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Combination of ovarian tissue harvesting and immature oocyte collection for fertility preservation increases preservation yield

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Abstract The aim of this study was to evaluate the safety and efficacy of combined ovarian tissue cryopreservation and oocyte aspiration just before ovarian tissue cryobanking. A retrospective cohort study of fertility preservation patients treated in 2007–2013 in one tertiary centre was performed. A total of 255 cancer patients were admitted for fertility preservation: 142 patients underwent ovarian tissue cryopreservation only (OTC), 56 underwent OTC plus oocyte retrieval from ovarian tissue ($_{OT}$IVM), nine underwent oocyte aspiration and in-vitro maturation ($_{A}$IVM) and 48 underwent all three procedures. The total number of oocytes, total number of metaphase II (MII) oocytes, maturation rate, fertilization rate and total number of cryopreserved oocytes between groups were compared. The study found significantly more oocytes ($P < 0.001$), more MII oocytes ($P < 0.001$), better maturation rate ($P < 0.01$) and more cryopreserved oocytes ($P < 0.05$) with all three compared with $_{OT}$IVM or OTC. No adverse outcome was observed by performing oocyte retrieval before ovarian resection for cryopreservation. In conclusion, oocyte aspiration just before ovarian tissue cryobanking is safe and gains more oocytes with a better maturation rate than ovarian tissue oocyte cryobanking alone. Better results were obtained with 3 days of stimulation before oocyte retrieval.

KEYWORDS: embryo freezing, fertility preservation, in-vitro maturation, oocyte cryopreservation, ovarian tissue cryopreservation

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Introduction

New and current aggressive therapeutic modalities of cancer treatments have improved survival and cure amongst girls, young adolescents and women (Howlader et al., 2011). However, antineoplastic therapy is associated with significant morbidity, including gonadotoxicity (Meirow et al., 2010). Meanwhile, cancer survivors often have a strong desire to foster biological children. This natural desire to have a family may even increase after the experience of cancer (Kong et al., 2011; Scanlon et al., 2012). All these define the need for fertility preservation in patients at risk. Although several approaches for fertility preservation in young women diagnosed with cancer have been suggested and different guidelines were proposed (Chung et al., 2013; Jeruss and Woodruff, 2009; Lee et al., 2006; Lobo, 2005; Practice Committees of American Society for Reproductive and Society for Assisted Reproductive, 2013), there is no optimal fertility preservation protocol and the technology used should be individualized.

Embryo freezing after ovarian stimulation and oocyte retrieval is an established technology (Chung et al., 2013; Meirow et al., 2014), and success rates of oocyte cryopreservation following IVF are improving (Cobo and Diaz, 2011; Cobo et al., 2008, 2013; Garcia-Velasco et al., 2013). However, this technique requires the delay of cancer therapy (Lee et al., 2006) in order to undergo ovarian stimulation, and in some cancer conditions or in hypercoagulable state, exposure to high concentrations of oestrogens and progestins may be contraindicated. Some of the patients are too young to undergo the procedure or are not candidates for mature egg collection due to recent exposure to chemotherapy (Chung et al., 2013).

Although few studies suggested poorer yield of IVF in cancer patients (Domingo et al., 2012; Friedler et al., 2012), other reports failed to show differences in ovarian response between cancer and healthy patients (Chung et al., 2013; Garcia-Velasco et al., 2013). Oocyte cryopreservation may also be considered, specifically for cancer patients without a male partner, and encouraging results have indicated similar success rates with fresh cycles when used in young women (Cobo et al., 2010; Practice Committees of American Society for Reproductive and Society for Assisted Reproductive, 2013).

Ovarian tissue cryopreservation (OTC) and transplantation is a means of preserving fertility potential that has been contemplated by physicians and scientists for many years. This procedure offers women and prepubertal girls the possibility of attempting to preserve their fertility before acute insult to the ovary that can result in permanent sterility (Meirow et al., 2014).

