

Cryopreservation of Mammalian Gametes and Embryos: Methods and Protocols
Agarwal et al

Chapter on: Human Ovarian Tissue Vitrification

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Summary:

Ovarian freezing and transplantation has garnered increasing interest as a potential way of preserving fertility in cancer patients as well as for women who just wish to delay childbearing. This chapter spells out our techniques of ovarian cortex vitrification and results for frozen compared to fresh ovarian cortex transplantation (in one single series from one center for the sake of consistency), as well as potentially provide insight into the mechanism behind ovarian follicle recruitment [1-15]. We put an emphasis on ovarian tissue vitrification in preference to slow freeze. But slow freeze has also yielded good results, and we will explain the difference in this chapter. A comparison of fresh versus frozen transplantation techniques is presented, highlighting the similarity and differences between the fresh and frozen transplantation procedures. Much of the literature is scattered case reports with different patient populations and different techniques. This represents an effort to simplify and popularize an approach that has yielded favorable results (all cases recovered ovulation and 75% had successful spontaneous pregnancy) in one single, disciplined study. It should be clear that this is a review for the more general reader of our original scientific papers published in RBMOnline, New England Journal of Medicine, Fertility and Sterility, Human Reproduction, and Molecular Human Reproduction.

Keywords: Fertility, preservation, ovarian freezing, transplant, follicle recruitment

Patient characteristics and results are all summarized in Tables 1 and 2. All recipients of fresh or frozen ovarian transplants had the same robust return of FSH to pre-menopausal levels by 150 days, menstruation by 130 days, and massive AMH elevation by 170 days. The AMH then fell to below normal by 240 days and remained

at that level for many years with normal ovarian cycling and hormonal function. Seventeen babies resulted from 11 fresh and six frozen cycles. Grafts of 1/3 of an ovary cortex lasted as long as 8 years or more despite eventually low AMH. Our results support a hypothesis that ovary freezing and transplantation is clinically a robust method of preserving ovarian function, and it supports a hypothesis that ovarian stromal density gradient can account for the early meiotic arrest of fetal oogonia, and also for “resting” follicle recruitment in the adult.

Methodology for Ovarian Cortex Cryopreservation

Successful fresh as well as frozen ovarian cortex transplants in humans were first published in 2004 and 2005 as a case reports, and many other case reports have subsequently followed [1-2, 3-15]. There has now developed a rapidly growing interest in frozen ovarian cortical transplantation despite the absence of a clearly documented success rate for this procedure. The primary impetus for this procedure has been to cryopreserve ovarian tissue prior to sterilizing cancer treatment with the objective of transplanting the tissue back after cancer cure, after the proof of principle was reported in sheep in 1994 [16]. The possibility also loomed of preserving fertility and even hormonal function against the natural decline caused by aging [17, 18].

Oocyte freezing is often mentioned as another alternative for preserving fertility [19, 20, 21]. However, many different centers do it in different ways and most have not verified long-term viability of the oocytes. There could therefore be many disappointed women in years to come. Among the pitfalls to oocyte freezing are too rapid and changing osmolality with dangerously quick osmotic shifts. Also, there is a common failure to ensure a rapid enough freeze, and worse yet not a rapid enough thaw. The commercial kits that are designed to make this “easy” often fail in this regard, and

closed freezing is more problematic than open freezing for rapid freeze and thaw. It was not until 2005 that a highly efficient method was published, which stimulated a huge wave of enthusiasm, for this approach, also known as the “bridge” technique (Figure 1 a-f), but it also is very user-dependent, and none of the oocyte vitrification protocols are as fool proof and easy as freezing of ovarian tissue.

One extra benefit of ovary cryopreservation and transplantation over oocyte freezing, however, is the putting off of hormonal menopause as well as preserving fertility. Although oocyte freezing is now mostly performed with vitrification, there is still controversy regarding ovary tissue between slow freeze and vitrification. It is well known that unilateral oophorectomy does not diminish fertility, and does not hasten menopause. Thus, it is speculatively possible that grafts taken from young women could be used to delay menopause in the future [22, 23, 24].

Ovarian Cortical Transplantation

However the main benefit of ovary cryopreservation over oocyte cryopreservation is for cancer patients who need to start their chemotherapy and radiation promptly and cannot afford to wait around for two or three cycles of IVF, knowing the pregnancy rate per single egg is only about 4%. With ovary freezing, they can potentially get pregnant naturally, and preserve several hundred thousand eggs (Figures 2, 3 and 4).

Successful fresh and cryopreserved ovarian cortex transplants in humans were first published in 2004 and 2005, as case reports, and many other case reports have subsequently followed [3, 5, 6, 10-15, 17, 18, 25-29]. The first human applications were preceded by a long history of animal experimentation. As far back as 1954, Deanesly showed in rats and, in 1960, Parrott showed in mice, that ovarian tissue could be

successfully frozen and autografted resulting in live births [30, 31]. Interest in human applications began after Gosden's report of successful pregnancies in sheep in 1994 [16]. One of the most interesting case reports in humans involved rejuvenating menopausal ovarian cortex with cryopreserved autotransplantation [32]. Interest in cryopreserved ovarian cortical transplantation is rapidly growing, although only one report to date has clearly stated its success rate [8]. No systematic report has been published from one centre comparing fresh donor transplants and cryopreserved ovary autografts, and little has been gleaned from studies of these procedures on analysis of ovarian function and resting follicle recruitment [33].

The primary impetus for this procedure has been to cryopreserve ovarian tissue before sterilizing cancer treatment, with the objective of transplanting the tissue back after cancer has been cured, thus allowing patients to preserve their fertility. It is also possible that grafts taken from young women with cancer could be used to delay their menopause in the future [17, 18, 22-24, 34-41]. The possibility of preserving fertility and even hormonal function against the natural decline caused by ageing has also been speculated, but is considered of less importance [17-21].

Most published research in this field consists of case reports of cryopreserved transplants only. We conducted a worldwide survey of 37 babies born from cryopreserved transplants, but still could not establish a clear success rate [18]. Here, we report a single series of both fresh and cryopreserved transplants from one centre, carried out with the same technique and assessed uniformly over follow-up, with the aim of improving our understanding of resting follicle recruitment and demonstrating the clinical robustness of the procedure. All of the recipients had normal return of hormonal and menstrual ovarian function at about the same time after surgery (4.5 months) (Figures 5 and 6).

In all fresh and cryopreserved transplant recipients, day 3 FSH decreased from the menopausal range to normal levels in about 150 days, and menstrual cycling resumed by roughly 130 days. Since all developing follicles were destroyed or excluded from the thin (1-mm) donor ovarian cortical graft, this period seems to represent the number of days required for resting primordial follicles to be recruited and develop to the ovulatory stage, at which point they finally become sensitive to cyclic FSH and LH (Figure 5).

The duration of function of fresh grafts was directly related to the original ovarian reserve of the donor. In all cases, only one-quarter to one-half of the donor ovary was transplanted, and most of the tissue cryopreserved for future use. All grafts functioned for more than 2 years, over one-half of them for over 6 years, and two of them already for over 8 years (Table 1). Thus if the donor's ovarian reserve is high, these grafts can last for a long time despite reduced AMH levels [17, 42]

The relationships among FSH levels, menstruation and AMH levels in donor and recipient in fresh transplants are indicative of resting follicle recruitment and ovarian reserve [43]. As recipient FSH levels returned to normal within 130-170 days, the low AMH level of recipients then began to rise in response to an increasing number of mature gonadotrophin-sensitive follicles (Figures 5 and 6). The AMH of recipients continued to rise to well above the normal baseline AMH level of the donor. In the fresh allograft recipient shown in Figure 6, although FSH decreased to normal levels by day 133 and normal menstrual cycling resumed, AMH levels rose far above normal (higher than the donor) shortly thereafter. Despite the transplanted graft continuing function, AMH then returned to low levels. This analysis of both donor and recipient data after transfer is rare and useful in its demonstration of no significant long-term change in donor AMH levels despite loss of an ovary. The rise of recipient AMH levels

above donor AMH levels reflects over-recruitment of resting follicles in the recipient compared with the donor (Figure 6). Contrary to what might have been expected based on earlier studies [44], no evidence was found of significant loss of follicles from transplantation ischaemia, as each patient had FSH levels return to normal as AMH levels rose far above normal. Despite continued function of the transplanted graft, AMH then returns to low levels (Figure 7 a-c). Preservation of follicles was supported by our observation of substantial follicle recruitment with many eggs produced in the two patients who underwent IVF during the window of high AMH levels. Quantitative studies have also supported this finding in bovines [45].

The auto-transplantation of cryopreserved ovary tissue yielded results almost identical to fresh transplantation (Figure 5). This finding is consistent with the observed absence of histological damage from cryopreservation [42, 45, 46]. As with fresh ovary transplants, FSH levels returned to normal by about 150 days in all cases, and menstrual cycles resumed shortly before that. The return to normal did not differ between slow-freeze cases and vitrification cases. In all of the cryopreserved cases, just as with fresh transplants, AMH rose to high levels shortly after FSH returned to normal, at around 130-170 days (Figure 7 a-c). Then, exactly as with fresh transplant cases, AMH dropped to a lower baseline level by about 240 days and remained at that lower level [17].

