Cryopréservation par vitrification puis transplantation intra-utérine de tissu ovarien

ELBARBARY, Taher

Abstract
La cryoconservation de tissu ovarien est l'une des options envisagée pour la conservation de la fertilité de la femme après guérison de son cancer. Notre étude a pour objectifs, d'évaluer la vitrification comme une technique de conservation de tissu ovarien, et d'évaluer l'utérus comme site de la transplantation d'un fragment de tissu ovarien. L'ovaire gauche a été excisé chez des souris OF1 âgées de 12-20 semaines et les fragments ovariens répartis en deux groupes. Le groupe 1 n'a pas fait l'objet d'une congélation (groupe contrôle). Les fragments du groupe 2 (test) ont été congelés selon la technique décrite par Sugimoto et al., 1999. L'analyse histologique des fragments ovariens avant et après greffe, à l'état frais ou après vitrification indiquent une morphologie normale des follicules. Des grossesses ont été obtenues chez deux souris test et deux souris contrôle. La transplantation de tissu ovarien dans l'utérus peut permettre l'obtention de grossesses spontanées.

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CRYOPRESERVATION PAR VITRIFICATION PUIS TRANSPLANTATION INTRA-UTERINE DE TISSU OVARIEN

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LIST OF CONTENTS.............................................................................................................1
LIST OF TABLES ................................................................................................................4
LIST OF FIGURES ............................................................................................................5
RESUME .............................................................................................................................6
INTRODUCTION .................................................................................................................9
LITERATURE REVIEW .......................................................................................................11
1. Fertility preservation ........................................................................................................11
   1.1. Established practices .............................................................................................13
       1.1.1. Surgical translocation ..................................................................................13
       1.1.2. Embryo cryopreservation ..........................................................................13
   1.2. Experimental strategies .......................................................................................13
       1.2.1. Cryopreservation of oocytes ......................................................................13
       1.2.2. Cryopreservation of nuclear material ..........................................................14
       1.2.3. Gonadotrophin suppression .......................................................................14
       1.2.4. Cryopreservation of ovarian tissue..............................................................16
       1.2.5. Somatic cell cloning ....................................................................................18
2. History of cryopreservation ............................................................................................19
3. Indications of ovarian tissue cryopreservation ..............................................................23
   3.1. The first threat is the passage of time ....................................................................24
   3.2. The second threat is the abnormally rapid destruction of the follicular reserves ending in premature ovarian failure (POF) .........................................................25
       3.2.1. X-chromosome abnormalities ......................................................................27
       3.2.2. Galactosemia ..............................................................................................28
       3.2.3. Auto-immune POF ....................................................................................29
       3.2.4. Infections ....................................................................................................31
       3.2.5. Enzyme deficiencies ...................................................................................31
       3.2.6. Signal defects ............................................................................................32
   3.3. The iatrogenic threat, particularly among patients who need to undergo anticancer treatments: ........................................................................................................32
       3.3.1. Surgical treatment .......................................................................................33
       3.3.2. Radiation Therapy .....................................................................................35
       3.3.3. Chemotherapy ...........................................................................................37
4. Cryobiology of gonadal cells and tissues ........................................................................39
4.1. Freezing injury ................................................................. 39
4.2. Cryoprotective agents ......................................................... 39
4.3. Protocols of OTCP .............................................................. 41
  4.3.1. Slow cooling, rapid thawing protocol ................................... 42
  4.3.2. Ultra-rapid cooling (vitrification) protocol ......................... 43
    4.3.2.1. Definition of vitrification ............................................ 43
    4.3.2.2. History of vitrification .............................................. 43
    4.3.2.3. Vitrification of ovarian tissue ..................................... 44
5. Future of the cryopreserved ovarian tissues ............................. 49
  5.1. Autotransplantation .......................................................... 49
    5.1.1. Orthotopic transplantation .......................................... 49
    5.1.2. Heterotopic transplantation ........................................ 50
  5.2. Xenotransplantation .......................................................... 53
  5.3. In vitro growth and maturation ........................................... 55
6. Growth factors and OTCP ....................................................... 62
7. Evaluation of the viability of the cryopreserved ovarian tissues .... 63
  7.1. Histological evaluation ...................................................... 63
  7.2. Endocrinological evaluation .............................................. 64
  7.3. Auto-radiographic and in situ methods for detecting apoptotic DNA fragments ........................................... 65
MATERIALS AND METHODS .......................................................... 66
  1. Animals ................................................................................... 66
  2. Tissue collection and transplantation ....................................... 66
  3. Vitrification of the ovarian tissue ............................................. 66
  4. Thawing ..................................................................................... 67
  5. Assessment of the ovarian tissue viability and functionality ........ 67
    5.1. Viability of the ovarian biopsies ......................................... 67
    5.2. Functionality of the transplanted ovarian biopsies ............... 68
  6. Statistical analysis ....................................................................... 69
RESULTS ....................................................................................... 70
  1. Viability of the ovarian follicles after thawing .......................... 70
  2. Viability of the ovarian follicles after grafting ........................... 73
  3. Restoration of fertility .......................................................... 75
DISCUSSION ................................................................................. 77
LIST OF TABLES

Table (1): Clinical and experimental strategies for the preservation of reproductive function in oncological patients.................................................................12
Table 2: Review on the historical data of cryopreservation (Ludwig et al., 1999)......21
Table 3: Etiological classification of POF...............................................................26
Table 4: Autoimmune diseases associated with POF.........................................29
Table 5: Evidences suggesting an immunologic role in POF...............................30
Table 6: Indications for cryopreservation of ovarian tissue in cases of malignant disease........................................................................................................34
Table 7: The risk of sterilization according to the patient’s age and radiation dose...36
Table 8: Chemotherapeutic agents that affect sexual or reproductive function ....38
Table 9: Protocol for cryopreserving and thawing human ovarian tissue............43
Table 10: Summary of the primary benefits of vitrification.................................45
Table 11. Variables of vitrification that can profoundly influence its effectiveness.....47
Table 12: Comparison of follicle number in fresh and vitrified-thawed ovarian biopsies.................................................................70
Table 13: Comparison of follicle number in-4-week-old grafts after transplantation of fresh and vitrified-thawed ovarian biopsies.................................................73
Table 14: Evolution and outcome of pregnancies in mice after ovarian tissue transplantation..................................................................................................75
LIST OF FIGURES

Fig.1. Theoretic options for the use of banked human ovarian tissue .................. 48
Fig. 2. Schematic diagram of follicular development in the human ..................... 59
Fig. 3. Overview of follicle formation and growth in the human fetal ovary .......... 60
Fig. 4. Earliest stages of follicle growth in humans ........................................... 61
Figure 5: Light micrograph of Fresh ovarian biopsy ........................................... 71
Figure 6: Light micrograph of vitrified/thawed ovarian biopsy ............................. 72
Figure 7: Transplantation success of ovarian biopsies in the uterine cavity of bilaterally ovariectomized mice ................................................................. 74
Fig. 8 Autopsy picture and light micrograph of mouse pup with unilateral pulmonary hypoplasia ................................................................. 76
RESUME

INTRODUCTION

Les progrès dans le diagnostic et le traitement du cancer chez l'enfant, l'adolescent et l'adulte ont fortement augmenté l'espérance de vie des femmes pré-ménopausées atteintes de cancer. En conséquence, il existe une population croissante de survivants adolescents et adultes qui ont eu un cancer dans l'enfance. Pour la majorité des femmes les dommages ovariens causés par la radiothérapie ou la chimiothérapie peuvent provoquer une ménopause précoce. La cryopréservation des ovaires est un des moyens pour la préservation à long terme des cellules germinales de la femme. Avec cette méthode, les ovocytes en prophase I contenus dans les follicules primordiaux sont préservés. Ceci pourrait constituer une alternative attrayante car la différenciation cytoplasmique n’est pas complète dans les ovocytes immatures. Les noyaux sont bloqués au stade de la vésicule germinale. Il existe un nombre élevé d’ovocytes immatures dans le cortex ovarien, de sorte que la méthode peut être également utilisée pour des filles pré-pubères. Avec cette méthode, les ovocytes immatures peuvent être préservés en même temps que d’autres cellules ovariennes nécessaires au développement et à la maturation des ovocytes. Cette technique permettrait non seulement la préservation, de gamètes femelles à divers stades de développement, ainsi que celle des fonctions endocriniennes de l’ovaire. Notre étude vise à évaluer la vitrification comme méthode de cryopréservation des tissus ovariens, et évaluer l’utérus comme site de transplantation des tissus ovariens congelés-décongelés.

METHODOLOGIE

Des souris femelles OF1, et âgées de douze et vingt semaines ont été utilisées pour cette étude. Dans le groupe de vitrification, la technique opératoire a été réalisée en deux temps. Dans le premier temps, l’ovaire gauche a été extrait pour être cryopréservé, le pédicule ovarien gauche a été ligaturé avec du vicryl 4/0 pour assurer l’hémostase, le péritoine a été fermé par une suture continue et la peau a été fermée par des points séparés en utilisant soit du nylon 6/0 ou du vicryl 4/0. Deux semaines plus tard, une seconde laparotomie a été effectuée pour extraire l’ovaire droit et pour transplanter des biopsies de l’ovaire gauche congelées-
décongelées dans la partie la plus distale de la corne utérine gauche. Dans le groupe de contrôle, la technique opératoire a été effectuée en un seul temps opératoire. Par laparotomie, les deux ovaires ont été extraits et les pédicules ovariens ont été ligaturés avec du vicryl 4/0. Les ovaires ont ensuite été lavés dans une solution HB1, et immédiatement transplantés dans la partie la plus distale de la corne utérine gauche.

La solution mère de vitrification (VS 100%) est préparée en ajoutant 20.5% [V/V] de diméthyl sulfoxide [DMSO], 15.5% [W/V] d’acétamide, 10.0% [V/V] de propylène glycol et 6.0% [W/V] de polyéthylène glycol 6000, à une solution HB1. La dilution de la solution mère de VS (100%) avec la solution de HB1 permet la formation de concentrations de 12.5%, 25%, et 50% de VS. Les ovaires collectés ont été coupés en 2 à 4 pièces à l’aide d’un scalpel stérile, lavées dans une solution de HB1, puis immergées successivement à température ambiante dans des solutions VS, 12.5%, 25% (15 minutes/solution), puis en chambre froide (4°C) dans les solutions VS 50% et 100% (15 minutes/solution). Enfin, chaque fragment ovarien a été placé dans une ampoule de congélation avec 0.5mL de VS à 100%, puis transféré en stockage dans l’azote liquide (-196°C) jusqu’à utilisation. Les fragments ovariens ont été décongelés en immergeant les ampoules pendant quelques secondes dans de l’eau à 37°C. Les cryoprotecteurs sont éliminés par dilution successive dans des bains de VS 50%, 25% et 12.5% (10 min./bain, température ambiante). Les fragments ovariens sont finalement lavés dans une solution HB1.

La viabilité des tissus ovariens (tests) congelés-décongelés et celle des fragments ovariens des groupes contrôle, n’ayant pas subit la cryoconservation, a été évaluée par analyse histologique avant et après la transplantation. La fonctionnalité des biopsies ovariennes transplantées a été évaluée par comptage des grossesses.

RESULTATS

L’analyse histologique a montré qu’il y avait une différence entre le nombre de follicules normaux dans les biopsies fraîches et vitrifiées-décongelées. Au moyen du test-t, la différence se révèle statistiquement significative par rapport en
considérant tous les types de follicules. Cependant que la différence entre le nombre de follicules normaux dans des greffes ovariennes fraîches et vitrifiées-décongelées au moyen du test-t, se révèle non statistiquement significative (Valeur de P = 0.6).

Les résultats en terme de grossesse chez les souris après la transplantation des greffes fraîches ont été 7 souriceaux viables puis fertiles et 1 mort néonatale. Les résultats chez les souris après la transplantation des greffes congelées-décongelées ont été 2 morts in utero et l’analyse histologique posthume se révèle hypoplasies pulmonaires bilatérales, et 2 morts néonatales et l’analyse histologique posthume se révèle rien.

CONCLUSIONS

L’ovaire peut tolérer une exposition à la déshydratation osmotique et à la vitrification dans une solution concentrée de cryoprotecteurs.

La technique de vitrification atteint les follicules à tous les stades de développement mais dans une moindre mesure les follicules primordiaux.

L’utérus murin semble être un site de greffe adéquat pour la transplantation de tissus ovariens et peut conduire à des grossesses naturelles.
INTRODUCTION

In recent years, fertility preservation has been evolving rapidly as a result of clinical imperatives and advances in technology (Gosden and Nango, 2002). Advances in diagnosis and treatment of childhood, adolescent and adult cancer have greatly enhanced the life expectancy of premenopausal women with cancer. As a result, there is growing population of adolescent and adult long-term survivors of childhood cancer and for the majority of women, ovarian damage caused by radiotherapy or chemotherapy would result in premature menopause (Donnez et al., 2000). When the deleterious effects of the treatment are not reversible, mature oocytes, embryo cryopreservation and ovarian tissue cryopreservation may be considered to restore fertility following the completion of the treatment. Mature oocytes cannot be cryopreserved easily, this is because the cytoplasm volume and water content is much higher than in other cells, and the nucleus is blocked in metaphase II of meiosis which makes the spindle very fragile and sensitive to temperature and osmotic stress. Also, the collection of mature oocytes requires ovarian stimulation, which is not always possible because the high levels of estrogens induced may be deleterious in some cancers. Furthermore, this procedure should only be used in adult patients. Embryo cryopreservation is an established clinical technique that results in acceptable pregnancy rates, depending on the age of the patient. However, the patient must have a partner or be in a stable relationship at the time of the treatment and again women with estrogen-sensitive cancers cannot undergo the necessary ovarian stimulation before the in vitro fertilization (IVF) (Poirot et al., 2002). Cryopreservation of the ovaries (OTCP) is one of the means for long-term preservation of female germ cells (Donnez et al., 2000). In this method, prophase I oocytes contained in primordial follicles are preserved. This could be an attractive alternative because cytoplasmic differentiation is not complete in immature oocytes, nuclei are blocked in the germinal vesicle stage, and, there are large numbers of immature oocytes in the ovarian cortex. So, this method can be used as well for prepubertal patients as adult patients (Poirot et al., 2002). With this method, immature oocytes together with other ovarian cells necessary for development and maturation of oocytes can be preserved. This technique would enable not only the preservation of female gametes in diverse stages of
development but would also preserve the endocrine functions of the ovary (Sugimoto et al., 2000). Thus, the cryopreservation of ovarian tissue is a potential alternative or addition to the cryopreservation of embryos or mature oocytes for women at risk of premature ovarian failure (Poirot et al., 2002).

The recent surge in ovarian tissue banking reflects the importance and the need of fertility conservation for many women facing premature ovarian failure, particularly those with cancer (Kim et al., 2001). After the success of animal experiments, reports have been published about ovarian tissue cryopreservation for such patients (Law, 1996). Most previous studies on cryopreservation employed slow freezing rapid thawing technique (Gosden et al., 1994). It is a time consuming procedure, and it requires special equipment such as a programmable freezer. Vitrification is an alternative cryopreservation method that might be effective for organ cryopreservation as well as for cryopreserving embryos and small specimens. Several kinds of cells including oocytes have been successfully cryopreserved by vitrification and, in some cases; the results were comparable or superior to slow freezing rapid thawing technique (Dinnys et al., 1995). The majority of grafts have been to the kidney, since it has a good blood supply and high concentration of angiogenic growth factors, but the uterus has a high blood supply and is also immunoprivileged. Transplantation procedures would be enhanced if the ovarian tissue could be successfully cryopreserved and transplanted into the uterus. Beer et al., 1999 found that intrauterine skin homograft survive in the uterus of the rat for the period of pregnancy. Kagabu and Umezu transplanted vitrified-thawed rat ovaries in the uterus and demonstrated that it was immunologically acceptable to the uterus (Kagabu and Umezu, 2000). However, none of the previous studies tried to test the fecundability of the cryopreserved and transplanted ovarian grafts in the uterus. In the present study we aimed to evaluate ultra-rapid freezing technique (vitrification technique) as a method of ovarian tissue cryopreservation, and to evaluate the uterine cavity as a site of ovarian tissue transplantation.
LITERATURE REVIEW

1. Fertility preservation

As survival rates for young cancer patients continue to improve, protection against iatrogenic infertility caused by chemotherapy with or without radiotherapy assumes higher priority. Hodgkin's disease is the most common malignancy in the population aged 15–24 years. Prolonged survival of almost 90% of patients is now expected for young patients treated with cytotoxic chemotherapy for Hodgkin’s disease. This is due to the introduction of effective chemotherapy such as MOPP (mechlorethamine, vincristine, prednisone and procarbazine) and/or ABVD (adriamycin, bleomycin, vinblastine and decarbazine) and its variants. Similar rates of long-term survival have been reported for patients with non-Hodgkin lymphoma, as well as for patients with other types of tumours receiving chemotherapy (Blumenfeld et al., 1999). It was estimated that by the year 2010 one in 250 of the young adult population would be a long-term survivor of childhood cancer (Wallace, 1997). Moreover, cytotoxic agents have been also used as chemotherapy for various autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, and for the prevention of organ transplant rejection (Blumenfeld et al., 1999).