OTC has an important role in fertility preservation when ovarian stimulation is not possible due to time constraints or when hormonal stimulation is contraindicated for medical reasons (Meirow, 2008; Rodriguez-Wallberg and Oktay, 2012).

Since the first live births post spontaneous and IVF pregnancies following transplantation of cryopreserved thawed ovarian tissue (Donnez et al., 2004; Meirow et al., 2005), orthotopic re-implantation has led to the birth of over 30 healthy babies (Donnez and Dolmans, 2015). Before re-implantation, the risk of ovarian involvement from the underlying cancer should be considered and may prevent future transplantation (Meirow et al., 2008).

Another fertility preservation method is the emerging and promising use of in-vitro maturation (IVM) of human oocytes (Chian et al., 2004; Rao and Tan, 2005; Tan and Child, 2002; Trounson et al., 1994, 1998). The main advantage of this technique is that it does not require gonadotrophin stimulation, and therefore the treatment is shorter, and most importantly, safer, avoiding the risk of ovarian hyperstimulation syndrome (OHSS) (Jurema and Nogueira, 2006). Due to these advantages, the technique has been applied to fertility preservation (Cao et al., 2009; Huang et al., 2007, 2010; Nisker et al., 2006; Oktay et al., 2010; Prasath et al., 2014; Shalom-Paz et al., 2010; Smitz et al., 2011).

In this method oocytes are aspirated, most of them in the immature stage with or without prior mild ovarian stimulation. The oocytes are then matured *in vitro* and used for IVF or oocyte cryopreservation. It was shown that oocyte retrieval can be performed also in the luteal phase of the cycle (Maman et al., 2011) and that immature oocytes can also be collected from excised ovarian tissue from adults and even from prepubertal girls (Fasano et al., 2011; Revel et al., 2009). A live birth from an IVM oocyte retrieved during ovarian tissue processing for cryopreservation was recently reported (Prasath et al., 2014).

Prompted by the aforementioned observations and in an attempt to increase the likelihood of future fertility, cancer patients were offered a combined approach of ovarian tissue harvesting and maturation of oocytes originating from ultrasound-guided follicular aspiration or collected directly from the ovarian specimens during the same procedure.

The purpose of this study was to evaluate the yield of the combined approach and compare it with IVM of ovarian tissue oocytes and to IVM of aspirated oocytes only.

Materials and methods

Patients and fertility preservation methods

All cases of fertility preservation in cancer patients at Sheba Medical Center between 2007 and 2013 were reviewed. According to the centre's policy, patients were offered individualized fertility preservation methods, tailored according to parameters such as patient's age, marital status, type disease, treatment protocol, contraindication for hormonal treatment and time constraints.

Regular IVF for embryo cryopreservation was performed when hormonal treatment was allowed and time was not a constraint. All other patients were offered the combined procedure of ovarian tissue storing and immature oocyte retrieval and maturation. Whenever ovarian tissue cryopreservation was performed, isolation of oocytes from the ovary was attempted except for patients who had undergone chemotherapy during the 6 months preceding the fertility preservation procedure and thus could not have oocytes or embryos frozen (Meirow and Nugent, 2001; Meirow et al., 2001). These patients were offered fertility preservation by freezing ovarian sections only. In cases where chemotherapy was distant (more than 6 months previously) oocyte aspiration from the ovaries was performed. When possible, oocyte aspiration prior to ovarian tissue cryopreservation (OTC) by laparoscopy was done ($_{A}$IVM). In patients that could not undergo the oocyte aspiration procedure (young patients, virgin patients, patients with cervical cancer), oocytes were collected for further fertilization or freezing only during ovarian tissue processing (ovarian

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tissue oocytes–$_{OT}$IVM). Some patients elected not to undergo OTC, and thus oocyte aspiration ($_{A}$IVM) only was performed. Patients who underwent fertility preservation by regular IVF were excluded from the study. Therefore, the study group included the following subgroups: Group A – OTC only; Group B – OTC and $_{OT}$IVM during tissue processing; Group C – $_{A}$IVM procedure only; and Group D – OTC, $_{A}$IVM and $_{OT}$IVM.