Eight of the 11 cryopreserved autotransplant recipients had a follow-up of over 1 year, which allowed the assessment of pregnancy potential. Seven of these eight patients spontaneously conceived, although one spontaneously aborted (Table 2). The other six were healthy singleton pregnancies. Therefore, cryopreserved and fresh transplantation were similar in hormonal function and high pregnancy outcome. Functional hormonal results thus far have demonstrated a remarkable degree of repeatable concordance in all 22 cases of fresh ovary donor transplantation and

cryopreserved ovary autotransplantation. Thus we had a spontaneous pregnancy rate overall of 75%.

In conclusion, no difference was found in clinical and functional results between fresh and cryopreserved ovary cortical grafts, and both demonstrated a high success rate in preserving fertility as well as endocrine function for long periods of time. Therefore, aside from the benefit of fertility preservation for cancer patients, this procedure offers the benefit of relief from menopause without having to resort to exogenous hormone replacement [17].

Ovarian cryopreservation by original slow freeze technique

For slow freezing, after enucleating medullary tissue with a sharp scalpel dissection, the cortex was pared down manually to an ultrathin translucent shell with a thickness of ≤ 1 mm. Tissue for cryopreservation was divided into multiple strips and transferred to 1.5 ml cryovials after equilibration in 1.5 mol/l 1,2-propanediol and 0.1 mol/l sucrose at 37°C for 30 min, followed by 1.5 mol/l 1,2 propanediol and 0.2 mol/l sucrose for 5 min, then cooled at a controlled rate, as described previously [16, 46]. Thawing was achieved rapidly by agitating the vials in a warmed water bath. If tissue had thickened by contraction after thawing, it was pared down again to <1 mm under an operating microscope with microsurgical scissors before transplantation [46].

However, we prefer vitrification for cryopreservation because of our in vitro viability analysis studies as well as in vivo transplant studies in the bovine [45]. A total of 16 cancer patients requesting fertility preservation by ovarian banking consented to an oocyte viability test and histologic review of a small sample ($<10\%$) of their fresh or preserved tissue. In eight cases, the tissue had been preserved by vitrification, six by a

slow freezing protocol and in two only fresh tissue was analyzed. The goal of this in vitro study was to determine which method produced a higher cell survival rate [42].

The high viability (92%) of oocytes in both control (fresh) specimens and vitrified specimens indicated that disaggregation per se had only caused minimal damage to this cell type [42]. Overall, 2301 oocytes were examined from 16 specimens. The results within each of the three groups revealed no significant difference overall between fresh and vitrified tissue, but the viability of slow freeze-cryopreserved tissue was less than one-half (42%; $P < 0.01$). Transmission electron microscopy also has been used to analyze ovarian tissue that had been either cryopreserved by slow freezing or vitrified by ultrarapid freezing, showing vitrification to be superior (Table 3) [47].

We know there have certainly been successes with slow freeze, but vitrification is easier, and does less cryo damage.

Cryoinjury

Most of the stroma cells in the slow freeze-cryopreserved specimen were lysed and their nuclei compressed between dense bundles of extracellular fibers. The same cells were generally intact after vitrification. Small follicles were found in each specimen, all of which were intact within their basement membranes. The high viability (92%) of oocytes in control (fresh) specimens indicated that disaggregation per se had only caused minimal damage to this cell type. Overall, 2,301 oocytes were examined from 16 specimens. Results within each of the three groups were consistent and revealed no significant difference overall between fresh and vitrified tissue, although the viability of slow freeze-cryopreserved tissue was less than one-half (42%) and highly significant ($P < .01$; Table 3).

Cryopreservation

For slow freezing, after enucleating medullary tissue with sharp scalpel dissection, the cortex was pared down manually to an ultrathin translucent shell with a thickness of ≤ 1 mm. Tissue for cryopreservation was divided into multiple strips and transferred to 1.5 mL cryovials after equilibration in 1.5 mol/L 1,2-propanediol and 0.1 mol/L sucrose at 37°C for 30 minutes, followed by 1.5 mol/L 1,2-propanediol and 0.2 mol/L sucrose for 5 minutes, and then cooled at a controlled rate, as described previously [16, 46]. Thawing was achieved rapidly by agitating the vials in a warmed water bath. If tissue had thickened by contraction after thawing, it was pared down again to < 1 mm under an operating microscope with microsurgical scissors before transplantation. For vitrification, details have been described elsewhere [42, 45].

Tissue analyses

Small samples of cortical tissue from donor organs were assessed: 1) on a semiquantitative scale of relative follicle density in histology slides (0, +, ++, or +++, in order of increasing abundance); and 2) by testing viability after enzymatic disaggregation. Also, all original cortical tissue from transplant recipients who were in ovarian failure was removed and prepared histologically after excision to verify that total follicular depletion had occurred. For viability testing, tissues were incubated and pipetted in type I collagenase (1 mg/mL) for 10 minutes to isolate the small follicles and visualize their oocytes. The cells were briefly incubated in Hoechst 33342 and propidium iodide, then washed before viewing by fluorescence microscopy, for a total of 2,301 oocytes from 16 patients. Transmission electron microscopy was also used to analyze ovarian tissue that had been either cryopreserved by slow freezing or vitrified by ultrarapid freezing [47].

Ovarian tissue vitrification

Cortex tissue of each ovary is cut into slices of 1 × 0 × 10 mm. The precise 1-mm tissue thickness was guaranteed with a tissue slicer designed explicitly for this purpose (Kitazato Biopharma, Japan). The tissue slicer is put on the surface of ovary. Then another plate is placed over the tissue slicer, and the ovary is cut between the slicer and the surface of the ovary using a sharp blade. The cortical ovarian tissue is thus cut into 1 × 10 × 10 mm pieces. The ultra-thinness of the tissue is crucial, not only for the cryopreservation, but also for rapid revascularization after grafting.

Ovarian tissues are initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulphoxide (DMSO) in handling medium (HM:HEPES-buffered TCM-199 solution supplemented with 20% (v/v) synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) for 25 min followed by a second equilibration in 20% DMSO with 0.5 mol/l sucrose for 15 min. Ovarian tissues are then placed in a minimum volume solution (virtually 'dry') onto a thin metal strip (Cryotissue: Kitazato BioPharma, Fujinomiya, Japan), and submerged directly into sterile liquid nitrogen [48], following which the strip was inserted into a protective container and placed into a liquid nitrogen storage tank.

For thawing, the protective cover is removed and the Cryotissue metal strip is immersed directly into 40 ml of 37°C HM solution supplemented with 1.0 mol/l sucrose for 1 min. Then, the ovary tissues are transferred into 15 ml of 0.5 mol/l sucrose HM solution for 5 min at room temperature, and washed twice in HM solution for 10 min before viability analysis, or transplantation. No ice crystal formation occurs during any

of these vitrification procedures [45]. This approach is remarkably easier than slow freeze, and also more effective.

Cortical ovarian tissue transplantation technique

The recipients were prepared by minilaparotomy via a 3.5-cm incision above the pubis. For cortical tissue transplantation, recipient ovarian cortex was resected to completely expose medullary tissue; hemostasis was controlled with micro-bipolar forceps, and irrigation with heparinized saline was performed to avoid adhesion formation or micro-hematomas between donor and recipient tissues. This technique may be the most important reason for a minimal ischemic loss of oocyte viability. The tissue graft was trimmed to the dimensions of the exposed surface of the recipient and attached using 9-0 [42, 45, 49-55]. Since 1996, we have frozen ovary tissue for 100 young women with solid organ cancer, or with a great risk for POF, of whom 16 had spare frozen tissue subjected to detailed viability testing before cryopreservation and after thawing. All had histologic review by a variety of pathologists. Only one had ovarian metastasis, a young woman with widespread breast cancer metastasis throughout her entire body. Otherwise, none of our other cases had any tumor cells in their ovary. Andersen has also noted a complete lack of ovarian metastasis, even in the majority of leukemia cases. The reason for the remarkable absence of ovarian metastasis might possibly be due to the fibrous avascular nature of the ovarian cortex [56]. In fact, the reason why fetal ovarian tubules (which in the fetal male become seminiferous tubules) invade the fibrous cortex and become follicles, is that the dense fibrous tissue of the cortex (which in the fetal and adult testis is just tunica albuginea) is needed to suppress the resting follicles from developing all at once prematurely.

With the classical slow freeze technique (Figure 8 a-c), our in vitro viability testing showed only 41% of oocytes survived [42, 45, 46, 57]. However, with vitrification of the ovarian tissue there was no difference between fresh unfrozen controls and frozen tissue. It seems likely, therefore, that vitrified ovarian tissue would give better results after transplantation than tissue cryopreserved by slow freeze, but it is too early to state that with any certainty.

Although the clinical results seem promising from this large series of fresh and frozen ovarian transplants, the functional results may be even more interesting (Figures 5a, 6 and 7 a-c). Day 3 FSH came down from menopausal range to normal or near normal levels in all eleven cases by 150 days, and menstrual cycling resumed in all eleven recipients by 130 days. Since all developing follicles were destroyed or excluded from the thin (1mm) donor ovarian cortical graft, this would represent the number of days required for resting follicles to be recruited and develop to the ovulatory stage when they would finally become sensitive to cyclic FSH and LH.

