Spontaneous in vitro maturation of oocytes prior to ovarian tissue cryopreservation in natural cycles of oncologic patients

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Received: 30 May 2012 / Accepted: 30 August 2012 / Published online: 11 September 2012
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Abstract

Purpose To evaluate the recovery rate and spontaneous in vitro maturation (IVM) of immature oocytes enclosed within or released from follicles during the processing of ovarian tissue prior to its cryopreservation.

Methods Thirty-three oncologic patients who had not previously undergone chemo or radiotherapy underwent ovarian tissue cryopreservation (OTC) during natural menstrual cycles. Immature oocytes, enclosed within follicles or released during ovarian cortex processing, were collected and matured spontaneously in vitro for 48 h. Nuclear maturation was assessed every 24 h and the ability of the IVM oocytes to display a normal activation response following parthenogenetic activation was evaluated. The following outcome measures were also evaluated: disease, age, FSH, LH, E2,

P4 and AMH serum levels, menstrual cycle day, recovery and spontaneous IVM and parthenogenetic activation rates. **Results** Oocytes recovered per patient were 3.3 ± 0.7 (1.8–4.7 oocytes, 95CI), regardless of the menstrual phase. The mean number of IVM oocytes per patient was 1.3 ± 0.2 oocytes (95CI: 0.8–1.8), regardless of menstrual phase ($p=0.86$) and oocyte origin ($p=0.61$). Forty-one percent of oocytes extruded the second polar body and formed one pronucleus after parthenogenetic activation.

Conclusion Twenty-one of the 33 women (63.6 %) requesting OTC produced at least one mature oocyte.

Keywords Ovarian tissue cryopreservation · Immature oocyte · Spontaneous in vitro maturation · Human · Oncologic patient · Natural cycles

Capsule Immature oocytes enclosed within or released from follicles were recovered and spontaneously in vitro matured prior to ovarian tissue cryopreservation in natural cycles of oncologic patients.

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Introduction

Given current survival rates among oncologic patients, protocols of fertility preservation (FP) are of growing relevance.

According to the 2005 Ethics Committee of the American Society for Reproductive Medicine, the only accepted method of FP for oncologic patients is embryo cryopreservation. Ovarian tissue cryopreservation (OTC) is an alternative protocol that has attracted interest due to the fact that the only precondition for its application is the presence of ovaries, while variables such as age [9], menstrual cycle phase [12, 25] and subjacent disease do not have a bearing on whether or not it can be performed. In addition, the tissue reimplantation that follows depends purely on the absence of malignant cells [2, 13, 20, 21, 24].

Retrieval of immature oocytes from unstimulated ovaries followed by IVM is an effective treatment option for many infertile women [4, 5, 11, 25] and cancer sufferers. In oncologic patients, oocytes can be retrieved by means of standard

oocyte aspiration [12, 15] or in association with OTC [9, 10, 12, 19]. Collection of immature oocytes in association with OTC was first proposed by Revel et al. [19], who described a procedure in which nine pre-chemotherapy female patients underwent oophorectomy by unilateral laparoscopy. Visible follicles in the tissue were punctured and the immature oocytes aspirated. Immature oocytes that had been released from broken follicles during OTC process were also collected. In another study, Huang et al. [9] reported four cases of female oncologic patients in whom an oophorectomy (one) and wedge resections (three) were performed prior to cryopreservation of their ovarian tissue, after which immature oocytes were retrieved as described by Revel et al. [19]. More recently, Maman et al. [12] reported collection of compacted cumulus-oocyte complexes by transvaginal ovarian puncture of hCG-primed ovaries. In all these reports, recovered immature oocytes were matured in vitro in FSH supplemented media and subsequently cryopreserved or fertilized by ICSI.

In the present study, we report a series of 33 oncologic patients in which oocytes were obtained from the partially excised right ovarian cortex from either visible large follicles within the cortex or the detritus left over after tissue processing. Our aim was to explore the recovery rate of immature oocytes in association with our OTC protocol [22, 23] and in the absence of any additional treatment or intervention (e.g. transvaginal puncture or hCG-priming). The potential of oocytes to mature spontaneously in vitro and the ability of MII oocytes to respond to parthenogenetic activation were also assessed.

Materials and methods

The present study was approved by both the Hospital and the IVI Reproduction Clinic Research Ethics Boards. Thirty-three women enrolled in our OTC FP program gave their written consent to participate in the present study after being informed accordingly. The following data were recorded for each patient: age, oncologic disease, day of menstrual cycle, and serum hormonal levels of FSH (IU/l), LH (IU/l), estradiol (pg/mL), progesterone (ng/mL) and antimüllerian hormone (AMH, ng/mL) on the day of ovarian cortex extraction.

