Developmental potential of human oocytes matured in vitro followed by vitrification and activation

Patrick Imesch1†, David Scheiner1‡, Min Xie2, Daniel Fink1, Erwin Macas2, Raghvendra Dubey2 and Bruno Imthurn2

Abstract

Background: Oocyte in vitro maturation (IVM) and cryopreservation at the time of routine ovarian tissue freezing may be offered to cancer patients as an additional option for fertility preservation. This study aimed to investigate the developmental capacity of oocytes isolated from unstimulated ovaries.

Methods: Immature oocytes (n = 63) from seven consenting premenopausal patients were analysed. Oocytes were collected during routine laparoscopic examination with biopsy of an ovary (cystic adnexal mass, n = 3; cervical adenocarcinoma, n = 2) or oophorectomy (sex reassignment surgery, n = 2) without previous stimulation of the ovaries. The stage of the patient’s menstrual cycle was not considered. Oocytes in all visible antral follicles were aspirated from ovaries, cultured in IVM medium and vitrified at the MII stage before being kept in liquid nitrogen for at least one month. After warming, oocytes were subjected to parthenogenetic activation by chemical stimulus. Their further development was recorded at intervals of 24 hours for up to 6 days of culture.

Results: 61.9% of oocytes matured in vitro within 48 hours. The survival rate after vitrification and warming was 61.5%. A total of 75% of surviving oocytes were able to respond to artificial activation, 44.4% of the parthenotes developed to early embryonic stage. However, only 1 in 18 (5.6%) of the resulting embryos reached blastocyst stage.

Conclusions: Oocytes matured in vitro from unstimulated ovaries seem to have limited developmental potential after cryopreservation and artificial activation. Although the outcome of IVM for non-stimulated oocytes is poor, it is currently the only chance besides cryopreservation of ovarian tissue for women for whom ovarian stimulation is not possible due to life circumstances. Based on our preliminary results, we suggest that the use of cryopreserved ovaries for fertility preservation in women with cancer warrants further investigation.

Keywords: In vitro maturation, Fertility preservation, Vitrification, Ovarian tissue, Parthenogenesis

Background

Fertility preservation in women with cancer is increasingly in demand due to improvements in diagnosis, effective treatment and follow-up, leading to better patient survival, especially among younger women [1,2]. The deleterious effects on ovaries of aggressive chemo- and radio-therapy are well known. Depending on treatment regimen, individual circumstances and response to treatment, some patients recover and can lead a normal, fertile life, while others may suffer permanent loss of gonadal function and become sterile [3-5]. Thus, the preservation of reproductive potential in women prior to treatment for cancer is becoming increasingly important. Recently, awareness of fertility preservation has also been raised in women with non-oncological conditions such as haematological and auto-immunological diseases that require similar treatments as for cancer. Such treatments can also cause premature ovarian failure or even total loss of fertility [6].

Currently, one method used to potentially preserve a woman’s fertility is to freeze ovarian cortical tissue before the patient undergoes radio- and/or chemotherapy.
However, depending on the type and stage of cancer—especially blood-borne cancer and those with potential danger of metastasis to ovaries—there is a risk of transmission of malignant cells when ovarian cortical tissue is cryopreserved and later used to restore fertility by autografting (retransplantation) [7-9]. In the latter situation, isolation of oocytes from antral follicles at the time of freezing of the ovarian tissue, with maturation in vitro before the oocytes are cryopreserved, can be considered as an alternative approach. However, little is known about the developmental capacity of such oocytes following cryopreservation and fertilization. This study therefore aimed to investigate the developmental potential of oocytes matured in vitro after cryopreservation and artificial activation by chemical stimulus.