Females are generally less susceptible than males to the deleterious effects of chemotherapy on the gonad. Nevertheless, ovarian dysfunction is well-recognized following combination chemotherapy. Treatment of Hodgkin's disease with mechlorethamine, vinblastine, procarbazine and prednisolone (MVPP), or MOPP results in ovarian failure in 19-63%. Amenorrhoea is much more commonly encountered in women over 30 years than in younger women where ovarian function appears to be preserved in 48-100% (Mackie et al., 1996). Long-term follow-up will be necessary as a number of these young women may subsequently progress to a premature menopause (Thomson et al., 2002).

The major challenge faced by pediatric oncologists today is to sustain the excellent survival rates while striving to achieve optimal quality of life. One of the most frequently encountered and psychologically traumatic late complications of radiotherapy and chemotherapy for childhood cancer is infertility. Consequently,
attention is focusing on developing techniques to preserve the patient’s fertility (Chen et al., 1996)

Advances in assisted reproduction and increasing interest in gamete extraction and maturation have focused attention on preserving gonadal tissue from children before commencement of sterilizing chemotherapy or radiotherapy, with the realistic expectation that future technologies will be able to utilize their immature gametes. The options available are dependent upon the sexual maturation of the patient. A number of strategies for protecting the ovaries and preserving fertility during cancer therapy have been attempted with limited success. Some of these strategies are used clinically, while others are still in the experimental stage (Table 1). Limitation of radiation dose to the ovary was practised but was not very effective. In young, sexually mature females with partners, collection of mature oocytes for storage or fertilization and subsequent embryo cryopreservation is possible. For prepubertal girls, and the majority of young women, preservation of fertility remains experimental and harvesting and storage of gonadal tissue before commencing cancer therapy is the most promising option (Thomson et al., 2002).

**Table (1): Clinical and experimental strategies for the preservation of reproductive function in oncological patients.**

<table>
<thead>
<tr>
<th>Established practices</th>
<th>Experimental strategies</th>
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<tr>
<td>Oophoropexy</td>
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<td>Embryo cryopreservation</td>
<td>Cryopreservation of nuclear material</td>
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<td>Cryopreservation of ovarian tissue</td>
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<td>Somatic cell cloning</td>
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</table>
1.1. Established practices

1.1.1. Surgical translocation

Reducing the radiation dose to the ovary by shielding or removing the ovaries from the field of radiation (oophoropexy) may preserve ovarian function (Schlatt et al., 1999). Oophoropexy involves laparoscopic transposition of the ovaries (with intact blood supply) to a position behind the uterus, which acts as a shield, or to the paracolic gutters, away from the field of radiation to minimize exposure. The ovarian dose received during pelvic nodal irradiation for Hodgkin's disease can be limited by midline oophoropexy from 44 Gy to between 0.22 and 0.55 Gy, and in women who are less than 25 years of age at the time of treatment, ovarian failure is infrequent. However, even where ovarian function is preserved oocyte retrieval with assisted reproduction and surrogacy may be required to achieve pregnancy as the uterus may also have been damaged by the radiation therapy. This may compromise the ability of the women to carry a pregnancy to term (Thomson et al., 2002).

1.1.2. Embryo cryopreservation

The only strategy currently available for preservation of female fertility is cryopreservation of embryos. Mature oocyte retrieval and in vitro fertilization (IVF) may be offered to women before treatment of cancer. Embryo banking has the advantage that it allows a number of embryos to be stored without the need for further IVF cycles; however, this requires the patient to have a partner or to use donor sperm. On average, 10 oocytes are collected per cycle, limiting the number of embryos available for cryopreservation and the overall live birth rate from a single cycle of treatment is 11% (Porcu et al., 1997).

1.2. Experimental strategies

1.2.1. Cryopreservation of oocytes

Cryopreservation of oocytes is an alternative possibility for women without a partner but is much less successful, with less than one baby born per 100 oocytes
stored and as such remains experimental (Atkinson et al., 1994).

The main disadvantage of embryo cryopreservation or oocyte storage is requirement for superovulation with gonadotrophins that will inevitably delay the commencement of cancer therapy. Also, the high levels of estrogen induced may be deleterious in some cancers. Furthermore, this procedure should only be used in adult patients (Thomson et al., 2002).

1.2.2. Cryopreservation of nuclear material

Both second Polar Bodies (2PBs) and Female Pro-nuclei (FPNs) are sub-cellular components of the zygote. They are membrane-bound nuclear materials, free of meiotic microtubings or ooplasmic organelles, and more importantly, contain a full haploid complement of female chromosomes. In addition, they are smaller in volume and less complex in composition than oocytes, and could be less sensitive to cryoinjury. So, if these components can be safely cryopreserved, it may serve as an alternative method to preserve female fertility. However, the potential in this new approach for female fertility preservation still relies heavily on the efficiency of both cryopreservation and nuclear transfer of the thawed 2PBs or FPNs. Recently, He et al 2003, has proven that FPNs or 2PBs removed from a mouse two-pronuclei zygote can be cryopreserved–thawed and later used to generate embryos leading to live births. However, in mice, FPNs can be accurately selected based on their proximal location to the 2PBs and its small size relative to the male pronuclei. Unfortunately, in human zygotes, both female and male pronuclei are not distinguishable and can easily be confused. Clinically, a mistaken transfer of the wrong pronuclei could lead to major problems because it has been shown in mice that a zygote requires both a male and female pronuclei to develop to full term (He et al., 2003).

1.2.3. Gonadotrophin suppression

The mechanism of cytotoxic chemotherapy and radiotherapy induced gonadal damage is uncertain and may differ in males and females, between different
modalities of therapy and individual drugs. In contrast to older women, the prepubertal women appear to be more resistant to the cytotoxic effects of chemotherapy. Consequently it was hypothesized that inducing a prepubertal milieu during chemotherapy would decrease the risk of premature ovarian failure. Gonadotrophin releasing hormone analogues (GnRH-a) prevent follicular growth and mitosis by blocking gonadotrophin induction. Although the exact mechanism is uncertain, it may involve direct suppression of GnRH receptors in the ovary, with subsequent inhibition of recruitment of small follicles into the proliferating pool as well as atresia of the already developed follicles. Although a number of studies have demonstrated that GnRH-a inhibit chemotherapy-induced ovarian follicular depletion in rodents, there remains uncertainty about applicability in the human, particularly as the human ovary has significantly fewer numbers of GnRH receptors in the ovary (Glode et al., 1981). Ataya et al. demonstrated that GnRH-a co-treatment protected the Rhesus monkey from cyclophosphamide induced ovarian damage by significantly reducing follicular decline compared with cyclophosphamide alone (Ataya et al., 1995). These findings are supported by clinical studies which demonstrated that co-treatment of GnRH-a with chemotherapy resulted in premature ovarian failure (POF) in one out of 28 (3.6%) compared with 26 out of 40 (65%) in the group treated with chemotherapy alone (Blumenfeld et al., 1996). Adjuvant treatment with GnRH-a to limit the gonadal toxic effects of otherwise successful treatment regimens is potentially attractive. However, this must be viewed with some caution as although GnRH-a provided some protection against cyclophosphamide, no advantage was conferred against irradiation-induced damage. This may be in part explained by the different mechanism of gonadal damage induced by radiotherapy, namely, the destruction of primordial follicles, which are not under the influence of gonadotrophins. The judicious use of GnRH-a may play a role in the appropriate patient group, such as young women and children subjected to alkylating-agent-based chemotherapy for Hodgkin’s disease (Thomson et al., 2002).

1.2.4. Prevention of follicle atresia

Oocyte loss induced by anticancer therapy has been shown to occur by apoptosis, consequently, inhibition of the apoptotic pathways has been explored as a mechanism for preventing ovarian failure. Although the exact pathway remains to
be elucidated, accumulating data support the role of ceramide in signaling somatic cell death. Ceramide is a sphingolipid second messenger derived from sphingomyelinase-catalyzed hydrolysis, in addition to de novo synthesis. In turn, ceramide is metabolized and phosphorylated to give sphingosine-1-phosphate (SIP), which is believed to inhibit apoptosis in somatic cells. The role of the sphingomyelin pathway has also been studied in germ cells. Disruption of the gene encoding acid sphingomyelinase or treatment with sphingosine-1-phosphate attenuates apoptosis of primordial fetal oocytes with increased oocyte numbers present at birth. Treatment of mice oocytes with sphingosine-1-phosphate prevents chemotherapy-induced apoptosis in vitro. In vivo administration of sphingosine-1-phosphate confers resistance to radiation-induced apoptosis in mice, with pregnancy rates of 100%. While SIP may herald promise of a new approach to preservation of ovarian function, further studies are necessary to explore the detrimental effects of such treatment on normal neurological function as deletion of sphingomyelinase during normal fetal life leads to the development of Niemann-Pick-disease-like symptoms in post-fetal life (Morita et al., 2000).

1.2.5. Cryopreservation of ovarian tissue

Cryopreservation of ovarian tissue is the only option potentially available for prepubertal children and the majority of young women. With this method, immature oocytes together with other ovarian cells necessary for development and maturation of oocytes can be preserved. This technique would enable not only the preservation of female gametes in diverse stages of development but would also preserve the endocrine functions of the ovary (Sugimoto et al., 2000).

1.2.6. Stem cell research

The inner cell mass (ICM) of the 3.5 days post coitum (d.p.c.) mouse blastocyst comprises a pool of pluripotent cells, which gives rise to all the differentiated cell types that make up the embryo and adult, and many of the extra-embryonic tissues. By 4.5 d.p.c. the pluripotent ICM cells that lined the blastocoelic cavity have differentiated to form primitive endoderm, an extra-embryonic cell lineage which gives rise to both visceral and parietal endoderm. From 4.5 d.p.c. to 6.5
d.p.c. the remaining pluripotent cells, referred to collectively as 'epiblast', undergo extensive proliferation, selective apoptosis and migration to give rise to a columnar epithelial monolayer of pluripotent cells termed the primitive ectoderm. Remodelling of the pluripotent cell population during this time is accompanied by alterations in gene expression and differentiation potential. The primitive ectoderm, through the process of gastrulation, gives rise to the mesoderm, ectoderm and endoderm of the embryo, germ cells, and the remaining extraembryonic tissue. While this represents the normal course of embryogenesis, considerable developmental lability is evident from the observation that destruction of up to 85% of cells within the mouse embryo prior to organogenesis does not necessarily prevent formation of a normal embryo. This property is thought to reside in the capacity of pluripotent cells to reprogram their development in response to environmental signals. Pluripotent cell lines (embryonic stem or ES cells) can be isolated from the pre-implantation mouse embryo and maintained in the undifferentiated state in vitro. These cells exhibit gene expression profiles and differentiation potentials that are consistent with their embryonic origin, and can be used as a model system for the analysis of pluripotent cell biology (Lake et al., 2000). ES cells are characterized by several features, however, the two key properties that make these cells so remarkable are these:

First: ES cells can be grown in vitro and expanded in number indefinitely in the primitive undifferentiated state characteristic of the embryonic cells from which they are derived.

Second: throughout long periods of cultivation in vitro they retain a key property of those embryonic cells that is the pluripotency, or the ability to develop into any cell type in the adult body (Pera et al., 2000).

Recently, Schöler and colleagues 2003, showed that mouse embryonic stem cells in culture can develop into oogonia that enter meiosis, recruit adjacent cells to form follicle-like structures, and later develop into blastocysts (Hübner et al., 2003). The reported differentiation happened spontaneously over a period of nearly 50 days. Schöler and colleagues used bulk two-dimensional differentiation on tissue culture plastic, in which both male and female lines of ES cells yielded oocytes (Geijsen, 2004). They concluded that it is not surprising that the derivation of oocytes and blastocyst-like structures could be accomplished with both female and male ES cells. In the absence of appropriate SRY expression in the gonads, male primordial
germ cells enter the female pathway and often undergo the first step of oogenesis, entering meiotic arrest at prophase I (Hübner et al., 2003). The scope of even the more obvious applications envisioned for human cells with these properties is breathtaking: new approaches to the study of human embryonic development and disorders thereof, such as birth defects and embryonal tumours; access to hitherto-unexplored territories of human embryonic gene expression for modern genomic data mining; new tools for the discovery of polypeptide growth and differentiation factors that might find application in tissue regeneration and repair; new means to creating human disease models in vitro for basic research, drug discovery and toxicology; a potential answer to the issue of the chronic shortage of tissue for transplantation in the treatment of degenerative diseases, and an end to the use of immunosuppressive therapy in transplantation, if cloning techniques can be used to derive stem cells from a patient’s own tissue; new delivery systems for gene therapy (Pera et al., 2000).

1.2.7. Somatic cell cloning

Somatic cell cloning or somatic cell nuclear transfer is a technique in which the nucleus (DNA) of a somatic cell is transferred into an enucleated metaphase-II oocyte for the generation of a new individual, genetically identical to the somatic cell donor (Wilmut et al., 1997). From a biological and technical point of view, the cloning of mammals cannot be established exclusively from individual somatic cells in order to create directly from these cells a living being. In mammals, cloning requires the oocyte and early blastomeres (embryonic cells). The nuclear genome must be removed from the oocyte, “by microsurgical procedures” thus being enucleated. What remains is the ooplasmic envelope of this oocyte into which the nucleus from an embryonic or adult cell is transferred by different methods. Enucleated oocyte with donor genome can be cultured in vitro for several days. The cloned embryos are then implanted in the uterus of surrogate females. The surrogate mothers give birth to offspring, which are genetically identical with the original donor cells (Illmensee, 2001).

In 1997, a Scottish research group reported the birth of a mammal cloned for the first time from a somatic cell of an adult animal. The cloned sheep “Dolly”
originated genetically from an ewe’s udder cell. In the meantime, “Dolly” has given birth to viable and healthy offspring (Wilmut et al., 1997). The success of cloning an entire animal from a differentiated adult cell demonstrated that genes inactivated during tissue differentiation can be re-activated by a process called nuclear reprogramming that means the reversion of a differentiated nucleus back to a totipotent status. Somatic cloning may be used to generate multiple copies of genetically elite farm animals, to produce transgenic animals for pharmaceutical protein production or xeno-transplantation, or to preserve endangered species. With optimisation, it also promises enormous biomedical potential for therapeutic cloning and allo-transplantation. In addition to its practical applications, cloning has become an essential tool for studying gene function, genomic imprinting, genomic re-programming, regulation of development, genetic disease, and gene therapy, as well as many other topics (Tian et al., 2003).

Illmensee, 2001 mentioned a number of risk factors that may cause detrimental effects on normal development of cloned mammals:

- Cloning’s low efficiency. Currently the efficiency for nuclear transfer is between 0-10%, i.e., 0-10 live births after transfer of 100 cloned embryos.
- Alterations in number and structure of chromosomes (aneuploidy, deletions, inversions, and translocations).
- Alterations in telomeric regions of chromosomes (shortening of TTAGGGTTAGGG repeats).
- Alterations in methylation of genes (imprinting, IGF2 and IGF2R).
- Epigenetic changes of gene activity.
- Point mutation of genes.
- Influence of culture conditions on somatic donor cells and cloned embryos (large offspring syndrome).
- Pre- and postnatal congenital malformations.