At the time of intervention, the right ovarian cortex was partially excised from the medulla and removed as described earlier [22]. The left ovary was not manipulated in any way. The surgical intervention having been completed, follicles measuring ≥ 6 mm were sectioned from the excised ovarian cortex and placed in 5 mL DMEM medium at 25 °C. The ovarian tissue was then processed by scraping its medullar side until the piece was reduced to a thin sheet with a thickness of 1–2 mm. Inevitably, some follicles of < 6 mm were broken during scraping and their content released into the detritus. After processing, the thinned ovarian piece was divided in two and

placed in clearly identifiable tubes and transported in DMEM at 4 °C to the Centro de Transfusión de la Comunidad Valenciana for freezing and cryobanking [23].

Isolated ≥ 6 mm follicles (if present) and the detritus left over after processing the ovarian tissue (which is usually disposed of with other surgical residues) were collected and transported to the IVF Laboratory of IVI Valencia within the following 3 h.

Oocytes enclosed in ≥ 6 mm follicles were released using two sterile scalpels. Large cumulus-oocyte-complexes were collected (Fig. 1a), washed and individually cultured in 50 μ L CCM (Vitrolife, Göteborg, Sweden) under standard conditions (37 °C, 5%CO₂ in air) for 48 h, as in the case of GV oocytes recovered from stimulated cycles [6, 7].

The detritus obtained during ovarian tissue processing was inspected by two observers. Isolated cumulus-surrounded oocytes (Fig. 1b), presumably released from follicles that had been broken during processing, were collected, washed and cultured for 48 h in the abovementioned conditions. No cumulus-free oocytes were recovered and degenerated oocytes were discarded and not included in the study.

Oocytes were checked every 24 h for nuclear maturation under contrast phase microscopy observation at 400 $\times$ magnification. The presence of the germinal vesicle (GV stage), GV breakdown (GVBD, metaphase I stage) and first polar body extrusion (metaphase II stage -MII-) were confirmed. MII oocytes (Fig. 1c and d) that were healthy in appearance were artificially activated with calcium ionophore (A23184) and puromycin [6, 14]. In short, oocytes were exposed to A23187 (8 mM, Sigma, Barcelona, Spain) for 5 min and subsequently cultured in puromycin (10 μ g/mL; Sigma, Barcelona, Spain) for 5 h. Once activated, eggs were cultured in 50 μ L IVF medium (Vitrolife, Göteborg, Sweden) in the previously referred conditions for 16–20 h and then assessed for extrusion of the second polar body (2PB) and number of pronuclei.

Patients were confirmed to be in the follicular, ovulatory or luteal phase of their menstrual cycle according to their hormonal levels and the reference values of the Hospital Universitario Dr. Peset Laboratory.

Continuous variables (age and FSH, LH, E2, P4 and AMH serum levels) were compared between groups using the ANOVA test. Values of continuous variables were presented as the median and 95 % confidence interval (95CI).

The recovery rate of oocytes and their ability to mature in vitro — measured according to menstrual phase and oocyte origin — were compared between groups using the Chi-square test. The mean number of recovered/matured oocytes per patient and menstrual phase were compared by means of an ANOVA test.

All statistical analyses were performed using the Statistical Package for Social Sciences 17.0 (SPSS Inc., Chicago, IL) and MedCalc Software (Ghent, Belgium).

Fig. 1 Photographs of large cumulus-oocyte-complexes recovered from ≥ 6 mm follicles (a) and cumulus-surrounded oocytes recovered from < 6 mm follicles (b) on day of OTC or after 48 h of in vitro maturation (c and d, respectively)

Results

Thirty-three women aged between 15 and 38 years old (31.5 ± 5.2 years; 95CI: 29.7–33.3 years) and who had requested OTC prior to initiation of oncologic treatment for breast cancer ($n=28$), Hodgkin's disease ($n=3$), non-Hodgkin lymphoma ($n=1$) or leukaemia ($n=1$) agreed to participate in this study. The time lapsed between the first contact with the patient and surgery varied from 12 h to 6 days (mean 3.4 days).

As shown in Table 1, the groups organized according to menstrual phase exhibited significant differences with respect to day of the menstrual cycle ($p<0.01$), FSH ($p<0.01$), LH ($p<$

0.01), E2 ($p=0.031$) and P4 ($p<0.01$), while neither AMH levels (2.7 ± 2.3 ng/mL; $p=0.46$) nor age (31.5 ± 5.2 years, 95CI: 29.7–33.3 years; $p>0.05$) varied.