The study was approved by the local Institutional Review Board (IRB) committee (1512–14-SMC, 21/09/2014).

IVM protocol ($_{A}$IVM)

Patients underwent an IVM cycle according to accepted IVM protocols (Fadini et al., 2009). On day 3 of a spontaneous menstrual cycle, women underwent a baseline transvaginal ultrasound assessment to determine ovarian morphology, AFC, and endometrial thickness and basal blood concentration of oestradiol and progesterone. Following that, 150 IU/day recombinant FSH (rFSH) were administered to the patients for 3 days. A second evaluation was performed on day 6. An injection of 10,000 IU human chorionic gonadotrophin (HCG; Pregnyl; Organon, Oss, Holland) was administered subcutaneously when the leading follicle was 12 mm. Oocyte retrieval was performed 36 h later by transvaginal ultrasound-guided needle aspiration.

The follicular fluid was collected in culture tubes containing flushing medium with heparin (MediCult prod. no. 10760125, Denmark). In several cases due to time limitation, IVM was performed without the routine preparation. In these cases patients did not receive HCG. Several cases were performed during the luteal phase. Data regarding patients' distribution according to fertility preservation methods is presented in **Figure 1**.

Ovarian tissue biopsy

When performed the combined procedure, immediately following the $_{A}$IVM process, laparoscopy for ovarian tissue harvesting was performed. During laparoscopic surgery approximately two-thirds of the ovarian cortex from one ovary was harvested and immediately placed in gamete buffer (Cook Medical, Bloomington, USA) and transferred to the IVF laboratory (in the adjacent room) where it was prepared for cryopreservation. Bleeding during the procedure was controlled with cautery (Donnez et al., 2013; Meirow et al., 2005, 2014).

Ovarian tissue cryopreservation (OTC)

The tissue was transferred in medium to the laboratory. In the laboratory the ovarian tissue was cleaned from the medulla and prepared for cryopreservation. The cortical tissue was cut into thin pieces 1–2 mm thick and divided into slices that fit the size of the cryodevice (5–10 mm strips or smaller). The ovarian tissue pieces were then frozen by slow freeze method (Meirow et al., 2007).

Oocyte isolation from ovarian tissue

During the handling of the ovarian tissue pieces, oocytes were collected from the ovarian tissue ($_{OT}$IVM). Small follicles observed were aspirated and their oocytes observed in the follicular fluid were separated and saved for further maturation and handling in the laboratory. In addition, the medium in which the ovarian tissue pieces were prepared was searched