The relationship between FSH levels, menstruation, and AMH levels in these fresh transplants is indicative of resting follicle recruitment. As the FSH returned to normal by 130 days, the very low AMH level of the recipient began to rise in response to an increasing number of mature gonadotropin sensitive follicles [42, 43]. The AMH of the recipient then continued to rise to well above the normal baseline AMH of the donor (Figures 5 a, 6 and 7 a-c). Contrary to what might have been expected from earlier studies [44], this indicated no significant loss of follicles from the transplantation, which was also indicated by ultrasonographic examination of stimulated tissues at this period, which showed a very robust follicular response to gonadotropin stimulation when IVF was attempted (third identical twin case). Then by 240 days the AMH of the

recipient descended to well below baseline levels, and then remained steady at this level usually for many years (Figure 6) [42, 43, 45-47].

The auto-transplantation of frozen ovary tissue yielded results almost identical to fresh (as might have been guessed by failure to notice any histological damage from freezing) [42, 45-47]. As with fresh ovary transplants, the FSH always returned to normal in these cases by 150 days without exception, and menstrual cycles resumed shortly before that. The return to normal was no different for slow freeze cases than for vitrification cases. In all of these cases, just like with fresh transplants, the AMH rose to very high levels shortly after the FSH returned to normal, at about 170 days. Then the AMH, exactly as with fresh transplant cases, came down to a lower baseline level by 240 days and remained at that lower indefinitely.

Eight of the eleven frozen cases were over one year ago, and thus suitable to look at pregnancy potential. However, one of these eight has chosen not to get pregnant yet and is using barrier contraception. Of the remaining seven, six have gotten pregnant although one miscarried. Thus frozen results were similar to fresh in terms of hormonal function and pregnancy outcome. Quite remarkable was the repeatable concordance of functional results thus far seen in all 22 cases of fresh ovary donor transplantation and frozen ovary autotransplantation.

Future Perspective

Firstly, ovarian freezing and transplantation should no longer be considered experimental moving forward. Secondly, this could provide a unifying theory for adult recruitment and fetal arrest of primordial follicles. Without a steady controlled release of “resting” follicles in the adult, women would run out of eggs prematurely. Similarly, if fetal oocyte arrest did not occur after meiotic activation, there would be no oocytes

remaining at birth. Similarly, if there were not a slow, steady, monthly release of primordial (“resting” or better, “arrested”) follicles from the resting phase, without any hormonal regulation, related to tissue pressure gradients in the ovarian cortex (the same tissue as the dense stroma in the tunica albuginea of the male), all the adult follicles also would soon be gone (Figure 8 a-c). Thus we propose a unifying theory for both fetal oocyte arrest and adult resting follicle recruitment.

Figure Legends

- Figure 1 a-f (a) The “bridge” technique for oocyte freezing. (b) Setup for “bridge” equilibration. (c) First “bridge” between ES and isotonic HEPES media; three minutes. (d) Second “bridge” equilibration; three minutes (e) Transfer to full concentration ES; nine minutes. (f) Transfer from ES to VS; 60-90 seconds. Caption: High cryoprotectant concentration is not toxic. It is just the rapid osmotic shifts that kill the egg or embryo and give the incorrect impression of toxicity. To avoid over rapid osmotic shifts (that are more poorly tolerated by the egg than the embryo) the original “bridge” technique is best. ES solution droplets are first “bridged” over to the iso-osmotic solution the egg is in, and three minutes later another droplet of ES solution is “bridged” over to the original solution very gradually and continuously raising the osmolality of the solution the oocyte is resting in.
- Figure 2 Steps in the procedure of ovarian transplantation between MZ twin sisters: (a) preparation of donor ovarian cortex by dissection in a Petri dish on ice; (b) preparation of recipient ovarian medulla; (c) attaching donor cortical tissue to recipient ovarian medulla; (d) attaching thawed donor cortical tissue for re-transplant to the recipient medulla.
- Figure 3 Histology (a) pre and (b) post vitrification of ovarian tissue.
- Figure 4 Ovarian tissue slice.
- Figure 5 a-b Levels of FSH after fresh or cryopreserved ovary graft. Time of first menstruation and the duration of time required for FSH levels to revert to normal levels, allowing for ovulation to occur, is represented

for the 11 patients who received fresh ovary tissue transplants (a) and the 11 patients who received cryopreserved ovary tissue transplants (b).

Figure 6 Levels of FSH and anti-Müllerian hormone levels in second allograft donor and recipient. AMH = anti-Müllerian hormone.

Figure 7 a-c Levels of FSH and anti-Müllerian hormone levels after ovary transplant. AMH = anti-Müllerian hormone.

Figure 8 a-d (a-c) Stromal density gradient in ovarian cortex. (d) Follicles develop inward toward the softer ovarian medulla

Bibliography

- [1] Silber, S., Pineda, J., Lenahan, K., DeRosa, M., and Melnick, J. (2015). "Fresh and cryopreserved ovary transplantation and resting follicle recruitment." *Reprod Biomed Online*, 30(6), 643-50.
- [2] Silber, S. (2015). "Unifying theory of adult resting follicle recruitment and fetal oocyte arrest." *Reprod Biomed Online*.
- [3] Silber, S. J., Lenahan, K. M., Levine, D. J., Pineda, J. A., Gorman, K. S., Friez, M. J., Crawford, E. C., and Gosden, R. G. (2005). "Ovarian transplantation between monozygotic twins discordant for premature ovarian failure." *N Engl J Med*, 353(1), 58-63.
- [4] Silber, S. J. (2012). "Ovary cryopreservation and transplantation for fertility preservation." *Mol Hum Reprod*, 18(2), 59-67.
- [5] Demeestere, I., Simon, P., Englert, Y., and Delbaere, A. (2003). "Preliminary experience of ovarian tissue cryopreservation procedure: alternatives, perspectives and feasibility." *Reprod Biomed Online*, 7(5), 572-9.
- [6] Demeestere, I., Simon, P., Moffa, F., Delbaere, A., and Englert, Y. (2010). "Birth of a second healthy girl more than 3 years after cryopreserved ovarian graft." *Hum Reprod*, 25(6), 1590-1.
- [7] Donnez, J., Dolmans, M. M., Demylle, D., Jadoul, P., Pirard, C., Squifflet, J., Martinez-Madrid, B., and van Langendonck, A. (2004). "Livebirth after orthotopic transplantation of cryopreserved ovarian tissue." *Lancet*, 364(9443), 1405-10.
- [8] Donnez, J., Dolmans, M. M., Pellicer, A., Diaz-Garcia, C., Sanchez Serrano, M., Schmidt, K. T., Ernst, E., Luyckx, V., and Andersen, C. Y. (2013). "Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation." *Fertil Steril*, 99(6), 1503-13.
- [9] Donnez, J., Silber, S., Andersen, C. Y., Demeestere, I., Piver, P., Meirow, D., Pellicer, A., and Dolmans, M. M. (2011). "Children born after autotransplantation of cryopreserved ovarian tissue. a review of 13 live births." *Ann Med*, 43(6), 437-50.
- [10] Meirow, D., Levron, J., Eldar-Geva, T., Hardan, I., Fridman, E., Zalel, Y., Schiff, E., and Dor, J. (2005). "Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy." *N Engl J Med*, 353(3), 318-21.
- [11] Poirot, C., Abirached, F., Prades, M., Coussieu, C., Bernaudin, F., and Piver, P. (2012). "Induction of puberty by autograft of cryopreserved ovarian tissue." *Lancet*, 379(9815), 588.
- [12] Sanchez, M., Alama, P., Gadea, B., Soares, S. R., Simon, C., and Pellicer, A. (2007). "Fresh human orthotopic ovarian cortex transplantation: long-term results." *Hum Reprod*, 22(3), 786-91.
- [13] Silber, S. J., DeRosa, M., Pineda, J., Lenahan, K., Grenia, D., Gorman, K., and Gosden, R. G. (2008). "A series of monozygotic twins discordant for ovarian failure: ovary transplantation (cortical versus microvascular) and cryopreservation." *Hum Reprod*, 23(7), 1531-7.
- [14] Silber, S. J., and Gosden, R. G. (2007). "Ovarian transplantation in a series of monozygotic twins discordant for ovarian failure." *N Engl J Med*, 356(13), 1382-4.