At least one immature oocyte was obtained from 30 of the 33 women enrolled in the study (Table 2), which demonstrated that stage of menstrual phase had no bearing on outcome. In this way, 3.3 ± 0.7 (1.8–4.7, 95CI) oocytes were recovered per patient.

Analysis of the origin of the recovered oocytes confirmed that significantly more oocytes were obtained from < 6 mm follicles (1.2–3.7 oocytes, 95CI) than ≥ 6 mm follicles (0.7–1.6 oocytes, 95CI). However, mean numbers of oocytes were comparable in all the menstrual phase groups, regardless of their origin (Table 2).

In terms of spontaneous nuclear in vitro maturation, 21 women (6 out of 10) had at least one MII, which showed that stage of menstrual phase at OTC was not a relevant factor.

Analysis of the mean number of in vitro matured oocytes per patient did not reveal significant differences between menstrual phase groups ($p=0.86$) or oocyte origin ($p=0.61$). Thus, the mean number of MII oocytes obtained per patient was 1.3 ± 0.2 (95CI: 0.8–1.8, Table 2).

The average IVM rate was 36.1 % (95CI: 25.6–56.5 %; data not shown in Table 2) and no differences between menstrual phase groups ($p=0.78$) or oocyte origin ($p=0.71$) were detected, although it should be noted that none of the five oocytes retrieved from ≥ 6 mm follicles in the ovulatory phase group matured.

In global terms, 21 of the 33 women (63.6 %) originally requesting OTC had at least one mature oocyte.

In relation to the ability of IVM oocytes to respond to parthenogenetic activation, 16 out of 39 MII oocytes extruded

Table 1 Age, day of the menstrual cycle and serum hormonal levels of patients during surgery for ovarian tissue cryopreservation in follicular, ovulatory or luteal phase

	Follicular phase	Ovulatory phase	Luteal phase	<i>P</i> -value
Number of patients	12	3	18	
Age (years)	32.0 ± 6.7	29.7 ± 6.4	31.5 ± 3.9	>0.05
Menstrual cycle day (days)	7.4 ± 5.4^a	$13.0 \pm 4.6^{a,b}$	25.9 ± 12.0^b	<0.01
FSH (IU/l)	5.5 ± 1.7^a	7.9 ± 1.7^a	2.7 ± 1.4^b	<0.01
LH (IU/l)	4.2 ± 1.8^a	16.4 ± 5.9^b	2.6 ± 2.4^a	<0.01
E2 (pg/ml)	62.1 ± 49.1^a	160.3 ± 99.1^b	$105.2 \pm 59.4^{a,b}$	<0.05
P4 (ng/ml)	0.2 ± 0.2^a	$1.2 \pm 0.4^{a,b}$	7.5 ± 5.4^b	<0.01
AMH (ng/ml)	2.0 ± 0.6	2.4 ± 2.5	3.2 ± 2.9	>0.05

Values are presented as mean $\pm$ SEM (standard error of the mean)

^{a,b} Different superscripts in the same row indicate statistical significance between groups

Table 2 Number of immature oocytes recovered from <6 mm or ≥6 mm follicles and their corresponding in vitro maturation, according to menstrual cycle phase

	Follicular phase	Ovulatory phase	Luteal phase	Total
Number of patients	12	3	18	33
Number of recovered oocytes from:				
<6 mm follicles	33 (2.7±0.8)	17 (5.7±1.8)	58 (3.2±1.2)	108 (3.3±0.7)
≥6 mm follicles	20 (2.0±0.7)	12 (4.0±1.0)	41 (2.4±0.9)	73 (2.4±0.6)
Number of matured oocytes from:				
<6 mm follicles	13 (1.3±0.3)	5 (1.7±1.2)	17 (1.0±0.3)	35 (1.2±0.2)
≥6 mm follicles	13 (1.3±0.4)	5 (1.7±0.3)	21 (1.2±0.3)	39 (1.3±0.2)
<6 mm follicles	6 (0.7±0.2)	5 (1.7±0.3)	14 (1.2±0.4)	25 (1.1±0.2)
≥6 mm follicles	7 (0.9±0.2)	0	7 (0.6±0.2)	14 (0.7±0.1)

Values in brackets refer to mean ± standard error of the mean per patient

the second polar body and formed a single pronucleus (41.0 % displayed a normal activation response; 95IC:27.1–45.2 %), which means that 0.7 ± 0.1 oocytes per patient were activated normally (data not shown in tables).

Discussion

In the present study, we report a large series of oncologic patients undergoing OTC during natural cycles (without hCG priming) in whom an average of 3.3 oocytes were recovered after partial unilateral dissection of the ovarian cortex. Similarly, other authors have reported average numbers of 2.7 to 4.6 immature retrieved oocytes per patient (in natural cycles) following aspiration from visible follicles in surgically removed ovaries [9, 19] or in excised unilateral ovarian cortex [9].