**Methods**

Between October 2010 and January 2012, seven patients (age ranged from 18 to 41 years) were recruited at the Department of Gynecology, University Hospital of Zurich. Participation was voluntary, and written informed consent was obtained before the procedure. Inclusion criteria for patient recruitment were a premenopausal status and the feasibility of oocyte retrieval: all recruited patients were scheduled to undergo a laparoscopic procedure. Medical indications for laparoscopy were female-to-male sex reassignment surgery (SRS) \( (n = 2) \), benign cystic adnexal mass \( (n = 3) \), and cervical adenocarcinoma \( (n = 2) \). In the case of SRS, bilateral oophorectomy was performed, while in the other cases an ovarian biopsy was taken. Due to the required medical therapy, the stage of menstrual cycle at the time of oocyte collection could not be taken into account and was therefore ignored. In addition, no ovarian stimulation (FSH or HCG priming) was performed in order to avoid further physical burden to the patients. The two patients who underwent SRS had preoperative hormonal treatment with androgens.

**Oocyte retrieval**

Oocytes were retrieved by biopsy of the ovary. Ex vivo, visible (size not measured) follicles were aspirated under reduced pressure of 85 mm Hg using a 19-gauge single-lumen aspiration needle (K-OPS-7035-RWH-ET; Cook, Australia), according to the transvaginal aspiration technique described by Chian *et al.* [10]. The aspirated fluid was collected in 10 ml culture tubes containing 2 ml pre-warmed 0.9% saline solution with 2 IU/ml heparin. For bilateral oophorectomy, ovaries were transported immediately in IVM (in vitro maturation) medium containing HEPES (Sage; CooperSurgical, USA) supplemented with 75 mIU/ml FSH and 75 mIU/ml LH at 37°C in an atmosphere of 6% CO\(_2\) in air with high humidity for up to 48 hours. About 24 hours after the incubation, the oocyte-cumulus-complex was denuded with 80 IU/ml hyaluronidase solution (Vitrolife, Sweden) in order to assess maturity. Mature oocytes (MII) were vitrified immediately using a Kitazato Vitrification Kit (Japan). CryoTop was used as the carrier. Any immature oocytes were cultured for an additional 24 hours. Vitrification was repeated for any further mature oocytes observed.

**Oocyte activation and development**

Oocytes were kept in liquid nitrogen for at least one month before being warmed (Kitazato Warming Kit). Thawed oocytes were allowed to recover for a minimum of 1 hour at 37°C in a 6% CO\(_2\) incubator before being subjected to artificial activation (parthenogenesis). Only viable cells were subjected to chemical activation after warming. For ethical reasons, oocyte activation using chemical stimuli as described by Polak *et al.* [11] was conducted to simulate the process of fertilization. In brief, oocytes were incubated with 10 μM ionomycin (Sigma) in G-Mops medium supplemented with 5% HSA (Vitrolife, Sweden) at 37°C in room air for 6 minutes before placing in culture medium containing 2 mM 6-dimethylamimopurine (6-DMAP, Sigma) for 6 hours at 37°C / 6% CO\(_2\). The oocytes were analysed for successful activation 16–20 hours later by assessing the presence of a single pronucleus within the cytoplasm without a second polar body. Further development was recorded at intervals of 24 hours for up to 6 days of culture.

Statistical evaluation was undertaken using Intercooled Stata 10.0 (StataCorp LP, College Station, TX) by means of Fisher’s exact test for categorical data. P-values below 0.05 indicate statistical significance.

The study was reviewed and approved by the Ethics Committee of Canton Zurich (KEK-ZH-NR: 2010-0169/0).

**Results**

Table 1 shows the basic characteristics of the oocytes from the seven patients and summarises the results. From the seven patients, a total of 63 viable immature oocytes was obtained, with numbers ranging from 4 to 19 oocytes per patient. At the time of collection, all oocytes were at the germinal vesicle (GV) stage. In total,
61.9% (39/63) of immature oocytes (GV) reached stage MII following maturation for up to 48 hours in vitro, with a rate of 35.9% (14/39) IVM in the first 24 hours. The survival rate after vitrification and warming was 61.5% (24/39). 75% (18/24) of the surviving oocytes were able to respond to artificial activation, and 44.4% (8/18) of them developed to the 4- to 6-cell stage (blastomere). Only one (1/18, 5.6%) of the resulting embryos (parthenotes) reached the blastocyst stage. Analyzing for different patient diagnosis, no statistically significant differences (P=0.269) were found in regard to oocyte development (Table 2).