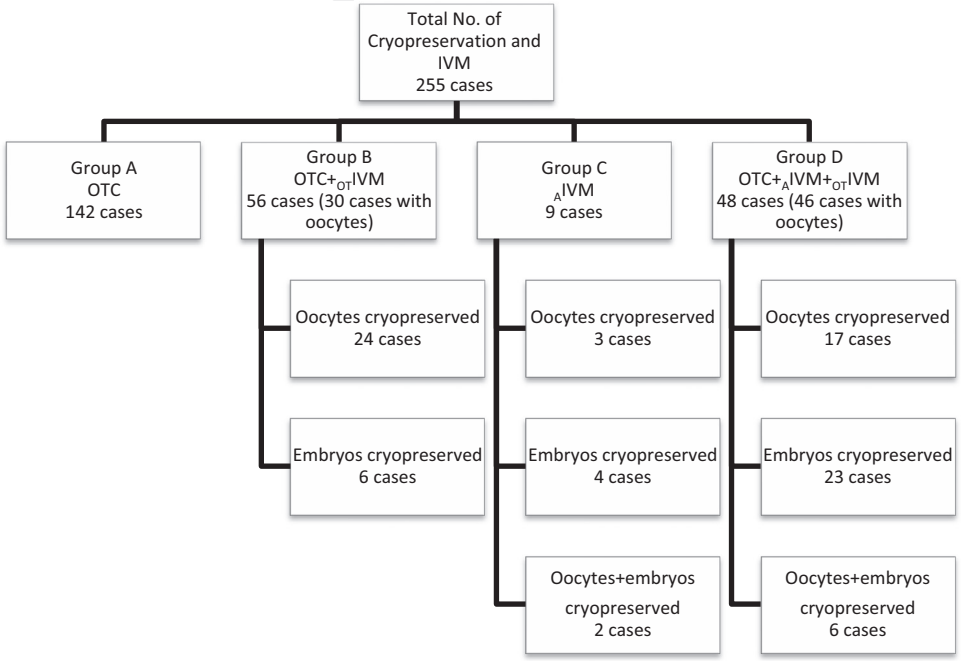

Figure 1 Organization chart of the patients' distribution according to the fertility preservation option. Ovarian tissue cryopreservation (OTC), in-vitro maturation of aspirated oocytes ($_{A}$IVM), in-vitro maturation of ovarian tissue oocytes ($_{OT}$IVM).

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for oocytes. Those were collected and saved for further treatment (Meirow et al., 2014; Revel et al., 2004).

IVM oocytes that were selected for fertilization were fertilized by intracytoplasmic sperm injection (ICSI). Oocyte vitrification was performed with a vitrification kit (SAGE Vitrification Kit, SAGE Media, USA) in the cryotop device. Embryos were frozen using slow-rate cryopreservation following traditional protocols using a cryopreservation kit (Quinn's Advantage Embryo Freeze Kit, SAGE media, USA) a programmable rate freezer (PLANER Kryo 360).

Statistical analysis

The following statistical tests were used to analyse the data in this study: independent-samples *t*-test was applied, for testing the statistical significance of the differences in treatment protocol results between two groups. One-way ANOVA was used to compare the patients' age. Fisher's exact test was used to compare maturation and fertilization rates between groups. All tests applied were two-tailed, and a *P*-value <0.05 or less was considered statistically significant. All statistical analyses were performed using the IBM SPSS Statistics, version 20 (IBM corp., Armonk, NY).

Results

Patient characteristics

A total of 255 patients (Figure 1) underwent fertility preservation in the centre between 2007 and 2013 and were included in the study. Table 1 summarizes the characteristics of the patients who participated in this study in each group.

One hundred and forty-two patients underwent laparoscopy and freezing of ovarian tissue without any other procedure and were not included in further data analysis. Patients who underwent OTC without $_{A}$IVM (groups A and B) were significantly younger, including some prepubertal girls in which oocyte aspiration was avoided ($P < 0.05$). The average number of oocytes and MII oocytes that were obtained in young (aged 10–16) compared with adult (aged 17–40) patients was not significantly different (data not shown).

About half of the patients (128/255, 50.2%) suffered from Hodgkin's disease or haematological malignancies. Among patients undergoing OTC + $_{OT}$IVM there were a relatively large number of sarcomas (11/56, 19.6%). Fifty-four per cent of the patients undergoing OTC + $_{OT}$IVM + $_{A}$IVM suffered from Hodgkin's disease (Table 1).

In all treated groups, there was no report of unusual complications during fertility preservation procedures. There were no signs of excessive bleeding from the aspirated ovaries. There was no difficulty in excision of the ovarian cortex and there were no problems in ovarian tissue preparation for cryopreservation following oocyte retrieval.

