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Fertility preservation in girls during childhood: is it feasible, efficient and safe and to whom should it be proposed?

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BACKGROUND: In prepubertal and adolescent girls, fertility may be impaired by gonadotoxic treatments, repeat ovarian surgery or genetic disorders. Cryopreservation of ovarian cortex is an existing option to preserve fertility in these young girls at risk of premature ovarian failure (POF). The efficacy, feasibility and risks of ovarian cryopreservation in children must be assessed in order to validate the technique.

METHODS: Here, we conducted a review of ovarian cryopreservation in adults and, more specifically, in children using the PubMed databases. In addition, our own experience with ovarian cryopreservation in children was evaluated and compared with the literature.

RESULTS: Analysis of the literature and six published series on ovarian cryopreservation in children, as well as our own series of 58 cases, show that there is no reason to doubt its efficacy in this young population. However, no consensus has yet been reached on the indications for the technique. Indeed, with existing models, the real risk of POF may be over- or underestimated.

CONCLUSION: Our review suggests that ovarian cortex cryopreservation is feasible and as safe as comparable operative procedures in children. Although no births have yet resulted from freeze-thawing of prepubertal ovarian cortex, the results of this approach in adults are encouraging. However, the absence of consensus on the indications for fertility preservation, as well as the optimal timing and quantity of ovarian cortex for cryopreservation, should be taken into consideration when discussing fertility issues with girls at risk of POF and their parents.

Key words: childhood / oncology / ovarian tissue cryopreservation / premature ovarian failure

Introduction

Preservation of fertility in women at risk of ovarian damage has been a concern for gynecologists and oncologists for more than 20 years now and although this is a priority in all women at risk, this review focuses on young girls, whose issues are biologically and socially unique. Ovarian damage is often the cause of premature ovarian failure (POF), manifesting as anything from immediate menopause to loss of ovarian function before the age of 40. At the end of the 20th century, several reviews described possible options for cryopreservation of embryos, mature and immature oocytes and ovarian cortex prior to gonadotoxic therapy (Aubard, 1998; Donnez and Bassil, 1998; Newton, 1998; Oktay *et al*., 1998). Cryobanking of embryos, oocytes and ovarian cortex was initiated in various fertility centers worldwide. Today, one and a half decades later, many of these techniques have yielded positive results (see for review Donnez *et al*., 2006).

One of the biggest remaining challenges is preservation of fertility in children, in whom most of these techniques are inapplicable. This article sets out to analyze current knowledge on preservation of fertility in prepubertal and adolescent girls:

Which girls are at risk of POF?
Which fertility preservation methods are available for prepubertal and pubertal girls?
Are these methods feasible, safe and efficient?

In order to answer these questions, the literature is reviewed and discussed.

Indications for fertility preservation in girls

Oncological indications

Childhood cancers are estimated to occur in ~13 of 100 000 children under 15 years of age (Belgian Society of Pediatric Haematology and Oncology, unpublished data), with 45% being cases of leukemia and lymphoma, 20% craniospinal tumors, 8% neuroblastomas, 8% soft-tissue tumors, 7% nephroblastomas, 3% retinoblastomas and 9% other rare tumors. Cancer treatment is the most frequent cause of ovarian damage in girls. Several large studies have evaluated the fertility outcome of childhood cancer survivors (Byrne *et al*., 1987; Sanders *et al*., 1988; Signorello *et al*., 2006; Green *et al*., 2009). In a recent study, the relative likelihood of female childhood cancer survivors ever conceiving was 0.81 (95% confidence interval 0.73–0.90; $P < 0.001$) compared with their female siblings (Green *et al*., 2009). Gonadal failure in these girls is caused by the accelerated and premature depletion of germ cells in the gonads as a result of the toxic effect of chemotherapy and radiotherapy. The extent of ovarian damage is related to age, exposure to increasing doses of radiation to the ovaries and dose and type of chemotherapeutic agents.

In girls exposed to radiotherapy, the degree of impairment depends on the radiation dose, radiation field, fractionation schedule and age at the time of treatment (Wallace *et al*., 1989; Sanders *et al*., 1996; Bath *et al*., 1999). Effective total sterilization doses are 20.3 Gy at birth, 18.4 Gy at 10 years and 16.5 Gy at 20 years (Wallace *et al*., 2005a). Wallace *et al*. were able to calculate 95% confidence limits for age at which POF occurs for estimated radiation doses to the ovary, from 1 Gy to the effective sterilization dose and from birth to 50 years (Wallace *et al*., 2005a). For example, irradiation of the ovaries at the age of 10 years, with 3, 6, 9 and 12 Gy, would result in POF at the age of 36.7, 26.5, 19.7 and 15.3 years, respectively (see Table I in Wallace *et al*., 2005a).

As far as chemotherapy is concerned, the nature and extent of ovarian damage depends on the drug given, the dose received and the age of the patient. Many drugs are gonadotoxic (see for review Donnez *et al*., 2006), with alkylating agents posing the greatest threat.

The relative contribution of every individual drug can be difficult to determine, however, because most treatments are given in the form of multidrug regimens.

The risk of subfertility after cancer treatment can be classified according to malignant disease type and its associated treatment (Table I, Wallace *et al*., 2005b). The authors of this list recommend fertility preservation interventions when there is a high risk (estimated >50%) of POF.

Non-oncological indications

Certain benign conditions (Table II) such as myelodysplasia, aplastic anemia, thalassemia, drepanocytosis and multiple sclerosis, as well as severe rheumatic diseases, such as Wegener's syndrome, polyarthritis and systemic lupus erythematosus, may also necessitate administration of high doses of chemotherapy with or without bone-marrow

Table I Assessement of risk of subfertility after treatment for common cancers in childhood and adolescence (from Wallace *et al*., 2005b).

Low risk <20%	Acute lymphoblastic leukemia
	Wilm's tumor
	Soft-tissue sarcoma: stage I
	Germ-cell tumors (with gonadal preservation and no radiotherapy)
	Retinoblastoma
	Brain tumor: surgery only, cranial irradiation <24 Gy
Medium risk	Acute myeloblastic leukemia
	Hepatoblastoma
	Osteosarcoma
	Ewing's sarcoma stage II or III
	Neuroblastoma
	Non-Hodgkin lymphoma
	Hodgkin's disease: alternating treatment
	Brain tumor: craniospinal radiotherapy, cranial irradiation >24 Gy
High risk	Whole-body irradiation
>80%	Localized pelvic radiotherapy
	Chemotherapy conditioning for bone-marrow transplantation
	Hodgkin's disease: treatment with alkylating-drugs
	Soft-tissue sarcoma: stage IV
	Metastatic Ewing's sarcoma

Table II Non-malignant pathologies with risk of POF.