Discussion

Our results show that IVM can be achieved in immature oocytes isolated from ovaries without taking account of the phase of the menstrual cycle in which they are collected, and without any hormonal stimulation. There are some reports on IVM and cryopreservation of immature oocytes retrieved during routine ovarian tissue cryo-banking for female patients with cancer, suggesting the possibility of combined fertility preservation [12-17]. Revel et al. were the first to demonstrate that, without any hormonal stimulation, oocytes retrieved from ovarian tissue dissection can be matured in vitro and cryopreserved, or fertilized by ICSI followed by freezing as an additional option for fertility preservation [12]. However, to our knowledge, no report has shown any further investigation of the fertilization and developmental potential of such oocytes after IVM and cryopreservation.

In our study, we achieved an IVM rate of 61.9%. A study by Fasano et al. [15] reported a rate of 31% only, using the same commercial IVM medium and same concentrations of gonadotrophin supplements. After vitrification and warming, 75% of the surviving oocytes in our study responded to activation, which is higher than the expected rate of 67% according to the findings of Paffoni et al. [18]. Together with the survival rate of 61.5% after vitrification and warming, our results indicate the

### Table 1 Outcomes for oocytes collected from ovaries

| Patient | Age | No. viable oocytes collected | IVM to MII for up to 48 h (%) | No. oocytes survived after warming (%) | No. oocytes activated (%) | No. oocytes further developed (up to 6 days) (%) | Patient diagnosis |
|---------|-----|-------------------------------|-----------------------------|---------------------------------------|--------------------------|-----------------------------------------------|------------------|
| 1       | 18  | 9                             | 2                          | 1                                     | 1                        | 0                                             | Sex reassignment surgery |
| 2       | 24  | 19                            | 14                         | 10                                    | 7                        | 4                                             | Sex reassignment surgery |
| 3       | 25  | 11                            | 9                          | 4                                     | 3                        | 0                                             | Cystic adnexal mass     |
| 4       | 29  | 4                             | 2                          | 2                                     | 2                        | 2                                             | Cystic adnexal mass     |
| 5       | 39  | 5                             | 2                          | 2                                     | 1                        | 1                                             | Cystic adnexal mass     |
| 6       | 37  | 5                             | 5                          | 4                                     | 3                        | 0                                             | Cervical adenocarcinoma |
| 7       | 41  | 10                            | 5                          | 1                                     | 1                        | 1                                             | Cervical adenocarcinoma |
| Total   | 63  | 39/63 (61.9%)                 | 24/39 (61.5%)              | 18/24 (75%)                           | 8/18 (44.4%)             | 8/63 (12.7%)                                  |                  |
| P value | 0.84 | 0.53                        | 1.0                        | 0.70                                  |                          |                                               |                  |

No statistically significant differences were found between different patient diagnosis (SRS, adnexal mass, and adenocarcinoma) at each step (IVM, oocyte activation and development).

| Age of patient at the time of oocyte collection. |
| All MII oocytes were vitrified after IVM. |
| One embryo reached the blastocyst stage. |
| Fisher’s exact test. |

IVM, in vitro maturation; MII, metaphase II.

| Table 2 Development of collected oocytes after IVM, vitrification, and parthenogenetic activation according to patient diagnosis |
|---------------------------------------------------------------|
| Patient diagnosis                                           | No. oocytes collected | No. oocytes further developed (up to 6 days) | Success (%) |
| Sex reassignment surgery                                    | 28 | 4 | 14.3% |
| Cystic adnexal mass                                         | 20 | 3 | 15% |
| Cervical adenocarcinoma                                     | 15 | 1 | 6.7% |
| P value                                                        | 0.269 |

No statistically significant differences were found for further development of oocytes between the different patient diagnoses.

