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Outcomes of immature oocytes collected from ovarian tissue for cryopreservation in adult and prepubertal patients

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KEY MESSAGE

Oocyte in-vitro maturation and vitrification procedures after ex-vivo collection from ovarian tissue could be considered as an additional option for fertility preservation.

A B S T R A C T

The efficiency of oocyte in-vitro maturation (IVM) and vitrification procedures after ex-vivo collection from ovarian tissue were assessed according to patient age, number of retrieved oocytes and tissue transport conditions. The combined procedure was performed in 136 patients: 130 adults (mean 27.6 ± 5.6 years) and six prepubertal girls (mean 8.7 ± 2.3 years). A higher mean number of oocytes were collected in girls compared with adults (11.5 ± 8.0 versus 3.8 ± 4.2, respectively, $P < 0.001$) but the percentage of degenerated oocytes was significantly higher in girls (35.5% versus 17.1%, respectively, $P < 0.001$). IVM rates were significantly lower in prepubertal than postpubertal population (10.3% versus 28.1%, $P = 0.002$). In adults, a negative correlation was observed between number of retrieved oocytes and age ($P = 0.002$; $r = -0.271$); the correlation was positive between anti-Müllerian hormone (AMH) and number of collected oocytes ($P = 0.002$; $r = 0.264$). IVM rates were not correlated with AMH levels ($r = 0.06$) or age ($r = -0.033$). At present, nine oocytes and one embryo have been warmed in four patients and one biochemical pregnancy obtained. This suggests the combined procedure could be an additional option for fertility preservation.

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Introduction

New therapeutic strategies for cancer have led to great improvements in cure and survival rates in young cancer patients (Howlader et al., 2011). However, antineoplastic therapies may be associated with significant gonadotoxicity and young cancer survivors usually express a strong desire to preserve childbearing potential (Letourneau et al., 2012; Meirow et al., 2010; Scanlon et al., 2012). Therefore, fertility preservation is an emerging field that offers new hope for women and girls treated for cancer before or during their reproductive years. All oncological guidelines address this issue by recommending that young patients be informed regarding possible fertility risks associated with treatment and that they be referred to a fertility centre if required (Knight et al., 2015; Peccatori *et al.*, 2013). Different approaches for fertility preservation in women diagnosed with cancer have been reported (Chung *et al.*, 2013; Demeestere et al., 2013; Jeruss and Woodruff, 2009). Among them, ovarian tissue cryopreservation (OTC) has emerged as an alternative to the standard oocyte or embryo vitrification option. This experimental procedure has several advantages as it can be rapidly performed without ovarian stimulation, even in prepubertal girls or when chemotherapy has already started. It also offers the possibility of storing a large number of follicles within ovarian fragments that can be later transplanted to restore fertility (Meirow, 2008; Meirow et al., 2014; Rodriguez-Wallberg and Oktay, 2012). Since the first live birth after transplantation of cryopreserved ovarian tissue (Donnez et al., 2004), this technique has led to the birth of over 80 healthy babies and has an estimated success rate of 25–30% (Donnez and Dolmans, 2015; Jensen et al., 2015; Meirow et al., 2016; Van der Ven *et al.*, 2016).

Despite these encouraging results, some advanced cancers or disseminated diseases, such as leukaemia, are considered to pose a high risk of recurrence after ovarian tissue transplantation due to the potential presence of neoplastic cells within the tissue (Bastings et al., 2013). Moreover, progress must still be made to improve the success rate of the procedure. OTC preserves the primordial and primary follicles, while the antral follicles, which contain immature growing oocytes, do not survive the procedure (Gosden, 2002). During the process, the ovarian cortex is dissected and frozen, while the medulla containing all the growing follicles is usually discarded or stored to assess the presence of neoplastic cells. These immature oocytes represent an additional source of gametes after in-vitro maturation (IVM). The vitrified oocytes can be directly used later for IVF and embryo transfer without any risk of disease transmission.

