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PhD THESIS IN

NOVEL TECHNOLOGIES FOR MATERIALS, SENSORS AND IMAGING

INNOVATIVE BIOMATERIALS FOR DEVELOPMENT OF NEW STRATEGIES TO PRESERVE FERTILITY IN CANCER WOMEN

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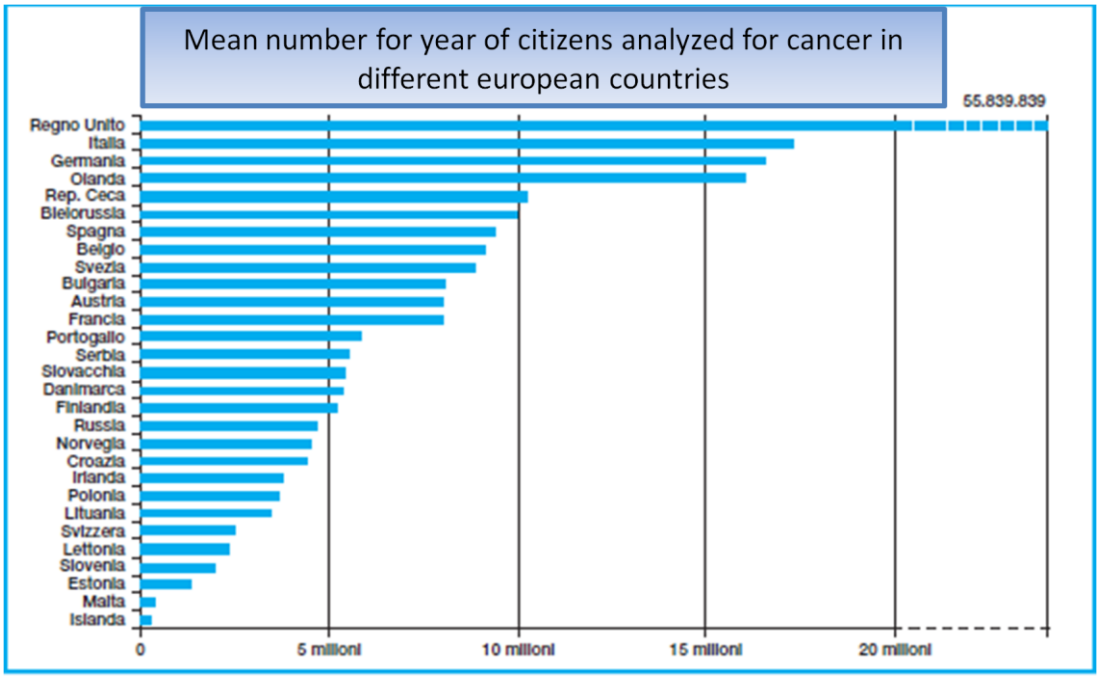
INTRODUCTION

1. Cancer incidence and mortality in Italy

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (World Health Organization, 2008). The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets. The total number of cancer deaths by country are collected annually and are made available by the World Health Organization (WHO) (World Health Organization Databank WHO Statistical Information System, 2010).

In the last edition of *Cancer Incidence in 5 Continents*, the presence of population-based cancer registries in European Countries varied widely. In some Countries (e.g.Greece) cancer registries were not present, in others they extended nationally. In Italy, as in several other Countries (Portugal, Spain, France, Belgium, Germany, Poland, Switzerland and The United Kingdom) cancer registries have a regional coverage. The Graph1 shows the mean number of citizens in each European Country who are observed by cancer registries. The United Kingdom ranks first with about 56 million citizens, Italy is second with more than 17 million. Italian cancer registries are part of the Italian Network of cancer registries – AIRTUM (www.registri-tumori.it). AIRTUM has a common centralized data-base where data from all the Italian cancer registries are uploaded after quality checks. After the publication of *Cancer Incidence in 5 Continents vol. IX*, cancer registration in Italy has also extended, with the support of AIRTUM, to include new registries. Nowadays, the cancer registration in Italy involves almost 20 million citizens and several others are under the control of Specialized (for age, or type of cancer) ones. In Italy there is no national cancer registry, but AIRTUM puts our Country among the European leaders with regard to the capacity to provide information on cancer epidemiology.



Graph 1: mean number of citizens in each European Country

A **cancer incidence rate** is the number of new cancers of a specific site/type occurring in a specified population during a year, usually expressed as the number of cancers per 100,000 population at risk. That is,

Incidence rate = (New cancers / Population) × 100,000

The numerator of the incidence rate is the number of new cancers; the denominator is the size of the population. The number of new cancers may include multiple primary cancers occurring in one patient. The primary site reported is the site of origin and not the metastatic site. In general, the incidence rate would not include recurrences.

In the area of the Italian Network of Cancer Registries there were, on yearly average during 1998-2002, 783.4 cancer cases diagnosed every 100,000 males and 613.1 every 100,000 females. The most frequently diagnosed cancers were non-melanoma skin cancers (15.2%), prostate (14.4%), lung (14.2%), urinary bladder (9.0%), and colon cancer (7.7%) among males and breast (24.9%), non-melanoma skin cancer (14.8%), colon (8.2%), lung (4.6%), and stomach cancer (4.5%) among females. In Italy, 162,756 new cancers among males and 129,247

among females have been estimated to be diagnosed yearly. As regards mortality, there were 89,561 cancer deaths among males and 66,471 among females in 2002. The cumulative risk (0-74 years) of developing a cancer is 1 case every 3 men and 1 case every 4 women.

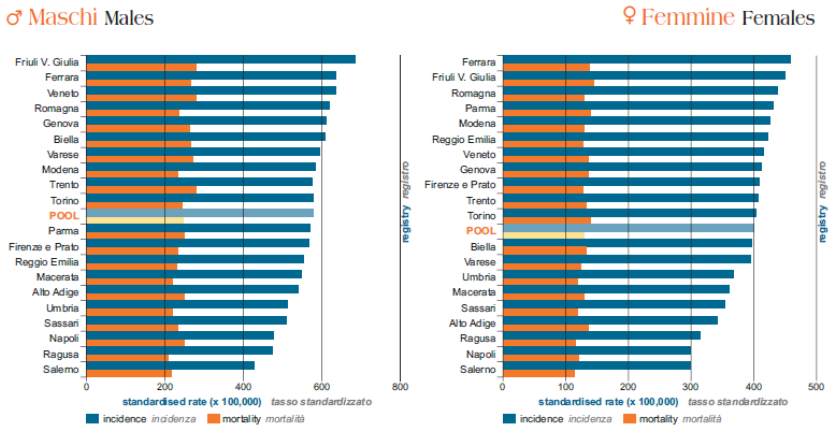


Fig. 1: incidence e mortality for cancer in Italy

1.1 Cancer in enfants

Recent data coming from AIRTUM Working group about children cancer incidence (Epidemiol Prev 2008; 32(2) Suppl 2: 1-112) indicate that there is a growth trend of both all new cases of cancer and mean tumor sites at 0-14 years. Particularly, there are 168,5 cancer cases diagnosed every 1.000.000 children and the most frequently diagnosed are leukemia (52,1) and acute lymphatic leukemia (40,5).

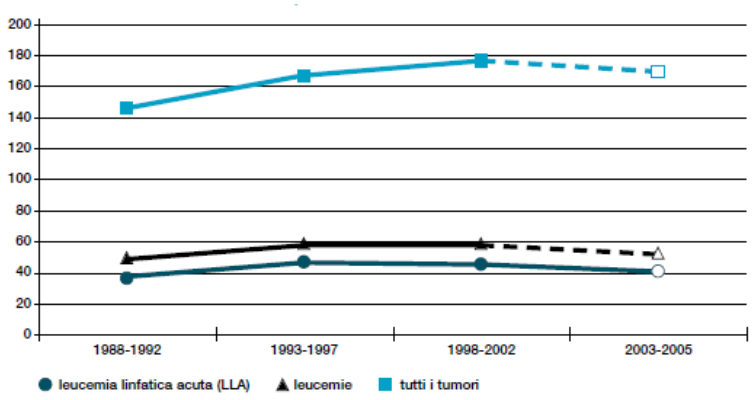


Fig. 2: cancer incidence at age 0-14 years (AIRTUM)

2. Chemotherapy and radiotherapy

Two-thirds of women <40 years at diagnosis will have a tumor is >2 cm in size and/or involved axillary lymph nodes (stage II or higher) (Rosenberg R. et al., 2003). Almost all women with stage II tumors and even most with stage I disease with a tumor greater than 1 cm in size will be advised to have gonadotoxic chemotherapy (Goldhirsch A. et al., 2009). At least two-thirds of women under 40 will have a hormone receptor positive tumor and in addition to chemotherapy (or as a single modality in women with favorable tumors) will be advised to undergo 5 years of antihormone therapy with a GnRH agonist. Adjuvant treatment is selected based on both the risk of recurrence and the biologic characteristics of the tumor. The 15-year risk of recurrence and death for women under 50 not receiving adjuvant systemic therapy is 53% and 42%, respectively, irrespective of stage or biologic characteristics according to Early Breast Cancer Trialists (EBCTCG) meta-analyses with the latest major published outcomes in 2005 (EBCTCG, 2005). These figures dramatically differ by stage and biomarker variables, with a 12.5% breast cancer mortality rate at 15 years for women under 50 with low-risk node-negative tumors, 25% for women with high-risk node-negative tumors, and 50% for node-positive tumors (EBCTCG, 2005). Biologic characteristics with the greatest impact on treatment selection include estrogen and progesterone receptors (ER and PR), proliferation (usually measured by Ki-67), and presence of the growth factor receptors, such as HER-2 neu (Perou CM et al., 2000).