Oocyte collection

Table 2 summarizes the results of oocytes retrieved from the three groups in which oocytes were collected (groups B, C and D). In 30 cases out of 56 cases of group B ($_{OT}$IVM only) ovarian tissue oocytes were found (53.6% of the cases). In the groups involving the $_{A}$IVM procedure, (groups C and D) oocytes were obtained in all but two cases (96.5% of the cases). As seen in Table 2, performing $_{A}$IVM or the combination of $_{A}$IVM + $_{OT}$IVM not only increased the chance of obtaining oocytes, but also yielded significantly more oocytes and more importantly, significantly more mature oocytes (both $P < 0.001$). Another contributor to the higher number of mature oocytes was the higher maturation rate observed in $_{A}$IVM compared with $_{OT}$IVM. Moreover, in the combined group (Group D, OTC + $_{A}$IVM + $_{OT}$IVM), most of the oocytes and mature oocytes obtained originated from the $_{A}$IVM procedure. Importantly, the number of mature oocytes on the day of fertility preservation ("in-vivo matured oocytes") known to be with the highest chance of resulting in a pregnancy (Fadini et al., 2009, 2015; Son and Tan, 2010), was higher in $_{A}$IVM compared with $_{OT}$IVM.

Table 1 Patient characteristics.

	Group A OTC	*Group B* OTC + $_{OT}$IVM	*Group C* $_{A}$IVM	*Group D* OTC + $_{A}$IVM + $_{OT}$IVM	*Total*
No. of cases	142	56	9	48	255
Age					
Mean ± SE[a]	23.8 ± 0.79	22.3 ± 1.26	27.2 ± 1.70	26.8 ± 0.95	24.2 ± 0.56
Range	(2–41)	(10–40)	(19–33)	(17–41)	(2–41)
Diagnosis *n* (%)					
Hodgkin's	33 (23.2)	15(26.3)	3(33.3)	26 (54.2)	77 (30.2)
Haematological	35 (24.6)	8 (14.0)	-	8 (16.7)	51 (20.0)
Sarcoma	7 (4.9)	11 (19.6)	-	2 (4.2)	20 (7.8)
Breast	22 (15.5)	4 (7.0)	3(33.3)	7 (14.6)	36 (14.1)
Cervix	18 (12.7)	6 (10.5)	-	1 (2.1)	25 (9.8)
Other	27 (19.0)	12 (21.1)	3(33.3)	4 (8.3)	46 (18.0)
Chemotherapy before FP (%)	46.8	20.0[b]	None	31.9[b]	34.1

FP = fertility preservation.
[a]$P < 0.05$.
[b]More than 6 months from chemotherapy to FP.

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Table 2 Characteristics of oocytes and embryos derived from $_{OT}$IVM and $_{A}$IVM.

	Group B *OTC + $_{OT}$IVM* n = *56*	*Group C* *$_{A}$IVM* n = *9*	*Group D* *OTC + $_{A}$IVM + $_{OT}$IVM* n = *48*
No. cases – oocytes obtained *n* (%)	30 (53.6)	9 (100)	46 (95.8)
No. total oocytes			
Mean ± SE[a]	6.95 ± 0.83[b]	12.33 ± 4.34	11.87 ± 1.22[b]
Range	(1–18)	(1–44)	(1–31)
Oocytes from $_{A}$IVM	–	12.33 ± 4.34	8.93 ± 1.04
Oocytes from $_{OT}$IVM	6.95 ± 0.83[b]	–	2.93 ± 0.51[b]
Total GV oocytes on day of FP			
Mean ± SE[a]	5.97 ± 0.80[c]	11.22 ± 4.31	9.93 ± 1.15[c]
Range	(0–18)	(0–44)	(0–31)
GV from $_{A}$IVM	–	11.22 ± 4.31	7.62 ± 0.99
GV from $_{OT}$IVM	5.97 ± 0.80[b]	–	2.31 ± 0.49[b]
Total MII oocytes on day of FP			
Mean ± SE[a]	0	0.44 ± 0.17	0.85 ± 0.24
Range	–	(0–1)	(0–7)
MII from $_{A}$IVM	0	0.44 ± 0.17	0.63 ± 0.20
MII from $_{OT}$IVM		–	0.22 ± 0.14
Total MII oocytes obtained after maturation			
Mean± SE[a]	2.47 ± 0.41[b]	7.33 ± 2.32	6.45 ± 0.81[b]
Range	(0–8)	(0–24)	(0–20)
MII from $_{A}$IVM	–	7.33 ± 2.33	5.57 ± 0.77
MII from $_{OT}$IVM	2.47 ± 0.41[b]	-	0.89 ± 0.23[b]
Total maturation rate (%)	34.7[c]	58.6	54.4[c]
Maturation rate $_{A}$IVM *n* (%)	–	65/111 (58.5)	256/411 (62.3)
Maturation rate $_{OT}$IVM *n* (%)	70/202 (34.7)	–	41/135 (30.4)