At present, only a few studies have investigated the feasibility of a combined option that preserves both ovarian tissue and in-vitro matured oocytes (Abir et al., 2016; Fadini et al., 2012; Fasano et al., 2011; Revel et al., 2009). Three live births from in-vitro matured oocytes retrieved during ovarian tissue processing for cryopreservation were recently reported (Prasath et al., 2014; Segers et al., 2015; Uzelac et al., 2015) but the factors involved in the success of the procedure, including oocyte collection, IVM and live birth rates are still unknown.

Previous studies have shown that the combined procedure is feasible in paediatric patients, including prepubertal girls (Abir et al., 2016; Fasano et al., 2011; Revel et al., 2009). However, the viability, developmental competence and fertilization potential of oocytes from paediatric patients must be confirmed as no oocytes have been warmed for fertility restoration yet.

Proper handling of ovarian tissue that originates from an operating room not immediately adjacent to the embryology laboratory constitutes another challenge. Animal studies have confirmed that follicles are subject to acute ischaemia during transport, leading to adverse changes such as a decrease in glucose concentration or increase in apoptotic index in granulosa cells (Ferreira et al., 2001; Pedersen et al., 2004; Sakamoto et al., 2006; Wongsrikeao et al., 2005). To avoid these adverse effects, time of transport and processing before cryopreservation should be as short as possible. However, the effects of timing or temperature during tissue transport on IVM rates have not been carefully investigated. One strategy used to reduce ischaemic damage during ovary transport is to decrease the median transport temperature. Animal studies have suggested that lowering the storage temperature of ovaries during transport is required to maintain the developmental competence of oocytes (Matsushita et al., 2004). However, hardening of the zona pellucida, impaired microtubules and cytoskeleton and damaged cytoplasmic membranes have also been described in oocytes exposed to low temperatures (Bianchi et al., 2014; Lee et al., 2006; Mandelbaum et al., 2004). Only one study reported oocyte maturation rates according to transport time in humans and did not show any negative impact of longer transport times (Yin et al., 2016). Therefore, the impact of the timing of ovarian transport on the developmental competence of oocytes remains uncertain and needs further evaluation.

The present study assessed the efficiency of oocyte IVM and vitrification procedures after ex-vivo collection from ovarian tissue according to the age of the patients, the number of retrieved oocytes, and the timing of tissue transport.

Materials and methods

Ethical approval

The cryopreservation of ovarian tissue, including immature oocyte collection when feasible, was approved by the central Ethical Committee from Erasme Hospital on 16 September 2008 (reference number: P2004/122). All patients or parents were informed about the procedure and provided written informed consent.

Cryopreservation of ovarian tissue

Ovarian tissue biopsies or unilateral oophorectomy were carried out at Erasme Hospital or in one of the participating Belgian centres. Unilateral oophorectomy was usually proposed for prepubertal patients, women who were at major risk of premature ovarian failure, or in cases where it was surgically indicated (Imbert et al., 2014). Tissues were transported in Leibovitz L-15 medium (Life Technologies, Merelbeke, Belgium) at 4°C and processed in the IVF laboratory at between 15 min (on-site) and a maximum of 3 h from removal. Three groups were compared according to the duration of transport (15 min, less than 1 h and between 1 h and 3 h).

Ovarian tissue freezing procedures have been described elsewhere (Demeestere et al., 2003; Imbert et al., 2014). Briefly, the medulla was gently removed and the cortex was cut into small fragments (≈5 × 5 × 1–2 mm). Ovarian fragments were incubated in a cryoprotectant solution containing 1.5 M dimethyl sulfoxide (DMSO) and 0.1 M sucrose for 30 min and then frozen using a slow freezing protocol in a programmable freezing machine (Kryo 360, Planer, UK) before storage in liquid nitrogen.

Cumulus–oocyte–complex (COC) retrieval and IVM procedures have been described elsewhere (Fasano et al., 2011). All visible antral

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Table 1 – Indication for ovarian tissue cryopreservation.