2.1 Drug effect and their toxicity

Specifically, anticancer drugs diminish the primordial follicle pool, cause ovarian atrophy, and harm the ovarian blood vasculature (Reulen RC et al., 2009). Possible mechanisms of damage include follicular apoptosis and cortical fibrosis. Recent hypothesis suggests increased activation of follicles from the resting pool, resulting in accelerated atresia, and eventually a premature ‘‘burn-out’’ of the primordial follicle reserve. However, the exact mechanism of injury still remains unclear. Importantly, the magnitude of anticancer drug-induced damage is variable: in some instances, therapy can be sterilizing, whereas often instead,

women suffer from partial ovarian injury. The extent of damage is related to patient's age, chemotherapeutic agent, and drug regimen used (Critchley HOD et al., 2005). When treated with radiation, variable effects are also related to the location of the irradiation field. Although achieving a pregnancy years after cancer treatment is safe (Wallace WH et al., 2010), exposure to anticancer drug therapies may harm the quality of maturing eggs and therefore, concern has been raised regarding pregnancy and the health of future offspring conceived with oocytes exposed to chemotherapy in a non dormant state. Radiation to the uterus can interfere with implantation and uterine growth during pregnancy, resulting in poor obstetric outcome (Nicosia S et al., 1985). As cancer treatment improves, the number of childhood cancer survivors wishing to have healthy children has grown. As a result, it has become imperative to fully understand the mechanisms that lead to ovarian damage in the prepubertal and adult state and the impact variables such as age and treatment regimen have on the magnitude of injury for the physicians to effectively counsel patients in future attempts at successful pregnancies.

2.2 Chemotherapy induced ovarian damage

At birth, the ovary contains a finite number of oocytes that are surrounded by a single layer of pregranulosa cells to form primordial follicles (about 2 millions). By 5 months of gestational age, the female ovary establishes a fixed number of primordial follicles and therefore, the number of primordial follicles is a direct indication of fertility reserve. Throughout the life cycle, there is an ongoing decline in the number of primordial follicles that is the result of apoptotic cell death. Eventually, this loss of primordial follicles results in menopause at an average age of 50 to 51 years. In a recent study, the first model of human ovarian reserve from conception to menopause that best fits the combined histologic evidence has been described (Wallace WH et al., 2010). This model suggests that 81% of the variance in primordial follicle population is exclusively owing to age and analysis showed that 95%of the fluctuation in follicular reserves is owing to age alone for ages up to 25 years. When ovarian functioning is disrupted by

anticancer drugs, the effects can be devastating. Clinically, patients may suffer from complete ovarian failure resulting in amenorrhea, climacteric symptoms, an increase in gonadotropins, and low estradiol levels (Meirow D, 2000). Histologic studies show that the end stage effects of chemotherapy are ovarian atrophy, a depletion of the primordial follicle stockpile, diminished ovarian weight, and stromal fibrosis (Nicosia S et al., 1985). Alternatively, chemotherapy's effects may be partial and patients may experience a reduction in their primordial follicle stockpiles, menstrual irregularities, and hormonal disturbances, but may still be able to maintain menses post-treatment. In animal studies, chemotherapy has been shown to cause a significant loss in both maturing follicles and dormant primordial follicles. In mice, chemotherapy causes the destruction of growing follicles (Utsunomiya T et al., 2008). Chemotherapy also results in diminished primordial follicle stockpiles in mice in a dose-dependent manner (Meirow D et al., 1999) and has been correlated to a reduction in primordial follicles in the rhesus macaque (Ataya K et al., 1995). Chemotherapy has differential effects on primordial, dormant follicles and growing, larger ovarian follicles. Chemotherapy targets actively dividing cells, and therefore, destroys mature ovarian follicles during treatment, specifically by inducing apoptosis in granulosa cells. Mice exposed to combination chemotherapy (Ironotecan HCl) show TUNEL positive granulosa cells in large ovarian follicles (Utsunomiya T et al., 2008; Meirow D et al., 1999; Ataya K et al., 1995; Philosof-Kalich L et al., 2009).However, the effects that chemotherapy has on primordial, dormant follicles are variable and the question remains as to whether the same effect is observed in these follicles. Clinically, patients exposed to chemotherapy initially stop menses as a result of the destruction of growing follicles and resume cycling after a period of recovery. Even low doses of chemotherapy can wipe out the population of maturing follicles, but partial ovarian reserve remains intact, allowing for the eventual resumption of menses. The means by which chemotherapy induces damage to the primordial follicle stockpiles, which represent future fertility potential, remains unclear. It is therefore, of paramount importance to understand the mechanisms by which chemotherapy injures the follicular stockpile to develop ways to improve fertility post anticancer drug exposure.

2.3 Follicular apoptosis

Chemotherapy has been suggested to induce damage to primordial follicles by inducing apoptosis. Electron microscopy has shown that within hours of chemotherapy exposure, primordial follicles become surrounded by abnormally thick basal lamina (Familiari G et al., 1993). A substantial body of evidence has documented in vitro evidence of primordial follicle apoptosis (Meirow D et al., 1999) and chemotherapy treatment in vitro has also been shown to cause primordial follicle architecture disruption and pregranulosa cell swelling. Preliminary in vivo studies using human ovarian xenografts in SCID mice have also shown indications of primordial follicle apoptosis (Oktem O et al., 2007). More research is needed to verify that primordial follicle apoptosis does indeed occur in true in vivo conditions, and whether the oocyte or surrounding granulosa cells are the primary target. Alternatively, other mechanisms of damage, such as cortical fibrosis and follicular ‘‘burn-out’’ have been suggested to explain the variable loss of follicular reserves.

2.4 Cortical fibrosis

It is clear that chemotherapy results in ovarian cortical fibrosis and blood vessel damage (Nicosia S et al., 1985). In a study conducted on human ovarian tissue exposed to combination chemotherapy in vivo, hyalinization of cortical blood vessels, neovascularization, and cortical fibrosis were observed. These modes of injury result in local ischemia, thereby affecting the growth and survival of primordial follicles. Triangular areas of fibrosis have been observed to coincide with a depletion of primordial follicles, indicating that blood vessel damage results in primordial follicle injury. This may also impair the processes of new vessel formation that are critical for normal follicle growth within the territory of the damaged vasculature. If apoptosis had initially led to a diminished need for blood vasculature, then a uniform pattern of primordial follicle loss would be expected; this however, is not the observed trend (Meirow D et al., 2007).

2.5 Follicular "BURN-OUT" hypothesis

A novel hypothesis to explain the loss of primordial stockpiles is the ''burn-out'' mechanism. When exposed to chemotherapy, growing follicles are destroyed and therefore, there is a possible decrease in granulosa cell-derived paracrine growth factors that inhibit primordial follicle recruitment, such as anti Mullerian hormone (AMH). AMH null mice show increased activation of primordial follicles, resulting in greater numbers of large follicles that undergo atresia, and eventually resulting in a premature ''burn-out'' of the primordial follicle reserve (Durlinger ALL et al., 1999). As a result, a possible mechanism by which chemotherapy may cause a depletion of primordial follicles is by increasing the recruitment of dormant follicles into the pool of actively growing follicles. An expected result of chemotherapy would, therefore, be an increase in large follicles and a decrease in primordial follicles. When mice were exposed to cyclophosphamide, an increase in the ratios of both primary and secondary follicles to primordial follicles was observed, thus supporting the ''burn-out'' hypothesis (Philosof-Kalich L et al., 2009). Premature activation may therefore, contribute to the deleterious effects of chemotherapy on the ovary.