[a]Mean ± SE calculated from cases in which oocytes were obtained.
[b]Group B versus Group D $P < 0.001$.
[c]Group B versus Group D $P < 0.01$.

Prior chemotherapy had no significant effect on the mean number of oocytes retrieved regardless of the retrieval method ($_{A}$IVM or $_{OT}$IVM) (data not shown).

Oocyte and embryo outcomes

Table 3 summarizes the outcomes of the collected oocytes. It is important to clarify that the decision of whether to fertilize the oocytes or to cryopreserve them was made according to the patient's wishes and was influenced by their age and marital status. Therefore, the focus was on the comparison of the total oocyte and total mature oocytes obtained. The average number of mature oocytes cryopreserved was significantly higher ($P < 0.05$) in the $_{A}$IVM + $_{OT}$IVM group (group D) compared with the $_{OT}$IVM group (group B).

The number of fertilized oocytes was greater for the $_{A}$IVM + $_{OT}$IVM procedure group (Group D) compared with the other two groups, although not significantly (possibly due to the small number of $_{OT}$IVM patients who chose to proceed to fertilization and embryo cryopreservation). The number of embryos that were frozen was lower in $_{OT}$IVM (Group B), in whom oocytes were collected only from ovarian tissue. The number was higher in the $_{A}$IVM only group (Group C) but was highest in the $_{A}$IVM+ $_{OT}$IVM group (Group D). Moreover, the highest total number of oocytes and embryos frozen was in patients who underwent combined treatment.

Combined procedure outcome according to treatment protocol

Two IVM treatment protocols were used prior to oocyte retrieval: stimulated and unstimulated, according to the time interval that was possible for fertility preservation. The results indicate that in spite of 3 days of hormonal stimulation prior to fertility preservation laparoscopic procedure (13 of the 15 cases with stimulation) the laparoscopic procedure to harvest ovarian tissue was not complicated by excessive bleeding.

In order to assess the effect of hormonal stimulation on the amount and quality of oocytes retrieved, all cases of oocyte retrieval were divided into stimulated or non-stimulated cases (Table 4).

Hormonal stimulation before the fertility preservation procedure in cancer patients was associated with a higher number of oocytes obtained, as well as higher number of MII oocytes obtained on day of oocyte collection and total number of MII oocytes. Maturation and fertilization rates were not affected by stimulation prior to oocyte retrieval.

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Table 3 Outcomes of the collected oocytes.