Fisher’s exact test.
development potential of oocytes matured in vitro followed by cryopreservation and activation. However, more than half of the activated oocytes in our study arrested at the pronucleus stage. Nearly all of the dividing parthenotes failed to develop beyond the 6-cell stage, and only one embryo reached blastocyst stage. The higher IVM rate in our study may be explained by the small cohort of seven patients representing a less oncologic collective, as some oncologic patients are found to exhibit impaired potential of oocyte development, although this remains controversial [19].

Using fresh, mature oocytes donated by patients from controlled ovarian hyperstimulation cycles, Paffoni et al. have shown that parthenogenetic activation using ionomycin and 6-DMAP as chemical stimuli is as effective as fertilization by ICSI, with 67.3% after activation and 62.3% after fertilization [18]. But these authors also observed that the rate of developmental arrest was significantly higher among activated oocytes (32.9%) than among fertilized oocytes (8.5%), and the blastocyst rate was 12.8%. Although Polak de Fried et al. have also reported an activation rate of 86.1% using noninseminated, cryopreserved human oocytes under similar conditions, the blastocyst rate was 16.7% [11]. Based on these results, we cannot exclude the possibility that insufficiency of artificial activation might affect embryo development, causing early developmental arrest.

The size of dominant follicles at the time of oocyte collection determines the outcome of in vitro maturation and embryo development to blastocyst stage [20]. HCG priming 36 hours before oocyte retrieval increases the rate of IVM and embryo development significantly, resulting in many live births [21,22]. Since the criteria of patient recruitment were based mainly on their age (pre-menopausal) and the feasibility of additional oocyte retrieval, i.e., that the required surgical procedure would not be compromised, the stage of the menstrual cycle at the time of oocyte collection was not considered. This in part reflects the real clinical situation when dealing with immediate ovarian tissue cryopreservation for cancer patients. Thus, while this could have contributed to the very low rate of blastocyst formation in our results, our procedure of retrieving oocytes irrespective of menstrual cycle stage or medical preconditioning reflects the actual clinical situation for cryopreservation of ovarian tissue for women with cancer.

The development of oocyte cryopreservation has made rapid progress since the introduction of vitrification techniques, and rates of fertilization, development and implantation have improved significantly, with survival rates of more than 85% and pregnancy rates of over 40% [23-25]. It has been further demonstrated that vitrification is superior to conventional slow freezing procedures in terms of meiotic spindle maintenance and recovery during and after the freezing process [26-28]. Therefore, in this study we used vitrification to cryopreserve oocytes matured in vitro. The relatively low survival rate of 61.5% could reflect oocyte quality before vitrification. Since the above-cited results by others were obtained from either oocyte donation programs or patients seeking fertility treatment, the oocytes were likely to be healthier than those from the present cohort of diseased patients in this study. In clinical practice, however, patients requiring fertility preservation due to cancer-related diseases are often in a most vulnerable state, as reflected in our study and also in a recent report by Escriba et al. [17]. The latter authors found that 36.1% of the immature oocytes isolated during routine ovarian tissue cryopreservation for oncology patients achieved spontaneous nuclear maturation in vitro. However, only 41% of them responded to parthenogenetic activation. It can be speculated therefore that the outcome of IVM and oocyte cryopreservation in such cases is less promising than would be predicted by the results of IVM used for infertility treatment.

The limitation of our study is the small number of patients and collected oocytes in this heterogenous patient collective. However, with the exception of the SRS group, this might in part reflect the real clinical situation when dealing with immediate ovarian tissue cryopreservation for cancer patients. We cannot ignore the fact that pretreatment with androgens for SRS patients might also have had a negative influence on oocyte quality, although the only blastocyst was generated from one of the two SRS cases. In addition, no statistically significant differences were found for the SRS group compared with the other subject groups in our study. Another limitation in the current study is that, for ethical reasons, we are not allowed to perform studies using fertilization. Using artificial activation only we were not able to reveal the true developmental potential of oocytes matured in vitro. An improvement on the study design would be to include a control group. In theory, a control could consist of immature oocytes from patients undergoing hormonal stimulation for IVF. Unfortunately, our daily routine has shown that these by-products are often in a state of degeneration, thus limiting their use as a control.