TABLE 1. Cytotoxic Agents According to Degree of Gonadotoxicity

High Risk	Intermediate Risk	Low/No Risk
Cyclophosphamide	Doxorubicin	Methotrexate
Busulfan	Cisplatin	Bleomycin
Melphalan	Carboplatin	5-Fluorouracil
Chlorambucil		Actinomycin D
Dacarbazine		Mercaptopurine
Procarbazine		Vincristine
Ifosfamide		
Thiotepa		
Nitrogen mustard		

Table 1: cytotoxic agents according to degree of gonadotoxicity

2.6 DRUG EFFECT

There are 6 main classes of chemotherapeutic drug groups: alkylating agents, platinum derivatives, antibiotics, antimetabolites, plant alkaloids, and the taxanes. The extent of ovarian damage is influenced by the type of drug being

administered (Lee SJ et al., 2006). Alkylating agents are often and effectively used in combination chemotherapy treatment but they are also associated with particularly high levels of premature ovarian failure. Indeed, alkylating agents are responsible for the highest age-adjusted odds ratio of ovarian failure rates, followed by other drug families (Meirow D., 2000) (Fig. 3).

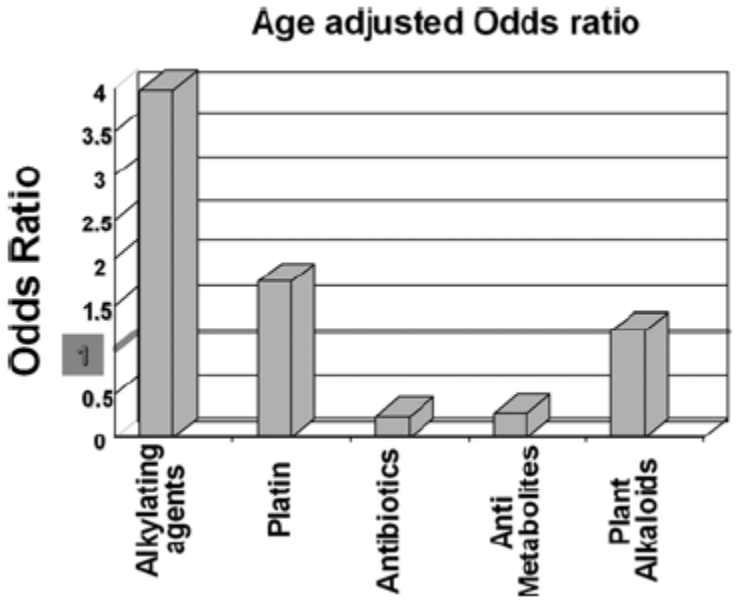


Fig. 3: age-adjusted odds ratio of ovarian failure rates

There are limited data surrounding the effects the taxane family has on ovarian failure rates, however; it has been documented that patients suffer from gonadal toxicity and high FSH levels (Anderson RA et al., 2006). In addition, the inclusion of paclitaxel, a member of the taxane family, to traditional AC (doxorubicin, cyclophosphamide) treatment significantly increases ovarian damage after treatment as measured by recovery of regular menstrual cycling (Petrek JA et al., 2006). Cancer patients usually undergo combination chemotherapy treatment, and a number of studies have reported the effects treatment protocols have on ovarian function. In breast cancer patients, individuals who underwent combination chemotherapy with regimens containing cyclophosphamide, methotrexate, and fluorouracil suffer from the highest amenorrhea rates. CAF (cyclophosphamide, doxorubicin, and fluorouracil)

treatment also induced high rates of ovarian failure whereas AC (doxorubicin and cyclophosphamide) treatment resulted in a greater eventual recovery of ovarian function after treatment (Meirow D et al., 2004). In a study of the incidence of menstrual bleeding after chemotherapy treatment in breast cancer patients, there was a 48% bleeding rate in individuals 1 month after cyclophosphamide, methotrexate, and fluorouracil treatment. This rate declined significantly over time. Alternatively, patients that underwent AC with or without paclitaxel had an initial significant decline of menstrual bleeding, slow recovery, and eventual restoration of regular menstrual bleeding in approximately 50% of patients (Petrek JA et al., 2006) (Table 2). Patients treated for Hodgkin lymphoma experience varying impacts on fertility potential based on the course of their treatment. Treatment with protocols containing alkylating agents such as MVPP (Nitrogen Mustard, vinblastine, procarbazine, and prednisolone), COPP (cyclophosphamide, vincristine, procarbazine, and prednisolone), or ChlVPP (chlorambucil, vinblastine, procarbazine, and prednisolone) had relatively high rates of ovarian damage, with amenorrhea rates between 38% and 57%. Alternatively, in treatment with ABVD chemotherapy, (doxorubicin, bleomycin, vinblastine, and dacarbazine) significantly less ovarian damage is reported (Meirow D et al., 2004; Brougham MFH et al., 2005). In the study of Hodgkin lymphoma survivors attempting to achieve pregnancy, it was found that ABVD treatment did not hinder fertility potential, as pregnancy rates were comparable with controls (Hodgson DC et al., 2007) (Table 2). Variable results have been reported regarding the effects treatment protocols have on non-Hodgkin lymphoma patients. Patients treated with a variety of drug regimens have documented relatively high ovarian failure rates, with sterilization rates of 44%. Patients treated for leukemia have low rates of reproductive failure after treatment and in a study of 47 patients, only 15% of the cohort experienced ovarian failure after treatment with combinations of the drugs ARA-C, daunorubicin, mitoxantrone, and VP-16 (Meirow D et al., 2004). Patients that undergo bone marrow transplantation (BMT) have extremely high ovarian failure rates. The risk of ovarian failure after treatment with total body irradiation (TBI) as conditioning for a bone marrow transplant is significant, but less predictable. In a large study of

718 long-term survivors, treated with chemotherapy and/or TBI as conditioning treatment before a bone marrow transplant, 532 had received TBI (10 to 15.75 Gy, single exposure or fractionated) and 186 chemotherapy, with either cyclophosphamide or busulphan. After TBI, 90% developed ovarian failure and after cyclophosphamide or busulphan 60% failed. There were 16 spontaneous pregnancies to 13 women who had received TBI, 6 of these women were prepubertal at the time of their radiotherapy (Sanders JE et al., 1996). In an additional study of 63 cancer patients who had undergone BMT, only 5 individuals did not experience a premature menopause, giving a failure rate of 92%.3 Additional studies further document this high risk of damage, with BMT inducing ovarian failure rates ranging from 72% to 100% (Meirow D. et al., 2004) (Table 2). It is clear that chemotherapeutic regimens, drugs, and age all have differential impacts on ovarian function. It is important for clinicians to fully realize the variable effects treatment can have on a patient's ability to resume menses after completing therapy to effectively consult patients in fertility preserving options, especially in the case of young patients that hope to continue building their families in the future.

Breast Cancer

Reference	Treatment	Age	Ovarian Failure
Lower et al[25]	Premenopause		45%
		< 35 y	28%
Bines et al[26]	Premenopause		68%
Meirow[27]		< 44 y	50%
Goodwin et al[28]	CMF	43.7 ± 5.2	65%
Burstein and Winer[29]	CMF	30	19%
		30-39	30-40%
	CAF	< 30	0%
		30-39	10-25%
	AC	< 30	0%
		30-39	13%
Jonat[30]	Premenopause		60%
Petrek et al[21]		< 35	15%
		35-39	39-55%
		> 39	Higher than 55%
Hodgkin Disease			
Howell and Shalet[31]	Aggressive treatment		38%-57%
Meirow[27]	Relapse post 1st treatment		32%
Bokemeyer[32]	Infradiaphragmatic Rx.		50%
Brusamolino et al[23]	Ovarian sparing protocol	< 25 y	0%
		< 45 y	30%
Behringer et al[33]	Dose-escalated BEACOPP	> 30 y	95%
		< 30	51%
Bone Marrow Transplantation (BMT)			
	No. patients	**Age**	**% ovarian failure**
Sanders et al[34]	73	38 (mean)	99%
Teinturier et al[35]	21	2-17	72%
Thibaud et al[36]	31	3.2-17	80%
Meirow[27]	63	29 (mean)	79%
Grigg et al[37]	19	30 (mean)	100%

AC indicates oxorubicin cyclophosphamide; CAF, cyclophosphamide doxorubicin and fluorouracil; CMF, cyclophosphamide, methotrexate, and fluorouracil.

