

1. Battaglia DE, Goodwin P, Klein NA, Soules MR (1996) Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. Human reproduction (Oxford, England) 11 (10):2217-2222
2. Chiang T, Duncan FE, Schindler K, Schultz RM, Lampson MA (2010) Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes. Current biology : CB 20 (17):1522-1528. doi:10.1016/j.cub.2010.06.069
3. Hasegawa H (2009) The Best Approach to Observe Microtubule Organization in Oocyte Cytoplasm Production of Oocyte Wole Mount Samples by Buffer M Fixation. Journal of Mammalian Ova Research 26 (4):232-233. doi:10.1274/jmor.26.232
4. Hassold T, Hunt P (2001) To err (meiotically) is human: the genesis of human aneuploidy. Nature reviews Genetics 2 (4):280-291. doi:10.1038/35066065
5. Humphrey MM, Apte SM (2009) The use of minimally invasive surgery for endometrial cancer. Cancer control : journal of the Moffitt Cancer Center 16 (1):30-37
6. Krey LC, Grifo JA (2001) Poor embryo quality: The answer lies (mostly) in the egg. Fertility and sterility 75 (3):466-468
7. Lim AS, Tsakok MF (1997) Age-related decline in fertility: a link to degenerative oocytes? Fertility and sterility 68 (2):265-271
8. Lister LM, Kouznetsova A, Hyslop LA, Kalleas D, Pace SL, Barel JC, Nathan A, Floros V, Adelfalk C, Watanabe Y, Jessberger R, Kirkwood TB, Hoog C, Herbert M (2010) Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2. Current biology : CB 20 (17):1511-1521. doi:10.1016/j.cub.2010.08.023
9. McLaren A (2007) Free-range eggs? Science (New York, NY) 316 (5823):339.

doi:10.1126/science.1142267

10. Morita J, Terada Y, Hosoi Y, Fujinami N, Sugimoto M, Nakamura S-I, Murakami T, Yaegashi N, Okamura K (2005) Microtubule organization during rabbit fertilization by intracytoplasmic sperm injection with and without sperm centrosome. Reproductive Medicine and Biology 4 (2):169-178. doi:10.1111/j.1447-0578.2005.00096.x

11. Revel A, Safran A, Benshushan A, Shushan A, Laufer N, Simon A (2004) In vitro maturation and fertilization of oocytes from an intact ovary of a surgically treated patient with endometrial carcinoma: case report. Human reproduction (Oxford, England) 19 (7):1608-1611. doi:10.1093/humrep/deh241

12. Selesniemi K, Lee HJ, Muhlhauser A, Tilly JL (2011) Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. Proceedings of the National Academy of Sciences of the United States of America 108 (30):12319-12324. doi:10.1073/pnas.1018793108

13. Subramanian VV, Bickel SE (2008) Aging predisposes oocytes to meiotic nondisjunction when the cohesin subunit SMC1 is reduced. PLoS genetics 4 (11):e1000263. doi:10.1371/journal.pgen.1000263

14. Vogt E, Kirsch-Volders M, Parry J, Eichenlaub-Ritter U (2008) Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. Mutation research 651 (1-2):14-29. doi:10.1016/j.mrgentox.2007.10.015

15. Wiser A, Son WY, Shalom-Paz E, Reinblatt SL, Tulandi T, Holzer H (2011) How old is too old for in vitro maturation (IVM) treatment? European journal of obstetrics, gynecology, and reproductive biology 159 (2):381-383. doi:10.1016/j.ejogrb.2011.09.009

Table 1. Total number and average of collected oocytes and their developmental stages

case	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**total**	**30s**	**40s**
age (y.o)	35	44	41	41	38	42	42	35			
Oocytes	25	1	4	1	14	11	5	26	87	65	22
GV, GVBD	4	1	4	0	4	3	1	6	23	14	9
MI	11	0	0	1	3	2	2	3	22	17	5
MII	6	0	0	0	0	0	0	5	11	11	0
Unclassified	4	0	0	0	7	6	2	12	31	23	8
Average of oocytes									10.9	21.7	4.4

Figure 1. Oocyte collection from resected ovaries.