	Group B $OTC + {}_{OT}IVM$	*Group C* ${}_{A}IVM$	*Group D* $OTC + {}_{A}IVM + {}_{OT}IVM$
No. cases oocytes obtained	30	9	46
No. cases of oocyte cryopreservation only	24	3	17
Total oocytes mean ± SE	2.42 ± 0.49[a]	4.33 ± 2.03	6.00 ± 1.37[a]
Range	(0–8)	(1–8)	(0–18)
Oocytes from ${}_{A}IVM$	–	4.33 ± 2.03	5.18 ± 1.29
Oocytes from ${}_{OT}IVM$	2.42 ± 0.49[a]	–	0.82 ± 0.31[a]
No. cases in which all oocytes injected (ICSI)	6 cases	4 cases	23 cases
Total oocytes mean ± SE	2.66 ± 0.56	4.50 ± 1.32	5.22 ± 1.01
Range	(1–4)	(3–6)	(1–16)
Oocytes from ${}_{A}IVM$	-	4.50 ± 1.32	4.77 ± 0.94
Oocytes from ${}_{OT}IVM$	2.66 ± 0.56	-	2.50 ± 1.01
Total fertilization rate[b] *n* (%)	10/16 (62.5)	19/35 (54.3)	121/163 (74.2)
Fertilization rate ${}_{A}IVM$ *n* (%)	–	19/35 (54.3)	105/139 (75.5)
Fertilization rate ${}_{OT}IVM$ *n* (%)	10/16 (62.5)	–	16/24 (66.7)
Total embryos cryopreserved[c]	1.67 ± 0.56	2.00 ± 0.93	3.39 ± 0.73

[a]Group B versus Group D $P < 0.05$.
[b]Fertilization rate was calculated for all cases.
[c]Mean number of embryos cryopreserved was calculated only for cases that chose to cryopreserve embryos only and therefore all their MII oocytes were injected.

Table 4 Combined procedure (Group C and Group D) outcome according to stimulation protocol.

	Stimulated[b]		*Non-stimulated*	
No. patients with oocytes retrieved	15		40	
	${}_{OT}IVM$	${}_{A}IVM$	${}_{OT}IVM$	${}_{A}IVM$
Mean no. oocytes retrieved	2.46 ± 1.00	13.13 ± 2.73[a]	3.03 ± 0.58	8.13 ± 1.06[a]
	13 cases	15 cases	34 cases	40 cases
Sum	15.27 ± 2.69		10.70 ± 1.30	
Total MII oocytes on day of procedure *n* (%)	1/32 (3.1)	22/197[a] (11.2)	9/103 (8.7)	11/325[a] (3.4)
Mean no. MII oocytes obtained	0.46 ± 0.29	8.27 ± 1.75[a]	1.06 ± 0.29	4.93 ± 0.74[a]
Sum	8.67 ± 1.72		5.80 ± 0.81	
Maturation rate *n* (%)	6/32 (18.8)	124/197 (62.9)	35/103 (34.0)	197/325 (60.6)
Fertilization rate *n* (%)	1/1	34/68	10/23	54/106
		(50.0)	(43.5)	(50.9)

[a]$P < 0.05$.
[b]In 2/9 of cases in Group C patients received IVM stimulation protocol.

Discussion

The main role of fertility specialists with cancer patients is to preserve gametes, embryos or gonadal tissue for use at a future time (Ethics Committee of American Society for Reproductive Medicine, 2013). There have been several publications that demonstrate efficacy of the different approaches for fertility preservation, including oocyte or embryo cryopreservation obtained by IVF (Chung et al., 2013; Garcia-Velasco et al., 2013), IVM (Shalom-Paz et al., 2010) and ovarian tissue cryopreservation (Donnez et al., 2013; Meirow et al., 2005, 2014), and the combination of ovarian tissue cryobanking with retrieval of immature oocytes followed by IVM has been previously described (Fasano et al., 2011; Prasath et al., 2014; Revel et al., 2009). In the present study, in addition to oocytes recovered from the excised ovarian tissue, oocyte aspiration was performed for IVM just before the laparoscopic ovarian harvesting. To the best of our knowledge, this is the first article evaluating the yield of these two techniques combined in the same procedure.