Table 2: ovarian failure rates tables

2.7 Radiation induced ovarian damage

High-dose ionizing radiation is used to treat many types of cancer, and central nervous system and hematologic malignancies. Whenever possible, the gonads are shielded from radiation, however, this is not possible when the radiation field overlaps with the ovaries or when total body irradiation is required as a conditioning treatment for bone marrow transplantation. Radiotherapy to the pelvis results in ovarian injury and diminished follicle reserve; the extent of damage is largely based on patient's age, treatment dose, and the irradiation field. The location of the radiation field impacts the degree of ovarian damage with total body irradiation observed to result in ovarian failure in 90% of patients in long-term follow-up (Sanders JE et al., 1996). After abdominal radiation, ovarian

failure rates may be as high as 97% (Wallace WH et al., 1989). Irradiation dosage and patient age also largely determine the degree of follicular loss and the likelihood of developing premature menopause. Approximately, half of the total number of nongrowing follicles are lost at doses of 2Gy (Wallace WHB et al., 2003) (LD50). In female cancer survivors, the relative risk of developing premature ovarian failure and infertility rates have both been observed to increase with increasing doses of abdominal pelvic radiation. In addition, older patients are more susceptible to radiation-induced damage. Lower doses of radiation cause ovarian failure in older patients whereas young patients are more resilient, and succumb to ovarian failure at higher doses.

2.8 Radiation induced Uterine Damage

In addition to ovarian injury, childhood cancer survivors that were exposed to radiotherapy reportedly suffer from uterine damage, resulting in an increase in premature deliveries and miscarriage rates. These effects are likely the result of disruption to uterine blood vasculature and a decrease in uterine weight and length; with the extent of damage related to the radiation field and dose. In women who were treated with whole abdominal radiotherapy, the mean uterine length of 10 women was 4.1 cm when compared with 7.3 cm found in controls (Critchley HOD et al., 1992). Endometrial thickness is diminished in women who had earlier undergone abdominal radiation and when treated with exogenous sex steroid replacement, no increase in thickness was reported. In a later study, women who had undergone total body irradiation did show some improvement in endometrial thickness and uterine volume in response to sex steroids (Bath LE et al., 1999). It is likely that the high irradiation doses used in abdominal and directed uterine irradiation result in irreversible damage to the uterus. This is particularly worrisome for women hoping to become pregnant, and even those individuals who decide to use oocyte donors as a result of ovarian failure must realize the higher risk of miscarriage and premature deliveries.

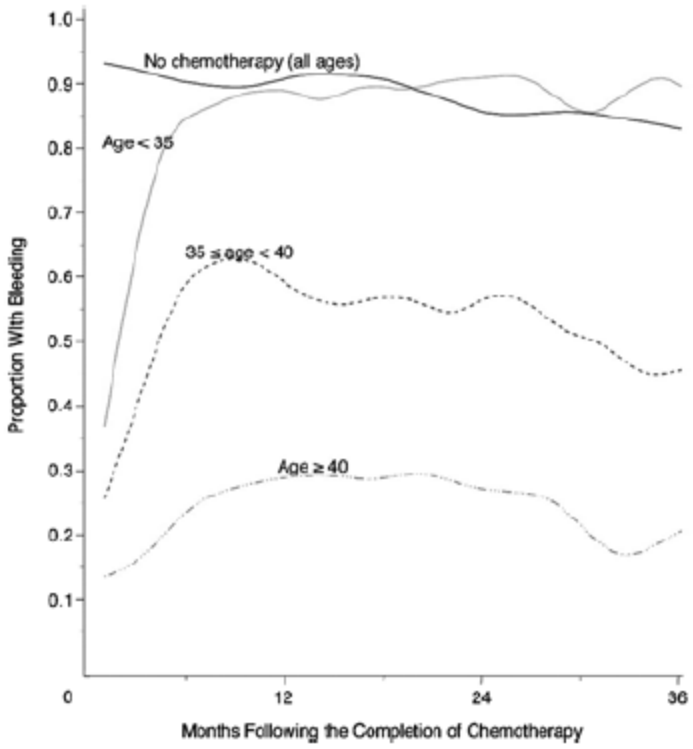
2.9 Determinants of ovarian damages

The primary determinants of chemotherapy-induced amenorrhea and/or loss of fertility are age of the woman at the time of chemotherapy, dose and number of cycles of the alkylating agent received, and, to a lesser extent, exposure to anthracyclines, taxanes, and platinum analogs (Petrek JA et al., 2006; Walshe JM et al., 2006; Gerber B et al., 2008; Hickey M et al., 2009). The aklylating agent, cyclophosphamide, is also one of the most potent in reducing ovarian follicular reserve. A woman who takes the equivalent of 2.4–3 g/m^2 of cyclophsophamide over 12–16 weeks can count on adding an approximate 10 years to her ovarian reproductive age or 1.5–3.0 years per cycle (Petrek JA et al., 2006; Walshe JM et al., 2006; Gerber B et al., 2008; Hickey M et al., 2009). A woman who is 30 at chemotherapy initiation may have an equivalent ovarian age of 40 after 4–6 cycles of cyclophosphamide containing polychemotherapy. Women with a good prognosis breast cancer (stage I node negative) who received either six cycles of CMF or four cycles of AC experienced an estimated 33% rate of amenorrhea (Goldhirsch A et al., 1990; Bonadonna G et al., 1995; Fisher B et al., 1990; Bines J et al., 1996; Jones S et al., 2009). Rates of amenorrhea for more aggressive regimens given to women with poorer prognosis tumors such as six cycles of fluorouracil plus epirubicin or doxorubicin and cyclophosphamide (FEC or FAC), six cycles of AC or four cycles of AC followed by four of docetaxel are higher ranging from 50% to 65% (Levine MN et al., 1998; Hortobagyi GN et al., 1986; Swain SM et al., 2009; Fornier MN et al., 2005; Tham YL et al., 2007). Fifteen to 50 percent of women younger than age 40 at diagnosis will recover menses. Amenorrhea is likely to be permanent in 90% of women over 40 and in 95% of women over 45 (Swain SM et al., 2009; Fornier MN et al., 2005; Tham YL et al., 2007; Perez-Fidalgo JA et al., 2010).

2.10 Age effect and fertility potential

Age plays a crucial role in determining how resilient the ovary will be to chemotherapy treatment. Older patients are more likely to develop a premature menopause after chemotherapy, than are younger individuals. Petrek et al (Petrek

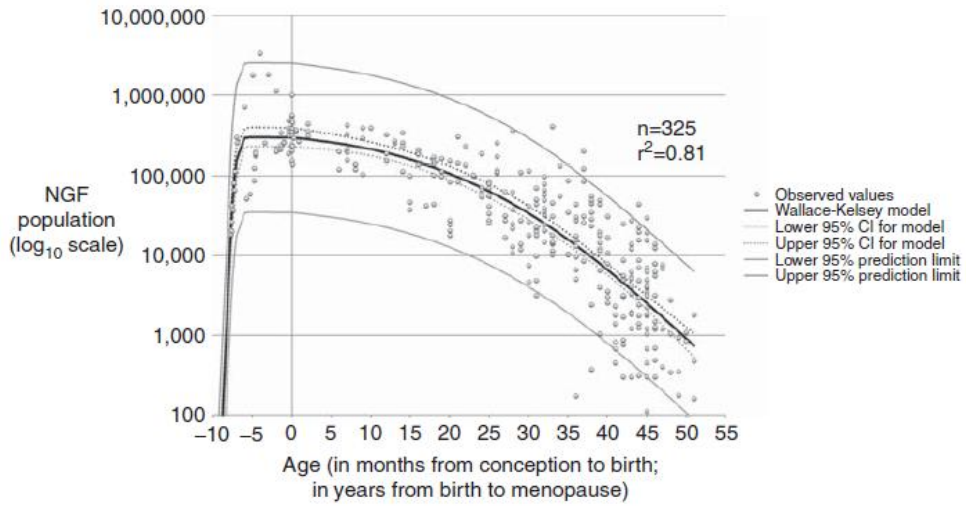
JA et al., 2006) found that age correlated to a decrease in the prevalence of menstrual bleeding as a result of chemotherapy exposure. Breast cancer patients younger than 35 had an approximately 85% recovery in monthly bleeding whereas women aged between 35 and 40 ranged from a 45% to 61% recovery rate and women over 40 years had an even lower recovery rate, with many individuals in this age group never regaining regular menses (Graph. 2).



Graph. 2: recovery in monthly bleeding after chemotherapy as a function of age

In an additional study of 168 individuals, patients who maintained ovarian function post treatment were found to be significantly younger (27.4+ 8.3 y) than those that lost ovarian function (34.7+ 8 y) (Meirow D et al., 2004). Patients treated for Hodgkin lymphoma suffer from significantly less gonadotoxicity when under the age of 25 (Brusamolino E et al., 2000). This age effect is mostly owing to the strong negative correlation between age and the nongrowing follicle (NGF) population, representing ovarian reserve (Wallace WH et al., 2010) (graph. 3).

Older women have diminished primordial follicle stockpiles and therefore, the effects of chemotherapy seem more marked.