**Conclusions**

This first attempt suggests that oocytes matured in vitro from unstimulated ovaries have limited developmental potential following cryopreservation and artificial activation. However, although the outcome of IVM for non-stimulated oocytes is poor, this method is currently the only chance besides cryopreservation of ovarian tissue for women for whom time-consuming hormonal stimulation is not possible due to life circumstances, e.g. cancer. We hope that our findings will provide a useful stimulus for further investigation aimed at modifying
and optimizing the methodology to achieve successful in vitro culture and development of oocytes isolated from ovaries during routine ovarian tissue cryopreservation, particularly from patients with malignant disease.

Abbreviations
FSH: Follicle-stimulating hormone; OV: Ovarian vesicle; hCG: Human chorionic gonadotropin; ICSS: Intracytoplasmic sperm injection; IVF: In vitro fertilization; IVM: In vitro maturation; MII: Metaphase II; SRS: Female-to-male sex reassignment surgery.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MX, EM, RD, and BI conceived and designed the experiments. MX and EM performed the experiments. PI, DS, and MX contributed reagents/materials/analysis tools. PI, DS, DF, RD, and BI analyzed the data. PI, MX, EM, RD, and BI conceived and designed the experiments. MX and EM contributed reagents/materials/analysis tools. PI, DS, and DF contributed reagents/materials/analysis tools. PI, DS, DF, RD, and BI contributed reagents/materials/analysis tools. PI, MX, EM, RD, and BI contributed reagents/materials/analysis tools. PI, MX, EM, RD, and BI contributed reagents/materials/analysis tools. PI, MX, EM, RD, and BI contributed reagents/materials/analysis tools.

Acknowledgements
Part of this study (chemicals, disposables and culture media) was supported by Hartmann-Müller Foundation for Medical Research, Switzerland.

Reference
Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update 2006, 12:519–535.