Indications	Total *n* (%)	Adults *n* (%)	Prepubertal *n* (%)
Breast cancer	62 (45.6)	62 (47.7)	–
Haematological diseases			
Lymphoma	24 (17.6)	24 (18.5)	–
Leukaemia	5 (3.7)	4 (3.1)	1 (16.7)
Haematological benign	8 (5.9)	4 (3.1)	4 (66.7)
Borderline tumours	3 (2.2)	3 (2.3)	–
Pelvic tumour (gynaecological carcinoma, urological carcinoma)	10 (7.4)	10 (7.7)	–
Solid tumour (carcinoma, sarcoma, medulo-blastoma)	16 (11.8)	15 (11.5)	1 (16.7)
Immunological disorders	8 (5.9)	8 (6.2)	–
Total	136	130	6

follicles were punctured before ovarian tissue dissection to collect COCs. After dissection, the discarded material was filtered through a cell strainer and transferred into a Petri dish to search for additional COCs. The maturation stage of oocytes with expanded surrounding cells was easily evaluated using the sliding technique while oocytes tightly enclosed in cumulus cells were considered to be immature. After staging, immature COCs were transferred into IVM Maturation Medium (Sage, IVM Kit media) supplemented with 75 mIU/ml FSH and 75 mIU/ml LH and incubated at 37°C in a 6% CO_2/20% O_2 humidified atmosphere. All COCs were denuded after 24 h and resting immature oocytes were subjected to an additional incubation of 24 h. The mature oocytes were directly vitrified or fertilized by ICSI and the embryos obtained vitrified. Standard protocols (Irvine, Vitrification Kit media) with aseptic devices (CryoBiosystem, VHS Kit) were applied for mature oocyte and embryo vitrification according to the manufacturer's instructions (Fasano et al., 2012, 2014).

Population

From November 2008 to August 2014, 162 patients between 1 and 36 years old underwent OTC procedures in Erasme Hospital to preserve their fertility. The inclusion criteria for the cryopreservation of ovarian tissue have been previously described (Demeestere et al., 2003).

Twenty-six patients were excluded from the analysis. Twenty-three of them were treated with chemotherapy before the OTC procedure (Dolmans et al., 2005). Most of these patients were diagnosed with haematological diseases (15/23). For these patients, ovarian tissue has been cryopreserved but no oocytes were collected for further IVM. The other three excluded patients were aged less than 5 years. As previously reported by others, no healthy oocytes were found in these very young girls (Revel et al., 2009). The combined oocyte vitrification procedure was performed for 136 patients, 130 postpubertal/adult patients (mean age 27.6 ± 5.6 years) and six prepubertal girls (mean age 8.7 ± 2.3 years). Indications for OTC were mostly breast cancer and haematological diseases in postpubertal/adult and prepubertal patients, respectively (**Table 1**). Ovarian biopsies were performed in 93.8% (122/130) of adults while 83.3% (5/6) of prepubertal patients underwent unilateral oophorectomy.

Statistics

Statistical analyses were performed using SPSS-21 software (IBM Corp., USA). Continuous variables were expressed as mean ± SD and compared using Student's *t*-test. Proportions were analysed using the chi-squared test. Linear correlations between two independent variables were analysed by calculation of the *r*-values (Pearson's moment-correlation coefficient); the significance (two-tailed probability values) of *r* coefficients were calculated on the basis of the correlation values. Values of $P < 0.05$ indicated statistical significance.

Results

Oocyte retrieval

A total of 705 oocytes were collected *ex vivo* during the OTC procedure. A total of 140 of them (19.9%) were degenerated while six oocytes were already at metaphase II (MII) stage and directly vitrified. Most of the MII oocytes were obtained after puncture of visible antral follicles (4/6) but two MII oocytes were collected in filtered material after dissection of the ovarian tissue. A higher mean number of oocytes were collected in prepubertal patients compared with adults (11.5 ± 8.0 versus 3.8 ± 4.2, respectively, $P < 0.001$) but the percentage of degenerated oocytes was also significantly higher in the younger population (35.5% versus 17.1%, $P < 0.001$) (**Figure 1**). For 31 patients out of 136 (22.8%), no oocytes were found during the combined procedure and for two patients only degenerated oocytes were collected (1.5%). All of them were postpubertal patients.