Bone-marrow transplantation
Sickle cell anemia
Thalassemia major
Aplastic anemia
Autoimmune diseases unresponsive to immunosuppressive therapy
Autoimmune diseases requiring chemotherapy
Systemic lupus erythematosus
Rheumatoid arthritis
Behçet's disease
Wegener's disease
Multiple sclerosis
Ovarian pathologies
Recurrent ovarian cysts
Ovarian torsion
Endocrine or genetic diseases
Turner syndrome
Galactosemia
Family history of premature ovarian failure

transplantation. In these patients too, fertility preservation options should be contemplated.

Repeat ovarian surgery may also be associated with POF. Ovarian cysts in girls are often diagnosed as a result of ovarian torsion. Diagnosis of torsion is not always easy and late diagnosis may increase the risk of irreversible necrosis and the need for aggressive surgery, such as oophorectomy. Moreover, mature teratomas, the most frequently encountered ovarian cysts in prepubertal girls, can recur in 15% of cases, requiring repeat surgery. Ovarian torsion may also occur without underlying cysts, with an increased risk of recurrence. Ovarian cryopreservation could therefore be discussed in case of repeat ovarian surgery in young girls, especially if only one ovary remains, or when there is an increased risk of ovarian torsion: in this case, ovariopexy should be performed during the same procedure.

POF may also be related to endocrine or genetic diseases, such as galactosemia and Turner syndrome. Turner syndrome is estimated to occur in 40 of 100 000 girls (Stochholm *et al.*, 2006). Although 30% of girls with Turner syndrome undergo spontaneous pubertal development, only 2–5% experience spontaneous menses with the potential to achieve pregnancy without medical intervention (Saenger *et al.*, 2001).

Gonadal dysgenesis in Turner syndrome is caused by oocyte loss from the 18th week of pregnancy onwards or over the first few postnatal months and years. In most 45× Turner syndrome patients, this oocyte loss happens rapidly and results in streak ovaries composed of white fibrous stromal tissue containing no oocytes or follicular derivates (Abir *et al.*, 2001). Ovarian function appears to be preserved for longer in patients with mosaic Turner syndrome (Pasquino *et al.*, 1997; Saenger *et al.*, 2001). Cryopreservation of ovarian tissue has therefore been proposed for girls with Turner syndrome, especially the mosaic type (Saenger *et al.*, 2001; Donnez *et al.*, 2006; Oktay and Oktem, 2009; Borgström *et al.*, 2009). A recent large study (Borgström *et al.*, 2009) evaluated 57 girls with Turner syndrome aged from 8 to 19.8 years, who underwent laparoscopy for ovarian biopsy, evaluation of the presence of follicles and cryopreservation. Ovarian biopsy was feasible in 47 of the 57 patients, and histological analysis of the tissue pieces found follicles in 15 of the 57 girls (26%). Follicles were identified in six of seven girls (86%) with mosaicism, 6 of 22 (27%) with structural chromosomal abnormalities and 3 of 28 with karyotype 45× (10.7%). Fifty-eight percent of girls with signs of spontaneous puberty showed follicles (11/19) and 62% of girls with spontaneous menarche (8/13). Borgström *et al.* (2009) concluded that signs of spontaneous puberty, mosaicism and normal hormone concentrations [FSH and/or anti-Mullerian hormone (AMH) levels] are positive and statistically significant signs, but not exclusive prognostic factors for finding follicles in case of Turner syndrome. They recommend ovarian cortex cryopreservation in girls with mosaic Turner syndrome, girls with spontaneous onset of puberty (45× or mosaic), and those with normal serum FSH and/or AMH levels, with or without spontaneous onset of puberty.

Options for fertility preservation in girls

Oocyte cryopreservation

Oocyte cryopreservation may be considered in some adolescent patients, but gonadotrophins must be administered at low doses to avoid ovarian hyperstimulation in these young girls with a large ovarian reserve (Oktay and Oktem, 2009). However, oocyte retrieval may be difficult in these usually sexually immature subjects and therefore not applicable in the case of younger patients. Cryopreservation of oocytes has been described in prepubertal girls in association with ovarian cortex cryopreservation (Revel *et al.*, 2009). At the time of surgical biopsy, antral follicles observed on the ovarian surface are aspirated and the retrieved immature oocytes are then matured *in vitro* and cryopreserved. Using this method, Revel *et al.* (2009) were able to cryopreserve 11 mature oocytes from three prepubertal girls aged 5, 8 and 10 years.

Ovariopexy and ovarian transposition

When radiotherapy is indicated, ovariopexy or ovarian transposition can be proposed in order to displace the ovaries away from the radiation field. Several techniques can be used (Bisharah and Tulandi, 2003; Cowles *et al.*, 2007; Jadoul *et al.*, 2007).

In the case of craniospinal irradiation, the ovary can be fixed laterally as far as possible from the spine (Fig. 1). The anatomic relations of the ovary with the Fallopian tube and uterus are thereby maintained and natural fertility can be preserved.

In the case of pelvic irradiation, the ovary could be moved outside the pelvis, which may require section of the utero-ovarian ligament and Fallopian tube. The ovary is anchored, as high as possible, to the anterior abdominal wall, laterally in the paracolic gutter. Titanium clips are placed on the two opposite borders of the ovary to allow radiological identification prior to radiotherapy (Fig. 2). The success of ovarian function preservation by means of ovarian transposition prior to radiotherapy ranges from 16 to 90% (Thibaud *et al.*, 1992; Morice *et al.*, 2000; Aubard *et al.*, 2001; Scott and Schlaff, 2005). Success rates are affected by the degree of scatter radiation, vascular compromise, patient age, radiation dose and use (or not) of

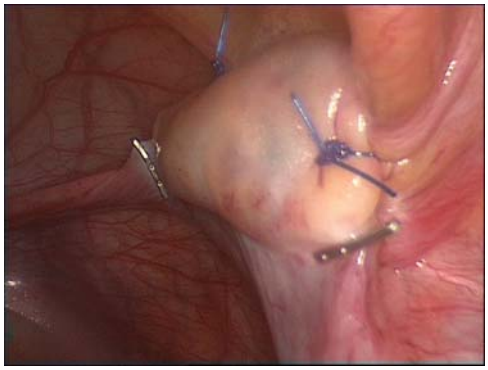

Figure 1 Ovariopexy: in the case of craniospinal irradiation, the ovary is fixed as far as possible from the midline. Clips are placed to allow ovary localization during radiotherapy. This patient is an 8-year-old girl (ovary size 2 cm).

concomitant chemotherapy (Sonmezer and Oktay, 2004). When the ovaries are transposed to an abdominal position, spontaneous pregnancy may not be possible, unless a second procedure is performed to relocate the ovaries back to the pelvis. Furthermore, should these patients need IVF in the future, oocyte retrieval may be technically more challenging. Candidates for ovarian transposition should therefore be selected carefully, taking into account all variables that may affect its success rate. It should also be borne in mind that when gonadotoxic chemotherapy is associated with radiation there is no strong rationale for this procedure.