2. History of cryopreservation

The first reports on the cryopreservation of different tissues were published as early as 1776 (Luyet and Rapatz, 1970). More than 200 years ago, the Scottish surgeon and anatomist, John Hunter, pioneered cryopreservation in the hope that
tissues could be revived after a prolonged period of suspended animation. Hunter speculated that life could be rekindled after freezing of the human body. Several attempts were made to cryopreserve living cells and tissues earlier this century, but they met with little success until cryoprotectants were discovered in a London laboratory (Oktay et al., 1998). Important milestones on the road to modern cryopreservation techniques are shown in Table 2 (Ludwig et al., 1999) and include studies on cryopreservation and subsequent thawing of rabbit embryos (Chang, 1948), which resulted in the first reported pregnancy by this means. The pioneering attempts to cryopreserve gonadal tissue were discovered in 1950s when biologists successfully grafted rodent ovarian and testicular tissues after freezing and thawing in a glycerol solution. Although these tissues were implanted without vascular anastomosis, follicle development and spermatogenesis resumed in castrated host animals and in some cases the females were mated and delivered small litters (Nugent et al., 2000). Chang was also one of the first authors to describe better survival rates of fertilized compared with non-fertilized oocytes in rabbits (Chang, 1953). The first pregnancy after cryopreservation of a human embryo ended in abortion (Trounson and Mohr, 1983). The first deliveries were reported in 1984 (Zeilmaker et al., 1984). A later report demonstrated that a genuine opportunity exists for successful ovarian tissue banking in mammals (Gosden et al., 1994). The main problem of the cryopreservation procedures is the damage to cells and tissues, i.e. the formation of intracellular ice crystals and cell destruction. This has been extensively described (Mazur, 1990). For each cell, there exist an optimal cryopreservation procedure, defined by the cryoprotectant used, and the freezing and thawing protocol. However, advances in instrumentation and automation to control freezing rates finely and a better theoretic understanding of cryobiology have helped to underpin progress in the banking of frozen cells (Oktay et al., 1998). Until now, the pregnancies and births yielded by cryopreservation of unfertilized prophase I and metaphase II oocytes have been limited. More experience has been gained using pronuclear stage oocytes and embryos. Intracytoplasmic sperm injection (ICSI) and pre-implantation genetic diagnosis (PGD) with the necessary embryo biopsy have introduced a new dimension to the different areas of cryopreservation (Ludwig et al., 1999).
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Date</th>
<th>Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spallanzani</td>
<td>1776</td>
<td>Freezing of spermatozoa in snow and subsequent thawing, and retrieval of motile spermatozoa.</td>
</tr>
<tr>
<td>Ponchet</td>
<td>1866</td>
<td>Freezing of red blood cells.</td>
</tr>
<tr>
<td>Jahnel</td>
<td>1938</td>
<td>Retrieval of human spermatozoa after cryopreservation to –196°C or –269°C.</td>
</tr>
<tr>
<td>Chang</td>
<td>1947</td>
<td>Freezing of rabbit oocytes.</td>
</tr>
<tr>
<td>Chang</td>
<td>1948</td>
<td>Pregnancy after cryopreservation/thawing of rabbit embryos.</td>
</tr>
<tr>
<td>Smith and Polge</td>
<td>1952</td>
<td>Cryopreservation and subsequent in vitro culture of rabbit zygote using glycerol.</td>
</tr>
<tr>
<td>Bunge and Sherman</td>
<td>1953</td>
<td>First established pregnancy after insemination using cryopreserved human spermatozoa.</td>
</tr>
<tr>
<td>Chang</td>
<td>1953</td>
<td>Better results of cryopreservation after using fertilized rather than unfertilized rabbit oocytes.</td>
</tr>
<tr>
<td>Ferdows et al.</td>
<td>1958</td>
<td>Pregnancy after cryopreservation of rabbit oocytes.</td>
</tr>
<tr>
<td>Sherman and Lin</td>
<td>1958</td>
<td>Cooling of unfertilized mouse oocytes using glycerol and subsequent in-vitro fertilization.</td>
</tr>
<tr>
<td>Parrott</td>
<td>1960</td>
<td>Pregnancy after ovarian tissue cryopreservation and transplantation in mice.</td>
</tr>
<tr>
<td>Mazur</td>
<td>1963</td>
<td>Theory on cryopreservation damage of cells.</td>
</tr>
<tr>
<td>Whittingham</td>
<td>1971</td>
<td>Cryopreservation of mouse embryos at the blastocyst stage and birth of living young.</td>
</tr>
<tr>
<td>Wilmut</td>
<td>1972a ,b</td>
<td>Cryopreservation of mouse embryos and storage in liquid nitrogen.</td>
</tr>
<tr>
<td>Authors</td>
<td>Year</td>
<td>Description</td>
</tr>
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</tr>
<tr>
<td>Whittingham et al.</td>
<td>1972</td>
<td>Cryopreservation of mouse embryos and subsequent delivery of live young.</td>
</tr>
<tr>
<td>Wilmut and Rowson Bank</td>
<td>1973</td>
<td>Cryopreservation of calf embryos and subsequent delivery of live young.</td>
</tr>
<tr>
<td>Whittingham et al.</td>
<td>1974</td>
<td>Using different cryoprotectant (sodium acetate, glycerol, PVP, and DMSO).</td>
</tr>
<tr>
<td>Maurer and Hansemann</td>
<td>1976</td>
<td>Freezing of non-fertilized mouse oocytes, IVF, pregnancy and living young.</td>
</tr>
<tr>
<td>Whittingham</td>
<td>1976</td>
<td>Slow and fast two step freezing.</td>
</tr>
<tr>
<td>Trounson and Mohr</td>
<td>1977</td>
<td>First successful cryopreservation at –196°C of mammalian (mouse) oocytes, subsequent fertilization, pregnancy and birth of living young.</td>
</tr>
<tr>
<td>Zeilmaker et al.</td>
<td>1983</td>
<td>Pregnancy and abortion after cryopreservation of a human embryo after IVF.</td>
</tr>
<tr>
<td>Cohen et al.</td>
<td>1984</td>
<td>First deliveries after cryopreservation of human embryos.</td>
</tr>
<tr>
<td>Chen et al.</td>
<td>1985</td>
<td>Cryopreservation of human blastocysts and subsequent birth.</td>
</tr>
<tr>
<td>Mandelbaum et al.</td>
<td>1986</td>
<td>Twin pregnancy and subsequent delivery after cryopreservation of human non-fertilized oocytes and subsequent IVF.</td>
</tr>
<tr>
<td>Van der Elst</td>
<td>1988</td>
<td>Cryopreservation of human immature unfertilized oocytes.</td>
</tr>
<tr>
<td>Gosden et al.</td>
<td>1994</td>
<td>Cryopreservation of human embryos and subsequent pregnancy after ICSI.</td>
</tr>
<tr>
<td></td>
<td>1994</td>
<td>Autografting of cryopreserved ovarian tissue and subsequent live born in sheep.</td>
</tr>
</tbody>
</table>
3. Indications of ovarian tissue cryopreservation

Within the ovary the follicular reserve has dual functions: endocrine and exocrine. Although the endocrine function can be substituted by hormone replacement therapy, the exocrine function is irreplaceable and substitution by a donor oocyte does not allow the patient to transmit her genome to her progeny. The follicular reserve is a precious asset that diminishes throughout life, and once it is lost, it cannot be reconstituted. The key goal of OTCP is to preserve the patient’s fertility by protecting her ovarian tissue from threats to the follicular reserve (Aubard et al., 2000).
Three types of threats exist for the ovary, these are:

1. The first threat to the ovarian reserve and the most inevitable one is the passage of time. From the time of birth and even before, the follicular population undergoes exponential diminution, which accelerates at approximately the age of 40 years; the follicles are virtually exhausted at menopause (Aubard et al., 2001).

2. The second threat to the ovarian reserve is the abnormally rapid destruction of the follicular reserves that is observed in certain pathologic conditions responsible for premature menopause (Aubard et al., 2001).

3. The third threat to the ovarian reserve is the iatrogenic one, particularly among patients who need to undergo anticancer treatments (Aubard et al., 2001).

3.1. The first threat is the passage of time

In the embryo, germ cells first appear in the urogenital ridge. These germ cells then migrate to the primitive ovary. Once within the ovary, the germ cells multiply to form 3.5 million potential oocytes in each ovary. From this time, oocytes are held in suspended animation -meiosis- until required for ovulation perhaps 40 years later (Conway, 2000). Throughout the reproductive life of a woman, only approximately 500 follicles achieve maturation with release of a mature oocyte (Aubard et al., 2001). The body through apoptosis destroys most germ cells. Before birth, two thirds of the 7 million eggs are destroyed, presumably as part of quality control mechanism. Between infancy and the age of 40 years, eggs are gradually reduced from approximately 1 million to 10,000 in each ovary. Around the age of 40 years, the process of egg destruction is accelerated and few are left by the age of 50 years (Conway, 2000).

Might not OTCP constitute an alternative to this enormous wastage? If unilateral ovariectomy was performed in a 20-years-old woman and its ovarian cortex was cryopreserved, it has been estimated that her age at menopause will only be reduced by several years. If the ovarian tissue extracted at the age of 20-years was re-grafted in the patient at the time of her menopause (at approximately 45 years). By this way, the duration of ovarian cycling might then be extended beyond the usual age of menopause, thereby delaying the period when hormone replacement therapy may be needed. The crucial problem is knowing how long an
ovarian cortex that has been cryopreserved and then autografted would be functional. By how many years could one thereby delay menopause? We are, for the time being, unable to answer this question (Aubard et al., 2000).

3.2. The second threat is the abnormally rapid destruction of the follicular reserves ending in premature ovarian failure (POF)

Coulam et al. examined the medical records of 1,858 women in Rochester, Minnesota, and estimated the age-specific incidence of POF to be 1 in 100 by age 40 and 1 in 1,000 by age 30. In women with primary amenorrhea, the prevalence of POF is 10–28%; in those with secondary amenorrhea, POF occurs in 4–18%. POF is not uncommon, considering these incidence rates and the potential underreporting from women who fail to perceive the end of monthly bleeding as a medical problem (Coulam et al., 1986).

The cause of POF is unknown in the majority of women in whom the diagnosis is made (karyotypically normal spontaneous premature ovarian failure) and on the basis of serial blood sampling it was found that nearly half of these patients have ovarian follicles remaining in the ovary. The follicles function intermittently, and nearly 20% of patients ovulate during 4 months of observation (Nelson et al., 1994).

However, Patients with POF can be classified into 2 different categories as in Table 3 (Anasti, 1998) patients with follicle depletion and patients with follicle dysfunction. In patients with follicle depletion there is either an initial deficiency in primordial follicles or an accelerated rate of follicular atresia that will result in premature depletion of the initial follicle endowment. While in patients with ovarian follicle dysfunction there are normal appearing oocytes and follicles, yet they fail to function properly despite an adequate level of gonadotropins. Thus, the mere presence of oocytes does not ensure normal ovarian function. However, in some of these situations it is possible to predict premature menopause and, in theory, the ovarian tissue from these patients could be extracted for use in a later pregnancy. The major difficulty would be precisely estimating the risk of premature menopause and the age at which it would occur (Davis, 1996).
Table 3: Etiological classification of POF.

<table>
<thead>
<tr>
<th>Ovarian Follicle Depletion</th>
<th>Ovarian Follicle Dysfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td>♦ Deficient ovarian follicle number</td>
<td>♦ Enzyme deficiencies</td>
</tr>
<tr>
<td>Pure gonadal dysgenesis</td>
<td>17α-Hydroxylase</td>
</tr>
<tr>
<td>Thymus aplasia/hypoplasia</td>
<td>17-20 Desmolase</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>Cholesterol desmolase</td>
</tr>
<tr>
<td>♦ Accelerated follicle atresia</td>
<td>Galactose-1-phosphate uridylyltransferase</td>
</tr>
<tr>
<td>X-chromosome related</td>
<td>♦ Auto-immunity</td>
</tr>
<tr>
<td>Turner’s syndrome</td>
<td>Lymphocytic oophoritis</td>
</tr>
<tr>
<td>♦ X mosaics</td>
<td>Gonadotrophin receptor blocking immunoglobulins</td>
</tr>
<tr>
<td>X deletions</td>
<td>Antibodies to Gonadotrophins</td>
</tr>
<tr>
<td>Galactosemia</td>
<td>♦ Signal defects</td>
</tr>
<tr>
<td>Iatrogenic</td>
<td>Abnormal Gonadotrophins</td>
</tr>
<tr>
<td>♦ Viral agents</td>
<td>Abnormal Gonadotrophin receptor</td>
</tr>
<tr>
<td>Auto-immunity</td>
<td>Abnormal G protein</td>
</tr>
<tr>
<td>Oocyte-specific cell-cycle regulation defect</td>
<td>♦ Iatrogenic</td>
</tr>
<tr>
<td>♦ Idiopathic</td>
<td>Idiopathic (resistant ovary syndrome)</td>
</tr>
</tbody>
</table>
3.2.1 X-chromosome abnormalities

Specific chromosomal defects are associated with impaired ovarian development and functions. The most common chromosomal anomalies causing POF are Turner’s syndrome (45XO) and superfemale (47XXX). Mosaicism (multiple cell lines of varying sex chromosome composition as (45,XO/46,XX, 46,XX/47,XXX) and a variety of other X-chromosome anomalies are associated with early follicular atresia. Abnormalities of the short arm of the X-chromosome (Xp) generally don’t affect the ovarian function whereas deletions or translocations of the long arm (Xq) do (Davis, 1996).

Singh and Carr, examined the ovaries of eight (45,XO) stillborn infants, and noted that their germ cells migrated to the gonadal ridge but that primary oocytes underwent accelerated atresia during late prenatal life. This and other reports led to the postulation of the requirement of 2 intact, active X-chromosomes to ensure normal follicle endowment. The deletion or addition of an entire sex chromosome in patients with POF adds little to the understanding of the individual genes responsible for ovarian function. Thus, investigators have turned to the study of patients with POF with subtle X-chromosome deletions and translocations in the hope of isolating specific ovarian-regulating genes on the X-chromosome. Eight patients with aberrant ovarian function and balanced translocations to an autosome t (X;A) have had cells analyzed by molecular methods. On the basis of cytogenetic and clinical studies from these patients [t (X;A)] and from patients with deletions of the X-chromosome, a “critical region” for normal ovarian function has been proposed for Xq13-q26. Additional reports have suggested that a gene (POF1) localized to Xq21.3-q27 or within Xq26.1-q27 may be important in defining ovarian function. Powell et al. using molecular techniques discovered a second gene (POF2) of paternal origin located more proximal to the Xq locus at Xq13.3-q21.1. Of clinical interest is the younger onset of ovarian dysfunction in those patients with deletions in the POF2 region as opposed to individuals with POF1 deletions. This supports the theory that these regions (POF1 and POF2) are distinct from one another. Although it is possible that these translocations and subtle deletions may lead to POF by disrupting normal meiosis, a positional effect in which the functions of specific ovarian regulatory genes are changed as a result of their altered location may also cause ovarian failure. Future analysis, using
sophisticated molecular techniques, will permit further delineation of the specific regions and genes responsible for X-chromosome related POF (Anasti, 1998).

All patients under the age of 30 who have been assigned the diagnosis of ovarian failure on the basis of high gonadotrophins must have a karyotype determination. The presence of mosaicism with Y chromosome requires excision of the gonadal areas because the presence of any testicular component within the gonad carries with it a significant chance of malignant tumour formation. These are highly malignant secondary tumours from germ cells: gonadoblastomas, dysgerminomas, yolk sac tumours, and choriocarcinomas. As approximately 30% of patients with Y chromosome will not develop signs of virilization, therefore even the normal appearing adult women with elevated gonadotropin levels must be karyotyped (Speroff et al., 1999).

3.2.2. Galactosemia

Galactosemia is a rare autosomal recessive disorder due to a deficiency in the enzyme galactose-1-phosphate uridyltransferase (GALT). These patients develop hepatocellular, ocular, renal, and neurological damage as a result of the accumulation of galactose and its metabolites. According to one study, 81% of the 47 affected female patients developed ovarian failure, with primary amenorrhea noted in 8 patients, and the majority experienced POF shortly after puberty (Waggoner et al., 1990). There is controversy as to the ovarian pathophysiology of these disorders. In the murine model, gestating rats fed a high-galactose diet during the premeiotic phase of fetal oogenesis formed reduced numbers of oocytes. This suggests that the ovarian failure in patients with galactosemia is due to a galactose-induced decrease in the initial number of oogonia (Anasti, 1998). However, an autopsy report of a neonate who had galactosemia revealed normal ovarian histology, implying that accelerated follicle atresia depletes the oocytes after birth and before puberty (Levy et al., 1984). Furthermore, ovarian biopsy in a patient with galactosemia and POF disclosed primordial follicles but no follicle growth or development. There is similar confusion about the effect of galactosemia on the glycosylation of gonadotropins, hence reducing their biologic activity. A report found that the FSH isoforms from female galactosemic sera had a neutral isoelectric point. This neutral form of FSH was found to have higher affinity for its receptor, but it was unable to stimulate adenylate cyclase (Prestoz et al., 1997).
Other investigators have found normal biologic activity of the gonadotropins from galactosemic patients. A genetic marker has been identified in some patients with galactosemia, GALT 188Q (Kaufmann et al., 1994). Individuals heterozygous for GALT 188Q mutations are not at increased risk of developing ovarian dysfunction. Thus, the exact mechanism (follicle depletion, follicle dysfunction, or abnormal gonadotropins) of ovarian failure in galactosemic patients has yet to be elucidated (Anasti, 1998).

3.2.3.Auto-immune POF

A link between POF and other autoimmune diseases is well established as mentioned in (Table 4) (Anasti, 1998).

**Table 4: Autoimmune diseases associated with POF**

| ♦ Auto-immune thyroid disease  |
| ♦ Adrenal insufficiency        |
| ♦ Vitiligo                     |
| ♦ Myasthenia gravis           |
| ♦ Systemic lupus erythematous  |
| ♦ Hypoparathyroidism           |
| ♦ Autoimmune haemolytic anaemia|
| ♦ Candidiasis                  |
| ♦ Idiopathic thrombocytopenic purpura |
| ♦ Diabetes mellitus           |

The frequency of concurrence of these autoimmune conditions varies somewhat between different study populations, although anti-thyroid antibodies are consistently the most commonly observed tissue-specific antibodies (Belvisi et al., 1993). POF is detected in 10-20% of women with idiopathic Addison’s disease and often occurs in autoimmune polyglandular syndromes (APS) type I and II (Davis,
Circulating anti-ovarian antibodies to ovarian tissue are present in only some patients and its low frequency in women with POF may indicate that such antibodies are only present during a limited phase of the disease process (Belvisi et al., 1993). Several lines of evidence suggest possible roles of the immune system in the etiology of POF are summarized in Table 5 (Anasti, 1998).