IVM rate

A total of 559 oocytes were subjected to IVM: 491 at germinal vesicle (GV) stage (87.8%) and 68 at metaphase I (MI) stage (12.2%). After IVM for 24–48 h, at least one mature oocyte or embryo was vitrified in 52.9% of the patients (72/136). The overall IVM rate, excluding MII oocytes retrieved during the procedure, was 25.9% (16.1% at 24 h and 9.8% at 48 h). The IVM rate obtained was significantly lower in the prepubertal population compared with the postpubertal population (10.3% versus 28.1%, $P = 0.002$) (**Tables 2 and 3**). The maturation process

Table 2 – Results obtained for adult patients according to the diseases.

Indication	Age (mean ± SD)	Number of ovarian fragments/patient (mean ± SD)	Number of oocytes retrieved /patients (mean ± SD)	MII oocytes obtained *n*/total (%)
Breast cancer (*n* = 62)	30.2 ± 3.7[a]	20.1 ± 7.8[a]	3.4 ± 4.3	56/209 (26.8)
Haematological diseases (*n* = 32)	23.4 ± 6.3[b]	23.9 ± 7.2[b]	4.2 ± 4.4	42/133 (31.6)
Others (*n* = 36)	26.6 ± 5.4[c]	18.4 ± 6.7[c]	4.3 ± 3.6	45/154 (29.2)
Total (*n* = 130)	27.6 ± 5.6	20.6 ± 7.6	3.8 ± 4.2	143/496 (28.8)

P-values (age): a vs b; a vs c < 0.001; b vs c < 0.05.
P-values (fragments): a vs b < 0.05; b vs c < 0.01.

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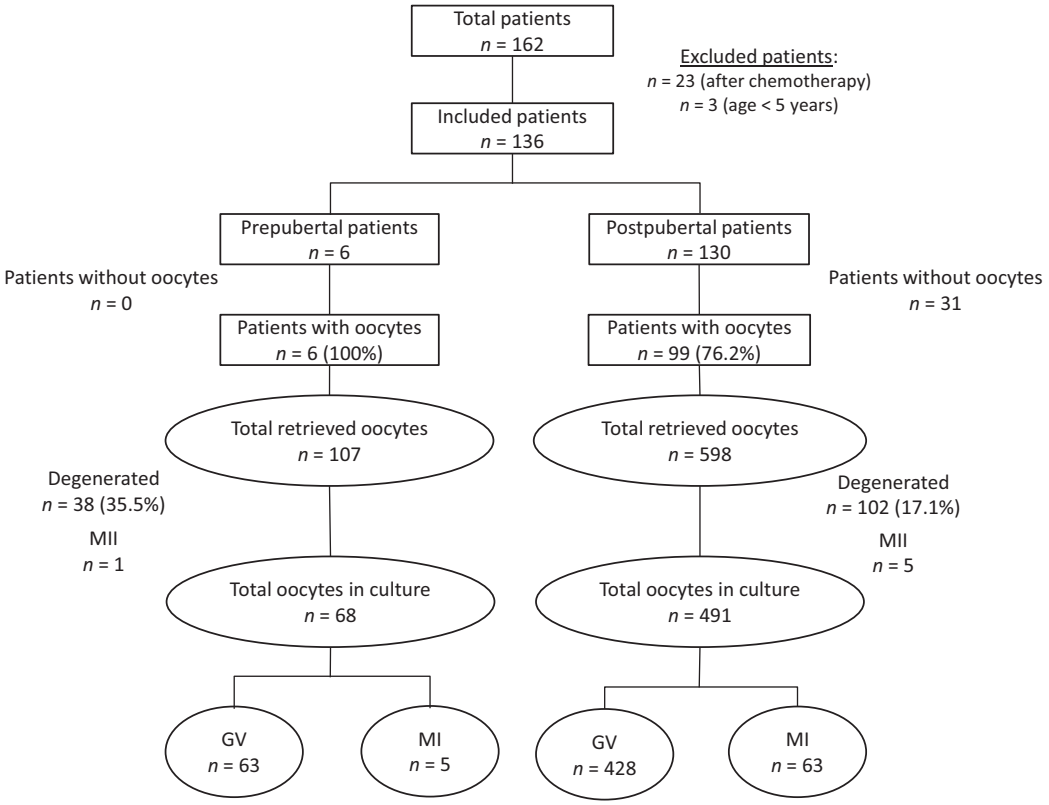