Ovarian cortex cryopreservation

Lessons learned from ovarian cryopreservation in adults

Ovarian cortex cryopreservation was initiated more than 10 years ago and performed with a view to either future reimplantation or follicular isolation and *in vitro* maturation (IVM) (Donnez and Bassil, 1998). IVM of follicles isolated from cryopreserved ovarian cortex has not yet resulted in pregnancy, but the reimplantation technique has yielded pregnancies and live births. At least ten children have been born after orthotopic reimplantation of frozen–thawed ovarian cortex (Donnez *et al.*, 2004; Meirow *et al.*, 2005; Demeestere *et al.*, 2007; Andersen *et al.*, 2008; Silber *et al.*, 2008; Piver *et al.*, 2009, Sánchez-Serrano *et al.*, 2009).

It is important to note that a vast majority of patients undergoing ovarian cortex reimplantation have demonstrated restoration of ovarian function, experiencing follicular development and ovulation.

In our series of 13 cases, only two patients failed to recover ovarian function owing to a very poor ovarian reserve in the cryopreserved ovarian tissue (Donnez *et al.*, 2006, Donnez *et al.*, 2008). In these two patients, as no ovarian function recovery was observed after reimplantation, histological analysis of several fragments of remaining cryopreserved ovarian cortex was performed and showed an almost complete absence of primordial follicles.

With this reimplantation method, there are some concerns about the possible presence of malignant cells in the frozen–thawed ovarian tissue, which could lead to a recurrence of the primary disease after grafting. Indeed, transmission of lymphoma via grafts of ovarian tissue from diseased donor mice to healthy recipients was reported by Shaw *et al.* (1996).

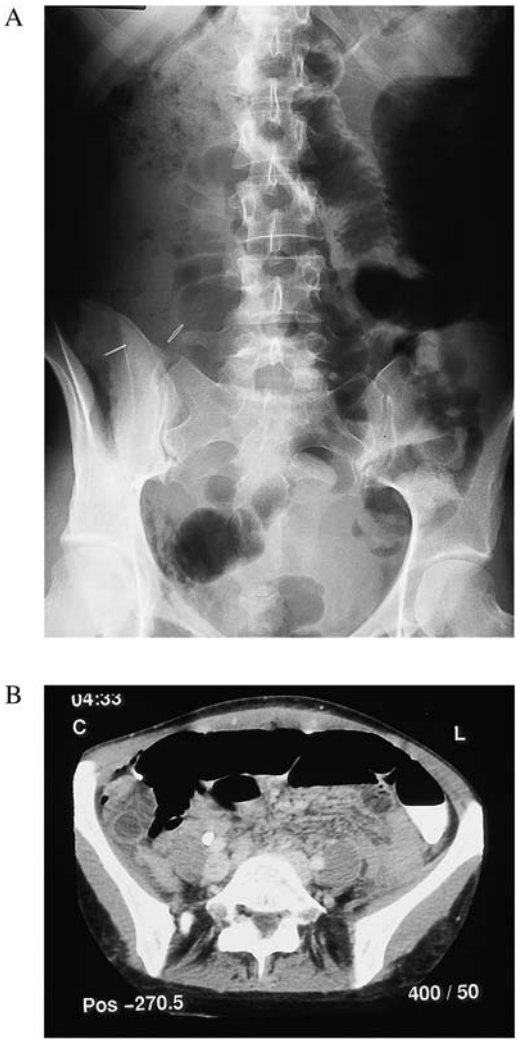

Figure 2 Post-operative identification of a transposed right ovary before radiotherapy using (**A**) radiography and (**B**) computed tomography.

Besides histological analysis of part of the cryopreserved cortex, several methods are now available to exclude the presence of malignant cells. Xenotransplantation of frozen–thawed ovarian tissue from patients suffering from Hodgkin's lymphoma to severely compromised immunodeficient mice (Kim *et al.*, 2001) did not result in the development of the disease. Specific and sensitive (Gabert *et al.*, 2003) methods for minimal residual disease (MRD) monitoring have also been developed over recent years. Currently available methods include PCR amplification of fusion transcripts, rearranged immunoglobulin or T-cell receptor genes, and flow cytometric detection of aberrant immunophenotypes.

Chromosome aberrations provide tumor-specific PCR targets for MRD detection that offer reliable estimates of residual malignant cells and are currently used as markers of molecular response to treatment during clinical remission (Meirow *et al.*, 2008).

Another way of avoiding the risk of reimplanting contaminated tissue is grafting isolated follicles (Dolmans *et al*., 2007, 2008). Research is under way to design a scaffold to support these follicles.

IVM of follicles within pieces of cryopreserved ovarian tissue has not yet yielded pregnancies, but protocols for long-term *in vitro* culture of human ovarian cortical tissue have been developed (Abir *et al*., 1997; Hovatta *et al*., 1999). Systems have been improved and some of the factors regulating follicular development have been identified (Hreinsson *et al*., 2002; Scott *et al*., 2004; Abir *et al*., 2006; Carlsson *et al*., 2006). It has been shown that secondary and often early antral follicles can be obtained from fresh, slowly frozen or vitrified-thawed ovary tissue in long-term culture (Rodriguez-Macias Wallberg *et al*., 2009). It was recently demonstrated that it is possible to achieve accelerated maturation and development of primordial and primary follicles using a two-step culture system (Telfer *et al*., 2008). After culture of human ovarian cortical strips in serum-free medium for 6 days, pre-antral follicles were isolated and cultured for 4 more days. Ninety percent of follicles cultured separately in the presence of activin A grew in size, confirming further development. Woodruff's studies reveal that early secondary follicles, once removed from the restricted environment of the ovary, have the capacity to survive and grow in culture. Using a three-dimensional (3D) supportive 360 matrix, follicles were shown to maintain bidirectional communication between somatic cells and germ cells, creating an environment conducive to oocyte growth and normal steroid production (Jeruss and Woodruff, 2009).

Our preliminary results indicate that alginate hydrogels may be a suitable system for *in vitro* culture of isolated human pre-antral follicles. A total of 159 small pre-antral follicles (mean $\pm$ SD: 42.98 $\pm$ 9.06 μm diameter) from frozen–thawed tissue were incubated in a 3D system (alginate hydrogel) and, after 7 days, all of them showed an increase in size (final size 56.73 $\pm$ 13.10 μm diameter). The survival rate of the follicles was 90% (oocyte and all granulosa cells viable) (Amorim *et al*., 2009).

The next frontier will be maturation of oocytes grown *in vitro* to the metaphase II stage, fertilization and embryo development, and the adaptation of these techniques to primordial follicles and cryopreserved tissues.