Received: 21 January 2013 Accepted: 15 April 2013
Published: 18 April 2013

References
1. Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonck A, Demytule D, Dolmans MM: Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update 2006, 12:519–535.
2. Deepinder F, Agarwal A: Technical and ethical challenges of fertility preservation in young cancer patients. Reprod Biomed Online 2008, 19:126–140.
3. Wallace WHB, Thomson AB, Kelsey TW: The radiosensitivity of the human oocyte. Hum Reprod 2003, 18:117–121.
4. Kim SS: Fertility preservation in female cancer patients: current developments and future directions. Fertil Steril 2006, 85:1–11.
5. Varghese AC, Nagy ZP, Agarwal A: Current trends, biological foundations and future prospects of oocyte and embryo cryopreservation. Reprod Biomed Online 2009, 19:126–140.
6. Gidoni Y, Holzer I, Tulandi T, Tan SL: Fertility preservation in patients with non-ondiational cancer. Reprod Biomed Online 2008, 16:792–800.
7. Meirov D, Hardan I, Dor J, Fridman E, Elzur S, Ra’anani H, Shysayevsky E, Amargilo N, Schiff E, Rechavi G, Nagler A, Ben Yehuda D: Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. Hum Reprod 2008, 23:1007–1013.
8. Dolmans MM, Marinucci C, Saussoy P, Van Langendonck A, Amorim C, Donnez J: Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. Blood 2010, 116:2008–2914.
9. Bittinger SE, Nazaretnian SP, Gook OA, Parmar C, Hanup RA, Stem CJ: Detection of Hodgkin lymphoma within ovarian tissue. Fertil Steril 2011, 95:803, e3–6.
10. Chian RC, Buckett WM, Abdul Jall AH, Son JW, Sylvestre C, Rao D, Tan SL: Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential option in infertility treatment. Fertil Steril 2008, 89:1675–1678.
11. de Fried EP, Ross P, Zang G, Divita A, Cunniff K, Derady F, Salamone D, Kessling A, Cicelli J: Human parthenogenetic blastocysts derived from nonstimulated cryopreserved human oocytes. Fertil Steril 2008, 89:5–5.
12. Revel AA, Koler MM, Simon AA, Lewin AA, Laufer NN, Safran AA: Oocyte collection during cryopreservation of the ovarian cortex. Fertil Steril 2003, 79:1237–1239.
13. Revel A, Revel-Vilk S, Azzenen E, Porat-Katz A, Safran A, Ben-meir A, Weintraub M, Shapiro M, Achache H, Laufer N: At what age can human oocytes be obtained? Fertil Steril 2009, 92:6–5.
14. Iach enko EE, Rahimi GG, Iach enko W, Nawroth FF: In-vitro maturation of germinal-vesicle oocytes and cryopreservation in metaphase II: a possible additional option to preserve fertility during ovarian tissue cryopreservation. Reprod Biomed Online 2004, 8:553–557.
15. Facaro G, MoFF, D, Dechêne J, Engieryt L, Demester E: In-vitro maturation of oocytes matured oocytes collected from antral follicles at the time of ovarian tissue cryopreservation. Reprod Biomed Online 2010, 26:150–200.
16. González C, Devesa M, Biada M, Corelló B, Veiga A, Bant PN: Combined strategy for fertility preservation in an oncologic patient: vitrification of in vivo matured oocytes and ovarian tissue freezing. J Assist Reprod Genet 2011, 28:1147–1149.
17. Escrivá M-J, Grau N, Escrich L, Novella-Maestre E, Sánchez-Serrano M: Spontaneous in vitro maturation of oocytes prior to ovarian tissue cryopreservation in natural cycles of oncologic patients. J Assist Reprod Genet 2012, 29:1261–1265.
18. Raffini AA, Brevini TALT, Sonzogni AA, Restelli LL, Gandolfi FF, Ragini GG: In vitro development of human oocytes after parthenogenetic activation or intracytoplasmic sperm injection. Fertil Steril 2006, 876–5.
19. Pal L, Luykin L, Schiffen RL, Isaacson KB, Chang YC, Niskui NN, Chen Z, Toth TL: Malignancy may adversely influence the quality and behaviour of oocytes. Hum Reprod 1998, 13:1837–1940.
20. Colombo ACA, Requena AA, Neuspiller FF, MMA, Mercader AA, Navarro JJ, Simón CC, Remohi JJ, Pellicer AA: Maturation in vitro of human oocytes from unstimulated cycles: selection of the optimal day for ovum retrieval based on follicular size. Hum Reprod 1999, 14:1864–1868.
21. Chian RC, Galieki B, Buckett WM, Tan SL: Priming with human chorionic gonadotropin before retrieval of immature oocytes in women with infertility due to the polycystic ovary syndrome. N Engl J Med 1999, 341:1624–1626.
22. Chian R-C, Buckett WM, Tan SL: In-vitro maturation of human oocytes. Reprod Biomed Online 2004, 8:148–166.
23. Kushaya WM, Vajta GG, Kato OO, Leibo SPS: Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online 2005, 11:300–308.
24. Colombo A, Kuszyk WM, Rez SP, Ruiz A, Pellicer A, Remohi J: Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryoprot method. Fertil Steril 2008, 89:8–5.
25. Chian R-C, Huang YJ, Gilbert L, Son W-Y, Holzer H, Cui SJ, Buckett WM, Tulandi T, Tan SL: Obstetric outcomes following vitrification of in vitro and in vivo matured oocytes. Fertil Steril 2009, 91:2391–2398.
26. Larmar MG, Miniari MG, Rienzi L, Gardner DK: Maintenance of the meiotic spindle during vitrification in human and mouse oocytes. Reprod Biomed Online 2007, 15:692–700.
27. Colbo A, Pérez S, Santos MJ, Zulategui J, Domínguez M, Remohí J: Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. Reprod Biomed Online 2008, 17:350–359.
28. Ciotti PM, Porcu E, Notarangelo L, Magrini O, Bazzocchi A, Venturoli S: Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. Fertil Steril 2009, 91:2399–2407.