**Table 5: Evidences suggesting an immunologic role in POF**

- Association with other auto-immune diseases
- Presence of circulating antibodies
  - Antiovarian antibodies
  - Gonadotropin receptor antibodies
  - Steroid cell antibodies
  - Zona pellucida antibodies
  - Other organ- and non-organ-specific antibodies
- Histologic evidence of oophoritis
- Association with infectious etiologies
- Altered cell-mediated immunity
- Recovery of ovarian function after immunosuppressive therapies
- Murine neonatal model of thymectomy-induced ovarian failure

A postulated autoimmune mechanism for the development of POF has been antibodies against the FSH or LH receptor (Davis, 1996). All of the cited studies used non-human gonadotropin receptors in their search for receptor antibodies and thus suffer from a potentially fatal flaw in experimental design. An important work by several investigators has suggested that human gonadotropin receptors are species specific, binding human gonadotropins with higher affinity than nonhuman ligand. Thus, human receptors may exhibit species-specific affinity for human antibodies. On the basis of this premise, Anasti et al., used a recombinant
human gonadotropin receptor bioassay (LH and FSH) to identify immunoglobulin G (IgG) antibodies to gonadotropin receptors. After testing the IgG extracted from the sera of 38 patients with POF and 14 controls, they were unable to demonstrate the existence of an IgG-inhibiting immunoglobulin. The data thus remain inconclusive as to the existence of an inhibiting antireceptor antibody as a cause of POF (Anasti et al., 1995).

3.2.4. Infections

The true incidence of ovarian failure due to viral illness is unknown, however Investigators noted an incidence of oophoritis of 3-7% in patients who contracted mumps during an epidemic, with an inconsistent onset of oophoritis in relation to infection. Cytomegalovirus-related oophoritis has been described in patients with a compromised immune system such as caused by AIDS or lymphoma and in transplant recipients receiving anti-rejection drugs (Anasti, 1998). In a retrospective review of medical histories in patients with POF, Rebar and Connolly noted that 3.5% of patients had indicated a previous infection such as varicella and other infections such as shigellosis or malaria (Rebar and Connolly, 1990).

3.2.5. Enzyme deficiencies

Several specific enzyme defects can disrupt estrogen synthesis, resulting in pubertal delay, primary amenorrhea, and elevated gonadotropin levels despite the existence of normal-appearing primordial follicles in the ovary. Defects in the cholesterol desmolase, 17α-hydroxylase, 17-20 desmolase, and aromatase enzymes can cause these clinical and histological abnormalities. Patients deficient in cholesterol desmolase are unable to produce any biologically active steroids and thus rarely survive to adulthood. Patients with 17α-hydroxylase deficiencies develop hypertension, hypokalemia, and ovarian failure secondary to impaired adrenal and ovarian steroid synthesis. Although the same p450 cytochrome complex as 17α-hydroxylase catalyzes it, 17-20 desmolase deficiencies can occur without a concomitant defect in17α-hydroxylase. Although these patients present
with POF, they exhibit no clinical manifestations of adrenal insufficiency (Yanase et al., 1990).

Galactose-1-phosphate uridyltransferase (GALT), as discussed earlier, induces POF by an as yet undefined mechanism. Studies suggest that GALT deficiency may cause POF by follicle dysfunction, abnormal gonadotropin bioactivity, or accelerated oogonial atresia. Because of the rarity of these enzyme disorders, there is a paucity of correlating histologic information (Anasti, 1998).

### 3.2.6. Signal defects

Stimulation of the G-protein-coupled gonadotropin receptors induces second messengers that initiate specific cellular functions. Defects in the gonadotropins or their receptors could result in ovarian failure. Although they are not associated with elevated gonadotropins, several inherited defects of gonadotropin synthesis and secretion causing hypogonadism have been described. These include mutations of the FSH-subunit, KAL, and DAX-1 genes. The KAL mutation, which results in Kallman’s syndrome, and the DAX-1 mutation, which results in X-linked adrenal hypoplasia, causes a deficiency of GnRH. In addition, the DAX-1 mutation results in defective pituitary production of gonadotropins (Layman et al., 1997).

### 3.3. The iatrogenic threat, particularly among patients who need to undergo anticancer treatments:

Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly enhanced the life expectancy of premenopausal women with cancer. As a result, there is a growing population of adolescent and adult long-term survivors of childhood cancer (Donnez and Bassil, 1998).

Frequently, however, the price that is paid by female patients for successful cancer treatment is ovarian failure and infertility. Most infertility associated with cancer results from the treatment and not the disease. The main lines of treatment against cancer are surgery, radiotherapy and chemotherapy (Meirow et al., 1999).

After the success of animal experiments, reports have been published about ovarian tissue cryopreservation for such patients. The storage of ovarian tissue may provide a means of restoring long-term fertility to patients undergoing
treatment that may irreversibly damage the oocyte population. Also, tissue obtained before any systemic or local treatment may provide more security with regard to genetic abnormalities for future offspring (Donnez and Bassil, 1998).

3.3.1. Surgical treatment

Surgical interventions that either remove the ovaries or alter blood flow to them affect estrogen level as well as gonadotropin (FSH and LH) levels. Gynecologic procedures, such as bilateral salpingo-oophorectomy and pelvic exenteration, result in a sudden disruption of estrogen production and a dramatic change in the hormonal dynamics. It can be disrupted also during any major abdominal surgery, if the blood flow to the ovaries is altered and follicles die (Lin et al., 1999). In some cases it is surgically necessary to remove part or all of the ovarian tissue, as, for example, with ovarian tumors. If a curative ovariectomy is performed because a primitive or metastatic neoplasia has involved the ovary itself, zones of ovarian cortex that appear normal at a macroscopic level may exist beside the tumor. As this ovarian cortex is lost to the patient in any case, its freezing may be useful. The choice of cortical tissue to cryopreserve must be made in consultation with a pathologist, and the pathologist should be given the entire ovarian medulla. If the histopathological analysis of the medulla and cortex has negative results, autografting appears to be possible. In other cases the ovary is not cancerous, but the risk of microscopic or future involvement has led to the decision to perform ovariectomy as prophylaxis. Here also, freezing of ovarian tissue appears a good solution. Patients with certain mutations of BRCA1 and BRCA2 genes has a greatly increased risk of developing ovarian cancer, the risk is sufficiently high that prophylactic ovariectomy is recommended at approximately age of 35 years. One could certainly consider removing one ovary from the patient at approximately age of 20 years, which would enable the woman to have an available reserve of ovarian tissue that she could use later at any age (Aubard et al., 2001).

The indications for OTCP in cases of malignant disease are listed in Table 6 (Donnez and Bassil, 1998). However, in the case of gynecological malignancies, a conservative fertility approach is valuable only if the uterus can be spared during surgery. This includes cases of early cervical carcinoma, early vaginal carcinoma,
ovarian tumours of low malignancy, and some selected cases of unilateral ovarian carcinoma, stage IA1 (Donnez and Bassil, 1998).

Table 6: Indications for cryopreservation of ovarian tissue in cases of malignant disease.

- **Extra-pelvic diseases**
  - Bone cancer (osteosarcoma-Ewing’s sarcoma)
  - Thyroid and kidney cancers
  - Breast cancer
  - Melanoma
  - Neuroblastoma
  - Bowel malignancy

- **Pelvic diseases**
  - Non-gynecological malignancy
    - *Pelvic sarcoma*
    - *Sarcomblastoma*
    - *Rhabdomyosarcoma*
    - *Sacral tumours*
    - *Recto-sigmoid tumours*
  - Gynecological malignancy
    - *Early cervical carcinoma*
    - *Early vaginal carcinoma*
    - *Early vulvar carcinoma*
    - Selected cases of ovarian carcinoma
    - Ovarian borderline tumours

- **Systemic disease**
  - Hodgkin’s disease
  - Non-Hodgkin’s lymphoma
  - Leukemia
  - Melanoblastoma
3.3.2. Radiation Therapy

Total body, abdominal or pelvic irradiation may cause ovarian and uterine damage. This damage may be produced directly by destroying ovarian follicles or indirectly by altering blood flow to the ovaries and uterus, causing vascular fibrosis. The degree of impairment is related to radiation dose, fractionation schedule and age at time of treatment as mentioned in Table 7 (Speroff et al., 1999; Lin et al., 1999). The number of primordial follicles present at the time of treatment and dose of radiotherapy will determine the fertile window and dictate the age at menopause. This means that the younger the child at the time of radiotherapy the larger the oocyte pool and hence the later the menopause. The human oocyte is sensitive to radiation, with an estimated LD50 of less than 4 Gy (Wallace et al., 1989b). A permanent menopause may be induced in women over 40 years following treatment with 6 Gy while significantly higher doses are required to destroy the oocyte pool completely and to induce ovarian failure in younger women and children (Howell and Shalet, 1998). Ovarian failure has been observed in 97% (37 of 38) of females following whole abdominal irradiation in childhood (20-30 Gy). Of the 37 women, primary amenorrhoea was reported in 71% and premature menopause (median age 23.5 years) in the remainder (Wallace et al., 1989a).

Total body irradiation (TBI) either alone or in combination with cyclophosphamide (as conditioning for bone marrow transplantation) is associated with infertility. All women conditioned with total body irradiation (9.2-15.75 Gy) and cyclophosphamide (120 mg/kg) before bone marrow transplantation for leukemias develop amenorrhoea, with recovery seen in only nine of 144. TBI was found to be the only factor significantly influencing ovarian failure-the younger the age of the patient at the time of treatment the greater the probability of recovery of ovarian function (Sanders et al., 1988). In a long-term follow-up of 708 women, median 3 years (range: 1-17), after bone marrow transplantation, 532 had received TBI (10-15.75 Gy, single exposure or fractionated) and 176 were treated with cyclophosphamide (200 mg/kg), alone or with busulphan (16 mg/kg), as conditioning therapy. Ovarian failure was observed in 90% of patients following TBI and 68% following chemotherapy among an additional 82 patients treated
prepubertally. With the same regimens, ovarian failure was reported in 72% (Sanders et al., 1996).

**Table 7: The risk of sterilization according to the patient’s age and radiation dose**

<table>
<thead>
<tr>
<th>Ovarian Dose</th>
<th>Sterilization Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 rads</td>
<td>No effect</td>
</tr>
<tr>
<td>150 rads</td>
<td>Some risk over the age of 40</td>
</tr>
<tr>
<td>250-500 rads</td>
<td>Ages 15-40: 60% sterilized</td>
</tr>
<tr>
<td>500-800 rads</td>
<td>Ages 15-40: 60-70% sterilized</td>
</tr>
<tr>
<td>Over 800 rads</td>
<td>100% permanently sterilized</td>
</tr>
</tbody>
</table>

It was demonstrated that uterine function may be compromised following radiotherapy and reduced uterine volume and decreased elasticity of uterine musculature, were found in girls receiving pelvic, abdominal and total body irradiation prepubertally (Bath et al. 1999). Disruption of uterine characteristics may compromise implantation and continuation of pregnancy. Although successful pregnancies following radiotherapy are reported, the incidence of spontaneous miscarriage, premature delivery and intrauterine growth retardation is significantly increased (Wallace et al., 1989a; Sanders et al., 1988). Following whole abdominal irradiation (20-30 Gy) in childhood, all pregnancies occurring in women with preserved ovarian function resulted in mid-trimester miscarriage. In studies exploring the role of exogenous sex steroids, women with premature ovarian failure following TBI for childhood leukemias treated with physiological sex steroid replacement therapy have shown an increase in uterine volume and endometrial thickness (Bath et al. 1999). This is encouraging for future fertility prospects and may be a useful way of improving uterine function when assessing these women for assisted reproductive techniques (Thomson et al., 2002).
3.3.3. **Chemotherapy**

The efficacy of chemotherapeutic agents depends upon their ability to destroy rapidly dividing cells. Thus, the initial phase of ovarian destruction involves proliferating granulosa and theca cells, which are the primary components of developing follicles. Other chemotherapeutic agents, especially alkylating agents, destroy cells by altering cellular DNA. This alteration of genetic material by these agents may account for the second phase of ova destruction, which includes non-proliferating primordial follicles (Anasti, 1998).

The literature on the effects of chemotherapy on ovarian function shows that alkylating agents are most likely to have an impact on reproductive function and are most closely linked with infertility, although other agents also affect ovarian function as shown in Table 8 (Lin et al., 1999). The detrimental effect of a given chemotherapeutic agent on the ovaries can occur through impairment of follicular maturation and/or depletion of primordial follicles. The risk of permanent ovarian damage and dysfunction is dependent upon the patient’s age, chemotherapeutic agent (particularly the alkylating agents), drug dose, treatment schedule and duration, regimen combination, and time since treatment completion. Age appears to be the most important factor. Patients older than 40 years, experience a consistently higher rate of amenorrhea compared with younger patients. Rates vary from 21% to 71% in the younger age group and from 49% to 100% in the older group (Blumenfeld et al., 1999).

Unlike their older counterparts, prepubertal patients are relatively resistant to the ovarian effects of alkylating agents. This apparent resistance of the prepubertal ovary to the effects of chemotherapy has led investigators to attempt to suppress ovarian follicle growth during or before treatments (Anasti, 1998). Whereas the cytotoxic-induced damage is reversible in other tissues of rapidly dividing cells such as bone marrow, gastrointestinal tract, and thymus, it appears to be progressive and irreversible in the ovary, where the number of germ cells is limited, fixed since the fetal life, and cannot be regenerated (Blumenfeld et al., 1999). Chemotherapy does not appear to have any significant long-lasting adverse effect on uterine function. Successful pregnancy with no increased risk of
miscarriage, and healthy offspring are reported following treatment with multi-agent chemotherapy regimens (Salooja et al., 2001).

*Table 8: Chemotherapeutic agents that affect sexual or reproductive function*

- **Alkylating agents**
  - Busulfan
  - Chlorambucil
  - Cyclophosphamide
  - Melphalan
  - Nitrogen mustard

- **Antimetabolites**
  - Cytosine arabinoside
  - 5-Fluorouracil
  - Methotrexate

- **Antitumour antibiotics**
  - Doxorubicin
  - Plicamycin
  - Dactinomycin

- **Plant products**
  - Vincristine
  - Vinblastine

- **Miscellaneous agents**
  - Procarbazine
4. Cryobiology of gonadal cells and tissues

4.1. Freezing injury

Although cooling retards the process of degradation and therefore preserves the structure of cells, it can destroy their function irreversibly when it goes below the freezing temperature without cryoprotective agents (CPAs). When cells are cooled to temperatures between $-5^\circ$C and $-15^\circ$C, ice crystal formation is induced in the extra-cellular medium. As the temperature decreases further, the amount of ice increases, the solutes concentrate in the extra-cellular medium creating an osmotic gradient, and there is net water movement from the cytoplasm to the extra-cellular medium causing cellular shrinkage and dehydration. Cells undergoing cryopreservation are therefore prone to damage induced by the formation of intracellular ice and from the build-up of salts in the cells as they dehydrate. Moreover significant freezing injury can occur during the thawing (re-expansion) phase because of the changes in the composition of the surrounding milieu, possibly mediated by temporary leakage of the plasma membrane (Picton et al., 2000).

4.2. Cryoprotective agents

The discovery of CPAs and the birth of modern cryobiology, despite a short history, have significantly affected the course of long-term storage of tissues and cells in various scientific disciplines (Picton et al., 2000). CPAs are essential for cryopreservation but are potentially toxic at high concentration. Therefore, defining the required, and non-toxic concentrations of CPAs is the primary challenge that must be met in order to vitrify organs successfully. Not only does this toxicity prevent the use of fully protective levels of these additives, but it may also manifest in the form of cryoinjury over and above the classical cryoinjury. Therefore, the main target of any vitrification protocol must be the suppression of toxicity without any loss of CPAs effectiveness. Many different chemicals, such as alcohols, amines, sugars, and proteins can achieve protection of cells and tissues from damage during freezing and thawing. An effective CPA must have a number of key properties: high water solubility to depress the freezing point, high permeability to minimize the osmotic gradient, and low toxicity (Liebermann et al., 2002a). CPAs commonly used for vitrification of living cells, embryos and tissues
include dimethyl sulphoxide (DMSO), 1,2-propanediol (PROH), polyethylene glycol, dextrose and acetamide. DMSO is the primary component because of its great glass-forming ability, permeability and low toxicity. The addition of acetamide in the cryoprotectant mixture also lowers the toxicity of DMSO not only because of dilution, but also because it complexes with DMSO, preventing it from denaturing fructose diphosphatase and similar enzymes. By similar mechanisms, the presence of DMSO reduces the damaging effects of acetamide. Dextrose is thought to also specifically neutralise the toxicity of DMSO by preventing its irreversible binding to proteins (Friedler et al., 1988). The CPAs penetrate the cell membrane and are thought to stabilise intracellular proteins, reduce the temperature at which cells undergo lethal intracellular ice formation and moderate the impact of concentrated intra and extra-cellular electrolytes. Glycerol was used in the past but now the previous CPAs supersede it. These CPAs have high watersolubility, rapid penetrability and produce less osmotic damage at high concentrations normally used in cryopreservation protocols. However, their effects on cellular viability are still largely unknown (Picton et al., 2000). The toxicity of CPAs depends on the inherent characteristics of the chemical itself, duration of exposure, and temperature. Incubation time is as important as the concentration of the CPAs and depends on the type of the CPAs utilized and the incubation temperature. DMSO penetrates human ovarian tissue more rapidly than PROH at 4°C, while at 37°C, cell penetration is similar for both of them, but they are more toxic at this temperature (Demirci et al., 2002). However, rapid permeation of cells with CPAs at low temperatures is desirable to minimize toxicity. For example, CPAs exposure in mature oocytes has been implicated in alterations in the cytoskeleton, microtubular structure, and spindle organization. Such effects may disrupt the normal organization and traffic of molecules and organelles, impair embryonic development and increase the incidence of aneuploidy and other chromosomal anomalies. Also, CPAs exposure has been shown to promote the passive influx of Ca²⁺ across the plasma membrane, possibly by stimulating the release of Ca²⁺ from storage sites in the mitochondria and endoplasmic reticulum. Such increases in the intracellular Ca²⁺ can lead to parthenogenetic activation of mature oocytes and are known to activate intracellular phospholipases, proteases, ATPases and endonucleases, resulting in altered plasma membrane integrity, denaturation of cytosolic proteins, and chromosomal fragmentation, all of which
can lead to irreversible cell injury and apoptosis. In addition to the potential cytotoxic effects of CPAs exposure, they are hyperosmotic and penetrate cells more slowly than water. Video microscopy of human oocytes has revealed that CPAs exposure causes 40% shrinkage of cellular volume as water is withdrawn down the osmotic gradient, followed by a return to normal volume as the CPAs penetrates the cell. When the oocyte is returned to the isotonic solution after thawing, there is an inward osmotic flux with the possibility of excessive swelling to 140-150 % of the physiological volume. These volumetric changes may contribute to the mechanism for the induction of cytogenicetic damage during cryopreservation of mature oocytes, and in extreme cases, can cause cellular lysis (Picton et al., 2000).