One can therefore conclude at this point that ovarian cortex cryopreservation has proved to be a major breakthrough in the field of fertility preservation and that this technique must be considered a valid alternative when fertility preservation options are contemplated.

Ovarian cryopreservation in children

The American Society of Clinical Oncology (Lee *et al*., 2006) has recommended that oncologists address the possibility of infertility in patients treated during their reproductive years and be prepared to discuss fertility preservation options or refer patients to reproductive specialists, as indicated. However, there is no consensus or directive on the age at which reproductive potential is actually reached, making it unclear how these recommendations can be effectively applied to cancer patients under the age of 18 years.

A recent study among pediatric oncologists in the UK (Anderson *et al*., 2008a) revealed that, in a pediatric cancer population of 463 girls diagnosed with the disease between November 2003 and October 2004, the risk of fertility impairment was discussed in 62% of cases, fertility preservation options were offered to 38 girls (8.2%), and only four patients, thus <1%, were referred for assisted reproduction techniques (ART). The most common reasons given for not discussing fertility preservation options included 'not at significant risk' in 29% of cases, 'too young' in 27%, 'techniques unproven' in 22%, 'no facilities' in 10% and 'no funding' in 8%.

Ethical issues have also been advanced as a reason against proposing ovarian cryopreservation in young girls. Dudzinski (2004) conducted a normative analysis of ethical issues in the context of oocyte and ovarian tissue cryopreservation for pediatric and adolescent cancer patients and concluded that more research is required before it will be ethical to enroll adolescents in clinical trials.

However, a child's right to fertility preservation is now acknowledged in the bioethical literature as a 'right in trust', to be safeguarded until the child reaches adulthood. Unfortunately, such rights are sometimes violated in advance, before the child is in a position to exercise them (Feinberg 1992; Davis, 1997). If the medical risk is acceptable, and the resources are available, it is an individual's ethical right to request fertility preservation. On the other hand, it is our ethical obligation to act 'in the child's best interest'. In order to determine the child's best interest, it is important to analyze the efficacy, feasibility and risks of the technique applied, particularly as this technology may still be seen as experimental and unproven.

Efficacy. Pregnancies obtained after implantation of frozen–thawed ovarian cortex in adults, and progress made in the field of IVM and in the development of methods to exclude malignant cells from ovarian cortical tissue, have validated the technique of ovarian cryopreservation.

One could argue that none of these pregnancies were obtained by reimplantation of ovarian tissue harvested before puberty. However, there is no reason to doubt the capacity of prepubertal ovarian cortex to develop and function correctly after reimplantation. Indeed, animal studies demonstrate that gonadal function can be restored by reimplantation of fresh and frozen–thawed 'immature' tissue (Carroll and Gosden, 1993; Sauvat *et al*., 2008). In mice, puberty and cyclic hormonal activity were restored by transplantation of fresh or cryopreserved immature prepubertal ovarian tissue in both prepubertal and adult animals.

Moreover, as mentioned before, Revel *et al*. were able to cryopreserve 11 mature oocytes from three prepubertal girls aged 5, 8 and 10 years, showing that prepubertal oocytes can be matured when properly stimulated (Revel *et al*., 2009).

Increased density of primordial follicles in children compared with adults at the time of ovarian cryopreservation might increase the chances of obtaining pregnancy, either by ovarian cortex or follicle reimplantation, or IVM. Indeed, results from several studies have demonstrated a correlation between age and follicular density, with significantly increased follicular density in young girls (Faddy, 2000; Schmidt *et al*., 2003; Hansen *et al*., 2008).

Feasibility. A number of authors have addressed the question of ovarian cryopreservation during childhood (Brougham and Wallace, 2005; Poirot *et al*., 2007; Weintraub *et al*., 2007; Fallat *et al*., 2008; Martin and Patrizio, 2009; Sauvat *et al*., 2009), but only a few series of performing ovarian cryopreservation in children have been documented (Table III).

Table III Series of ovarian cortex cryopreservation in children.

Authors	Patients (*n*)	Age (years)		Patients under 16 years		Patients under 10 years	
		Range	Mean	*n*	%	*n*	%
Feigin *et al.* (2007)	23	5–17.5	13.5	NA	NA	NA	NA
Poirot *et al.* (2007)	47	0.8–15	6.1	47	100	38	81
Anderson *et al.* (2008b)	36	5–35	19.2	11	31	3	8
Revel *et al.* (2009)	19	5–20	15.3	8	42	2	11
Oktay and Oktem (2009)	26	4–21	14.3	9	35	6	23
Borgström *et al.* (2009)	57	8–19.8	14.4	40	70	4	7
Jadoul *et al.* (present publication)	58	0.8–15.8	10.4	58	100	21	36

Revel's team reported ovarian cryopreservation in nine patients aged 8–20 years (Weintraub *et al.*, 2007). In a more recent publication, they described 19 patients aged between 5 and 20 years, but only eight of them were under 16 years of age. In these patients, they performed ovarian cortex cryopreservation, as well as IVM and cryopreservation of aspirated immature oocytes (Revel *et al.*, 2009). Feigin *et al.* (2007) reported 23 cases of cryopreservation in girls and adolescents aged 5–17.5 years (mean 13.5 years). Oktay and Oktem recently published a series of 26 patients aged between 4 and 21 years (mean 14.3 ± 1.1), who underwent ovarian cryopreservation between 1999 and 2008 (Oktay and Oktem, 2009). Nine were under 16 years of age. Anderson *et al.* reported the indications and outcomes of 36 women and girls undergoing ovarian cortex cryopreservation, 15 of whom (42%) were aged 16 years or less (mean age: 19.2 years) (Anderson *et al.*, 2008b).

The largest series involving a pediatric cancer population was published by Poirot *et al.* (2007). From September 2000 to February 2005, 47 prepubertal girls with a median age of 6.1 years (range 10 months to 15 years) underwent ovarian cortex cryopreservation. The procedure was performed by laparoscopy in 49% of patients and laparotomy in 51%. No complications occurred during surgery.

Another large series of ovarian cryopreservation in girls is that of Borgström (2009), previously mentioned, involving 57 girls aged 8–19.8 years (mean 14.4 years) with Turner syndrome, who underwent tissue harvesting by laparoscopy.

Over the last decade, operative endoscopy and the concept of minimally invasive surgery have fundamentally changed the practice of surgery. With continued improvement and miniaturization of the required equipment, laparoscopic surgery has proved itself to be safe in infants and even neonates (Jawad and Al Meshari, 1998; Wedgewood and Doyle, 2001; Davenport, 2003; Mansuria and Sanfillipo, 2004). Laparoscopic oophorectomy can be performed in children, yielding a very low rate of complications (Davidoff *et al.*, 1996).