Figure 1 – Study population and oocytes retrieved (maturation stage details) in prepubertals and adults.

Table 3 – Results obtained for prepubertal patients.

Indication	Cases (*n*)	Age (years)	Fragments (*n*)	Oocytes retrieved (*n*)	MII oocytes obtained (*n*)
Leukaemia	1	8	36	14	2
Ewing sarcoma	1	13	17	2	0
Sickle cell anaemia	1	8	33	22	3
Sickle cell anaemia	1	6	25	19	1
Sickle cell anaemia	1	9	31	6	2
Sickle cell anaemia	1	8	21	6	0
Total (mean ± SD)	6	8.7 ± 2.3	27.2 ± 7.4	11.5 ± 8.0	8/69 (11.6%)

MII = metaphase II.

was also slower in prepubertal than in postpubertal patients with a majority of the oocytes matured after 48 h (**Figure 2**). In adults, a negative correlation was observed between the number of retrieved oocytes and age ($P = 0.002$; $r = -0.271$) while the correlation was positive between AMH and the number of collected oocytes ($P = 0.002$; $r = +0.264$). However, IVM rates were not correlated with AMH levels ($r = 0.06$) or age ($r = -0.033$).

Transport duration

The effects of transport time on maturation rates in postpubertal patients are reported in **Table 4**. IVM showed a trend towards higher rates with shorter transport times but the differences did not reach significance.

A total of 139 mature oocytes were vitrified in 72 patients and seven embryos (pronuclear stage embryos) were obtained after microinjection of 12 MII oocytes and vitrified in five patients. At present, four adults have asked to use their oocytes or embryos to try to conceive after cancer treatment. For three patients, nine oocytes vitrified in 2009/10 were warmed for fertility treatment. Only two oocytes survived (both vitrified after 48 h IVM) and were fertilized by ICSI, one low-quality three-cell embryo was obtained and transferred at day

Table 4 – In-vitro maturation rate according to transport time in adult patients.

Transport time (h)	Procedures (*n*)	Patient's age (mean ± SD)	Oocytes retrieved (mean ± SD)	In-vitro maturation rate (%)
15 min	39	26.0 ± 5.3	2.8 ± 2.9[a]	31.2
<1 h	72	28.2 ± 5.7	4.0 ± 4.3	29.1
1–3 h	19	28.2 ± 5.7	5.3 ± 5.3[b]	22.0

P-values a vs b = 0.02.

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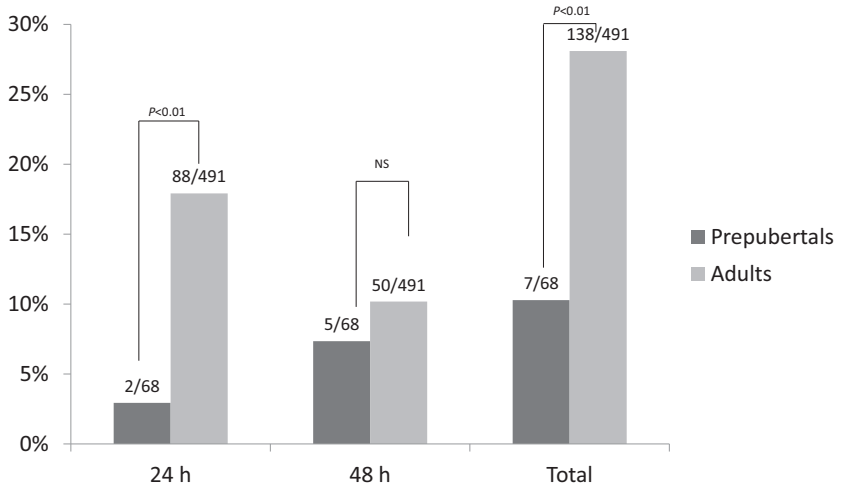