Cells naturally contain high concentrations of proteins, which are helpful in vitrification (Newton et al., 1998). Antifreeze proteins are mainly glycopeptides and probably act by an adsorption–inhibition mechanism to prevent the growth of ice crystals. It has been reported that an antifreeze protein from the common yellow mealworm beetle, Tenebrio larvae, has higher activity than fish antifreeze proteins. Natural antifreeze proteins may prove to be beneficial for ultra-rapid cooling (vitrification) in the presence of high concentration of CPAs to eliminate ice crystal formation. It was reported that the addition of fish antifreeze glycopeptides to vitrifying solutions increases post-thaw viability in cultured immature pig oocytes and embryos and appears to preserve cell membrane structural integrity. Antifreeze proteins have been found not only in animals but also in plants as well. Researchers in York have isolated antifreeze proteins from the taproot of cold-acclimated carrots (Daucus carota). This protein inhibits the re-crystallization of ice and exhibits antifreeze activity. Although antifreeze proteins appear to reduce freezing injury and improve cell viability, the practical value and application of antifreeze proteins needs further investigation (Poirot et al., 2002).

4.3. Protocols of OTCP

Unlike cryopreservation of isolated cells, freeze-storage of tissue presents new problems because of the complexity of tissue architecture and protocols must strike a compromise between the optimal conditions for each different cell type. For example, the cooling and warming rates together with dehydration conditions
which, result in optimal survival of one cell type may not be ideal for other cellular components of the same piece of tissue. In addition, problems can arise when ice forms extracellularly, because it can cleave tissues into fragments. Furthermore, rapid solute penetration of highly compacted tissue is vital to ensure high final concentration of CPAs at temperatures, which will minimise cytotoxicity. These requirements necessitate optimisation of freeze/thaw protocols for each cell type, as post-thaw survival of reproductive cells and tissues, is profoundly affected by both the type of CPAs used and the equilibration time required for CPAs uptake and removal. Despite the apparent difficulties compared with single cells, the storage of gonadal and particularly ovarian tissue has proved surprisingly successful. The suitability of ovarian tissue for freezing is enhanced by the developmental plasticity of the tissue as the ovary is capable of functioning even when its complement of follicles has been severely reduced such as naturally occurs during ageing, after partial ablation or after injury. Furthermore, the cortical distribution of primordial follicles permits the preparation of small strips of tissue that provide maximal surface area for rapid CPAs penetration as evidenced by NMR spectroscopy (Newton et al., 1998). In support of this recommendation there is the slow cooling, rapid thawing protocol and the ultra-rapid cooling protocol (vitrification).

4.3.1. Slow cooling, rapid thawing protocol

Where the cooling rate is fast enough to minimise exposure of cells to high intracellular concentrations of electrolyte and slow enough to avoid intracellular ice formation. Furthermore, these processes should be carried out in the presence of low concentrations of non-permeable osmolytes, such as sucrose and mannitol, to act as osmotic buffers against swelling during the addition and removal of CPAs. It is a time consuming procedure, and it requires special equipment such as a programmable freezer (Picton et al., 2000). Table 9 describes this protocol (Gosden et al., 1994).
Table 9: Protocol for cryopreserving and thawing human ovarian tissue

1. Equilibrate thin slices of ovarian cortex for 30 minutes on ice in buffered medium containing cryoprotectant (1.5 M DMSO), serum 10%, and 0.1 M sucrose/mannitol

2. Load the tissue in cryovials into an automated freezer starting at 0°C and cool at 2°C/min to –7°C

3. Soak for 10 minutes before seeding

4. Continue to cool at 0.3°C/min to -40°C

5. Cool at the faster rate of 10°C/min to -140°C

6. Transfer to liquid nitrogen dewar for storage

7. Thaw rapidly at ~100°C/min

8. Wash tissues stepwise in progressively lower concentration of medium

4.3.2 Ultra-rapid cooling (vitrification) protocol

4.3.2.1 Definition of vitrification

Vitrification can be defined as a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling. Equally important, the vitrification process completely avoids ice crystal formation in cryopreserved cells during warming to recover the cells for biological applications (Kuleshova and Lopata, 2002).

4.3.2.2 History of vitrification

Retrospectively, the phenomenon of vitrification was first investigated and described at the turn of the 19th century. The founder of cryobiology, Luyet, recognized the potential of achieving an ice free, structurally arrested state for cryopreservation 60 years ago and described it in his classical studies (Kuleshova
and Lopata, 2002). Luyet wrote that crystallization is incompatible with living systems and should be avoided whenever possible. The cooling of small living systems at ultrahigh speeds of freezing was considered to be possible, in that it could eliminate ice formation and create instead a glass-like (vitreous) state. This constituted the origin of the idea of vitrification but not, however, the beginning of the vitrification of organs, which was unthinkable at the rapidity of freezing and thawing demanded by Luyet (Rall et al., 1987). Subsequently, it was generally recognized that supporting solutions for vitrification would be better for the preservation of living cells and tissues than would solutions that crystallize and hence damage cells during cooling and warming. The procedure for successful cryopreservation by vitrification of mammalian embryos and oocytes, including human, has been the subject of intense research over many years. In 1985, Rall and Fahy showed for the first time that murine embryos could be successfully cryopreserved by vitrification (Rall et al., 1985). The initial solution (VS1) was very toxic, but those investigators found ways to overcome this problem. In these older studies, some elements of slow cooling procedures, rather than modern vitrification protocols were used so, researchers can consider Rall and Fahy studies as a bridge between vitrification and slow cooling (Kuleshova and Lopata, 2002).

4.3.2.3. Vitrification of ovarian tissue

Cryopreserving oocytes, embryos and ovarian tissues by vitrification has many advantages as mentioned in Table 10 (Liebermann et al., 2002a).
Table 10: Summary of the primary benefits of vitrification

- Direct contact between cells/tissue and liquid nitrogen
- No ice crystallization
- Utilizes higher concentration of cryoprotectant that allows shorter exposure times to the cryoprotectant
- Rapid vitrification/warming
- Small volume used provides a significant increase in the cooling rate
- Cooling rates from 15,000°C to 30,000°C/min
- Minimizes osmotic injuries
- Reduces the time of the cryopreservation procedure (duration from 2 to 10 min)
- Very simple protocols
- Eliminates the cost of expensive programmable freezing equipment

However, many variables can affect the effectiveness and success of vitrification and have an impact on the biological sample that is cooled from physiological to liquid nitrogen temperature (Table 11) (Liebermann et al., 2002a). The two most important parameters are the cooling rate and the concentration of the cryoprotectants.

A practical limit to attainable cooling speed exists, as does a biological limit on the concentration of cryoprotectant tolerated by the cells during vitrification. Therefore, a balance between the maximization of cooling rate and the minimization of cryoprotectant concentration is important. To achieve high cooling rates requires the use of high concentrations of the cryoprotectant solution, which depresses ice crystal formation. A critical concentration is required for vitrification. Furthermore, to facilitate vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution as much as possible (Liebermann et al., 2002a). To do that, special carriers are used during the vitrification process. These include open pulled straws (OPS) (Oberstein et al., 2002) or the flexipet-denuding pipette (FDP) (Liebermann et al., 2002c), microdrops (Papis et al., 2000), electron microscopic (EM) copper grids (Park et al., 2000), hemistraw
system (Liebermann et al., 2002b), small nylon coils (Kurokawa et al., 1996) or nylon mesh (Matsumoto et al., 2001), and the cryoloop (Liebermann and Tucker, 2002). These have all been used as carriers or vessels to achieve higher cooling rates. To minimise devitrification (the previously vitrified solution freezes upon rewarming), thawing must be rapid, because rapid thawing reduces the likelihood of devitrification, since the rate of ice crystal growth diminishes and eventually disappears totally above a certain rate of heating. The number of nucleation centres determines the position of the devitrification curve and the critical heating rate. Fahy et al., reported that heating rates no greater than 400-1000°C/min should be required to prevent devitrification (Fahy et al., 1984)

Various research groups have reported the successful vitrification of ovarian tissue from mice, rats, Chinese hamsters, rabbits, Japanese apes, cows, and human fetuses (Kagabu and Umezu, 2000; Van den Broecke et al., 2001). Vital follicles were still detected 4 days after the warming of vitrified fetal rat ovaries (Sugimoto et al., 1996). Miyamoto and Sugimoto (Miyamoto and Sugimoto, 1994) vitrified rat ovaries and removed the cryoprotectant stepwise. The histological examination of the follicles yielded positive results in surface area but revealed degenerative changes, such as pyknosis, vacuolization, and cell swelling, in the other remaining tissue. The comparison of slow freezing and vitrification of bovine ovarian tissue demonstrated, however, that a vitrification protocol (exposure to 5.5 M EG at 22°C for 20 min) could be just as effective as slow freezing (Yen et al., 2001).
Table 11. Variables of vitrification that can profoundly influence its effectiveness.

<table>
<thead>
<tr>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Type and concentrations of cryoprotectant (almost all cryoprotectants are toxic)</td>
</tr>
<tr>
<td>2. Media used as base media (holding media)</td>
</tr>
<tr>
<td>3. Temperature of the vitrification solution at exposure</td>
</tr>
<tr>
<td>4. Length of time cells/tissue are exposed to the final cryoprotectant before plunging into liquid nitrogen</td>
</tr>
<tr>
<td>5. Variability in the volume of cryoprotectant solution surrounding the cells/tissue</td>
</tr>
<tr>
<td>6. Device used for vitrification (size of the vapor coat and cooling rate)</td>
</tr>
<tr>
<td>7. Technical proficiency of the embryologist</td>
</tr>
<tr>
<td>8. Quality and developmental stage of the tested cells/tissue</td>
</tr>
<tr>
<td>9. Direct contact of the liquid nitrogen and the vitrification solution containing the biological material can be a source of contamination (Bielanski et al., 2000); and to eliminate this danger, using sterile liquid nitrogen for cooling and storage is essential</td>
</tr>
</tbody>
</table>
Fig. 1. Theoretic options for the use of banked human ovarian tissue

- Orthotopic Transplantation
- Heterotopic Transplantation
- Follicle Isolation
  - Resumption Of Cyclicity
  - Autograft
  - Xenograft
  - In Vitro Growth
    - Egg Retrieval
      - Spontaneous Conception
      - In Vitro Fertilization

*Fig. 1. Theoretic options for the use of banked human ovarian tissue*
5. Future of the cryopreserved ovarian tissues

Cryopreservation and banking of human ovarian tissue is now feasible, but the problem of ovarian tissue cryopreservation is that of the future of the frozen grafts. In theory, there are three possibilities: autotransplantation, xenografting and in vitro growth and maturation as shown in Figure 1 (Oktay et al., 1998).

5.1. Autotransplantation

Autotransplantation appears, at least in concept, to be a simple and practical method for maturation of oocytes in stored ovarian tissue. Cryopreserved ovarian tissue can be autografted either orthotopically or heterotopically (Salle et al., 1999).

5.1.1. Orthotopic transplantation

Orthotopic transplantation of cryopreserved ovarian tissue has been successfully performed in many animal models. These studies proved that frozen ovarian tissue could resume its cyclical function after transplantation without vascular reanastomosis, (mouse: Harp et al., 1994; sheep: Baird et al., 1999; human: Grischenko et al., 1987) and are capable of becoming pregnant (mouse: Cox et al., 1996), live youngs have been born in mice (Gunasena et al., 1997) and sheep (Gosden et al., 1994). In the rodent, cyclicity was restored by grafting in 75% of the mice and at the same time a similar percentage to that observed in controls carrying fresh ovarian grafts (Candy et al., 2000). Even more remarkably, frozen-thawed fetal mouse ovaries restored cycles to 100% of syngeneic adult ovariectomized mice, many of which became fertile (Cox et al., 1996). Studies of the sheep ovary have shown that ovarian tissue cryopreservation and orthotopic transplantation are feasible in a large species. This is of great interest because sheeps are large mammals whose ovarian architecture is very close to that of the human women. Furthermore, the ovarian cortex is thick, the size of the ovary approaches that of of the human ovary, and the number of subcortical primordial follicles is high. Because the whole ovary is too large to be cryopreserved, cortical slices were prepared from one organ for freezing to liquid nitrogen temperatures using a medium containing DMSO and a slow freeze, rapid thaw protocol. When the contralateral ovary was removed 3 weeks later, frozen-thawed and fresh slices
of tissue were grafted to opposite sides with 5-0 delayed absorbable suture to the ovarian pedicles. The first ovulations occurred approximately 4 months after the second operation (Salle et al., 2002). In a subsequent study, a further set of eight sheep was grafted with bilateral frozen-thawed autografts. The ovaries regained cyclicity and functioned as the controls for 22 months, until they were removed (Baired et al., 1999). Moreover, it has been reported that laparoscopic transplantation of frozen-banked ovarian tissue beneath the pelvic peritoneum in a patient 29 years whose ovarian tissue was previously removed and frozen with the slow-freeze technique resulted in ovulation and menstruation 4 months after the transplant in response to ovarian stimulation with menopausal gonadotropins (Oktay and Karlikaya, 2001). When an intact uterus and functional fallopian tubes are present, an orthotopic graft may restore normal fertility. However, the orthotopic graft sites are less numerous than in the case of heterotopic graft. The peritonium of the broad ligament has not given good results, whereas the contralateral ovary and uterus have accepted the graft without problem (Salle et al., 1999).

5.1.2. Heterotopic transplantation

Where the ovarian tissues can be grafted in different sites outside the pelvis, including the rectus muscle, abdominal subcutaneous tissue, the omentum, saphenous vein, and under the renal capsule (Salle et al., 1999).

The ideal site for heterotopic ovarian autotransplantation remains to be established. As a potential treatment for the menopause, the site of the graft seems not critical and with the advent of in vitro fertilization technology and high-resolution ultrasound, heterotopic grafting could also potentially form an alternative approach for the preservation of fertility (Callejo et al., 2001).

When fresh and frozen-thawed fetal murine ovaries were transplanted under the kidney capsule of adult mice, follicular development was identified in the grafts. When ovaries from fetal hamsters were transplanted under the kidney capsule of adult hamsters, the number of growing follicles increased after host treatment with estradiol or testosterone propionate (Abir et al., 2001). As a clinical application of this choice, Nugent et al. grafted fresh ovarian cortical biopsies from nine patients
into uterine subserosa. After 14 weeks, the histologic analysis showed preantral follicle development. On average, 25% of follicles survived after grafting in comparison with the ungrafted controls. The authors attributed the lack of antral follicle development to suppression by the patient’s functioning ovaries (Nugent et al., 1998). Oktay et al. performed a subcutaneous ovarian transplantation in a patient with squamous cell cervical carcinoma (Oktay et al., 2000). Placed in the forearm, the graft produced cyclical estrogen for more than a year. No spontaneous ovulation occurred, but a percutaneous retrieval after gonadotropin/human chorionic gonadotropin administration yielded a mature oocyte. Schnorr et al. reported restoration of ovarian/menstrual cyclicity in a majority of nonhuman primates after transplantation of 1 mm³ fresh and frozen-thawed ovarian pieces in the arm (Schnorr et al., 2000). Other work showed that gonadotropin administration could increase the survival of ovarian transplants to the body wall in mice (Imthurn et al., 2000).

In another landmark study in sheep, Aubard et al. (Aubard et al., 1999) compared the function of heterotopic and orthotopic autografts of frozen-thawed ovarian cortex. Fresh and frozen-thawed fragments of ovarian cortex were autografted onto the uterine horn of six ewes (orthotopic grafts) and under the skin of the belly in nine ewes (heterotopic grafts). In both fresh and frozen-thawed grafts, preantral and antral follicles were first detectable 4 and 10 weeks after grafting, respectively, but only 5% of the primordial follicles appeared to have survived. Although ovulation resumed in most ewes, none of the ewes that were grafted orthotopically became pregnant at a synchronized mating. Aubard et al. noted significant adhesion formation due to the transplantation procedure, explaining the lack of spontaneous conception. Seven months after grafting, immature oocytes were retrieved from heterotopic and orthotopic grafts. They were matured in vitro and some of them fertilized, but none developed to the blastocyst stage. Although in-vitro maturation alone might have contributed to the poor embryo development, lack of any blastocyst formation indicates the necessity for further study of the quality of the embryos generated from transplanted frozen-thawed ovarian tissue.