Moreover, the safety of neonatal and pediatric anesthesia has been significantly enhanced (Veyckemans, 2004), with laparoscopic procedures performed under the same anesthesia as that already required for other diagnostic or therapeutic purposes, such as bone-marrow aspiration or harvesting, central venous catheter insertion, imaging techniques and so on.

Experience of the authors. In our institution (Department of Gynecology, Université Catholique de Louvain, Belgium), we have so far performed ovarian cryopreservation in 58 girls under 16 years of age (from May 2001 to May 2009). Age and indications are detailed in Table IV.

Age. Patient age (± SD) ranged from 10 months to 15 years (10.4 ± 4.4 years). Twenty-one girls were under 10 years of age and 38 were prepubertal.

Indications. Forty-eight patients were suffering from cancer, five of whom had already received chemotherapy prior to cryopreservation. Three patients had undergone a complete course of chemotherapy and were referred to us at the time of recurrence, one was referred after one course of chemotherapy and one was referred before chemotherapy but cryopreservation was postponed after the anesthetic risk was deemed to be too high because of the presence of a large mediastinal mass. In all other cases, ovarian biopsy was performed before the start of chemotherapy. Three patients underwent cryopreservation because of genetic disorders, two for Turner syndrome and one for galactosemia. Two patients required cryopreservation prior to bone-marrow transplantation for benign disease, and one before chemotherapy for systemic disease. Four patients were suffering from benign ovarian pathologies. One girl underwent cryopreservation after torsion of a single remaining ovary, and one after ovariectomy for torsion of a normal ovary, with ovariopexy performed at the same time. Two girls underwent cryopreservation for recurrent ovarian cysts.

Techniques. All procedures were performed by laparoscopy without any complications. In girls under 10 years of age, we systematically carried out an open intraumbilical laparoscopy using a 5-mm or 7-mm laparoscope. Maximal intra-abdominal pressure was initially 8 mmHg, increased to 10 mmHg if possible. Two 5-mm lateral trocars were placed to perform biopsy or ovariectomy. In girls aged 2 years or younger, 3-mm trocars were used. In girls over age 10 years, classic infraumbilical laparoscopy was carried out using a 10-mm trocar, as in adults. In 38 patients, unilateral or bilateral cortical biopsies were taken (Fig. 3). In 20 patients, unilateral oophorectomy was performed (Fig. 4). The ovary was removed through the 5-mm trocar in most cases (Fig. 5), and through the central 10-mm trocar in a few cases.

Follow-up. For all oncological indications, chemotherapy was initiated from day 0 to day 5 after ovarian tissue cryopreservation. Histological

Table IV Personal series of 58 girls under 16 years of age who underwent ovarian cryopreservation from May 2001 to May 2009.

N	Age (years)	Indication	Quantity	POF risk according to Wallace
1	12.8	Ewing's sarcoma	Cortical biopsies	M
2	13.7	Hodgkin's lymphoma	Cortical biopsies	M
3	10.3	Non-Hodgkin's lymphoma	Cortical biopsies	M
4**	7.2	Rhabdomyosarcoma	Right oophorectomy	M*
5	12.9	Rhabdomyosarcoma	Right oophorectomy	M
6	12.9	Medulloblastoma	Right oophorectomy	M
7	15.3	Osteosarcoma	Cortical biopsies	M
8**	11.8	Non-Hodgkin's lymphoma	Cortical biopsies	M
9	6.2	Rhabdomyosarcoma	Cortical biopsies/ovarian transposition	M
10	12.1	Acute lymphoblastic leukemia	Cortical biopsies	L
11	11.4	Nasopharyngeal carcinoma	Cortical biopsies	M
12	12.2	45X0/46XX Mosaic Turner syndrome	Cortical biopsies	
13	15.8	Hepatosarcoma	Cortical biopsies	M
14**	12.2	Osteosarcoma	Cortical biopsies	M
15	13.5	Recurrent ovarian cyst	Cortical biopsies	
16**	13.6	Acute lymphoblastic leukemia	Cortical biopsies	L
17	12.8	Dysgerminoma	Cortical biopsies	L*
18	15.8	Hodgkin's lymphoma	Cortical biopsies	M*
19	3.4	Choroid plexus carcinoma	Right oophorectomy	M
20	6.4	Non-Hodgkin's lymphoma	Cortical biopsies	M
21	15.7	Hodgkin's lymphoma	Cortical biopsies	M
22	9.8	Hodgkin's lymphoma	Cortical biopsies	M
23	11.8	Stage IV nephroblastoma	Cortical biopsies	H
24	11.5	Osteosarcoma	Cortical biopsies	M
25	2.8	Acute lymphoblastic leukemia	Right oophorectomy	L
26	12.9	Torsion of unilateral ovary	Cortical biopsies	
27**	11.7	Osteosarcoma	Cortical biopsies	M
28	14	Synovial sarcoma	Cortical biopsies	L
29	15.5	Acute lymphoblastic leukemia	Cortical biopsies	L*
30	3.5	Medulloblastoma	Left oophorectomy	M
31	8.5	Non-Hodgkin's lymphoma	Cortical biopsies	M*
32	15	Rhumatoid polyarthritis	Cortical biopsies	
33	12.4	Non-Hodgkin's lymphoma	Cortical biopsies	M
34**	14.9	Non-Hodgkin's lymphoma	Cortical biopsies	M*
35	11.5	Rhabdomyosarcoma	Cortical biopsies	M
36	1.8	Vaginal rhabdomyosarcoma	Right oophorectomy/left ovariopexy	H
37	14.8	Acute lymphoblastic leukemia	Cortical biopsies	L
38**	15	Acute lymphoblastic leukemia	Cortical biopsies	L*
39	15.6	Non-Hodgkin's lymphoma	Cortical biopsies	M
40	2.8	Drepanocytosis	Left oophorectomy	H
41	11.6	Galactosemia	Left oophorectomy	
42	9.8	Unilateral ovary after oophorectomy for torsion	Cortical biopsies/ovariopexy	
43	10.3	Acute lymphoblastic leukemia	Right oophorectomy	L
44	15.2	Acute myeloblastic leukemia	Cortical biopsies	M*
45	10.3	Recurrent ovarian cyst	Cortical biopsies	
46	5.2	Non-Hodgkin's lymphoma	Left oophorectomy	M

Continued

Table IV *Continued*

N	Age (years)	Indication	Quantity	POF risk according to Wallace
47	3.3	Acute lymphoblastic leukemia	Right oophorectomy	L
48	5	Acute lymphoblastic leukemia	Left oophorectomy	L
49	8	Pinealoblastoma (PNET) grade IV	Left oophorectomy/right ovariopexy	M
50	11	Ewing's sarcoma	Cortical biopsies	M
51	14	Acute lymphoblastic leukemia	Cortical biopsies	L
52**	9.9	Ewing's sarcoma	Left oophorectomy	M
53	1.9	Drepanocytosis	Left oophorectomy	H
54	9.7	Acute myeloblastic leukemia	Left oophorectomy	M
55	14.6	45X0/46XX Mosaic Turner syndrome	Left oophorectomy	
56	5.5	Acute lymphoblastic leukemia	Cortical biopsies	L
57	2.9	Acute lymphoblastic leukemia	Left oophorectomy	L
58	0.8	Ependymoma brain tumor	Left oophorectomy	M

L, low risk; M, medium risk; H, high risk; POF, premature ovarian failure.
*Changed to high risk during cancer treatment.
**Died of disease.