Graph. 3: correlation between age and the nongrowing follicle

2.11 Alternative chemotherapy treatment regimens that may reduce follicular damage

Chemotherapy regimens can usually be altered somewhat to reduce gonadotoxicity. For example, three cycles of FEC followed by three cycles of docetaxel provides similar chemotherapeutic efficacy as six cycles of FEC with less ovarian damage due to reduced amount of the alkylating agent (Berliere M et al., 2008). For most Her- 2þ tumors, preliminary evidence suggests that taxane and carboplatin are as effective as anthracycline, cyclophosphamide, and taxane combinations, completely avoid cyclophosphamide, and are probably not as likely to result in sterility (Slamon D et al., 2001; Costa RB et al., 2010). Triple negative breast cancers (ER-, PR-, Her-2 negative) often have multiple deficits in DNA repair pathways, and women with these tumors may selectively benefit from treatment with cis- or carboplatin regimens in combination with Poly-(ADP-ribose) polymerase (PARP) inhibitors (Ismail-Khan R et al., 2010). Trials are

ongoing, but again platin-based regimens in combination with PARP are likely to be less gonadotoxic than cyclophosphamide-based regimens.

3. Preservation of fertility

3.1 Preserving ovarian tissue and function

Three core strategies are employed to preserve fertility and to protect ovaries from the insults discussed earlier. First, the cryopreservation of immature oocytes, mature eggs, or embryos is a favored strategy because high survival rates post-thawing are becoming commonplace (Saragusty J. et al., 2011). However, this strategy can be unavailable as embryo production necessarily requires a partner. Further, the production of significant numbers of mature eggs requires time for a stimulation cycle, and can require hormonal stimulation that is incompatible with treatment (Rodriguez-Wallberg K.A. et al., 2010). Even considering these contraindications, freezing eggs and embryos remains the most effective course of action, as women who survive their treatment with global reproductive tract function intact routinely achieve pregnancy. If these options are unavailable, the removal and cryopreservation of ovarian tissue is a reasonable but experimental strategy. In the second strategy, ovarian tissue containing primordial and primary follicles can be isolated, cryopreserved, and replaced after treatment and recovery. Here, it is hoped that the grafted tissue will allow the resumption of ovarian function and possible conception. The freezing of whole human ovaries and the freezing of small pieces of ovarian cortex, referred to as “cortical strips” are considered in more detail later (Table 3). The third strategy involves protecting intact ovaries from damage. The most common example of this “ovarian transposition,” where an ovary is surgically and mechanically displaced from its normal position so as to minimize direct irradiation during treatment (Chargari C et al., 2009; Dursun P et al., 2009; Bloemers M C et al., 2010).

Tissue	PRO	CON
Oocytes/embryos	No partner required (oocytes) Minimally invasive	Time for ovarian stimulation Oocyte number may be limited Stimulation contraindications Risk of ovarian hyperstimulation
Whole ovary	Full function restored (?)	Invasive Daunting surgery Reseeding cancer One chance!!
Ovarian cortical strips	Rapid Less invasive Proven cryosurvival Multiple chances	Follicle loss Surgical technique Reseeding cancer Variable follicle reintroduction

Table 3: fertility preservation strategies

3.2 Oocyte cryopreservation

3.2.1 Cryobiology

Historically, interdisciplinary researches networked together with the aim of maintaining long-term viability in living cells after cryopreservation. It is well known that biological material show a decrease or loss of viability above – 135°C because of biochemical reactions; consequentially the temperature that is generally used for mammalian cell storage is – 196°C, the same as liquid nitrogen. This should prevent reactions from taking place, because water at this temperature just exists in a solid state and the only possible alteration may be related to DNA damage caused by background reactions. This does not seem to compromise the chance of survival and development of human oocytes or embryos, anyhow. As cells are alive at 37°C and are almost totally inactive at – 196°C, the difficult steps to overcome are related to temperature decrease and the rewarming phase.

These passages represent the key points responsible for cell survival. Chemically, when water is cooled below its freezing point, it solidifies, thus becoming ice. This can cause damages to the cells mainly because of intracellular ice crystal formation. As ice takes more space inside the cell than liquid water and spreads through, it may cause stress and subcellular alterations, eventually, resulting in a

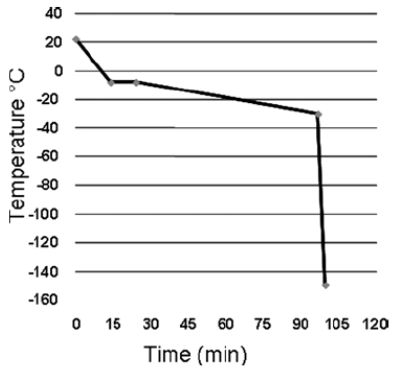
loss of viability after rewarming. The main issue to overcome in any cryopreservation procedure is to consequentially avoid ice crystal formation. All biological systems respond to lowering of the temperature and water solidification in a different manner. Mazur in 1984 (Mazur P., 1984) was the first author who determined, through specific equations, the kinetics of water exchange in the cell and predicted the likelihood of intracellular freezing as a function of the cooling rate. This quantitative description can be explained qualitatively considering that, by definition, water below its freezing point is supercooled showing higher vapor pressure, activity, and chemical potential at a given subzero temperature than that of ice or of an ice-water equilibrium solution. As a consequence, as far as the cell remains supercooled the vapor pressure or the chemical potential difference will allow the water to leave the cell and freeze externally. This causes dehydration during cryopreservation with a rate and extent dependent on the permeability of the specific cell to water and the cooling rate. Generally speaking the slower is the cooling rate the higher extent of dehydration will be obtained. Thus, it is obvious that one of the most important points in cryopreservation procedures is to dehydrate the cell correctly before or during the freezing procedure to reduce the damages caused by intracellular ice formation. If dehydration is inadequate, large, intracellular ice crystals may form, which can be lethal to the cells. Although the avoidance of intracellular freezing is important to improve survival rates, it is not the only aspect to overcome. In fact, ice also forms outside the cell leaving the residual unfrozen medium to form channels and increase solute concentration causing shrinkage. To overcome these issues, cryoprotectans (CPAs) have been introduced in laboratory practices. This family of compounds can be classified into two main categories:

- Permeating agents: can enter the cell and includes glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and 1,2-propanediol (PROH) and generally have a molecular weight lower than 100.
- Nonpermeating agents: cannot enter the cell because of their size and polarity. They include large sugar molecules such as sucrose, ficoll, and raffinose, and also proteins and lipoproteins.

The protective properties of cryoprotectants are related to their ability to lower the freezing point of the solution in a concentration-dependent way and to their chemical property. They form hydrogen bonds with water molecules eliminating ice formation. Moreover, they prevent damages caused by high salt concentration. The search for suitable cryoprotectants and their appropriate concentrations has been a priority in all the freezing protocols. It seems that low molecular weight agents such as glycerol have a protective effect because of their ability to increase the unfrozen fraction and reduce cell volume excursion during cooling and rewarming procedures. Moreover, they also reduce the toxic effects of high concentrations of other compounds (colligative properties). The cryoprotectants enter the cells by osmosis while the water leaves. This might cause shrinkage as the water rapidly leaves the cells to dilute the high concentration of extracellular solutes or more serious issues when cryoprotectants need to be removed. On the contrary, high molecular weight cryoprotectants cannot enter the cells and are, instead, involved in the stabilization of the plasma membrane by solute-specific interactions with the bilayer phospholipids. This is the main reason why in almost all the slow freezing protocols the cryoprotectant mixture is made up of a penetrating agent and a non penetrating agent.

3.2.2 Slow freezing

The slow cooling protocol is based on a very slow rate of decreasing temperature (< l°C/min) over time (graph 4).



Graph 4: slow freezing curve

Freezing solutions are cooled from room temperature (around 20°C) to – 8°C at a rate of 2°C/min. Manual seeding within straws is performed at near – 8°C and this temperature is maintained for 10 minutes to allow uniform ice propagation. This process prevents supercooling and starts the dehydration process. If ice formation is not initiated by seeding, the solution will remain unfrozen until a much lower subzero temperature is reached, which can be detrimental for oocyte survival. After this hold ramp the temperature is decreased to –30°C at a rate of 0.3°C/min and then rapidly brought to – 150°C at a rate of 50°C/ min. The straws are then directly plunged into liquid nitrogen at – 196°C and are stored. The thawing procedure consists of rapid rewarming, subsequent stepwise dilution of the cryoprotectants, and finally, return to 37°C for culture. The solutions that are commonly used during slow cooling procedures are a mixture of a penetrating cryoprotectant (usually PROH) and a nonpenetrating one (usually sucrose).

3.2.3 Vitrification

Vitrification is a recently developed alternative protocol that might become the elective method to freeze oocytes and ovarian tissue. It is based on the concept of avoiding ice crystal formation by using higher cryoprotectant concentrations, which causes the water to form a glassy state around the cell rather than ice crystals.