- [15] Silber, S. J., Grudzinskas, G., and Gosden, R. G. (2008). "Successful pregnancy after microsurgical transplantation of an intact ovary." *N Engl J Med*, 359(24), 2617-8.
- [16] Gosden, R. G., Baird, D. T., Wade, J. C., and Webb, R. (1994). "Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196 degrees C." *Hum Reprod*, 9(4), 597-603.
- [17] Andersen, C. Y., Silber, S. J., Bergholdt, S. H., Jorgensen, J. S., and Ernst, E. (2012). "Long-term duration of function of ovarian tissue transplants: case reports." *Reprod Biomed Online*, 25(2), 128-32.
- [18] Stoop, D., Cobo, A., and Silber, S. (2014). "Fertility preservation for age-related fertility decline." *Lancet*, 384(9950), 1311-9.
- [19] Homburg, R., van der Veen, F., and Silber, S. J. (2009). "Oocyte vitrification--women's emancipation set in stone." *Fertil Steril*, 91(4 Suppl), 1319-20.
- [20] Nagy, Z. P., Chang, C. C., Shapiro, D. B., Bernal, D. P., Kort, H. I., and Vajta, G. (2009). "The efficacy and safety of human oocyte vitrification." *Semin Reprod Med*, 27(6), 450-5.
- [21] Cobo, A., Domingo, J., Perez, S., Crespo, J., Remohi, J., and Pellicer, A. (2008). "Vitrification: an effective new approach to oocyte banking and preserving fertility in cancer patients." *Clin Transl Oncol*, 10(5), 268-73.
- [22] Lass, A., Paul, M., Margara, R., and Winston, R. M. (1997). "Women with one ovary have decreased response to GnRHa/HMG ovulation protocol in IVF but the same pregnancy rate as women with two ovaries." *Hum Reprod*, 12(2), 298-300.
- [23] Zhai, A., Axt, J., Hamilton, E. C., Koehler, E., and Lovvorn, H. N., 3rd. (2012). "Assessing gonadal function after childhood ovarian surgery." *J Pediatr Surg*, 47(6), 1272-9.
- [24] Gosden, R. G., Telfer, E., Faddy, M. J., and Brook, D. J. (1989). "Ovarian cyclicity and follicular recruitment in unilaterally ovariectomized mice." *J Reprod Fertil*, 87(1), 257-64.
- [25] Anderson, E. L., Baltus, A. E., Roepers-Gajadien, H. L., Hassold, T. J., de Rooij, D. G., van Pelt, A. M., and Page, D. C. (2008). "Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice." *Proc Natl Acad Sci U S A*, 105(39), 14976-80.
- [26] Dittrich, R., Mueller, A., Maltaris, T., Hoffmann, I., Magener, A., Oppelt, P. G., and Beckmann, M. W. (2009). "Hormonal and histologic findings in human cryopreserved ovarian autografts." *Fertil Steril*, 91(4 Suppl), 1503-6.
- [27] Revel, A., Laufer, N., Ben Meir, A., Lebovich, M., and Mitrani, E. (2011). "Micro-organ ovarian transplantation enables pregnancy: a case report." *Hum Reprod*, 26(5), 1097-103.
- [28] Revelli, A., Marchino, G., Dolfen, E., Molinari, E., Delle Piane, L., Salvagno, F., and Benedetto, C. (2013). "Live birth after orthotopic grafting of autologous cryopreserved ovarian tissue and spontaneous conception in Italy." *Fertil Steril*, 99(1), 227-30.
- [29] Stern, C. J., Gook, D., Hale, L. G., Agresta, F., Oldham, J., Rozen, G., and Jobling, T. (2013). "First reported clinical pregnancy following heterotopic grafting of cryopreserved ovarian tissue in a woman after a bilateral oophorectomy." *Hum Reprod*, 28(11), 2996-9.
- [30] Deanesly, R. (1954). "Immature rat ovaries grafted after freezing and thawing." *J Endocrinol*, 11(2), 197-200.
- [31] Bruce, H. M., and Parrott, D. M. (1960). "Role of olfactory sense in pregnancy block by

- strange males." *Science*, 131(3412), 1526.
- [32] Kawamura, K., Cheng, Y., Suzuki, N., Deguchi, M., Sato, Y., Takae, S., Ho, C. H., Kawamura, N., Tamura, M., Hashimoto, S., Sugishita, Y., Morimoto, Y., Hosoi, Y., Yoshioka, N., Ishizuka, B., and Hsueh, A. J. (2013). "Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment." *Proc Natl Acad Sci U S A*, 110(43), 17474-9.
- [33] Oktay, K., Economos, K., Kan, M., Rucinski, J., Veeck, L., and Rosenwaks, Z. (2001). "Endocrine function and oocyte retrieval after autologous transplantation of ovarian cortical strips to the forearm." *JAMA*, 286(12), 1490-3.
- [34] Aydin, Y., Celiloglu, M., Koyuncuoglu, M., and Ulukus, C. (2010). "Follicular dynamics and apoptosis following unilateral oophorectomy." *Syst Biol Reprod Med*, 56(4), 311-7.
- [35] Faddy, M. J., Gosden, R. G., Gougeon, A., Richardson, S. J., and Nelson, J. F. (1992). "Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause." *Hum Reprod*, 7(10), 1342-6.
- [36] Kaaijk, E. M., Hamerlynck, J. V., Beek, J. F., and van der Veen, F. (1999). "Clinical outcome after unilateral oophorectomy in patients with polycystic ovary syndrome." *Hum Reprod*, 14(4), 889-92.
- [37] Kim, S. S. (2003). "Ovarian tissue banking for cancer patients. To do or not to do?" *Hum Reprod*, 18(9), 1759-61.
- [38] Koskas, M., Uzan, C., Gouy, S., Pautier, P., Lhomme, C., Haie-Meder, C., Duvillard, P., and Morice, P. (2011). "Fertility determinants after conservative surgery for mucinous borderline tumours of the ovary (excluding peritoneal pseudomyxoma)." *Hum Reprod*, 26(4), 808-14.
- [39] Meredith, S., Dudenhoefter, G., Butcher, R. L., Lerner, S. P., and Walls, T. (1992). "Unilateral ovariectomy increases loss of primordial follicles and is associated with increased metestrous concentration of follicle-stimulating hormone in old rats." *Biol Reprod*, 47(2), 162-8.
- [40] Saiduddin, S., Rowe, R. F., and Casida, L. E. (1970). "Ovarian follicular changes following unilateral ovariectomy in the cow." *Biol Reprod*, 2(3), 408-12.
- [41] Yasui, T., Hayashi, K., Mizunuma, H., Kubota, T., Aso, T., Matsumura, Y., Lee, J. S., and Suzuki, S. (2012). "Factors associated with premature ovarian failure, early menopause and earlier onset of menopause in Japanese women." *Maturitas*, 72(3), 249-55.
- [42] Silber, S., Kagawa, N., Kuwayama, M., and Gosden, R. (2010). "Duration of fertility after fresh and frozen ovary transplantation." *Fertil Steril*, 94(6), 2191-6.
- [43] La Marca, A., Giulini, S., Tirelli, A., Bertucci, E., Marsella, T., Xella, S., and Volpe, A. (2007). "Anti-Mullerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology." *Hum Reprod*, 22(3), 766-71.
- [44] Baird, D. T., Webb, R., Campbell, B. K., Harkness, L. M., and Gosden, R. G. (1999). "Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196 C." *Endocrinology*, 140(1), 462-71.
- [45] Kagawa, N., Silber, S., and Kuwayama, M. (2009). "Successful vitrification of bovine and human ovarian tissue." *Reprod Biomed Online*, 18(4), 568-77.
- [46] Newton, H., Aubard, Y., Rutherford, A., Sharma, V., and Gosden, R. (1996). "Low temperature storage and grafting of human ovarian tissue." *Hum Reprod*, 11(7), 1487-91.
- [47] Keros, V., Xella, S., Hultenby, K., Pettersson, K., Sheikhi, M., Volpe, A., Hreinsson, J., and Hovatta, O. (2009). "Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue." *Hum Reprod*, 24(7), 1670-83.

- [48] Fuentes, F., Dubettier, R. (2004). Air Separation Method and Plant, United States Patent. Patent No. US 6,776,005 B2.
- [49] Bleyer, W. A. (1990). "The impact of childhood cancer on the United States and the world." *CA Cancer J Clin*, 40(6), 355-67.
- [50] Ries, L. A. G., and SEER Program (National Cancer Institute (U.S.)). (1999). *Cancer incidence and survival among children and adolescents : United States SEER program 1975-1995* / [edited by Lynn A. Gloecker Ries ... et al.], Bethesda, MD: National Cancer Institute, SEER Program.
- [51] Larsen, E. C., Muller, J., Schmiegelow, K., Rechnitzer, C., and Andersen, A. N. (2003). "Reduced ovarian function in long-term survivors of radiation- and chemotherapy-treated childhood cancer." *J Clin Endocrinol Metab*, 88(11), 5307-14.
- [52] Anderson, R. A., Themmen, A. P., Al-Qahtani, A., Groome, N. P., and Cameron, D. A. (2006). "The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer." *Hum Reprod*, 21(10), 2583-92.
- [53] Lee, S. J., Schover, L. R., Partridge, A. H., Patrizio, P., Wallace, W. H., Hagerty, K., Beck, L. N., Brennan, L. V., Oktay, K., and American Society of Clinical, O. (2006). "American Society of Clinical Oncology recommendations on fertility preservation in cancer patients." *J Clin Oncol*, 24(18), 2917-31.
- [54] Anderson, R. A., and Cameron, D. A. (2007). "Assessment of the effect of chemotherapy on ovarian function in women with breast cancer." *J Clin Oncol*, 25(12), 1630-1; author reply 1632.
- [55] Jeruss, J. S., and Woodruff, T. K. (2009). "Preservation of fertility in patients with cancer." *N Engl J Med*, 360(9), 902-11.
- [56] Greve, T., Clasen-Linde, E., Andersen, M. T., Andersen, M. K., Sorensen, S. D., Rosendahl, M., Ralfkiaer, E., and Andersen, C. Y. (2012). "Cryopreserved ovarian cortex from patients with leukemia in complete remission contains no apparent viable malignant cells." *Blood*, 120(22), 4311-6.
- [57] Gook, D. A., Edgar, D. H., and Stern, C. (1999). "Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1, 2-propanediol." *Hum Reprod*, 14(8), 2061-8.