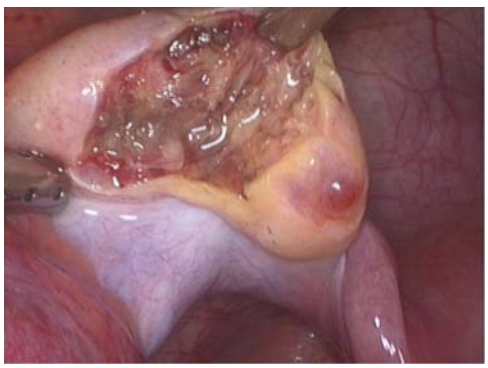

Figure 3 Cortical ovarian biopsy from a 12-year-old girl. The posterior part of the left ovarian cortex (2.5 × 1.5 cm) is removed.

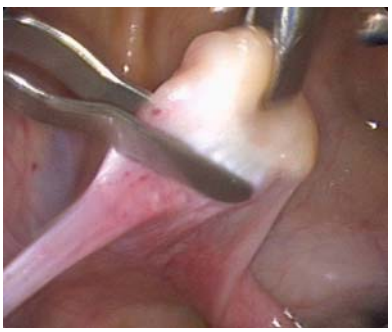

Figure 4 Laparoscopic oophorectomy in a 3-year-old girl.

analysis of a small piece of ovarian cortex showed the presence of follicles in all cases (mean = 43 primordial follicles per mm^2 in analyzed sections), except in the patient suffering from galactosemia, in whom FSH levels were as high as 18.7 mUI/ml at the time of ovarian cortex harvesting. In one patient with lymphoma, laparoscopy revealed tumoral infiltration of the ovary by a 10-cm mass, confirmed by histological analysis. No ovarian involvement was observed in any other cases.

When classified according to Wallace's list of risk factors for fertility impairment (Wallace *et al.*, 2005b), 14 patients were found to be at low risk, 32 at medium risk and 4 at high risk of premature menopause. During the subsequent months after cryopreservation, four girls changed categories from low to high risk and four from medium to high risk (asterisk in Table IV). Indeed, five girls initially at low or medium risk needed bone-marrow transplantation, two girls required further gonadotoxic drugs for recurrence of disease, and one patient had to undergo pelvic radiotherapy.

Eight girls have died from their disease to date (14%). If we exclude the one patient in whom no follicles were found, the 8 girls who died, 4 who had no further gonadotoxic treatment, 17 who have not yet reached pubertal age, 3 lost to follow-up and 4 pubertal girls still undergoing chemotherapy, 21 patients remain in whom ovarian function can be clinically assessed. The mean age of these 21 girls is now 17.6 years. Two girls are showing signs of premature menopause (FSH levels 117 and 90 mUI/ml), 15 have persistent ovarian function and four have been taking oral contraceptives since chemotherapy.

Our series shows that ovarian tissue cryopreservation is feasible at a young age using a laparoscopic approach, without complications and without postponing cancer treatment. We also demonstrate that it is difficult to give the patient or her parents an accurate assessment of the risk to fertility. Although we can define treatment regimens associated with varying degrees of risk, disease evolution is never completely predictable. Indeed, in our series, 14% of patients initially at low or medium risk of POF needed more aggressive gonadotoxic

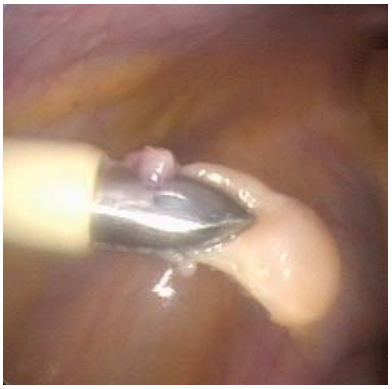

Figure 5 The ovary (15 mm in length) from the 3-year-old girl (as in Fig. 4) is removed through a 5-mm trocar.

treatment in the months following cryopreservation. On the other hand, a large majority of girls in whom ovarian function is assessable show persistent ovarian function. However, persistent ovarian function does not exclude the risk of subsequent POF.

Risks of the technique. There is no evidence to suggest that fertility preservation options used today directly compromise the success of cancer therapy or adversely affect a survivor's health (Lee *et al*., 2006). Laparoscopic surgery has been shown to be safe in infancy and childhood, and the safety of neonatal and pediatric anesthesia has significantly improved (Veyckemans, 2004). One should bear in mind, however, that anesthesia-related complications remain more common in pediatric patients than in adults. The risk of complications is greatest in children under 12 months of age, it significantly decreases after the age of 3 years and then continues to slowly decline (Weintraub *et al*., 2007). Anesthetists and surgeons should be fully aware of the effects of increased abdominal pressure and use of CO_2 in children. As a rule, low insufflation pressures should be used to reduce respiratory and hemodynamic consequences in CO_2-inflated body spaces, to allow for easier elimination of absorbed CO_2 and reduce the risk of mortality in case of accidental CO_2 embolism.

Another important factor is the health status of the child at the time of the procedure. Hematological, respiratory, infectious, biochemical and other disorders should be appropriately addressed prior to surgery.

As previously mentioned, reimplantation of ovarian cortex carries the risk of transmission of neoplastic cells. However, methods to detect residual tumoral involvement in cryopreserved tissue are becoming increasingly effective and research into other techniques, such as reimplantation of isolated follicles and IVM, continues to progress (Meirow *et al*., 2008; Dolmans *et al*., 2008).

Risks of pregnancy after fertility preservation treatment. Pregnancy after gonadotoxic treatment. Concerns have been raised that potentially mutagenic chemotherapy and radiotherapy may cause germline mutations and pose an increased risk of genetic abnormalities in offspring born to cancer survivors (Boice *et al*., 2003; Winther *et al*., 2004). If ovarian tissue cryopreservation is performed after induction of chemotherapy, this increased risk might apply.

Encouraging results were nevertheless achieved in two large international studies from the USA and Denmark, involving a cohort of almost 15 000 childhood cancer survivors, who gave birth to or fathered children. In the US series, genetic abnormalities were reported in 157 of 4214 (3.7%) childhood cancer survivors compared with 95 of 2339 (4.1%) children of sibling controls. Similar findings were reported in the Danish series, providing further reassurance that cancer therapies do not confer a greater risk of inherited genetic disease in offspring. Numerous studies (Hawkins, 1991; Byrne *et al*., 1998; Nagarajam and Robison, 2005; Rees *et al*., 2006) have investigated the incidence of cancer in the offspring of cancer survivors and, excluding known cancer predisposition syndromes, found minimal or no increased risk of cancer development.