Nevertheless, although these higher concentrations of cryoprotectants may prevent mechanical damages, they can also be toxic for the cell or create an osmotic shock. To design a vitrification protocol the main points to consider are the cooling and warming rates, the cryoprotectant concentrations, and the sample volume. Reducing the volume and raising the freezing rate requires a lower concentration of CPAs in the solution leading to lower side damages. On the contrary, if the volume is larger the likelihood of ice nucleation is higher causing the entire specimen to freeze instantaneously. Yavin and Arav (Yavin S et al., 2007) expressed the probability of vitrification as a direct relationship between the cooling/warming rate and viscosity and, as an inverse relationship with the volume. Increasing the viscosity or the cooling/warming rates or decreasing the

volume will raise the probability of vitrification. It is well known that CPAs at high concentrations might have toxic consequences. This was, in fact, one of the main controversial aspects in vitrification procedures. To improve the outcome without using very high concentration of cryoprotectants, it is suitable to lower the cooling rate or to reduce the volume of sample storage to a very small drop that will prevent ice crystal formation. The cooling rate can be increased using liquid nitrogen slush at – 210°C whereas the loading volume can be reduced to less than 1 mL using newly developed devices. The cryoprotectants in the vitrification procedure are involved in two main actions: they should remove the water from the cell and, at the same time, enter the cell to form the amorphous state in the cytoplasm and prevent the cell from damage caused by low temperature. Initially, only penetrating agents were used in vitrification mixtures but, more recently, the protocols have been changed and the solutions are made by using both penetrating and nonpenetrating agents, and this has increased the survival rate and made significant advancements in the procedure. The general methodology involves a 2-step sequential exposure to vitrification solutions containing one or more cryoprotectants in increasing concentrations up to 40% (vol/vol), loading the cerlls in a minimal volume (<1 mL) of solution onto a carrier device (open or closed system), and very rapid cooling by plunging directly into liquid nitrogen. The time and temperature of exposure to such solutions are critical to avoid toxicity. Conversely, warming rates must also be rapid to prevent ice nucleation during the warming process and achieve optimal results. After warming, the cells are then moved through at least three solutions with decreasing concentrations of sucrose to effectively remove the cryoprotectants and rehydrate the cells. Vitrification can be defined as a physical process by which a highly concentrated solution of cryoprotectants creates a glasslike state during rapid cooling without the formation of ice crystals. This glassy state is an extremely viscous supercooled solution. Vitrification shows certain advantages over conventional slow freezing because it avoids damages caused by intracellular ice crystals and osmotic effects caused by extracellular ice formation. Moreover, it is a very fast procedure that does not require any electronic equipment and allows the freezing of specimens in a very short time.

3.3 Freezing of mature oocytes

The major issue with the MII oocyte is related to the particular cell characteristics. The mature human oocyte is distinguished by the presence of the first polar body in the perivitelline space and the meiotic spindle in the cytoplasm. Several studies using slow freezing and vitrification protocols have been conducted to analyze the possible damages to the subcellular structures such as meiotic spindle, mitochondria, or cortical granules. It has been shown that the meiotic spindle is a very dynamic and sensitive structure, which is able to disappear and reform during cryopreservation and after thawing. This is regulated by fine polymerization and depolymerization of the tubulin, a very delicate equilibrium; if altered, it can lead to abnormal configuration of the spindle after thawing. The chromosomes are aligned on this structure tightly in contact with the microfilaments and even when the spindle disassembles, they are not found to be dispersed in the cytoplasm. Possible damages to the meiotic spindle are more related to abnormal fertilization than chromosomal abnormalities in the embryos. Another feature is represented by the zona pellucida and cortical granules, which are responsible for the correct oocyte fertilization by preventing multiple sperm penetration. Normally, the zona pellucida hardens after penetration of one sperm to block polyspermy as a consequence of the release of the cortical granules. The sperm, in fact releases a protein that causes an increase in intracellular calcium, which leads to the release of the cortical granules. The cryopreservation affects the normal process because it causes premature release of the cortical granules and zona pellucida hardening, which is why ICSI (intra-cytoplasmatic sperm injection) has been used routinely for the insemination of thawed oocytes. Although the zona pellucida seems to be compromised by cooling, the membrane of mature oocytes show a different lipidic composition compared with immature oocytes giving a better resistance to low temperature and cryogenic injuries. The cumulus is another feature of the mature oocyte: it is involved in the fine communication between the inside and outside of the egg. Even though evidence suggested that its maintenance does not improve the freezing outcome, this is still under debate. Over the past 10 years several satisfactory results have been published, yet there is not a defined, generally accepted approach that guarantees

a safe routine application. The first protocol used to cryopreserve oocytes was based on a slow cooling/rapid thawing method that had already been applied successfully for embryo cryopreservation. Since the first pregnancy in 1986, several advancements have been made with the aim of improving the original protocol. Most of the studies were focused around the choice of cryoprotectant and concentration or the exposure time. The freezing curve designed for the programmable freezer is basically unchanged from the original protocol first designed by Lassalle et al (Lassalle B et al., 1985) for embryo freezing. The CPAs generally used are PROH and sucrose. These two CPAs have been modified in concentration and exposure time during freezing/thawing procedure in different protocols developed in the last two decades. Clinical outcomes have been determined for all the different protocols. As already said the first protocol used was exactly the same as formulated for embryo freezing. It is based on 1.5MPROH (equilibration solution) and 1.5M PROH+0.1M sucrose (loading solution) in the freezing mixture and a stepwise dilution of PROH (1.0M to 0.5 M) with an unvaried 0.2M sucrose concentration in the thawing solutions. This protocol applied on 68 patients resulted in poor survival (37%), poor fertilization (45.4%), but good cleavage rate (86.3%). The pregnancy rate was relatively high (22% per patient) even though the implantation rate per oocyte was only 2.3% (Borini A et al., 2004).

As regards the first report of vitrification in embryology, it was with mouse embryos in 1985 (Rall WF et al., 1985), followed by the successful vitrification of oocytes in 1991 (Kono T et al., 1991), yet the general application of vitrification in assisted reproduction has been rather limited until recently. The use of vitrification has been described in the literature for several mammalian species, including humans, with varying degrees of success depending upon the wide variety of tools and procedures applied (Liebermann J et al., 2002; Kuwayama M et al., 2005). Different recent publications have shown outstanding results for survival and clinical outcomes using vitrification compared with slow cooling (Vajta G et al., 2006). Vitrification methods have been modified over the years to optimize results in humans, by using minimal volumes and very rapid cooling rates, allowing lower concentrations of cryoprotectants to reduce injuries related

to chemical toxicity, osmotic shock, chilling sensitivity, and ice nucleation (Vajta G et al., 2006; Kuwayama M et al., 2005). The most widely used vitrification protocol involves gradual exposure of oocytes to the equilibration solution [7.5% ethylene glycol, 7.5% DMSO, and 20% serum substitute supplement (SSS) in HEPES buffered medium 199 (M199- H)] for approximately 8 minutes and then in the vitrification solution (15% ethylene glycol, 15% DMSO, 0.5M sucrose, and 20% SSS in M199-H) for up to 110 seconds. Samples are then loaded onto a carrier device and plunged into liquid nitrogen. The thawing solutions are based on a series of solutions with decreasing sucrose concentrations (1.0, 0.5, and 0M) with 20% of SSS in M199-H.

3.4 Cryopreservation of immature oocytes

To circumvent issues associated with the MII structures, immature oocyte cryopreservation may represent an alternative. Oocytes arrested at prophase I of the meiotic process have different features than MII oocytes: they display a prominent nucleus called germinal vesicle (GV) that contains the chromosomes that are still decondensed even though they are transcriptionally active. Immature oocytes do not have microtubules organized in the spindle but rather dispersed mainly around the GV; this avoids possible damage during freezing procedures as opposed to MII oocytes. The cumulus and granulosa cells play a key role here because they are still tightly connected to the egg through transzonal projections and the plasma membrane is characterized by a reduced content in cholesterol and fatty acids so its permeability to CPAs is very different than that of MII oocytes. Immature oocyte cryopreservation may represent an alternative for fertility preservation in a selected group of patients who cannot undergo ovarian stimulation because of cancer-related issues. Generally speaking, this technique has not been extensively used because of the low success rates. This might be related either to the difficulties to overcome during freezing procedure that probably affect the transzonal projections negatively leading to irreversible damages or the poor results obtained with in vitro maturation (IVM) methods after freezing/ thawing. The immature oocytes are, in fact, characterized by this strict

communication between the cumulus and the egg and consequently, the freezing process can cause stress that destroys the tight gap junctions and corresponding communication. The penetration of cryoprotectants and any extracellular ice formation can result in cumulus-granulosa cell loss.