Figure 1 a-f

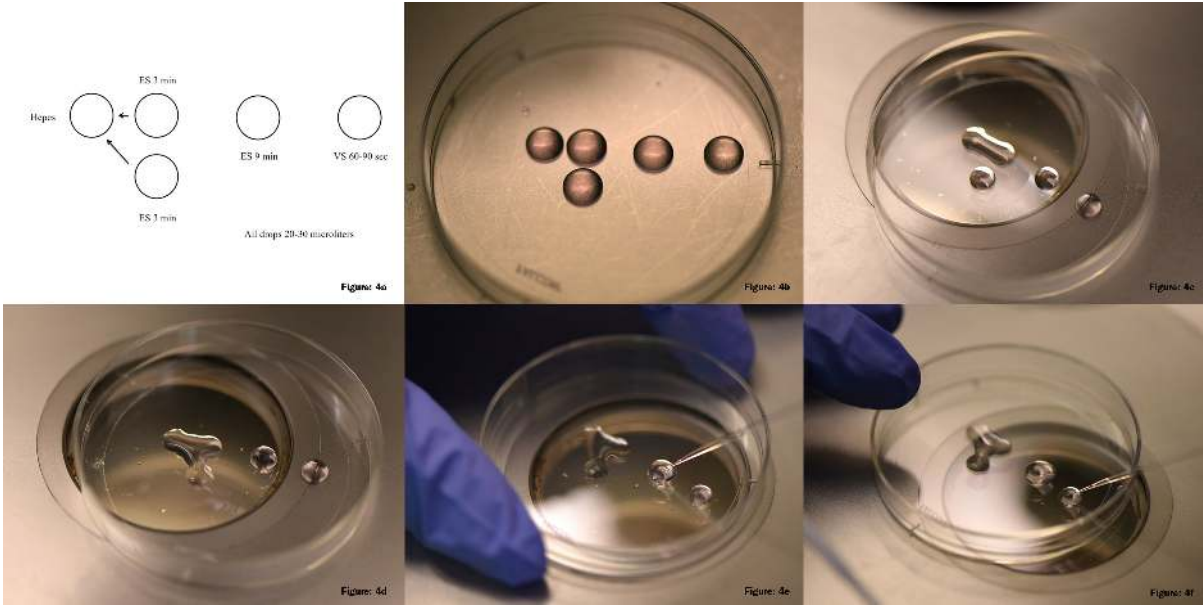


Figure 1 a

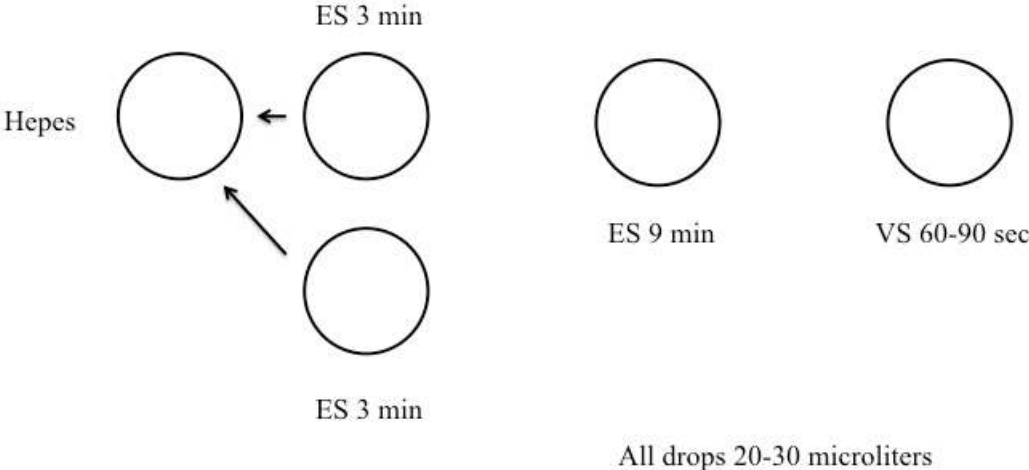


Figure 1 b

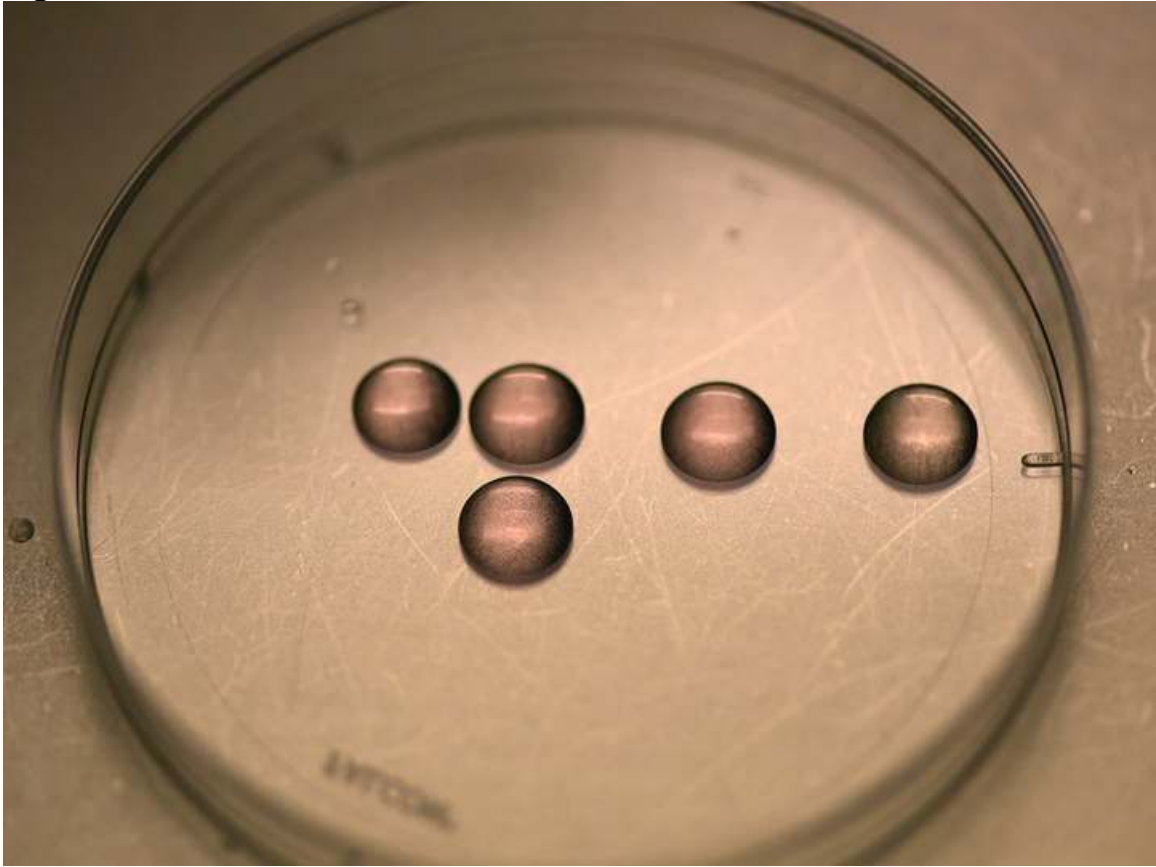


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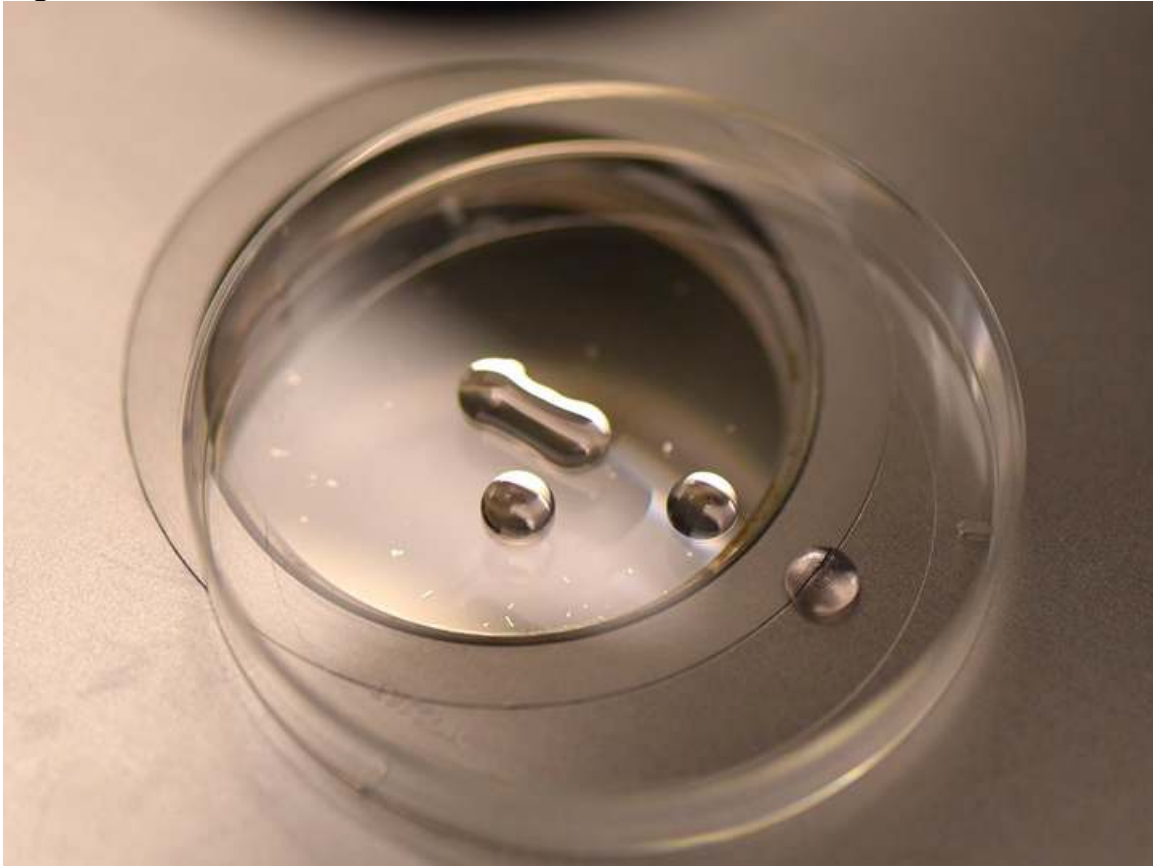