Although autografting into an orthotopic or heterotopic site seems most promising, its clinical application is problematic because it carries the following risks:
* The potential risk of disease transmission. The grafts can transmit viruses including human immunodeficiency virus (HIV), and hepatitis viruses.

* The risk of cancer recurrence (Shaw et al., 1996). There is a legitimate concern regarding the potential for reseeding cancer cells with ovarian transplantation, depending on the type of cancer. Although many cancer types never metastasize to ovaries, leukemias are systemic in nature and pose a greater risk to the recipient. Likewise, neuroblastomas pose a significant risk to the transplant recipient. In autopsy series, ovaries contained metastasis in 25-50% of neuroblastoma cases (Oktay, 2001). Shaw et al. has reported that small pieces (around 1mm³) of fresh and frozen ovarian grafts from AKR mice can transfer lymphoma to recipient animals (Shaw et al., 1996). However, the risk of transferring cancer cells depends on the disease type, activity, and stage. In another study (Kim et al., 1999), human frozen-thawed ovarian tissue from patients with Hodgkin's disease (n = 5), non-Hodgkin’s lymphoma (NHL) (n = 13), acute lymphoblastic lymphoma (n = 2), and acute myelocytic leukemia (n = 2) were xenografted into immunodeficient mice. Although none of the tissues from patients with NHL resulted in cancer recurrence, one xenograft from a patient with Hodgkin’s disease resulted in recurrence (one out of five). The results with tissues from patients who had acute myelocytic leukemia and acute lymphoblastic lymphoma were inconclusive. A study using a NUDE/SCID xenograft model tested the safety of cryopreserved human ovarian tissue from cancer patients, and although this study suggested that ovarian tissue transplantation in Hodgkin’s disease patients was safe, it did not exclude the risk of cancer transmission in other types of cancer (especially hematogenous or systemic neoplasm). Breast cancer carries a low to intermediate risk of ovarian involvement. In the absence of clinical and radiological evidence of distant metastasis, ovarian involvement has been reported in 2-11% of cases. In most cases, a thorough clinical and radiological evaluation rules out ovarian involvement. Ovarian involvement in Wilm's tumour, lymphomas (with the exception of Burkett's lymphoma), osteosarcomas, Ewing's sarcoma, and extragenital rhabdomyosarcomas is extremely rare. Likewise, in squamous cell cervical cancer, ovarian involvement is below 0.2%, including in the most advanced stages (Oktay, 2001). In order to further minimize the risk of cryopreserving ovarian tissue with metastasis, it is
necessary to develop screening methods to detect minimal residual disease (MRD) in ovarian tissue to eliminate the risk of cancer cell transmission with transplantation. Attempts to confirm the safety of ovarian tissue based on the absence of malignant cells by light microscopy may not be sufficient. Currently, molecular genetic techniques such as nested polymerase chain reaction (PCR), flow cytometry, fluorescence in situ hybridization (FISH), and cytogenetics have been applied to detect MRD before autologous peripheral stem cell or bone marrow transplant. The detection of specific gene translocation and immunoglobulin gene rearrangement in leukemia has been successful using PCR amplification. MRD in B cell lymphoma can be detected by a PCR-mediated RNase protection assay (Poiret et al., 2002). A consultation with the patient's medical oncologist and the oncologist's approval are required (Oktay, 2001).

5.2. Xenotransplantation

More controversially, immunodeficient animals could be used to grow the follicles to produce oocytes approaching ripeness. The growth and maturation of cryopreserved human primordial and primary follicles have been achieved by this method. However, the best site for ovarian tissue transplantation, capable of revascularization and providing easy access to mature follicles, has not yet been determined. The kidney capsule of SCID mice serves as a site for the transplantation of ovarian slices from various species. Sheep and cat ovarian cortical slices were transplanted to this site and developed to late antral stages. Candy et al. demonstrated that freezing and thawing do not substantially damage marmoset ovarian tissue, and that the cryopreserved tissue retains its ability to support the development of follicles at all stages of folliculogenesis, including large antral follicles, 21-32 weeks after transplantation (Donnez et al., 2000). Xenografts of ovarian tissue implanted under the renal capsule of immunodeficient mice have demonstrated viability of the human and monkey ovarian tissue. By studying the rate of fibrosis in fresh and frozen-thawed cortical slices xenografted into NUDE/SCID mice, an average of 40% of the graft was found to be fibrotic after 24 days. The authors observed a significantly higher fibrosis relative to surface area (68%) in frozen-thawed xenografted tissue, regardless of the site of transplantation (either subcutaneous or intraperitoneal) (Donnez et al., 2000).
In a preliminary study, when freshly dissected fragments from human fetuses, 16 gestational weeks of age, were transplanted under the kidney capsule of immunodeficient mice, germ cells in mitosis and at all stages of meiotic prophase were abundant, and initial follicular formation was observed 6 months after grafting. In addition, a pilot report showed development of antral and secondary follicles 6 months after transplantation in a FSH-treated host grafted with PROH frozen-thawed ovaries from a human fetus 21 gestational weeks of age. Transplantation of fresh or frozen-thawed human ovarian fragments from women under the kidney capsule of immunodeficient mice has also been described. Antral development was achieved in grafts from fresh and PROH frozen-thawed tissue. Four studies have been published on subcutaneous transplantation of fresh or frozen-thawed human ovaries from women. All showed active angiogenesis in the grafts and follicular survival. Administration of FSH to mice grafted with fresh or PROH or DMSO frozen-thawed tissue resulted in growth to antral stages. However, the grafts were reduced in size and fewer subcutaneous than kidney grafts were recovered. However, no differences were observed in follicular recovery, density, or ultrastructural quality at both grafting sites (Oktay et al., 2001).

Neovascularization of the graft remains one of the key factors for its survival. Ovarian tissue is a rich source of angiogenic factors that encourage the rapid migration of endothelial cells into the grafts and the early restoration of blood circulation. The transplanted ovary is able to produce substances that promote direct angiogenesis, and it has been demonstrated in animal models that the autotransplantation of ovaries can result in prompt ovarian revascularization. According to Dissen et al., 1994 gonadotropin secretion after ovarian transplantation contributes to the revascularization of grafts in the rat by upregulating the gene expression of two major angiogenic factors, vascular endothelial growth factor (VEGF) and transforming growth factor-alpha (TGF-α).

Using a morphometric study, Nisolle et al., 2000 demonstrated a vascular network located at the periphery of the graft, but macroscopic and microscopic differences were observed according to the site of transplantation. Indeed, when ovarian grafts were implanted subcutaneously, the authors observed macroscopic differences between fresh and frozen-thawed grafts. Indeed, fresh ovarian grafts were systematically well revascularized and small vessels were visible on the graft surface after 3 weeks, confirming that small pieces of fresh ovarian tissue (2 mm)
rapidly become revascularized (Donnez et al., 2000).

5.3. In vitro growth and maturation

The risks of ovarian tissue transplantation could be eliminated by obtaining primordial follicles (PMFs) from frozen-thawed tissue and, growing them in vitro, followed by routine IVF (Donnez et al., 2000). The development of follicles in the ovary is a complicated event. Primordial germ cells arise at about 3 weeks after fertilization and migrate by amoeboid movements from the epithelium of the yolk sac via the connective tissue of the hindgut to the genital ridge. During their journey, the primordial germ cells multiply rapidly. In humans, the genital ridge is formed at about 3.5–4.5 weeks of gestation by a knot of mesenchyme overlaid by coelomic epithelium. At about week 7 of gestation, the cells derived from the mesonephros and the coelomic epithelium migrate inward from the genital ridge, forming primitive medullary cords and sex cords, respectively. The cords, which are anatomically less well defined in females than in males, are compacted in the cortical region of the primitive gonad. The primitive cords are colonized by the primordial germ cells. In the female fetus, the primordial germ cells are named oogonia upon arrival in the primitive gonad. The oogonia are still interconnected by cytoplasmic bridges. The primitive medullary cords degenerate to be replaced by highly vascularized ovarian stroma. The cord cells proliferate and mesenchymal cells condense around the oogonia to form the individual primordial follicles. Follicle formation in humans begins in the inner part of the ovary, near the rete ovarii, between week 16 and week 18 of fetal life. In the primordial follicles, the mesenchymal cells secrete an outer basement membrane and the same cells will give rise to granulosa cells in the growing follicle. Meanwhile, the mitotic activity of the oogonia ceases and the oogonia enter meiosis (Byskov, 1986).

In mammals, this process of envelopment of oogonia occurs either before birth (humans, cows, and sheep) or shortly thereafter (mice, rats, and hamsters). A meiosis-initiating factor is derived from the cells of the ingrowing mesonephric tissue when the first meiotic division is initiated. Once the meiotic process is initiated, the oogonial germ cells are defined as primary oocytes. As a consequence of initiation of meiosis, multiplication is prohibited and the store of female gametes is set definitively at that stage of life. Once primordial follicles are
formed the first meiotic division is arrested at the diplotene stage. The chromosomes decondense and are packed within a nucleus known as the germinal vesicle. In humans, the follicle may remain at this stage of development for 40–50 years up to the moment at which a signal initiates oocyte and follicle growth. All remaining oogonia that are not surrounded by somatic cells are expelled from the ovary (Baker, 1963). Follicles develop from primordial follicular stage (inactive) to primary follicular stage (active) under an unknown initiation signal. Human primordial follicles grow from a size of 30-60 μm to 15-20 mm in preovulatory follicle as shown in figure 2 (Macklon and Fauser, 1998).

During this process, zona pellucida develops to 20-25 μm in diameter as the oocyte matures. Granulosa cells proliferate and become steroidogenic. A fluid-filled antrum forms between granulosa layers. The surrounding stromal cells transform to theca interna and externa outside the basal lamina of follicles (Qu et al., 2000). The maximum number of germ cells is present at about 4–5 months after conception, and decreases from $8 \times 10^6$ to 1–2 $\times 10^6$ at birth by a process called ‘attrition’ (Baker, 1963). During prepubertal life continuous initiation of follicle growth leads to further depletion of the store of gametes available. There are few reports on the number of primordial follicles in fetuses. The data from (Baker, 1963) and (Sforza et al., 1993) indicate that there is a trend towards increasing numbers of primordial follicles with gestational age. The limited data on early follicle growth stages in human fetal ovaries show that at birth in humans < 1% of follicles have developed further than the primary stage. Antral follicle stages can be observed in human fetal ovaries (Fig. 3) (Smitz and Cortvrindt, 2002).

The earliest stages of ovarian folliculogenesis are morphologically similar in different mammalian species. However, each species has its own specific timescale for development. In rodents (mice, rats) the time span between initiation of follicle growth and formation of the antral cavity is a few weeks; in large domestic animals it takes several months. During the preantral growth phase, the oocyte grows rapidly and reaches almost its maximum volume when the first accumulation of fluid is observed within the granulosa. In humans, Gougeon (1986) estimated that the maturation phase from primordial to primary follicle takes > 120 days as shown in figure 4 (Smitz and Cortvrindt, 2002). Once in the growing pool, the follicle requires 65 days to reach the early antral phase (follicle of 2–5
mm diameter), at which point it becomes dependent on gonadotrophins for further growth. Eppig and O’Brien were the first to report the development of primordial oocytes from newborn mouse ovaries to mature oocytes in vitro. The transfer of two-cell-stage embryos resulted in live birth of a mature pup. In this study a two-step strategy was developed: first, the whole ovaries of newborn mice were cultured for 8 days to allow the development of secondary follicles within the ovarian tissue. The second step was to enzymatically isolate the oocyte-granulosa cell complexes and culture them for an additional 14 days. This basic two-step strategy will probably serve as the essential framework for application to other species, for several reasons:

First: it is difficult to isolate and culture intact primordial follicles.

Second: the mechanism involved in the recruitment of PMFs into the pool of preantral follicles is unknown and may require the presence of ovarian factors external to the follicle itself.

Third: properly timed isolation and culture of oocyte-granulosa cell complexes probably enhances the development and nutrition of larger antral follicles.

Alternatively, final oocyte maturation could be achieved by a combination of in vivo transplantation of PMFs, followed by in vitro culture of growing follicles (Liu et al., 2000). Although the culture of ovarian follicles to various stages of maturity is well established in species such as mice, rats, sheep, and cows, growth of human follicles has met with limited success (Hardy et al., 2002). Techniques to grow human follicles in vitro are currently being developed and have been, in general, adapted from those used successfully in other species. Preantral follicles have been isolated enzymatically and mechanically and grown for several weeks, and some follicles have undergone antrum formation. Long-term culture of primordial follicles from fetal ovaries has resulted in follicle growth, oocyte maturation and even first polar body expulsion. In contrast, PMFs isolated from adult ovaries initiated growth, but did not survive beyond 24 h in vitro, and sustained high rate of oocyte loss (Hardy et al., 2002). One of the most promising techniques for preantral follicle culture is that of the tissue-slice culture system designed for the growth of human primordial, primary, and secondary follicles to large preantral and
occasionally early antral stages. Follicles are cultured within small pieces of ovarian cortex, which has the advantage of maintaining all the normal ovarian intercellular contact and support. Most PMFs initiate growth in human ovarian cortex, although many follicles undergo atresia during culture. Studies using this approach have shown that supplementation of the culture with FSH significantly reduces atresia, and stimulates follicle development, demonstrating that follicles are responsive to FSH before antrum formation (Wright et al., 1999). However, for the future of the IVG and IVM in human, a three-stage system encompassing initiation of growth in cortex pieces, follicle isolation and further in vitro growth followed by oocyte retrieval and IVM is envisaged for the production of oocytes competent to be fertilized (Abir et al., 1999)].
Fig. 2. Schematic diagram of follicular development in the human.

Primordial follicles entering the growth phase form primary follicles (class 1). This is followed by gonadotropin-independent (tonic) growth (class 1-4) and eventually gonadotropin-dependent growth. The overall development takes three cycles.
Fig. 3. Overview of follicle formation and growth in the human fetal ovary.

(a) Section through a fetal ovary at week 18 of gestation. Follicles are not yet organized. The large clear cells with the largest nuclei, observed mainly in the central portion of the section, are oogonia. (b) Part (a) at larger magnification. Oogonia are dispersed within mesenchymal cells. Cells from the coelomic epithelium form cords. At this stage oogonia are still interconnected by cytoplasmic bridges and appear as strings (arrow). The fetal ovary is delineated by coelomic epithelium. (c) Ovary from a fetus at week 24 of gestation. Oogonia can be observed which are entirely surrounded by pregranulosa cells and form the primordial follicle. The tissue already shows lytic changes. Follicles are embedded within mesenchymal tissue, which is highly vascularized. (d) Ovary from a fetus at week 25 of gestation. Follicles at the primordial stage with flattened pregranulosa cells are present. There is a follicle containing a growing oocyte (arrow) showing the cuboidal transformation of the pregranulosa cells. (e) Fetal ovary (week 30 of gestation) showing lytic changes. Primordial follicles are observed. In the right upper quadrant there is a follicle with three to four layers (arrow) surrounded by a theca interna containing a collapsed oocyte. (f) Fetal ovary (week 34 of gestation). The left half of the figure contains large (atretic) multilayered follicles, the right half contains very dense tissue populated with primordial follicles and oogonia. Scale bars represent (a) 20 mm, (b) 10 mm, (c,d) 25 mm, (e) 30 mm, (f) 150 mm.
Fig. 4. Earliest stages of follicle growth in humans.

The relationship between follicle diameter (x-axis) and oocyte diameter (y-axis) is shown and major milestones in follicle formation and differentiation are indicated. AR: androgen receptor; ER: oestrogen receptor; FSHr: FSH receptor; GC: granulosa cells; LHR: LH receptor; TC: theca cells.
6. Growth factors and OTCP

Qu et al., 2000, found that transforming growth factor-alpha (TGF-α), epidermal growth factor (EGF) and EGF receptors were expressed in human follicles and this leads to the assumption that TGF-α or EGF may be useful to add to the culture of cryopreserved ovarian tissue to promote the maturation of follicles in vitro. The possible influence of freezing and thawing on the immunoreactivities of TGF-α, EGF and EGF receptor in frozen ovarian tissue was investigated. The authors observed that there was no significant difference in immunostaining for TGF-α, EGF and EGF receptor between fresh and frozen ovarian tissues, indicating that human ovarian tissue could be frozen without substantially altering the immunoreactivities of TGF-α, EGF and EGF receptor follicles. TGF-α and EGF receptor were simultaneously expressed in the early follicles of human ovarian tissue. TGF-α might play a role in the regulation of folliculogenesis through binding to EGF receptor by an autocrine or paracrine mechanism. EGF was expressed in a different pattern from TGF-α and EGF receptor in follicles. These growth factors may be useful to promote the in-vitro maturation of follicles from cryopreserved ovarian tissue, for potential uses in the treatment of infertile patients and in in-vitro fertilization programmes in the future (Qu et al., 2000). Like insulin-like growth factor (IGF), TGF-β is an important intra-ovarian regulator of folliculogenesis. TGF-β was localized immunocytochemically in ovarian granulosa and theca-interstitial cells. The dominant form present in granulosa cells is TGF-β. Both theca-interstitial and granulosa cells secreted TGF-β in culture. An increase in TGF-β levels was recently observed in ovarian follicular fluid in women after ovarian hyperstimulation for IVF. TGF-β differentially regulated DNA synthesis and the proliferation of granulosa cells, and induced apoptosis in rat theca-interstitial cells. TGF-β enhanced basal and gonadotropin-stimulated steroidogenesis in granulosa and thecal cells although both inhibin and TGF-β suppressed luteinizing hormone-induced oocyte maturation in preovulatory follicles (Donnez et al., 2000).