3.5 Embryo cryopreservation

The successful cryopreservation of surplus embryos after IVF and resultant pregnancy following frozen-thawed embryo transfer (FET) was first reported in 1983, and the first child after embryo freezing was born in 1984 (Trounson A et al., 1983; Zeilmaker GH et al., 1984). For more than two decades, embryo cryopreservation (EC) has played an important role in assisted reproduction treatment (ART), providing couples with more than one attempt at embryo transfer after a single ovarian stimulation cycle with IVF, thus improving cumulative pregnancy rates while decreasing exposure to gonadotropins and reducing treatment costs. It is estimated that almost one quarter of the children born after ART are born following cryopreservation of mostly cleavage-stage embryos and, less commonly, blastocysts and oocytes (ICMART, 2008). Most recent data from the Society for Assisted Reproductive Technology and the European IVF Monitoring Program report a pregnancy rate of 34% following FET in women younger than 35 years and an overall pregnancy rate of 19%, respectively (Society for Assisted Reproductive Technology, 2009; Nyboe Andersen A et al., 2005). Slow-freezing has been the most widely applied technique for the cryopreservation of embryos, while vitrification has been used more frequently recently. A recent review assessing the medical outcome of ART children born after cryopreservation reported reassuring results (Wennerholm UB et al., 2009). The rate of preterm birth, birth defects and chromosomal abnormalities was not significantly different between children born after transfer of fresh or cryopreserved embryos. Similarly, these children demonstrated similar growth and mental development (Wennerholm UB et al., 2009). A recent systematic review of randomized trials comparing laboratory and clinical outcome with SF or vitrification conclude that pregnancy rates were not statistically

significantly different between the two methods (odds ratio (OR): 1.66, 95% confidence interval (CI): 0.98 –2.79, in favour of vitrification) (Kolibianakis E et al., 2009). However, vitrification was associated with significantly higher post-thawing survival rates, both for cleavage-stage embryos (OR: 6.35, 95% CI: 1.14–35.26) and for blastocysts (OR: 4.09, 95% CI: 2.45–6.84). Moreover, post-thawing blastocyst development of embryos cryopreserved at the cleavage stage was significantly higher with vitrification than with slow freezing (OR: 1.56, 95% CI: 1.07–2.27). Similar to IVF embryos, we achieved higher survival and pregnancy rates following vitrification of cleavage stage IVM embryos as compared with slow-freezing (Son WY et al., 2009). Although the cryopreservation of embryos following a stimulated IVF cycle is considered the only established method of fertility preservation for female cancer patients, several points raise concern about this option. As reported before, these are: (1) a possible delay of two to five weeks in treatment of the primary disease due to ovarian stimulation depending on the timing of the first consultation with the reproductive endocrinologist in relation to onset of the next menstrual cycle (2) exposure to supraphysiologic estrogen levels induced by ovarian stimulation (3) the requirement for a male partner or willingness to use donor sperm for embryo production (4) legal, ethical, religious issues related to cryopreservation of embryos in general.

3.5.1 Is it possible to overcome the limits of IVF-embryo cryopreservation?

A retrospective study addressing the above question concluded that having a reproductive medicine consult and subsequent ovarian stimulation followed by oocyte collection did not significantly delay the start of adjuvant chemotherapy in young patients with breast cancer (Baynosa J et al., 2009). However, the actual time required for completion of the fertility preservation procedure, which starts with the initial reproductive medicine consultation and technically ends with oocyte collection, depends on the conditions of any particular clinic. Ovarian stimulation takes between 2 and 5 weeks, depending on the stimulation protocol employed and the timing of the following menstrual cycle of the patient. Studies

assessing the effect of the length of time between surgery and the initiation of chemotherapy on the survival of women with breast cancer report no detrimental effect of a delay in treatment if chemotherapy is started within 12 weeks after surgery (Cold S et al., 2005; Jara Sanchez C et al., 2007; Lohrisch C et al., 2006). However, it must be emphasized that the external validity of these studies is limited to their inclusion criteria, and the potential effect of any delay in oncologic treatment due to fertility preservation procedures must be evaluated on a case-by-case basis together with the treating oncology team. In order to minimize any preventable delay, it is prudent to inform patients about the effects of treatment on fertility and the options for fertility preservation as early as possible in the course of oncologic diagnosis and treatment procedures. The effect of elevated estrogen levels on underlying disease. The risk of breast cancer is consistently found to be associated with persistently elevated blood estrogen levels (Yager JD et al., 2006). Serum estradiol (E2) levels are increased during ovarian stimulation for IVF and can reach levels twenty times higher than those of a natural cycle (Cahill DJ et al., 2000). Although the effect of a temporary increase in serum E2 levels on the risk of recurrence of breast cancer is controversial, these facts cause concern among both physicians and patients. Such concerns should not be limited to women with estrogen receptor positive breast cancer, because recent findings also suggest the presence of an indirect mitogenic effect of estrogen on hormone receptor negative breast cancer (Gupta PB et al., 2006). Moreover, increased E2 levels can be relevant for patients undergoing fertility preservation treatment due to other oncologic or non-oncologic diseases considered to be estrogen sensitive, such as desmoids tumours, systemic lupus erythematosus or severe endometriosis. In order to minimize the rise in estradiol levels in breast cancer patients undergoing IVF, Oktay et al. developed an ovarian stimulation protocol involving the concomitant use of an aromatase inhibitor, letrozole, with gonadotropins (Azim AA et al., 2008). Briefly, letrozole was started at a dose of 5mg/day on the second day of the menstrual cycle and gonadotropins were initiated two days later. A gonadotropin releasing hormone antagonist was used to prevent premature ovulation, and human chorionic gonadotropin (HCG) was administered when at least two follicles reached 19mm in diameter. Letrozole was reinitiated on the day

of oocyte collection in order to prevent a rebound increase in E2 level. Themeanpeak E2 level was 406 pg/ml (range 58 to 1,166 pg/ml) in 79 women with breast cancer undergoing ovarian stimulation for embryo or oocyte cryopreservation (Azim AA et al., 2008). An average of 10.3±7.75 oocytes were retrieved and 5.97±4.97 embryos/oocytes cryopreserved per patient. Compared to 136 women who opted against ovarian stimulation, the recurrence and relapse-free survival rates were similar after a median follow up of 23.4 months after definitive surgery. However, it is interesting to note that with the same centre 63.3% of breast cancer patients referred for REI consultation declined ovarian stimulation and IVF due to concerns about delay of chemotherapy, effect of ovarian stimulation on cancer or costs associated with treatment.

3.5.2 Ethical and legal issues associated with embryo cryopreservation

When embryos are cryopreserved in a fertility preservation program, the patient/couple should make an advance decision on the fate of these embryos in the event that they are not transferred for any reason including the patient's failure to survive cancer. It should be documented whether the remaining partner is entitled to use the embryos for his own reproductive end or whether they are to be donated to a third party, used for research or discarded. Considering these issues and making such decisions can be particularly difficult for a patient who has been recently diagnosed with a life-threatening disease and is facing a demanding treatment period. Therefore, patients should be given appropriate counselling using a multidisciplinary approach involving a psychologist and a legal advisor.

3.6 Whole ovary and cortical strip freezing

3.6.1 Whole ovary transplantation

It has been almost two decades since fresh whole ovary autotransplantation was reported in human studies. In those reports, ovaries were removed from their pelvic location and immediately transplanted into other sites. The use of heterotopic sites for ovarian autotransplantation dates back to 1988, when the first

case was reported (Muller G et al., 1988). Whole ovary transplantation with a vascular anastomosis was proposed as a mechanism to reduce ischemic time and, in theory, prolong the longevity of the graft (Jeremias E et al., 2002). In this technique, the whole ovary with its vascular pedicle is removed, cryopreserved, thawed, and then transplanted with a microvascular anastomosis into a heterotopic or orthotopic site. Transplantation of an intact ovary with vascular anastomosis reduces the ischemic interval between transplantation and revascularization by allowing immediate revascularization of the transplanted tissue (Jadoul P et al., 2007).

3.6.2 Whole Fresh Ovary Transplantation

Fresh whole ovary transplantation with vascular anastomosis has been successfully performed experimentally in animal models using a wide variety of orthotopic and heterotopic-recipient sites. In addition, a number of vessels have been used in a wide variety of animal models. These include pelvic vessels such as the ovarian artery and iliac artery, parietal vessels such as the inferior epigastric vessels and extrapelvic vessels such as the carotid vessels (Wang X et al., 2002; Goding JR, 1966). The revascularization process was compromised in approximately 50% of the cases when fresh ovaries were transplanted (Jeremias E et al., 2002) . A limited number of human studies with transplantation of fresh whole ovaries in orthotopic (Silber SJ et al., 2008; Mhatre P et al., 2005) and heterotopic sites (Leporrier M et al., 1987; Hilders CG et al., 2004) have been attempted with some success. Silber et al culminated his efforts in ovarian transplantation in monozygotic twins discordant for premature ovarian failure (POF) by reporting the first full-term pregnancy obtained using orthotopic whole fresh ovary transplantation with microvascular anastomosis. A fresh ovary from the fertile twin was implanted in her monozygotic twin with POF.