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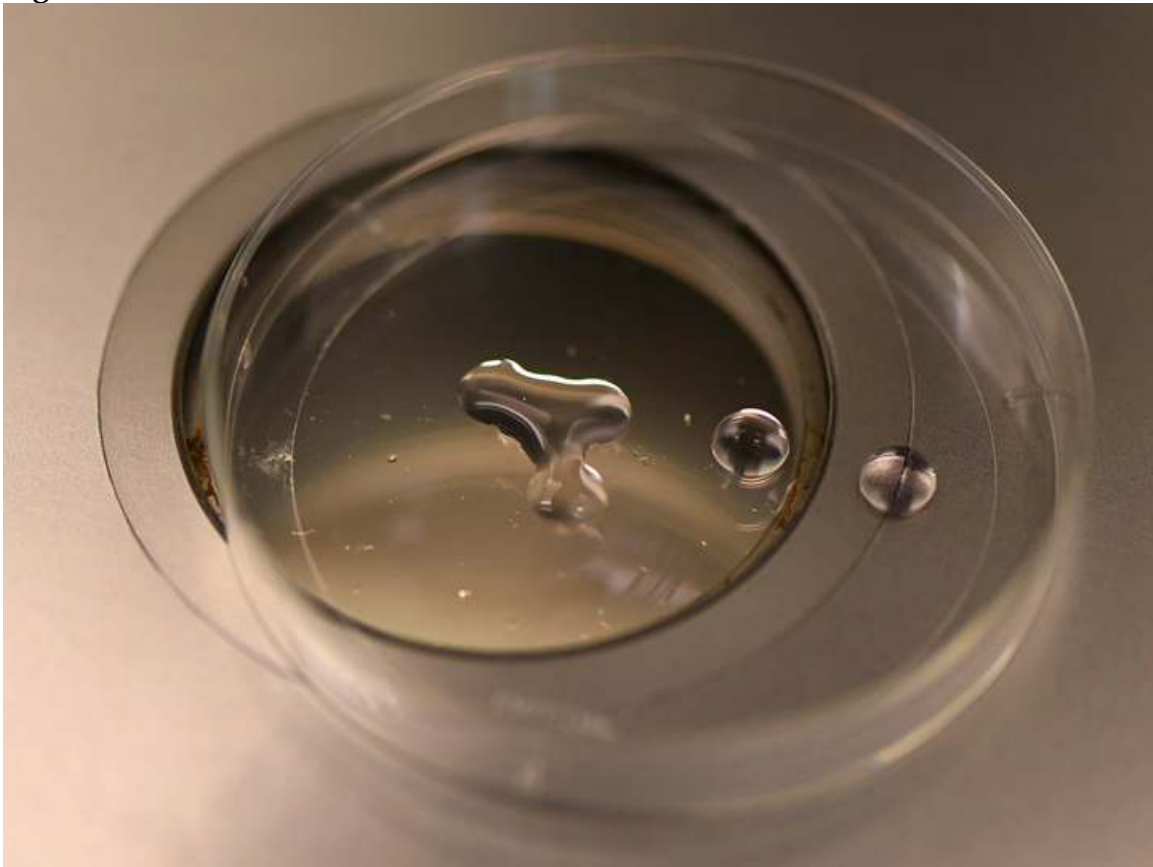


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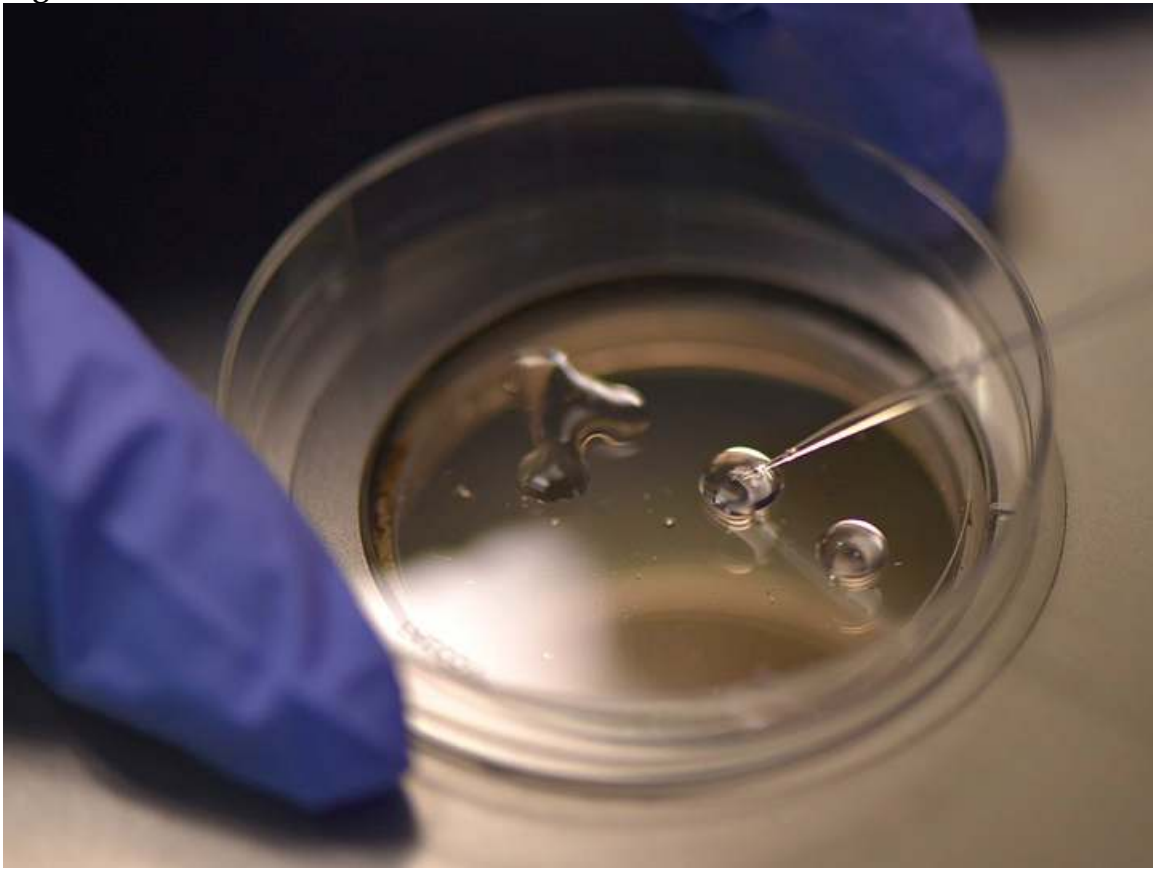