In another study, Qu et al. observed that the expression of TGF-β type II receptor was distinct from the type I receptor with respect to cellular distribution in ovarian tissue. TGF-β type II receptor stained only in the oocytes of primordial and primary follicles, faintly in the thecal cells, and no significant staining occurred in the
oocytes and granulosa cells in preantral and antral follicles. Their results are not consistent with the report by Roy and Kole on the expression of TGF-β type II receptor in granulosa, thecal and interstitial cells in the ovary. The reason for the discrepancy is unknown. The significance of the concomitant presence of TGF-β receptors and IGF type I receptor in early follicles has yet to be illuminated. However, it has been suggested that both IGF-I and TGF-β may be involved in a sophisticated paracrine-autocrine network in the regulation of oocyte maturation and follicular growth in the ovary, and it could be worthwhile to investigate further the possibility of using IGF-1 and TGF-β to regulate the in vitro or in vivo growth of oocytes in early follicles from cryopreserved ovarian tissue (Donnez et al., 2000).

Results from Qu et al. showed that there was no significant difference in immunostaining for either IGF type I receptor or TGF-β type I and II receptors in follicles of frozen ovarian tissues, compared with fresh tissues from pair-controlled samples. It seems that freezing does not significantly alter the immunoreactivities of IGF type I receptor and TGF-β type I and II receptors in follicles after ovarian tissue cryopreservation (Donnez et al., 2000).

7. Evaluation of the viability of the cryopreserved ovarian tissues.

There are many ways to examine the viability of the cryopreserved ovarian tissue.

7.1. Histological evaluation

Using the light microscope, different scoring systems were proposed to evaluate the structural normality of the cryopreserved ovarian follicles. Paynter’s et al. proposed the following scoring system (Paynter et al., 1999):

*Primary follicles* (small follicles containing a germinal vesicle-stage oocyte surrounded by 1-2 layers of cuboidal granulosa cells) were graded 1-3:

**Grade 1:** being spherical in shape with even distribution of granulosa cells, intact theca and a spherical oocyte.

**Grade 2:** having theca and granulosa cells pulled away from the edge of the follicle but the oocyte still spherical.
Grade 3: having theca and granulosa cells pulled away from the edge of the follicle with severe pyknotic nuclei in the granulosa cells and oocyte mis-shapen with/without vacuolation.

*Pre-antral follicles* (larger follicles surrounded by several layers of granulosa cells and containing a germinal vesicle-stage oocyte) were also graded 1-3:

Grade 1: is an intact spherical follicle with evenly distributed granulosa and theca cells, small spaces between cells being acceptable with a normal spherical oocyte.

Grade 2: is an intact spherical follicle with intact theca cells but with disruption of the granulosa cells and apparent loss of cells and containing a normal spherical oocyte.

Grade 3: shows great disruption and loss of granulosa cells with theca cells pulled away from the follicle edge with many pyknotic nuclei visible and mis-shapen vacuolated oocyte.

Using the electron microscope, the ultrastructural changes can be evaluated by examining the different organelles as the microvilli, mitochondria, cortical granules vesicles and zona pellucida (Fuku et al., 1995).

7.2. Endocrinological evaluation

Follow up of the endocrine functions in animals transplanted with the cryopreserved ovarian tissue can give an idea about the viability of the cryopreserved ovarian tissues. Sugimoto et al., transplanted vitrified-thawed left ovarian tissue, under the right renal capsule of 10-12-days-old rats and in the same sitting the right ovary was completely removed. Vaginal opening is checked daily on postnatal day 26, and after that until postnatal day 84. Vaginal smears were examined daily by light microscopic observation of Giemsa-stained smears to detect the phase of estrous cycle (Sugimoto et al., 2000). Direct measurement of the gonadotrophin level or steroid hormone level in the sera of the transplanted animals or human is a direct way to evaluate the graft viability. Bedaiwvy et al. measured the level of serum estradiol and follicle stimulating hormone after heterotopic transplantation of the ovarian tissue in ewes (Bedaiwvy et al., 2003). Callejo et al. evaluated estradiol and follicle stimulating hormone levels in 4
7.3. Auto-radiographic and in situ methods for detecting apoptotic DNA fragments.

These methods depend upon the fact that apoptosis is associated with activation of the calcium/magnesium-dependent endonuclease activity, which specifically cleaves cellular DNA between regularly spaced nucleosomal units. The end result is the generation of DNA fragments in size multiples of 185-200 base pairs, which can be visualized as a distinct ladder of DNA bands after agarose gel electrophoresis and ethidium bromide staining. After extraction of DNA, terminal transferase is used to attach labeled dideoxy-ATP (dd-ATP) to free 3´-ends of all DNA fragments, followed by size fractionation using agarose gel electrophoresis. The qualitative aspect of DNA degradation is revealed by autoradiography, whereas the quantitative estimation of apoptosis is determined by β-counting of gel fragments containing low molecular weight DNA (<15 kilo-bases) (Aaron et al., 1994). Cell death can be identified by TUNEL analysis as well. A kit designed for this purpose is used. Briefly, the tissues to be examined for apoptosis are immerse fixed in 4% paraformaldehyde, processed for routine paraffin histology, sections (5 um) were deparaffinized, pretreated with proteinase K (20 ug/mL), quenched with 3% hydrogen peroxide, and the TUNEL procedure conducted according to the manufacturer’s instructions. TUNEL-positive cells were visualized with diaminobenzidine and counterstained with methyl green (Brendan et al., 2000).
**MATERIALS AND METHODS**

1. **Animals**

Twelve- to twenty-week-old OF1 female mice were used in this study, and housed in the animal house (Geneva University). All mice were housed under a 12-h light/12-h dark regime at 22-24°C with food and water supplied ad libitum. The animals were classified into two groups, the vitrification and the control groups.

2. **Tissue collection and transplantation**

The mice were anaesthetized by intra-peritoneal injection of Ketarom in a dose of 0.01mL/gm/body weight (Ketarom is prepared by adding 2.4 mL of ketamine hydrochloride and 0.8mL of rumpun to 6.8mL of normal saline and kept at +4°C). Laparotomy was done through a midline or transverse incision in the ventral aspect of the mouse.

**In the vitrification group**, the operative technique was done in two settings at two weeks interval.

In the first setting, the left ovary was excised, cut into nearly 4 equal pieces by sterile and sharp knife, subjected to the vitrification technique as will be mentioned, and the left ovarian pedicle was ligated with vicryl 4/0 to ensure hemostasis. Continuous sutures closed the peritoneum while Interrupted sutures using either nylon 6/0 or vicryl 4/0 closed the skin.

In the second setting, another laparotomy was done two weeks later to remove the right ovary and to transplant one cryopreserved left ovarian quarter in the cavity of the most distal part of the left uterine horn.

**In the control group**, the operative technique was done in only one setting. Laparotomy was done as described before, both ovaries were removed, both ovarian pedicles were ligated with vicryl 4/0, and the left ovary was cut into nearly 4 equal pieces by sterile and sharp knife. One ovarian quarter was washed in HB1 solution and then immediately transplanted in the cavity of the most distal part of the left uterine horn.

3. **Vitrification of the ovarian tissue**

The vitrification solution (VS) is prepared by adding to HB1 solution the following
cryoprotectants to form 100% concentration of VS:

Dimethyl sulfoxide (DMSO): 20.5% V/V, acetamide: 15.5% W/V, propylene glycol: 10.0% V/V, and polyethylene glycol 6000 (average molecular weight 7500): 6.0% W/V.

HB1 solution consists of a modified Dulbecco’s saline solution containing: phosphate buffer: 1mM, sodium pyruvate: 0.33 mM, glucose: 5.56 mM, bovine serum albumin: 300 mg, and penicillin G: 100 IU/mL.

Dilution of 100% concentration of VS with HB1 solution allows the formation of 12.5%, 25%, and 50% concentrations of VS.

The ovaries were collected, cut into nearly 4 equal pieces by sterile and sharp knife, washed in HB1 solution and then exposed successively to an ascending concentration of the cryoprotectant. Firstly at room temperature, the ovarian fragments were exposed to 12.5% VS for 15 min, then to 25% VS for another 15 min to allow the tissues to be completely infiltrated by the cryoprotectants.

The ovarian suspension was then transferred to a cold room (at +4°C) where the ovarian fragments were exposed to 50% VS for 15 min. and then finally to 100% VS for another 15 min. then each ovarian fragment was placed in a freezing tube with 0.5 mL 100% VS, then transferred to the liquid nitrogen, and preserved until used.

4. Thawing

The ovarian suspension was re-warmed by putting the freezing tubes for few seconds in water warmed to 37°C. The remaining cryoprotectants were then washed away by passing the ovarian fragments in a descending concentration of the cryoprotectant as soon as the samples were liquified, firstly in 50% VS, secondly in 25% VS, thirdly in 12.5% VS, and lastly the ovarian peices were put in HB1 solution.

5. Assessment of the ovarian tissue viability and functionality

5.1.Viability of the ovarian biopsies

Viability of the previously vitrified-thawed ovarian fragments and that of the control
group was assessed by histological examination of the ovarian fragments before and after intrauterine transplantation. The ovarian biopsies and grafts were collected, fixed in acetic formol solution and embedded in paraffin. Serial sections (5 um) were cut, stained with hematoxylin and eosin, and examined under light microscope. Viable and atretic follicles were assessed according to Braw and Tsafriri criteria (Braw and Tsafriri, 1980):

Non-atretic follicles: contain oocytes in the resting stage of prophase, and pyknotic nuclei in the granulosa cell layer are absent. Follicular fluid is clear, i.e. without cell debris or macrophages. The theca interna consists of a few layers of elongated cells.

Stage 1 atresia: the oocytes of these follicles are in the resting stage of prophase and 1-10% of the nuclei in the granulosa cell layer are pyknotic. Mitotic figures are seen. The follicular fluid contains some cell debris.

Stage II atresia: in most of the follicles meiosis-like changes such as chromosomes at metaphase or a polar body could be observed. In the granulosa layer 10-30% of the nuclei are pyknotic, and mitotic figures could also be seen. Follicular fluid contains much cell debris.

Stage III atresia: the oocyte is fragmented or pseudocleaved. Most of the granulosa cells disappeared and macrophages are seen in the follicular fluid. The theca interna cells are hypertrophied. As atresia progresses the follicles collapse.

5.2.Functionality of the transplanted ovarian biopsies

Functionality of the transplanted ovarian biopsies was evaluated by restoration of fertility in the grafted animals. One month after transplantation, the grafted animals were stimulated by PMSG in a dose of 10 IU followed 48 hours later by hCG injection in a dose of 5 IU and mating was allowed with previously known fertile males. Successful mating was checked by the presence of vaginal plug. Fecundity of the ovarian grafts was calculated and expressed as the total number of live pups/ total number of the grafted ovarian tissues.
6. Statistical analysis

The unpaired t-test was used to analyse the numbers of viable follicles in fresh and vitrified-thawed biopsies as well as in fresh and vitrified-thawed grafts. The descriptive data were presented as mean ± SD. Differences at a probability of $p \leq 0.05$ were considered significant.
RESULTS

1. Viability of the ovarian follicles after thawing

Samples of fresh ovarian tissue were fixed within 30 minutes of collection to compare with vitrified-thawed ovarian tissue fixed immediately after thawing. Freshly excised ovarian tissue showed the presence of primordial, to cavitary follicles as well as corpus luteum (Figure 5). The vitrified–thawed ovarian tissue showed also primordial, primary, pre-antral follicles and corpora lutea (Figure 6). The nuclei and cytoplasm are unaltered by freezing at -196°C. Table 12 shows the mean number of morphologically normal follicles present in pieces of fresh (n = 7) and vitrified-thawed (n = 7) ovarian biopsies. The mean number of primordial, primary, pre-antral and antral follicles in the control group was significantly higher than those observed in the vitrified-thawed ovarian biopsies.

Table 12: Comparison of follicle number in fresh and vitrified-thawed ovarian biopsies.

<table>
<thead>
<tr>
<th>Ovarian biopsy</th>
<th>Primordial follicles</th>
<th>Primary follicles</th>
<th>Pre-antral follicles</th>
<th>Antral follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh biopsy (n = 7)</td>
<td>389 ± 55.6</td>
<td>80 ± 17</td>
<td>41 ± 8.3</td>
<td>7 ± 1.9</td>
</tr>
<tr>
<td>Vitrified-thawed biopsy (n = 7)</td>
<td>294 ± 44.9*</td>
<td>53 ± 7.5†</td>
<td>25 ± 2.6‡</td>
<td>3 ± 1.6§</td>
</tr>
</tbody>
</table>

P value * = 0.0040, P value † = 0.0025, P value ‡ = 0.0005, and P value § = 0.0017
Figure 5: Light micrograph of fresh ovarian biopsy

a. Fresh ovarian biopsy had primary follicle (notched arrow), mid-antral follicle (green arrow head), and corpus luteum (black arrow head) (magnification, X50).

b. Higher magnification of fresh ovarian biopsy showing primordial follicle (notched arrow), primary follicle (black arrow head), and pre-antral follicle (green arrow head) with visible oocytes (magnification, X200)
**Figure 6: Light micrograph of vitrified/thawed ovarian biopsy**

a. Vitrified-thawed ovarian biopsy showing primordial follicle (green arrow head) and primary follicle (black arrow head) (magnification, X50).

b. Higher magnification of Vitrified-thawed ovarian biopsy showing primordial follicle (green arrow head), and primary follicle (black arrow head) with visible oocytes (magnification, X200)
2. Viability of the ovarian follicles after grafting

The ovarian grafts were retrieved from 3 control and 4 test mice one month after grafting. Histological examination showed that transplanted grafts in both the fresh and frozen groups contained follicles at all stages of folliculogenesis and corpora lutea (Figure 7). Table 13 showed a comparison between the follicle number in 4-week-old grafts after transplantation of fresh and vitrified-thawed ovarian biopsies. The mean number of different types of follicles in both fresh and vitrified-thawed grafts was not significantly different (P value = 0.6)

Table 13: Comparison of follicle number in 4-week-old grafts after transplantation of fresh and vitrified-thawed ovarian biopsies

<table>
<thead>
<tr>
<th>Ovarian grafts</th>
<th>Primordial follicles</th>
<th>Primary follicles</th>
<th>Pre-antral follicles</th>
<th>Antral follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh grafts (n = 3)</td>
<td>197 ± 18.7</td>
<td>77 ± 8.1</td>
<td>42 ± 7.7</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Vitrified-thawed grafts (n = 4)</td>
<td>212 ± 44.3</td>
<td>69 ± 13.4</td>
<td>45 ± 7.3</td>
<td>6 ± 1.7</td>
</tr>
</tbody>
</table>
Figure 7: Transplantation success of ovarian biopsies in the uterine cavity of bilaterally ovariectomized mice.

a. Fresh ovarian transplant had primary follicle (notched arrow), early antral follicle (green arrow head), mid antral follicle (black arrow head), and corpus luteum (white arrow) (magnification, X200).

b. Vitrified-thawed ovarian transplant had primordial follicle (notched arrow), preantral follicle (white arrow), early antral follicle (green arrow head), and mid antral follicle (black arrow head) (magnification, X200)
3. Restoration of fertility

The functionality of the transplanted ovarian grafts was evaluated by restoration of fertility in the transplanted animals. One month after transplantation, PMSG was injected followed by hCG and mating was allowed at the expected time of ovulation with previously known fertile males. Fecundity of the ovarian grafts was expressed as the total number of live pups/ total number of the grafted ovarian tissues. The values were 0.2 and 0.42 for the cryopreserved and fresh ovarian grafts respectively. A similar number of fertile recipients of fresh and frozen grafts had obtained as shown in table 14. However, the number of pups obtained from fresh graft recipient was significantly different from that of the recipients of vitrified-thawed grafts. Interestingly, the mice carrying fresh grafts gave birth to 7 viable pups and one stillbirth. After 12 weeks, the fertility of the living pups was confirmed by producing second generation of mice on natural mating, demonstrating that these mice were anatomically normal in terms of fertility. While the recipients of vitrified-thawed grafts gave birth to 2 fresh stillbirths and 2 neonatal deaths. Autopsy of the 2 fresh stillbirths revealed unilateral pulmonary hypoplasia in both of them (Figure 9), while the autopsy of the 2 neonatal deaths revealed no abnormality.