Pelvic radiotherapy may cause damage to uterine vascular and muscular structures, resulting in diminished uterine blood flow, reduced uterine volume, decreased endometrial thickness and loss of distensibility. Irradiation affecting the uterus in childhood and adolescence is therefore associated with a raised incidence of spontaneous miscarriage and intrauterine growth retardation (Critchley *et al*., 1992; Wallace *et al*., 2003). The extent of the impact of radiation on the uterus may be more pronounced if administered before puberty. Women exposed to pelvic radiation after puberty have larger uteri and a greater likelihood of live birth than those exposed prior to puberty (Bath *et al*., 2002). In the Bath study (2002), women who had previously undergone total body irradiation were administered a physiological regimen of sex steroid replacement for 3 months, after which uterine volume significantly increased from 6.5 to 16.3 ml, but remained significantly lower than measurements in the control group (controls: median, 41.5 ml; range 28.1–57.9 ml).

Fortunately, there were also demonstrable increases in endometrial thickness, and endometrial tissue collected at the time of physiological sex steroid replacement exhibited an appropriate functional response, as determined by the immunohistochemical demonstration of endometrial sex steroid receptors (Critchley *et al*., 2002).

In another study (Larsen *et al*., 2004), there was no significant increase in uterine volume, endometrial thickness or uterine artery blood flow after hormonal treatment in three subjects receiving high-dose (30–54 Gy) whole-abdominal or pelvic irradiation. These authors concluded that higher irradiation doses, such as those delivered with abdominal and pelvic irradiation, compared with the lower doses with total body irradiation, are more likely to cause damage (possibly irreversible) to the uterine myometrium, endometrium and vasculature.

Even the prospect of a non-functional uterus in the case of pelvic irradiation during childhood does not imply that ovarian cryopreservation should not be discussed. Indeed, IVF surrogacy can be an option in countries where this practice is legal.

Pregnancy in girls with Turner syndrome. Girls with Turner syndrome should be counseled on the risk of pregnancy related to their disease. Several deaths with aortic dissection have been reported in Turner syndrome patients, who became pregnant through oocyte donation (Lin *et al*., 1986; Garvey *et al*., 1998; Weytjens *et al*., 2000; Beauchesne *et al*., 2001; Karnis *et al*., 2003). Karnis *et al*. (2003) estimated that at least four deaths occurred in ~200 donor oocyte pregnancies in women with Turner syndrome: preconception and postconception cardiac screening is therefore imperative for women with this disorder who wish to conceive. In addition, some investigators have reported spontaneous miscarriage rates as high as 50–60% in patients with Turner syndrome treated by oocyte donation

(Press et al., 1995; Yaron et al. 1996; Khastgir et al., 1997). Some have hypothesized that this risk is associated with the presence of a hypoplastic or bicornuate uterus in these patients (Khastgir et al., 1997). The low rate of spontaneous miscarriage (7%) in the Karnis et al. (2003) study is very reassuring, however, and questions the validity of these hypotheses.

Natural pregnancies occur in ~2–3% of patients with Turner syndrome (Pasquino et al., 1997; Hovatta et al., 1999; Abir et al., 2001). Not only are such pregnancies rare, but rates of miscarriage (29%), stillbirth (7%) and malformation (20%) are very high (Abir et al., 2001). Researchers believe that miscarriages are the result of fetal chromosomal abnormalities, mostly trisomy 21 and Turner syndrome, which have been observed in both aborted fetuses and live born children of these patients at a much higher rate than in the general population (4 versus 0.4% for trisomy 21 and 15 versus 0.5% for Turner syndrome, respectively) (King et al., 1978; Nielsen et al., 1979; Swapp et al., 1989). This increased risk of abnormalities and malformations leads some investigators to actively discourage unassisted pregnancies and even encourage egg donation (Kawagoe et al., 1993; Pasquino et al., 1997).

Ethics. On the basis of this analysis of the efficacy, feasibility and risks of ovarian tissue cryopreservation in children and our own experience, it appears wholly appropriate to discuss fertility issues and fertility preservation options with young patients facing the prospect of future fertility impairment. However, it is important in order to allow parents and children to make informed decisions, to clearly communicate the difficulty of estimating the real risk of ovarian failure. Our study shows that based on Wallace's list of risk factors (Wallace et al., 2005b), the risk of infertility may be underestimated in 14% of patients. On the other hand, Anderson et al. found, in their series of 36 patients, that the risk of infertility may also be overestimated (Anderson et al., 2008b): indeed, at least five of their patients who underwent ovarian cryopreservation for a pathology associated with a high risk of ovarian failure experienced spontaneous conception in the years following cryopreservation and chemotherapy. However, even if ovarian function persists after treatment, these patients remain at risk of subsequent POF and we are not able to predict the age at which this may happen. As social reasons often affect the timing of attempted conception, even a risk of ovarian failure at 35 or 40 years of age should be taken into account for young girls.

One of the most important ethical issues is to ensure that the intervention does not harm the patient by dangerously delaying cancer treatment or impairing quality of life, and that no remnant malignant cells are reintroduced by subsequent transplantation.

Respecting the code of good practice, all patients who may become infertile, whatever their age, have the right to receive proper consideration of their interests for future possibilities in the field of ovarian function preservation. Cancer treatment obviously takes priority over potential restoration of fertility, but offering the chance to preserve fertility may greatly enhance the quality of life of young cancer patients and their parents.

As stated by Dudzinski (2004), policies to protect the patient's future rights to her gametes should be developed. Due consideration must be given to the disposition of the ovarian tissue, regardless of whether the child lives or dies. If the child lives, a decision must be taken as to when she will have the necessary maturity and level of moral development to make an informed personal decision about what to do with the cryopreserved material. If the child should die, the parents should not have discretion over her biological material, and it should be destroyed. Besides ethical considerations, decisions should always be made according to existing laws of consent and according to local health policies, which vary from country to country.