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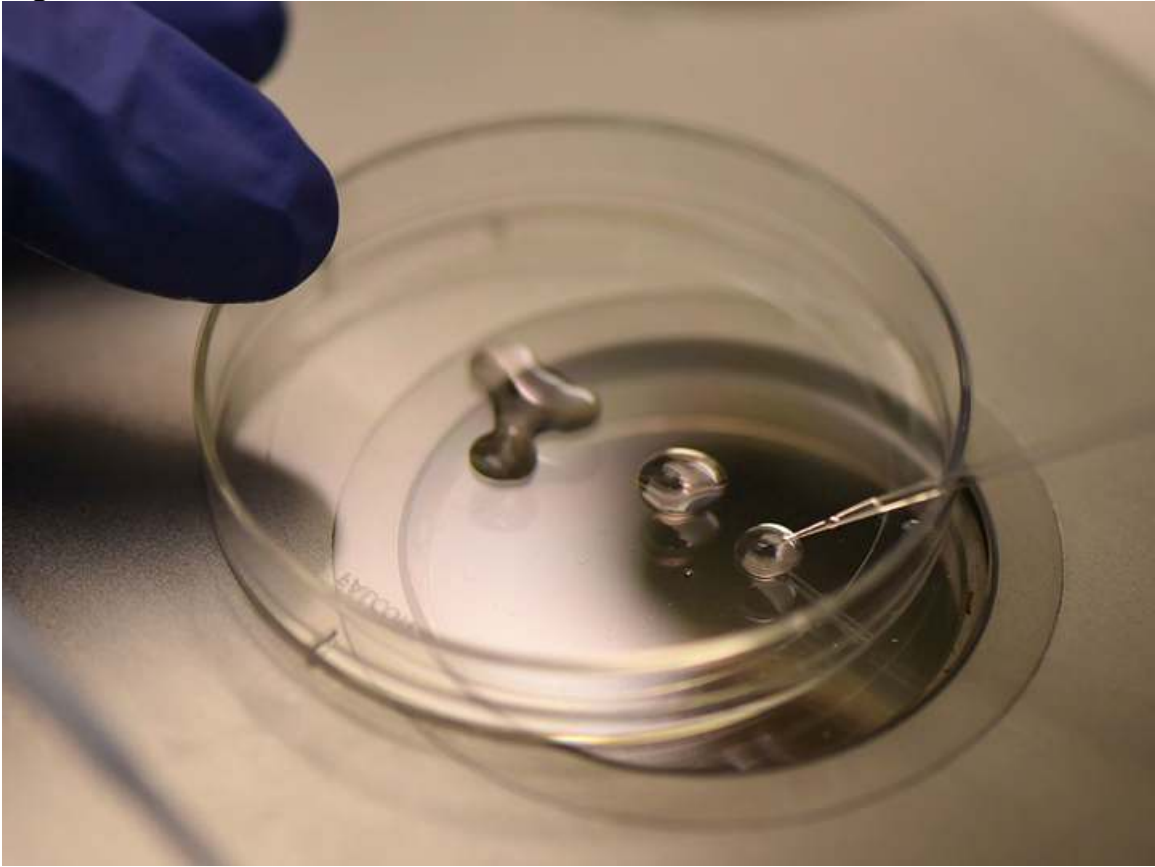