Figure 2 – In-vitro maturation rate in prepubertal patients and adults at 24 h and 48 h.

2 without pregnancy. For the fourth patient, a pronuclear stage embryo was warmed and a good quality four-cell embryo was transferred at day 2. Unfortunately, the patient had a biochemical pregnancy.

Discussion

The currently available options for preservation of fertility for cancer patients are to store gametes, embryos or gonadal tissue for later use. When OTC is offered, the combined ex-vivo retrieval of immature oocytes followed by IVM offers an additional chance to conceive, especially for patients who carry the potential risk of the presence of malignant cells in the ovary (Fadini et al., 2012; Fasano et al., 2011; Garcia-Velasco et al., 2013; Rosendahl et al., 2013).

While mature oocyte vitrification is now considered a standard procedure (Stoop et al., 2012), the efficiency of vitrification after IVM is less well established. However, previous studies have reported high cumulative clinical pregnancy rates using in-vitro matured oocytes in polycystic ovary syndrome (PCOS) patients (Junk and Yeap, 2012; Walls et al., 2012). Nevertheless, many aspects regarding IVM of ex-vivo retrieved oocytes need to be further addressed. COCs are exposed to suboptimal conditions during transport and dissection processes that may affect their competence and survival.

Researchers have recently reported cases of successful pregnancies and live births after the transfer of cryopreserved embryos obtained from oocytes collected *ex vivo* during OTC, matured *in vitro*, and fertilized by ICSI (Prasath et al., 2014; Segers et al., 2015; Uzelac et al., 2015). Segers et al. (2015) reported results on 34 patients who underwent a combination of OTC and IVM on ex-vivo collected oocytes. Most patients (32/34) underwent unilateral oophorectomy and the ovary was transported on ice to the IVF laboratory within 10 min (except for three patients). A mean of 14.7 ± 2.2 oocytes were collected per patient and the overall maturation rate reached 36.1 ± 4.3%. For one patient, 13 immature oocytes were retrieved, of which three embryos were cryopreserved. After a first unsuccessful attempt, the patient became pregnant using a second warmed embryo. Despite these encouraging results, the competence of these oocytes must be further investigated.

In our study, around 25% of the oocytes from adults were successfully in-vitro matured but only two out of nine oocytes survived the warming procedure. Almost all of them were obtained from ovarian biopsies. We expect that preservation of the whole ovary would provide the opportunity to retrieve more oocytes. Moreover, larger follicles can be punctured in intact ovaries to obtain potentially higher quality oocytes. The heterogeneity of the oocytes harvested *ex vivo* from cortex biopsies or whole ovaries may explain the lower IVM rates observed compared with classical COC protocols that nevertheless rarely exceed 50% (Sánchez et al., 2015). Finally, selection of the COCs in our study was less strict than in others as we included denuded oocytes and metaphase-I that may be of lower competence and quality.

However, we were able to obtain a biochemical pregnancy after transfer of a warmed embryo, suggesting that it might be feasible to obtain competent COCs from ovarian biopsies too.