(A) A 19 gauge needle was used to puncture the ovary approximately 15 times, and follicular fluid containing oocytes was aspirated. (B) Immature oocytes collected from follicular fluid. (C) Oocytes after *in vitro* maturation (IVM).

Figure 2 Oocyte staging by DNA and microtubule assessment.

Nuclear staining by Hoechst 33342 and immunofluorescence staining for microtubules after IVM revealed the spindle apparatus of oocytes in various nuclear phases. Microtubules (green) and chromatin (blue) were observed. (A) An oocyte in the germinal vesicle (GV) stage. (B) An oocyte in metaphase I (MI). (C) An oocyte in MI telophase. (D) An oocyte in completed metaphase II (MII).

Figure 3 Assessment of meiotic spindle formation in oocytes after IVM.

(A–E) Oocytes that have completed metaphase II (MII) after IVM. We used immunofluorescence staining to detect meiotic spindle arrangements of oocytes in MII. Both normal (A) and deranged configurations (B–E) were observed.

Figure 1

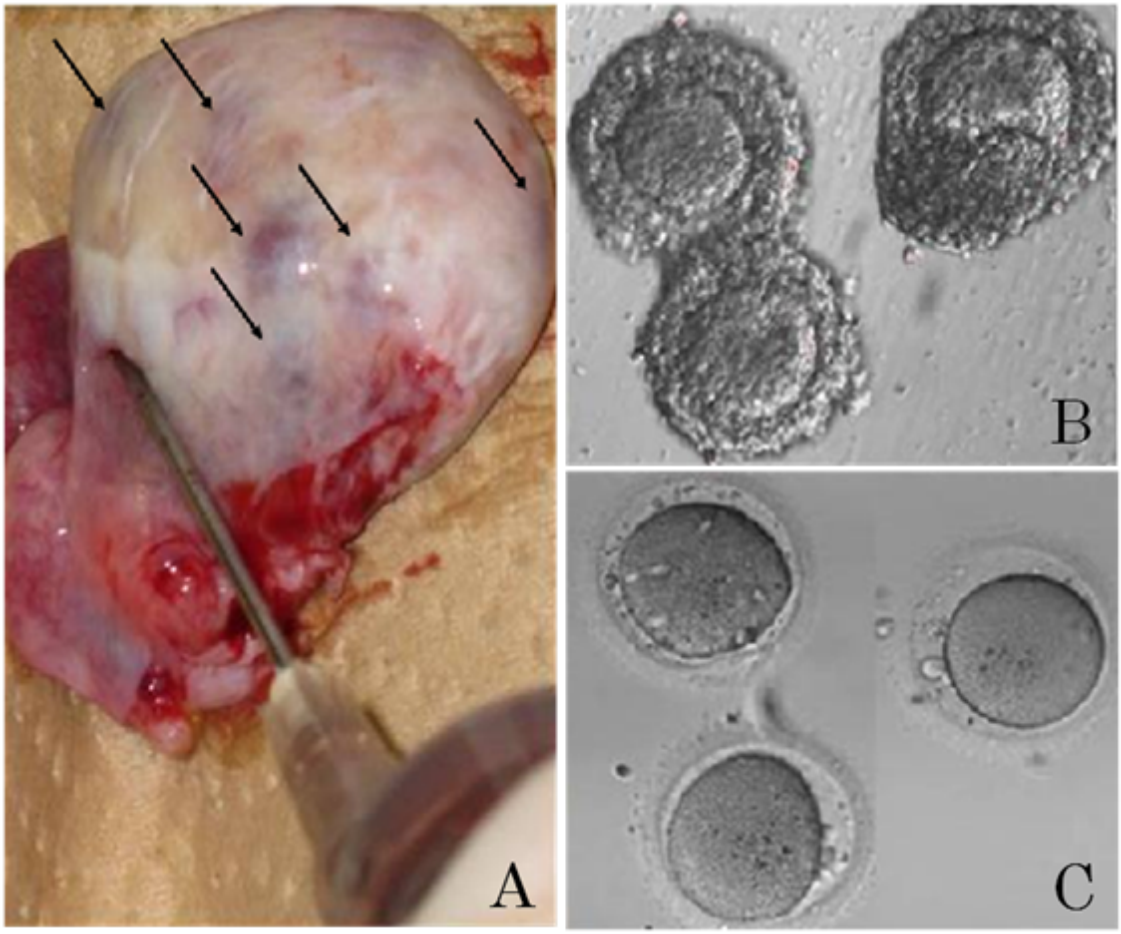

Figure 2

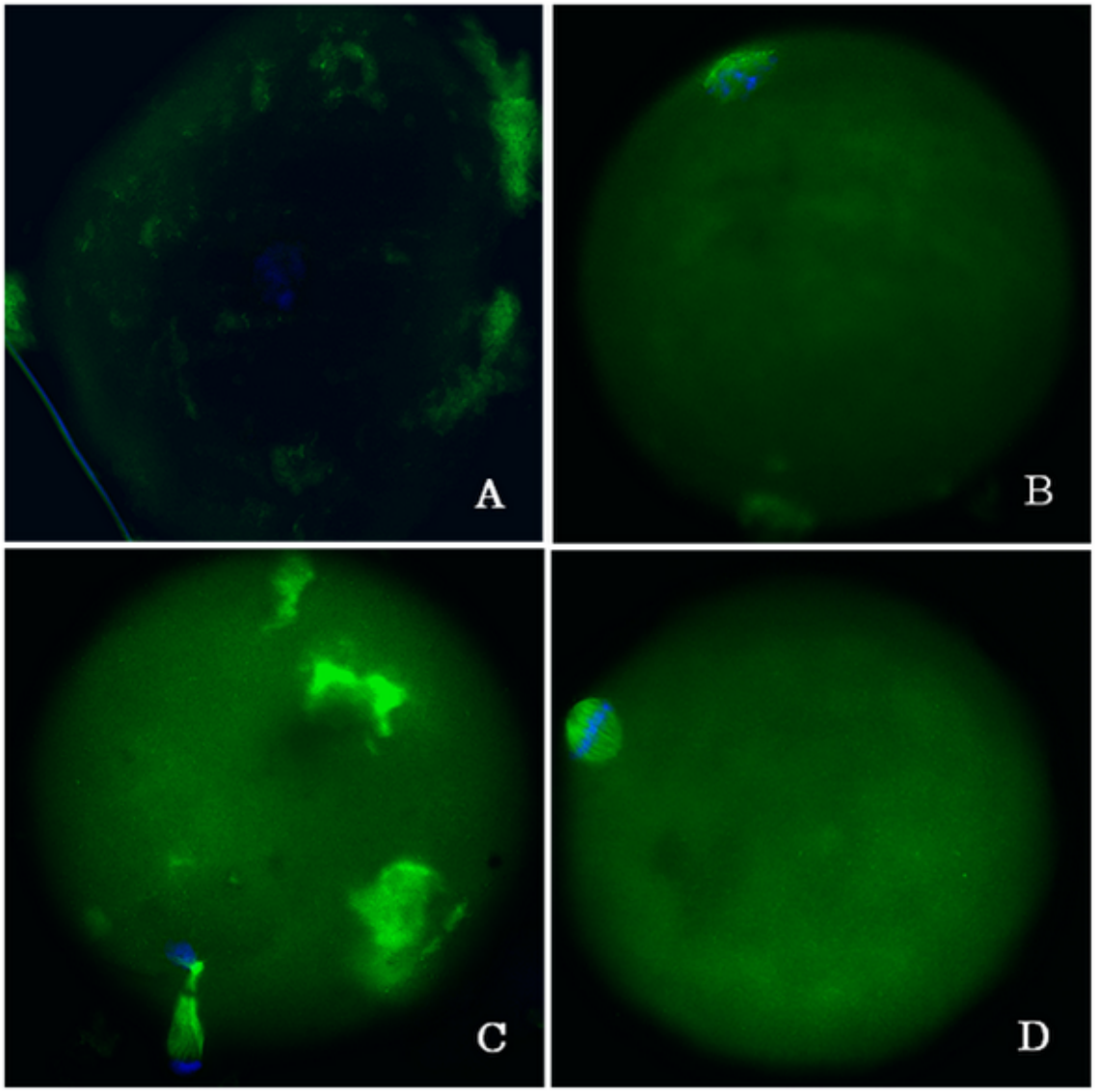

Figure 3