Questions still remaining. It is important to note that all patients in the Poirot (2007) series had already received several courses of chemotherapy by the time cryopreservation was undertaken. On the contrary, in our series, 46 of 51 patients requiring gonadotoxic treatment underwent ovarian tissue cryopreservation before administration of any chemotherapeutic agent. An argument in favor of giving chemotherapy prior to cryopreservation might be the hope of eradicating neoplastic cells in the ovary before the tissue is cryopreserved, especially in hematological cancers. However, one cannot exclude a gonadotoxic effect of this initial chemotherapy. Indeed, in adults, we demonstrated that even one course of chemotherapy impairs ovarian response to stimulation in an IVF setting (Dolmans et al., 2005). Moreover, morphometric studies of primordial follicles by transmission electron microscopy showed that, in patients with Hodgkin's disease treated with multidrug chemotherapeutic regimens, the quality of follicles was impaired and many follicles underwent atresia (Familiari et al., 1993). A study by Abir et al. (2008) also demonstrated increased oocyte vacuolization and granulosa cell nuclear abnormalities after chemotherapy, supporting the practice of proposing cryopreservation before treatment, if the general health of the patient allows it. However, as stated by Anderson et al. (2008b), even if a girl has already undergone chemotherapy, cryopreservation of ovarian cortex could be proposed if treatment carrying a greater gonadotoxic risk is subsequently required. Indeed, as follicle density is higher in children, there is a high probability of recovering intact follicles, even after several courses of chemotherapy.

In girls with genetic disorders, such as Turner syndrome, it is more difficult to determine the best timing for cryopreservation. Moreover, Turner syndrome is often diagnosed only at the time of puberty. Indeed, in the Stochholm (2006) review of women with Turner syndrome in Denmark, the median age at diagnosis was 15.1 years. In Borgström's study (2009), the youngest girl to undergo ovarian tissue cryopreservation was 8 years old. Since more follicles are found in younger girls with Turner syndrome, one of the main challenges for pediatricians and gynecologists is to diagnose Turner syndrome as early as possible and investigate markers of ovarian function in prepubertal girls. Indeed, if we were able to evaluate the ovarian reserve and quality before puberty, we would be better positioned to define the best timing for fertility-sparing methods in patients with Turner syndrome, and more accurately assess the gonadotoxic effects of oncological treatments.

In adults, a number of tests are used in clinical settings to evaluate ovarian function: ultrasonography with the evaluation of ovarian volume and antral follicle count, as well as serum measurements of FSH, LH, estradiol (E_2), inhibin and AMH (Broekmans et al., 2006). At present, serum AMH levels appear to be the most reliable marker to determine ovarian reserve (La Marca and Volpe, 2006; Visser et al., 2006). Measurement of AMH levels is thus widely used to evaluate ovarian reserve and responsiveness in ART (Fanchin

et al., 2005). It is also increasingly utilized to assess ovarian function after gonadotoxic therapy (Bath et al., 2003; Anderson et al., 2006; Loverro et al. 2007; Lutchman Singh et al., 2007; van Beek et al., 2007; Lie Fong et al., 2008, 2009; Rosendahl et al., 2008; Williams et al., 2008).

In prepubertal girls, FSH and E_2 levels are low and not representative of the ovarian reserve, while little has been published on AMH levels in this population.

Further studies are required to ascertain normal serum AMH levels in children and analyze the usefulness of AMH measurements in young girls undergoing potentially gonadotoxic treatments and those with a genetic risk of POF, such as Turner syndrome. Moreover, more research is needed to determine the quality of oocytes obtained after ovarian cryopreservation, which might not completely correlate with the quality of granulosa cells. The oocyte itself could be damaged by freezing, thawing and transplantation, while granulosa cells may be more resistant (Camboni et al., 2008). While some granulosa cell loss can be compensated for by mitosis of the surviving granulosa cells, this is impossible in case of oocyte damage. At this stage, oocyte quality can only be assessed by transmission electron microscopy by analysis of cytoplasm homogeneity, perivitelline space size and content and zona pellucida integrity (Nottola et al., 2008, Dolmans et al., 2009).

With appropriate markers for the various factors which may influence outcome of the cryopreservation/transplantation procedure, we will be able to pinpoint the indications and best timing for ovarian tissue cryopreservation.

Another important issue is the quantity of ovarian cortex to be harvested for cryopreservation. In Poirot's series (2007), a whole ovary was removed in all cases, whereas in Anderson's series (Anderson et al., 2008b), the procedure involved biopsies of cortical tissue. In our series, 20 girls (mean age 6.5 years) underwent unilateral oophorectomy and 38 girls (mean age 12.4 years) had cortical biopsies without oophorectomy. Our decision as to how much ovarian cortex to remove was influenced mainly by the estimated risk of ovarian failure related to the planned treatment and existing ovarian volume. For cases with pelvic irradiation, total body irradiation and high doses of alkylating agents, oophorectomy was performed. In very young girls, we were more likely to perform oophorectomy owing to the small size of the ovaries. Although biopsies might have been sufficient to cryopreserve a large number of ovarian follicles with the high ovarian reserve in young girls, coagulation is sometimes necessary for hemostasis when biopsies are performed. As the ovaries are small, damage to the remaining cortex is inevitable when coagulation is carried out. Moreover, in order to be able to reimplant the cortex, the tissue pieces must be large enough to allow suture. We therefore feel that when the ovaries are small (<15 mm in length), oophorectomy should be performed. Future research on reimplantation techniques and pregnancy after freeze–thawing of ovarian tissue will shed more light on optimal amounts of ovarian cortex to be used for cryopreservation purposes in younger patients.

Conclusions

Cryopreservation of ovarian cortex is currently the only option to preserve fertility in prepubertal girls when fertility is impaired by gonadotoxic treatments, repeat ovarian surgery or genetic disorders. At the time of ovarian cortex harvesting, aspiration of immature oocytes for IVM and cryopreservation is feasible. Although no pregnancies have yet ensued from the use of frozen–thawed ovarian cortex in children, results in adults are encouraging. As follicle density is higher in children than in adults, reimplantation of ovarian cortex might prove even more successful after cryopreservation of ovarian cortex during childhood, inversely proportional to the child's age. Laparoscopic removal of ovarian cortex for cryopreservation is feasible at any age, without complications in reports published so far and does not delay cancer treatment. It is important to inform the parents and, where possible, the child, about the experimental nature of the technique and all associated fertility issues. Indeed, all factors potentially compromising fertility must be addressed.

No consensus has yet been reached on the indications for ovarian cryopreservation: there are a number of different, and all legitimate, points of view. In children, who usually have a high ovarian reserve, the risks of POF might be overestimated and the procedure may be performed unnecessarily in a large number of cases. However, since disease evolution cannot always be predicted and additional gonadotoxic drugs might be needed at a later stage, the risk of POF can also be underestimated.

In the case of genetic disorders associated with POF, such as Turner syndrome, ovarian cryopreservation is at present mainly considered when there is proof of ovarian function. One of our goals should be to develop accurate tests of ovarian function in children in order to determine the indications and best timing for fertility-sparing treatments, especially in girls with genetic disorders.

Authors' roles

P.J. was responsible for the study design, surgical procedures, collection of data and writing of the manuscript. M.M.D. was responsible for cryopreservation of cortical biopsies and was involved in the discussion of the results. J.D. was involved in the discussion and revision of the manuscript.

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