An improvement in the recovery rate of oocytes is observed after controlled ovarian stimulation (COS) and hCG priming. COS usually includes gonadotropin administration (FSH and LH), which increases the oocyte recovery rate and concomitant serum estradiol levels which can induce cancer cell proliferation and/or dissemination in some malignancies [1, 16–18]. Moreover, COS requires time, which can mean delaying the initiation of oncologic treatment (around 30 days: [15]). On the other hand, the administration of hCG is usually included in protocols for oocyte retrieval from stimulated and non-stimulated ovaries (natural cycles), allowing between 1.7 and ~15 oocytes per patient to be retrieved by bilateral ovarian aspiration under transvaginal ultrasound guidance [10, 12, 15].

Not many studies have explored oocyte retrieval from unstimulated or non-hCG-primed ovaries and subsequent in vitro maturation. In the few that have been published [9, 19], age, pathology and day of menstrual cycle were found to be factors that influenced the number of immature oocytes retrieved. Conversely, the recovery rate in our study was not affected by such factors. The lack of differences in the oocyte recovery rate according to age, ovarian reserve (indirectly estimated by AMH serum levels) and pathology may have been due to the homogeneity of our patients, since their age

ranged from 29.7 to 33.3 years, their AMH levels were between 1.8 and 3.6 ng/mL (95CI) and 28 of the 33 women suffered breast cancer. In addition, the number of immature oocytes retrieved per patient did not depend on stage of menstrual phase (3.3 ± 0.7 oocytes), which is accordance with findings reported by Maman et al. [12].

In relation to in vitro maturation, 36.1 % of retrieved oocytes matured spontaneously in vitro, regardless of stage of menstrual phase at OTC and oocyte origin. The IVM rate achieved was low, but comparable to that reported by Cha and Chian (8.8 % to 34.5 %, [3]) and that published by Revel et al. (50 %, [19]), who also spontaneously matured in vitro oocytes retrieved from non-hCG-primed ovaries. Higher maturation rates can be achieved by supplementing the maturation media with gonadotropins (80 %, [9]). The inadequacy of the in vitro system we employed is also evident in the low percentage of IVM oocytes that displayed a normal activation response following parthenogenetic activation (41.0 %), especially in comparison to results obtained with in vivo matured oocytes (76 %; [6]).

It is of note that, in the ovulatory phase group, none of the oocytes recovered from ≥6 mm follicles matured. This result is surprising, since oocytes from large follicles generally exhibit good maturation and developmental competence. This could have been due to the fact that these oocytes, rather than originating in the largest follicle (and thus being destined to ovulate) could have originated in the large subordinate non-ovulatory follicles that become atretic as a result of the follicle dominance that takes place during the late follicular phase in humans [8].

In the present work, more immature oocyte were recovered from <6 mm follicles (broken during processing) than those which measured ≥6 mm. However, differences were not observed according to stage of menstrual phase, which was probably due to the heterogeneous origin of the oocyte population (from large follicles burst during manipulation of the tissue and/or from small antral follicles scraped during ovarian tissue processing). Thus, in order to draw sound conclusions, indirect indicators, such as oocyte diameter, should be used to identify follicle size at origin. From a practical point of view, the aspiration of visible follicles

should be performed before ovarian tissue dissection using an 18- or 21-gauge needle attached to a syringe [9, 12, 19]. Such a procedure would allow us to access the large follicles that are present in the contralateral ovary, which was not done as part of our OTC protocol [22, 23].

In conclusion, following the basic OTC protocol described herein, we were able to recover oocytes from 85 % patients and at least one mature oocyte was obtained in 75 % of cases. Thus, oocyte recovery and subsequent in vitro maturation associated with OTC may need to be mandatory, since it seems to increase the reproductive chances of patients without causing any physical discomfort or a delay in the initiation of oncologic treatment. Our protocol can be applied to virtually all patients, regardless of their age, sexual maturity or stage of menstrual cycle, and without prior controlled ovarian stimulation or hCG-priming. Nevertheless, further developments in protocols of in vitro maturation are necessary to improve the final outcome of maturation.

Acknowledgments The authors would like to thank Mr. Normanly for editing this manuscript. Authors would also like to thank the Hospital Universitario Dr. Peset de Valencia for providing the biological samples and analyzing blood samples. This work was funded by IMPIVA (Instituto de la Mediana y Pequeña Empresa Valenciana; IMIDTF/2009/133; IMIDTF/2010/82, IMIDTF/2011/203 and Generalitat Valenciana) and Instituto Universitario IVI Valencia. The authors declare that they have no conflict of interest.

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