Table 14: Evolution and outcome of pregnancies in mice after ovarian tissue transplantation

<table>
<thead>
<tr>
<th>Ovarian graft</th>
<th>Number of females transplanted</th>
<th>Number of fertile females</th>
<th>Number of pups/pregnancy</th>
<th>Outcome of deliveries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh graft</td>
<td>19</td>
<td>2</td>
<td>4</td>
<td>7 viable and fertile 1 Neonatal death</td>
</tr>
<tr>
<td>Vitrified-thawed graft</td>
<td>20</td>
<td>2</td>
<td>2</td>
<td>2. Fresh stillbirth 2. Neonatal death</td>
</tr>
</tbody>
</table>
Fig. 8 Autopsy picture and light micrograph of the lung of mouse pup with unilateral pulmonary hypoplasia

a. Mouse stilbirth showing normal development of all body systems namely the central nervous system, the heart, the urinary system, and the gastro-intestinal system, which are coincident with full term mouse. However one lung appears smaller than the other and collapsed, which means the presence of unilateral pulmonary hypoplasia.

b. Histological examination of the collapsed lung, revealed deficient septation and thick-walled mesenchyme (arrows) a findings coincident with lung hypoplasia together with large amounts of transudate filling both lungs (black arrow heads) which means that it was borne dead and never breath before (magnification, X200).
DISCUSSION

Assessment of various ovarian functions after transplantation is a direct way to examine viability of the preserved ovarian tissue. In many previous studies, viability of the cryopreserved ovaries and ovarian tissue was assessed by functional or morphological analysis after transplantation into orthotopic (Gosden et al., 1994; Cox et al., 1996; Harp et al., 1994; Aubard et al., 1998) or heterotopic sites (Nugent et al., 1998; Schnorr et al., 2000; Newton et al., 1996). In this study, survival of the cryopreserved ovarian tissues was assessed by histological examination and restoration of fertility in the grafted mice after natural mating and we have shown that ovarian tissue from mice containing follicles at different stages of development can be cryopreserved and is capable of surviving freezing and thawing. This is the first study that showed that this cryopreserved ovarian tissue could restore fertility to ovariectomised mice after heterotopic transplantation and natural mating without the need for medical assistance.

In this study, we found a statistically significant difference between the fresh and cryopreserved biopsies regarding all types of ovarian follicles. This is in agreement with Migishima et al. 2003 that showed significant difference between fresh and vitrified–thawed ovaries (Migishima et al., 2003). Jeremias et al. 2003 indicated that the number of primordial follicles per high power field was significantly reduced in the cryopreserved samples as compared to the freshly fixed group while changes in the percentage of primary and secondary follicles were not statistically significant (Jeremias et al., 2003). Our findings are not in agreement with Salle et al. 1999 that concluded that histological analysis of the fresh ovarian fragments showed primordial to cavitary follicles. Freezing and thawing of the ovarian fragments did not damage the ovarian structure and the number and distribution of follicles were identical to those in the fresh control sample (Salle et al., 1999). Fabbri et al. 2003 found statistical insignificant difference between the fresh and frozen/thawed ovarian tissue regarding the follicle morphology, the follicular density, the follicular distribution and the mean follicle diameter (Fabbri et al., 2003). Hovatta et al. 1996 indicated in a comparative study of fresh and frozen/thawed ovarian tissue using DMSO or PROH as cryopretectants, that there was no essential difference in the morphological appearance before and after
freezing and thawing (Hovatta et al., 1996). Paynter et al. 1999, also showed insignificant difference between different types of follicles in the fresh and cryopreserved ovarian biopsies using the slow cooling technique (Paynter et al., 1999). Candy et al. 1995 showed that freezing and thawing don’t substantially damage marmoset ovarian tissues and that the cryopreserved tissues retain its ability to support the development of large antral follicles after transfer beneath the kidney capsule of immunodeficient mice (Candy et al., 1995).

In this study, the significant reduction in the number of different types of follicles could be due to inadequate penetration of the cryoprotectants especially in the cells at the center of the tissues. Could also be due to the toxic cellular effect of the cryoprotectants. This cellular toxicity of the cryoprotectants can vary from non-specific impairment of cellular components (e.g., disruption of membranes, denaturation of proteins) to a specific action on distinct enzymes.

On the electron microscopic level, after vitrification and thawing of ovaries, Salehnia et al. 2002 concluded that, no noticeable changes occurred in the organelles of oocytes and follicular cells and the integrity of subcellular structure was well preserved (Salehnia et al., 2002). The same results were confirmed by Oktay et al. 200 when they showed that most cells lacked ultra-structural signs of damage but after cryopreservation by slow cooling technique (Oktay et al., 2001). Gook et al. 1999 showed that reasonable survival of both pregranulosa cells and oocytes can be achieved with the slow cooling procedure (Gook et al., 1999), while Nisolle et al. 2000 reported that ovarian tissue cryopreserved by slow cooling showed follicles that seemed to be degenerating and that these signs are more frequent in secondary follicles than in primordial follicles (Nisolle et al., 2000).

In this study, we have shown also an insignificant difference between the number of different types of ovarian follicles in the cryopreserved and fresh grafts. This was in agreement with Kagabu and Umezu 2000 who evaluated by histological examination the effects of vitrification, thawing and transplantation on the ovarian tissues from mouse, Chinese hamster, rabbit, Japanese monkey and rat and concluded that their results suggest that vitrification, thawing and transplantation do not greatly damage the ovarian tissue (Kagabu and Umezu, 2000). Candy et al. 1995 also showed no difference between grafts of fresh and cryopreserved
ovaries by slow freezing (Candy et al., 1995). Wang et al. 2002 indicated that there was no statistical significant difference between the grafts in the control and cryopreserved groups, with respect to the follicle number (Wang et al., 2002).

Our findings were not in agreement with Nisolle et al. 2000 who showed that the follicular density was significantly lower in transplanted cryopreserved ovarian grafts than in the fresh ovarian grafts (Nisolle et al., 2000). Salle et al. 1999 indicated that the frozen/thawed autografts contained cavitary and numerous primordial, primary and secondary follicles. However, the number of primordial follicles was greatly diminished compared with the fresh control sections (Salle et al., 1999).

Neovascularization of the graft remains one of the key factors for its survival. Ovarian tissue is a rich source of angiogenic factors that encourage rapid migration of endothelial cells into the grafts and early restoration of blood circulation. The stress of cryopreservation may stimulate the vitrified-thawed ovarian tissue to produce high levels of the angiogenic growth factors allowing rapid revascularization of the cryopreserved grafts and minimizing as much as possible the ischemic injury. Qu et al 2000 investigated the possible influence of freezing and thawing on the immunoreactivities of TGF-α, EGF and EGF receptor in frozen ovarian tissue and the authors observed that there was no significant difference in immunostaining for TGF-α, EGF and EGF receptor between fresh and frozen ovarian tissues, indicating that human ovarian tissue could be frozen without substantially altering the immunoreactivities of TGF-α, EGF and EGF receptor in the follicles (Qu et al., 2000). However, further investigations are thus needed in order to evaluate the influence of cryopreservation, thawing, and transplantation on the angiogenic growth factors. Equally important, is to evaluate the influence of the angiogenic growth factors on the viability of the transplanted ovarian tissue.

The idea of intra-uterine implantation of the ovarian tissue is not a new one, it dates back to year 1895. Estes operation was employed quite widely during the first half of this century. This operation consists of bilateral salpingectomy and unilateral oophorectomy followed by implantation of the cut surface of the remaining ovary (still attached to its pedicle) into the resected stump of one
oviduct and portion of the uterine horn. In 1961, Ikle reported 29 pregnancies out of 270 patients underwent Estes operation for tubal problems (Adams, 1979). In the rabbit, fertilization in utero is possible, as demonstrated by Chang (1955), Bedford (1969), Adams (1970) and Glass (1972). Chang transferred 26 freshly ovulated eggs to the uterus of a mated doe; at recovery 6 hours later, the eggs were found fertilized but some shows signs of degeneration. A finding which the other investigators has each confirmed (Adams, 1979). An orthotopic autograft of the ovarian biopsy may allow spontaneous pregnancy while heterotopic autograft can restore ovarian secretion, however, medical assistance would be necessary to obtain a pregnancy (Salle et al., 1998). In this study we have shown for the first time that heterotopic autograft of cryopreserved as well as fresh ovarian tissue without vascular pedicle can lead to spontaneous pregnancy without medical assistance. Fecundity of the ovarian grafts was expressed as the total number of live pups/ total number of the grafted ovarian tissues. The values were 0.2 and 0.42 for the cryopreserved and fresh ovarian grafts respectively. Despite the partial reduction in fecundity, the present results indicate that the fresh as well as cryopreserved/thawed ovarian tissues were functional and fertile after heterotopic transplantation into the recipients. This reduction in fecundity may be due to expulsion of the ova from the uterus soon after ovulation as stated by Estes and Heitmeyer (Adams, 1979). In the present study, we tried to investigate the possible cause of low fecundity, so, 5 grafted uterine horns, were excised from mice in which pregnancy have failed to occur. Histological examination revealed disappearance of the graft from one uterine horn. This is in agreement with Candy et al. 1995 who recovered only 10 grafts out of 13 in the first series and 4 grafts out of 6 at autopsy in the second series (Candy et al., 1995). Newton et al. 1996 also identified and recovered 100 out of 102 grafts from the original graft sites (Newton et al., 1996). The grafts were covered by endometrium in 3 uterine horns. This layer of the endometrium acts probably as a barrier, thus preventing the ovum from reaching the uterine cavity. This finding was also reported by Beyth and Polishuk, 1979 in their study to evaluate the ovarian implantation into the uterus (Beyth and Polishuk, 1979). Also, Salle et al. 1998 found, after orthotopic ovarian transplantation and failure of pregnancy, that the grafts were covered by peritoneum (Salle et al., 1998). In the last uterine horn, no explanation was found.
**Ethical and legal considerations of clinical applications**

The clinical application of ovarian cryopreservation and transplantation contains potential legal and ethical issues, as has happened with other new reproductive technologies (Aubard, 2000). However, elective oophorectomy and autologous replacement to preserve reproductive and hormonal function in women facing ovarian disease or cancer appears to be an ethically acceptable procedure in medically appropriate cases. Its goal of enabling an otherwise infertile woman to produce oocytes that could lead to the birth of a healthy child is the goal underlying most infertility treatment. If the surgical retrieval and replacement is safely performed and there is a reasonable basis for concluding that the oocytes are healthy, there is no ethical objection to providing this procedure as a way to preserve fertility for medically suitable candidates (Aubard, 2000). This is the beginning of a new technology that has numerous unresolved ethical and legal issues, including clinical indications, safety, age limits, time limit for storage, and tissue custody. Defining clinical indications and resolving safety issues will be an ongoing effort, along with improvement of the technology. In addition, there is a high possibility of extending the application of the technology beyond the present indication (John and Robertson, 2000).

The problem of informed consent leads directly to the next ethical problem. The child facing castration by anticancer treatment constitutes one of the best present indications for OTCP. But how can truly informed consent for this procedure be obtained from a child? How can the usefulness be weighed of a technique that she will only benefit from in 10 or 20 years and that will be performed by doctors who likely will not be the same as who extracted the tissue. Parents are often completely overwhelmed by the choices they have to make for their child (Aubard, 2000).

Is it ethical to harvest and freeze ovarian tissue without a certainty of success in transplantation? This technique still in a phase of clinical research and because it is unknown now how in the future the ovarian tissue, that was extracted from patients today, will be used, it is essential to very clearly inform patients of this situation and of the limits of the present knowledge to have a truly informed consent (Aubard, 2000).
If oophorectomy-biopsy and cryopreservation occur to preserve ovarian tissue, a decision will have to be made later whether to use that tissue for reproduction. In the case reported by Oktay and colleagues the woman who had requested cryopreservation had the tissue replaced in her pelvis 2 years after removal. Now that ovarian function has been confirmed, she may seek to have children through IVF of oocytes produced by the replaced ovary. If in vitro maturation of eggs becomes feasible, the preserved tissue could be induced to mature in vitro and obviate the need for autologous replacement. In either case, her eggs will have to be fertilized in vitro for conception to occur (John and Robertson, 2000).

A major ethical issue would arise with this procedure if a woman requesting use of her preserved ovarian tissue has reached the customary age of menopause in her culture or has exceeded a designated age limit, e.g., 55 or 60. Perhaps the most important ethical factor in this situation is the health of an older woman requesting autologous replacement or in vitro maturation of her oocytes for the purpose of initiating a pregnancy in herself. She should not be free to establish a pregnancy in herself via these techniques unless she meets the health and responsibility criteria for postmenopausal pregnancy that programmes set for egg donation or other assisted reproductive techniques for older women. Aside from concerns of physical health, some people have argued against the use of medical technologies to extend the reproductive age of women beyond normal age of menopause on the ground that it is unnatural or culturally inappropriate. Such an argument is difficult to sustain if gender-based distinctions in access to assisted reproductive technologies are rejected, for some men reproduce at comparable or older ages. Nor would protecting the welfare of resulting children be a convincing argument against postmenopausal replacement or use of preserved ovarian tissue, if the circumstances show that the woman and her partner are likely to be responsible child rearers. Although reasonable people might differ on this issue, if there is reason to think that the woman or couple would be responsible childrearers, there is no compelling ethical reason beyond health to deny postmenopausal women this option (John and Robertson, 2000).

Women electing ovarian preservation who face oophorectomy for cysts, endometriosis, or other noncancerous conditions will incur no further risk from the procedure, because they would in any case be undergoing an operative
procedure. Women facing treatment for non-ovarian cancers, on the other hand, would have to undergo oophorectomy prior to beginning cancer therapy, which could be highly burdensome for many patients. However, the risks and burdens of elective oophorectomy for later replacement would clearly appear to be within the range of risks and burdens that competent adults facing cancer therapy may choose to undergo. If a newly diagnosed cancer patient competently assesses the risks and benefits of the procedure and concludes that the benefits of preserving her ovarian function are worth the burdens of elective oophorectomy prior to cancer treatment, she and her physician should be free to elect this procedure (John and Robertson, 2000).

It is unlikely that otherwise healthy women would request such a procedure. Elective oophorectomy would have to be performed in a woman’s 20s or early 30s to ensure viability of oocytes. Few healthy women in this age group are likely to undergo the costs and burdens of elective oophorectomy merely to hedge against the unknown future risk of gametic infertility. Unless they know to a high degree of certainty that they will not reproduce in their fertile years, the burdens of elective oophorectomy would seem so much greater than the benefits that a physician who performed an oophorectomy in this situation might well be violating the medical ethical principles. A dramatic reduction in the costs and burdens of ovarian removal might increase the demand for ovarian preservation from healthy women. For example, proposals to obtain ovarian tissue by biopsy followed by autologous replacement or in vitro maturation of follicles and oocytes might increase the number of women in their 20s who choose some form of ovarian preservation. Still, few healthy women in this age group are likely to be so concerned about their future infertility that they would seek a surgical intervention not covered by insurance merely to hedge against the possibility that they might have lost the ability to produce viable oocytes at a future time when they wish to reproduce. If ovarian biopsy proves to be a safe and effective way to obtain ovarian tissue for preservation, the main beneficiaries would be women facing cancer treatment, not healthy women generally (John and Robertson, 2000).

Ovarian tissue could not be transplanted into other women on medical grounds, as it would require use of drugs such as cyclosporin to prevent rejection, increasing the risk of malignant disease. The use of such drugs for transplantation of organs
on grounds other than a threat to a person’s life was not accepted by an ethics committee at the Epworth Hospital, Melbourne, when tubal transplantation was proposed to assist in the treatment of infertility (John and Robertson, 2000).

Finally, xenomaturation of human follicles is already possible up to antral follicles and it is probable that this procedure will be functional well before in vitro growth and maturation. However, ethical concerns raised by this technique are not at all trivial, because of the possibility of alterations in the human genome or the transmission of hitherto unknown infectious agents (Donnez et al., 2000). Although engaging in ovarian banking may be a personal choice of the patients who desperately want to preserve fertility with no other option, it is also the responsibility of the medical and scientific communities to prevent misunderstanding and misuse of this emerging technology. There are many reasons, after all, to proceed with care in developing this technology (Aubard, 2000).
CONCLUSION

1. The ovary can tolerate exposure to osmotic dehydration and vitrification in a concentrated solution of cryoprotectants.

2. Vitrification affects all types of follicles in the cryopreserved biopsies.

3. The murine uterus is a suitable place for ovarian tissue transplantation and can lead to natural pregnancy.
REFERENCES


Aubard Y, Poirot C, Piver P, Galinat S, and Teissier MP. Are there indications for


Fabbri R, Venturoli S, Errico AD, Iannascoli C, Gabusi E, Valeri B, Siracchioli R,
and Grigioni WF. Ovarian tissue banking and fertility preservation in cancer patients: histological and immunohistochemical evaluation. Gynecology Oncology 2003; 89:259-266.


Fuku E, Xia L, and Downey BR. Ultra-structural changes in bovine oocytes cryopreserved by vitrification. Cryobiology; 1995:139-156.


Gosden RG, and Nagano M. Preservation of fertility in nature and ART. Reproduction 2002; 123:3-11.


Harp R, Leibach J, Black J, Keldahl C and Karow A. Cryopreservation of murine
Kuleshova LL, and Lopata A. Vitrification can be more favorable than slow cooling.


Macklon NS, and Fauser BCJM. Follicle development during the normal menstrual cycle. Maturitas 1998; 30:181-188.


Sanders JE, Hawley j, and Levy W. Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. Blood 1996; 87:3045-3052.


Thomson AB, Critchley HOD, Christopher JHK, and Wallace WHB. Late reproductive sequelae following treatment of childhood cancer and options for fertility preservation. Best Practice and Research Clinical Endocrinology and Metabolism 2002; 16:311-334