The results of this study indicate that performing oocyte aspiration is safe and that there are several advantages. First, it was possible to obtain oocytes in over 95% of the cases, compared with 54% when prior oocyte aspiration was not performed. We assume that this is due to the fact that IVM aspiration, performed under ultrasound visualization enables a better yield of available oocytes in the ovary. Another explanation is probably the fact that when performing IVM it was

possible to aspirate follicles from the contralateral ovary that was not operated on, too. Second, using the combined method allowed us to retrieve significantly more oocytes with better maturation rate. The maturation results for oocytes obtained by aspiration compared with ovarian tissue oocytes are similar to previous reports indicating that aspirated oocytes (Shalom-Paz et al., 2010) have better maturation potential than ovarian tissue oocytes (Fasano et al., 2011; Revel et al., 2009). We assume that $_{A}$IVM allows us to choose better follicles than the random collection of oocytes during $_{OT}$IVM. As expected, the number of $_{OT}$IVM oocytes was lower in the combined $_{A}$IVM + $_{OT}$IVM group compared with the $_{OT}$IVM only group, probably due to the fact some oocytes were aspirated prior to the ovarian resection; however, the total number of oocytes was higher using the combined procedure. Consequently, using this approach has allowed us to cryopreserve significantly more oocytes. A relatively small number of patients undergoing OTC without oocyte aspiration chose to freeze embryos, so although the number of embryos cryopreserved was higher for the combined approach, it did not reach statistical significance.

It must be noted that combining ovarian tissue cryopreservation with oocyte retrieval using an IVF protocol is not possible because the ovary is stimulated and hyperaemic, which makes the ovarian resection too risky.

However, the present results demonstrate the efficacy of using the mild IVM stimulation protocol prior to the fertility preservation procedure. The short, mild stimulation did not delay the procedure by more than a few days and allowed the retrieval of more oocytes and consequently more mature oocytes without any observed complications during fertility preservation by laparoscopy.

It must be noted that the total number of oocytes and mature oocytes that were obtained was significantly higher in $_{A}$IVM + $_{OT}$IVM (Group D) compared with $_{OT}$IVM (Group B), even when the cases that received mild stimulation were excluded ($P < 0.001$, data not shown).

Ten cases in this series were treated on the luteal phase due to time constraints. Even in these cases it was possible to obtain mature oocytes and embryos for cryopreservation in most of the cases, as previously shown (Maman et al., 2011). This demonstrates the benefits of applying this approach at any time during the menstrual cycle. Yet, the potential benefit regarding pregnancy results of one IVM cycle in normo-ovulatory patients for fertility preservation is still to be evaluated. Currently, improving IVM techniques is essential in order to improve the ability to mature oocytes *in vitro* and increase the number of good quality embryos per retrieved immature oocyte.

The use of the combined approach has allowed us to cryopreserve oocytes or embryos in over 95% of the cases compared with about 50% of the cases of using ovarian tissue oocytes, which is the reported rate for other centres (Fasano et al., 2011).

Our results emphasize the importance of performing the fertility preservation procedure in a facility that encompasses an operating room for laparoscopy and oocyte retrieval with a proximal IVF laboratory allowing the direct transfer of oocyte and ovarian tissue for processing. Transferring ovarian tissue on ice can be effective for fertility preservation regarding the cryopreserved tissue (Isachenko et al., 2012) but results in a poor outcome for isolated oocytes (Wilken-Jensen et al., 2014).

One shortcoming of this study is the lack of long-term follow-up data (including pregnancy outcomes). This is due to the fact that most patients have not yet used the cryopreserved oocytes and embryos to achieve pregnancy. This is still to be evaluated.

The combined approach allows us to offer more flexibility, and more fertility preservation options to the patient. This is especially important in cases when the frozen tissue contains cancer cells, so that the frozen oocytes and embryos can be used.

In summary, the data shown in this work prove that performing oocyte aspiration just prior to ovarian tissue harvesting for cryopreservation is safe and has the advantages of achieving significantly more oocytes with better maturation rate than ovarian tissue oocyte cryobanking alone. Therefore, combining both methods for fertility preservation can be offered for patients undergoing ovarian tissue cryopreservation.

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