Figure 2

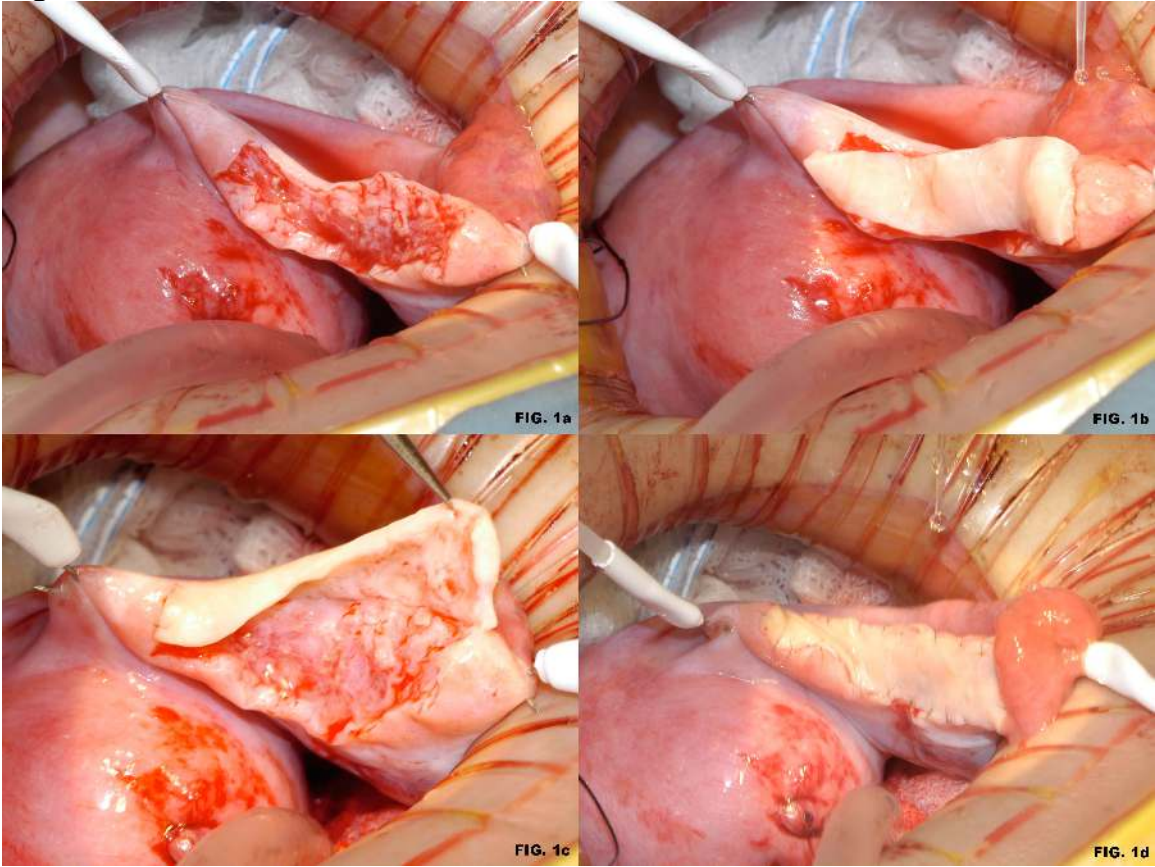


Figure 3



Figure 4



Figure 5

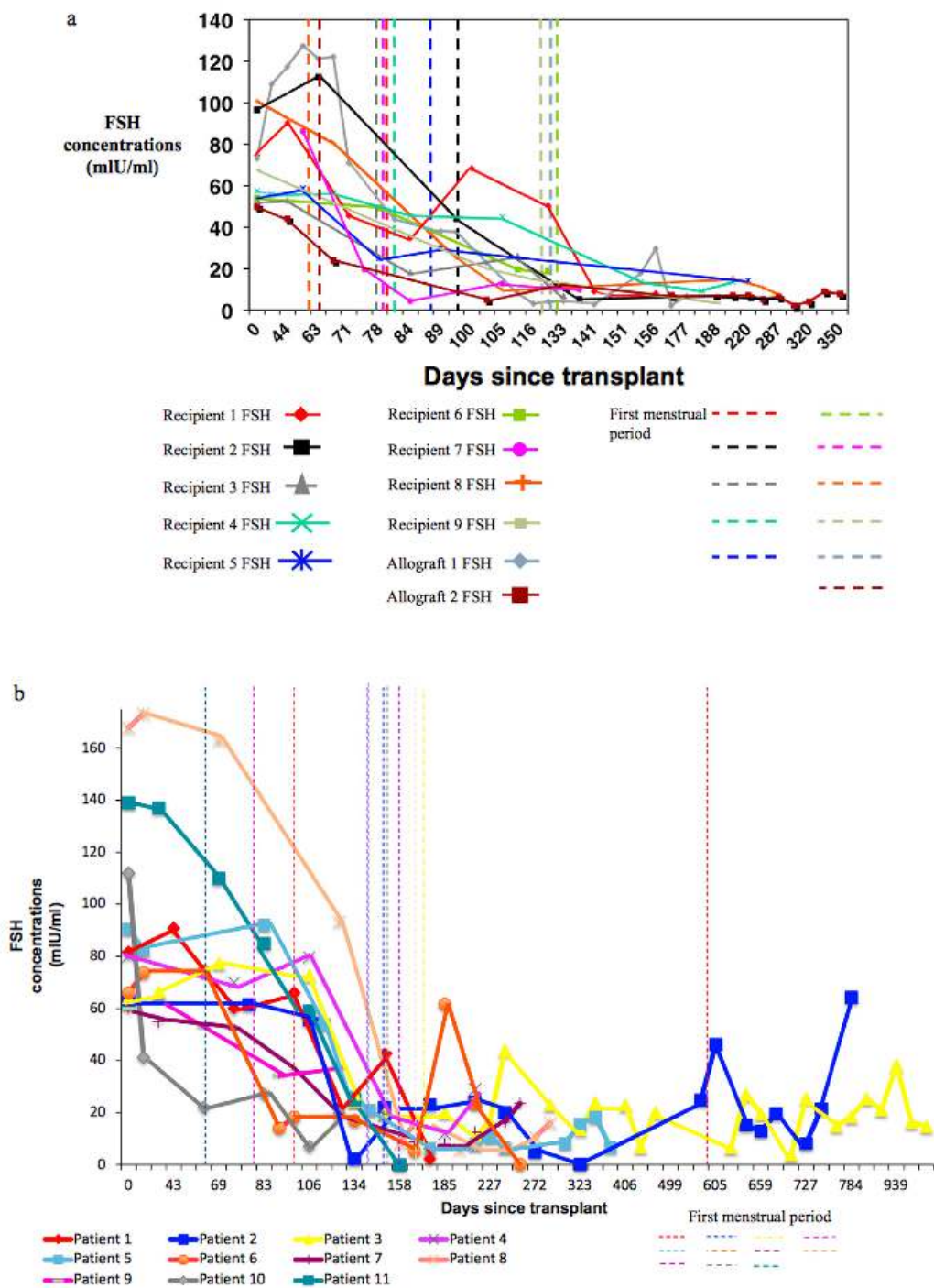


Figure 6

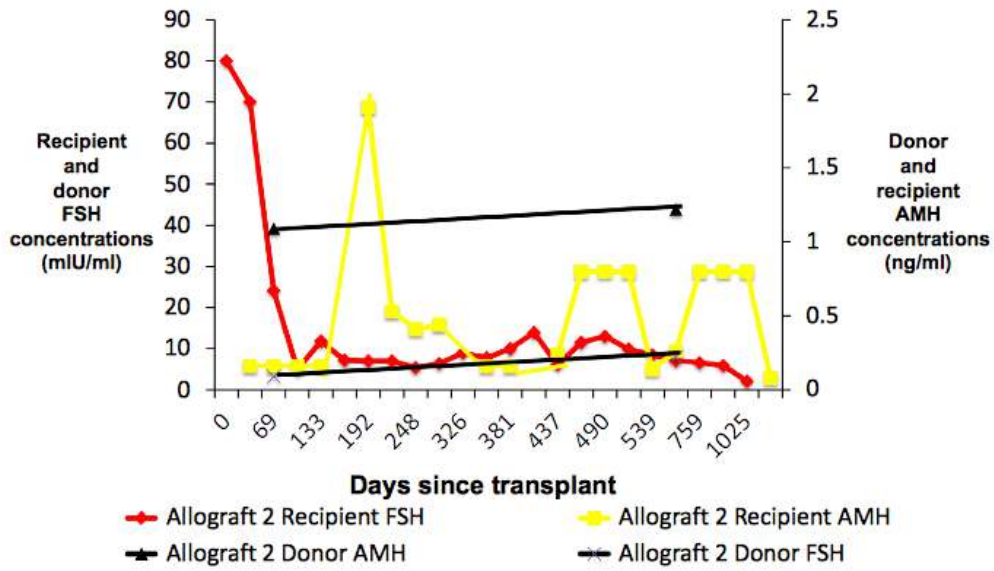
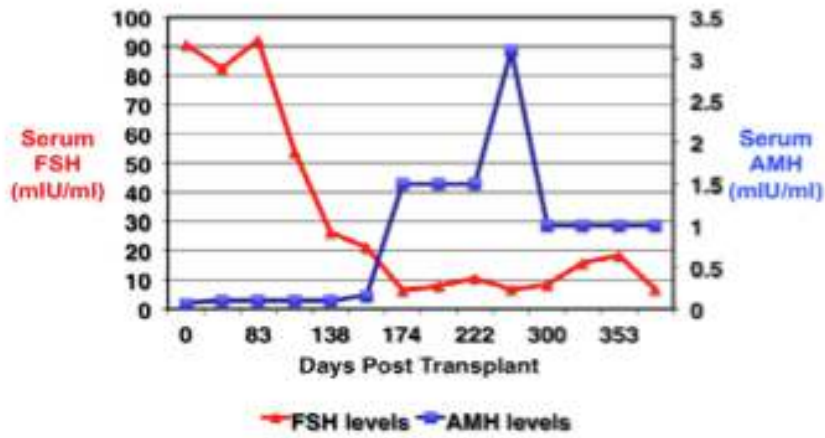
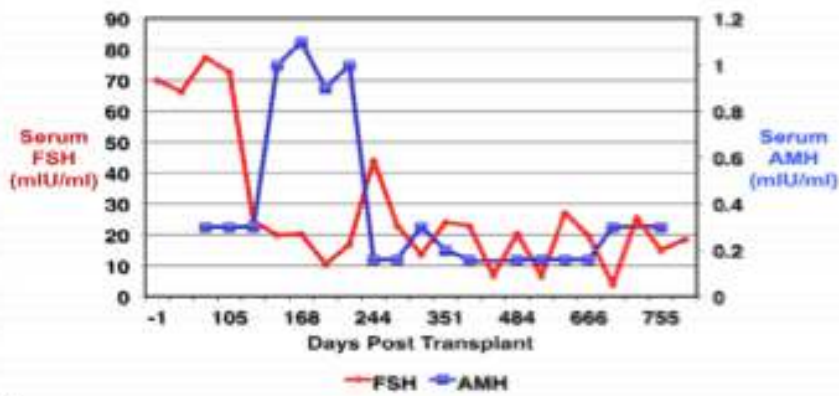


Figure 7 a-c



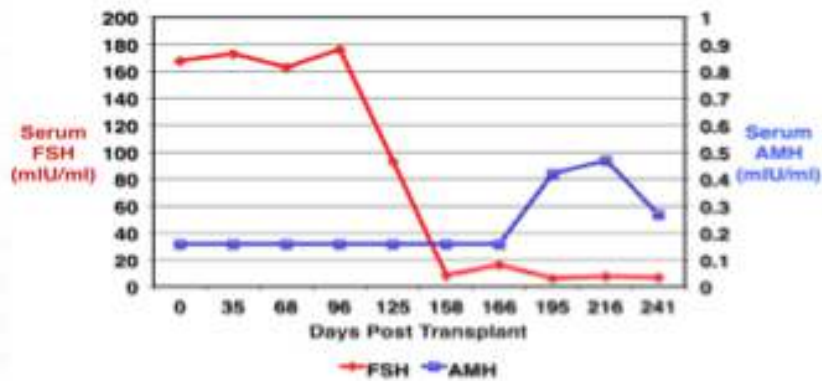
a

Transplant of Thawed Ovarian Tissue



b'

Transplant of Thawed Ovarian Tissue



c

Figure 8 a-d

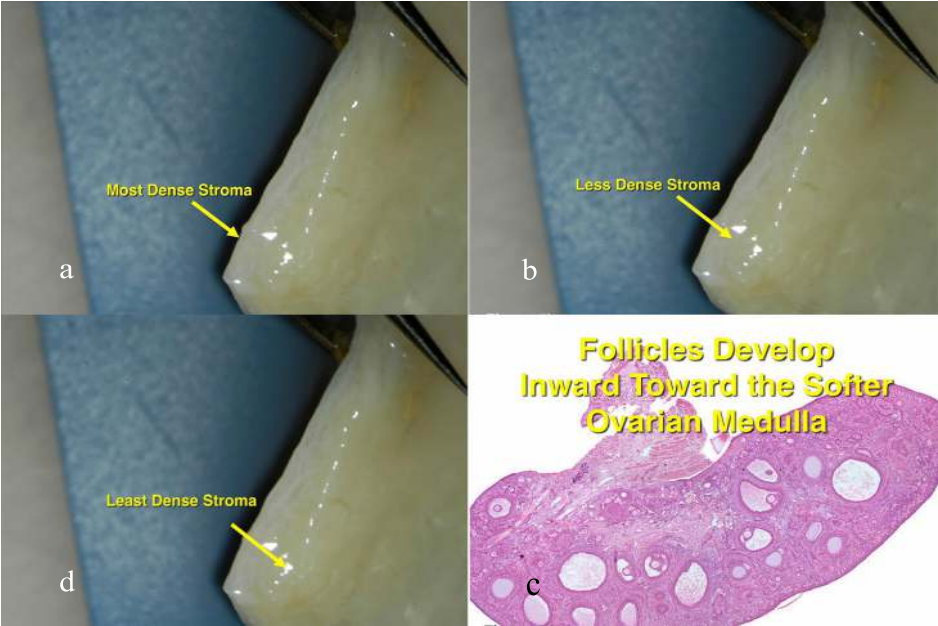


Table 1: Pregnancy and duration of function of fresh donor ovarian grafts from identical twin sister (9) or non-identical sister allograft (2)

Days of Ovarian Function	Date of Transplant	Ovarian Function End Date	Miscarriage	Baby Born	Girl	Boy
975	4/21/04	12/22/06	1	1	1	
3025	4/20/05	8/1/13		3	1	2
2285	6/7/05	9/9/11		2	1	1
1534	8/23/05	11/4/09	1	1		1
1107	9/20/05	10/1/08				
752	7/11/06	8/1/08		1	1	
2410+	8/16/06	n/a	2	2		2
1666	1/9/07	2/1/11		1	1	
618	4/21/08	12/30/09	1			
263 ^a	7/21/10 ^b	4/10/11				
1172+	2/11/11	n/a	1			
Total			6	11	5	6

Table 2: Pregnancy after frozen ovary autografts^a

Days of Ovarian Function	Date of Transplant	Date of First Menstruation post OT	Diagnosis	Miscarriage	Baby Born	Girl	Boy
804	3/6/07	9/19/2008 ^b	POF		1	1	
884	1/13/09	6/7/09	Hodgkins		1		1
997	6/9/09	11/28/09	POF	1	0		
1155+	6/17/11	11/5/11	Hodgkins		0		
564+	10/12/12	3/2/13	MS		1	1	
487+	3/29/13	7/5/13	POF		1	1	
496+	4/5/13	12/27/13	Brain Cancer		0		
502+	4/12/13	1/1/14	Blood Disorder		1		1
328+	10/1/13	12/19/13	Synovial Sarcoma		0		
294+	10/7/13	3/6/14	ALL		0		
306+	10/23/13	12/29/13	Breast Cancer		0		
Total				1	5	3	2

Table 3: Survival of small oocytes after enzymatic isolation from ovarian tissues following cryopreservation by vitrification or slow freezing.

Group	No. of ovaries	No. of oocytes harvested	No. of surviving oocytes (%)
Fresh	2	358	329 (91.9%) ^a
Vitrified	8	1,122	1,000 (89.1%) ^a
Cryopreserved	6	821	342 (41.7%) ^b
^{a,b} Groups with the same subscript are not significantly different ($P>.05$), whereas those with different superscripts are significantly different ($P<.01$). <i>Silber. Fertility after ovary transplantation. Fertil Steril 2010.</i>			