Our results suggest that the procedure used here could be offered to prepubertal children but the degenerated oocyte rate in this population was higher than that of the adults. Moreover, maturation rates were also low, suggesting that these oocytes might be less competent than those obtained from the postpubertal/adult population. In agreement with other authors, we also suggest that patients less than 5 years of age are not suitable for ex-vivo oocyte collection and maturation (Abir et al., 2016; Revel et al., 2009). During the study period, we performed the combined procedure in three patients aged <5 years with no oocytes retrieved for two of them and no matured oocytes for the third one. As these three patients had ovarectomy, we expected to retrieve a high number of oocytes, but no healthy oocytes were obtained, confirming previous reports.

It has been shown that COCs can be obtained in patients aged >5 years old and the efficiency of the combined procedure has been evaluated in prepubertal patients. Revel et al. (2009) obtained a total of 179 oocytes in 17/19 patients (89%) aged 5–20 years. They found 7, 8 and 17 oocytes in the youngest patients aged 5, 8 and 10 years, respectively. The overall maturation rate was 45/133 oocytes (34%). In contrast with our results, the maturation rate was in the same range for these three youngest patients compared with the older patients. Another prospective study (Abir et al., 2016) was conducted in a cohort of 42 paediatric females aged 2–18 years who underwent combined OTC and IVM of oocytes collected during the procedure. Oocytes were obtained from 20 patients before chemotherapy and from 13 after chemotherapy. The youngest patients were aged 2 years and 3 years but no healthy oocytes were obtained. In this study, authors reported

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a positive correlation between the number of vitrified oocytes and patient age ($P = 0.001$) but the percentage of cryopreserved oocytes over the total number of oocytes collected before chemotherapy ranged from 0% to 70% in patients less than 13 years old. In contrast, our data suggest that oocytes obtained after IVM in prepubertal children might be of lower quality than in the postpubertal population. However, the limited number of prepubertal patients in our study constituted a major limitation for the interpretation of the results and these data need to be confirmed in a larger cohort of patients. Nevertheless, they are supported by some experimental data. Recently, authors showed that prepubertal ovaries contained a high proportion of abnormal follicles (Anderson et al., 2014). Experimental results in animal models have confirmed that prepubertal follicles exhibited delays in meiotic progression and low developmental capacity (Leoni et al., 2015; Morton et al., 2008).

Most clinical protocols report that an oocyte IVM timing of between 24 h and 36 h provides the best balance between maturation rate and embryological outcome (Segers et al., 2015). In our study, IVM culture was extended up to 48 h for all the patients to rescue as many MII oocytes as possible, especially in prepubertal children, although the developmental capacity of 48 h in-vitro matured oocytes might be reduced (Ali et al., 2006; Chian et al., 2004; Sánchez et al., 2015). Overall, the discrepancy observed between studies raises questions about the future competence of vitrified oocytes in the prepubertal population but supports the potential benefit of this experimental procedure.

In our study, results showed that transport conditions did not significantly affect IVM rates despite a trend towards lower maturation rates with increasing transport times. Some authors have suggested that COC collection and maturation should only be attempted with oocytes from visible antral follicles and when the ovary is not subjected to a cooling period prior to recovery of immature oocytes (Wilken-Jensen et al., 2014). In the current study, the impact of transport duration was evaluated for a total of 130 procedures. The excised ovaries were transported to the laboratory either from the local hospital (15 min transport at 37°C) or from collaborating hospitals (1–3 h transport on ice). A higher maturation rate was obtained in immature oocytes collected from ovaries recovered at the local hospital compared with the other two groups, but the difference was not significant. Others have shown that transport up to 4–5 h on ice did not significantly affect oocyte maturation rates (Yin et al., 2016). Our results confirm that the transport of ovarian tissue should not be considered as a limitation for the combined procedure but it should be as short as possible to avoid potential impact on oocyte quality and survival.

This study on a large cohort of patients suggested that ex-vivo IVM should be considered as an additional fertility preservation option for women and children undergoing oophorectomy or ovarian tissue biopsy. Nonetheless, efficacy remains very low, especially for prepubertal patients. Future efforts should tailor and optimize not only cryopreservation, but also IVM protocols for use in either GV or MI oocytes, together with a comprehensive assessment of oocyte function and developmental competence to term.

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