3.6.3 Whole Frozen-thawed Ovary Transplantation

Whole frozen ovary transplantation with microvascular anastomosis was first described in rats by Wang et al (Wang X et al., 2002). They described successful vascular transplantation of frozen-thawed rat ovaries, which were transplanted along with the reproductive tract in 4 of 7 (57%) transplants; these transplants survived for Z60 days, were ovulatory and resulted in one pregnancy. Ovarian function was restored in 100% of cases when fresh organs were transplanted (Yin H et al., 2003). The first report of successful cryopreservation and transplantation of an intact ovary in sheep (defined as return of hormonal functions) occurred with a vascular anastomosis using the inferior epigastric vessels in 2003 (Bedaiwy MA et al., 2003). Successful pregnancy and delivery of a lamb in sheep was reported by Imhof et al in 2006 after autotransplantation of whole cryopreserved ovaries with microanastomosis of the ovarian vascular pedicle. The challenge of whole ovary cryopreservation and transplantation technology is not only the surgical technique but the cryopreservation protocol for an entire organ. Such a protocol should ensure that the cryoprotectant(s) evenly diffuses throughout the entire ovary. In addition, the frozen ovary should survive the thawing process and maintain functionality after transplantation. We have found evidence of endothelial cell damage caused by the freeze-thaw process or by the ischemic time until successful reanastomosis. Imhof et al reported that 18 months after transplantation, the follicular survival rate was less than an 8%. Other authors reported an even lower follicular survival rate (6%) and the depletion of the entire follicular population after whole ovary cryopreservation and transplantation (Courbiere B et al., 2009). Although ovarian vessel thrombosis is a potential complication of a vascular anastomosis, its incidence may be higher with different freezing techniques such as vitrification. Similarly, in a more recent study in ewes, it was shown that immediate vascular patency was achieved in all ewes and maintained in 7 of 8 cryopreserved and 3 of 4 control grafts. Functional corpora lutea were identified in 3 ewes (1 control and 2 cryopreserved) 18 to 25 weeks after grafting. In addition, inhibin-A levels indicated resumption of follicular development in 4 cryopreserved and 1 control ewes, however, castrate gonadotrophin levels persisted in 5 cryopreserved and 2 control ewes. The main

prominent feature of this whole ovary transplantation experiment is the fact that primordial follicle density was significantly reduced after grafting in both cryopreserved and nonfrozen ovaries (Onions VJ et al., 2009). Although transplantation of whole cryopreserved-thawed ovary was not performed in humans, cryopreservation of a whole ovary using a slow freezing protocol has been successfully attempted (Bedaiwy MA et al., 2006). The results showed both vascular and follicular integrity after freezing and thawing. More recently, a multigradient freezing device was recently used with promising results (Bromer JG et al., 2008). In that study, a high follicular viability, normal histologic architecture, and preserved vessel integrity were reported, supporting the potential for vascular reanastomosis. Martinez-Madrid et al in 2007 evaluated apoptosis by the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphates nick endlabeling method and by immunohistochemistry for active caspase-3 in fresh ovaries, after whole ovary freezing. Ultrastructure was also assessed by transmission electron microscopy in the thawed tissue. They found that primordial or primary follicles were not positive for either terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphates nick end-labeling or active caspase-3 after whole ovary freezing indicating the feasibility of whole ovary freezing.

3.6.4 Potential Techniques

An intact human ovarian autotransplantation was reported in 38-year-old monozygotic twins discordant for POF (Silber SJ et al., 2008). The donor ovary was removed laparoscopically from the fertile sister by dividing the infundibulopelvic ligament at its base to maximize the length. Using minilaparotomy, the donor's ovarian veins (3.0 mm in diameter) were anastomosed to the recipient's ovarian veins with 9-0 nylon sutures, and the donor's ovarian arteries (0.5mm in diameter) were anastomosed to the recipient's ovarian arteries with 10-0 nylon interrupted sutures. A normal- appearing blood flow through the ovarian vessels of the transplanted ovary was observed after an ischemic period of 100 minutes. Subsequently, the recipient twin had 11 regular menstrual cycles. At days 427 after transplantation, she became pregnant and gave

birth to a normal healthy baby girl. This case showed the feasibility of using whole-ovary transplantation between monozygotic twins who are discordant for POF to restore fertility in the affected twin. Should intact human ovary cryopreservation be optimized, the same approach could be adopted for autotransplantation of intact cryopreserved-thawed ovary with a vascular pedicle.
Although the likelihood of whole ovary cryopreservation and later reimplantation into a patient continues to increase, whole ovary freezing has two critical challenges to overcome. First, vascular re-anastomosis of the ovarian arteries in particular makes for very challenging surgery. Thus, an elevated risk of ischemia during reintroduction of the ovaries post-thaw is inherent in the procedure. Second, and most importantly, whole ovary handling means that there is only one chance for every step to go optimally. If handling, cryopreservation, or thawing and reimplantation are compromised at any step, the entire organ could be lost. For these reasons, whole ovary freezing and transplantation should be viewed as an experimental strategy for the time being. By comparison, the removal and storage of ovarian cortex ("cortical strips") has several advantages.

3.6.5 Prevention of Post transplantation Ischemic Ovarian Damage

Many strategies have been devised to minimize the initial post transplantation ovarian ischemia However, the majority of them lack standardization, reproducibility, and long-term success.

MECHANICALLY INDUCED NEOANGIOGENESIS

Surgically induced tissue injury is associated with neoangiogenesis and inflammation. Consequently, transplantation in the context of an inflammatory reaction could expedite the revascularization of ovarian grafts. This approach was tested by Donnez et al in 2004 who used a multistep process to create a peritoneal pocket 1 week before transplantation. Similarly, this hypothesis was further supported by the early perfusion of ovarian cortical strips upon their transplantation into granulation tissue (Israely T et al., 2006).

THE USE OF ANITOXIDANTS

Reactive oxygen species (ROS) are produced as a result of the ischemia perfusion process. They have the potential to damage cell membranes, endothelial membranes, and mitochondrial function (Kupiec-Weglinski JW et al., 2005). The use of antioxidants may reduce ROS-associated tissue damage and follicular loss after transplantation. The use of exogenous antioxidants to augment ovarian transplant resistance to ROS-associated damage has been evaluated by many investigators. In various animal models, a wide variety of antioxidants were attempted including ascorbic acid, mannitol (Sagsoz N et al., 2002), oxytetracycline (Sapmaz E et al., 2003), and vitamin E (Nugent D et al., 1998) with variable successes. In a human in-vitro model, Kim et al in 2004 found that incubating ovarian tissue with ascorbic acid for up to a maximum of 24 hours reduced apoptosis.

GROWTH FACTORS-INDUCED NEOANGIOGENESIS

Neoangiogenesis is an integral part in the establishment of graft function. Therefore, it is expected that neoangiogenic growth factors such as fibroblast growth factor, transforming growth factor, and vascular endothelial growth factor (VEGF) somehow aid in the establishment of graft function. In lower animals, it was shown that the invasion of the rat cortex by vessels was associated with a significant increase in the expression of mRNA in the outer cortex for both transforming growth factor and VEGF.11 However, systemically administered VEGF was not associated with improved graft function in an animal model (Schnorr J et al., 2002). Local administration at the transplant site may be more beneficial.

HORMONAL TREATMENT

Data from several animal experiments showed that pretreating the graft recipient and/or the donor with gonadotrophin stimulation before and after transplantation may have a positive effect on the viable growth follicle rate (Nugent D et al.,

1998; Imthurn B et al., 2000; Wang X et al., 2002). However, the impact of such treatments on long-term ovarian function and fertility is still questionable and needs further investigation.

3.7 Ovarian cortex and ovarian follicles

The transplantation of pieces of ovaries and ovarian cortex (which can contain thousands of immature follicles) has been performed successfully in different mammals since early in the last century (Lamond DR, 1959; Simmer HH et RT Morris, 1970). Use of the sheep, whose ovaries are anatomically similar to the human, resulted in important proof-concept studies (Gosden RG et al.; Baird DT et al., 1999) that later would be applied to the human. That work showed that pieces of sheep ovarian cortex could be removed, optionally cryopreserved, and then reintroduced surgically to the surface of a recipient's ovary. Strikingly, ovarian function resumed long-term in graft recipients, and pregnancies, ostensibly from oocytes in graft tissue, were achieved. Grafting human ovarian cortex back to the ovary has been similarly successful. The first notable example of this was shown in a patient treated for Hodgkin's lymphoma who had ovarian cortex removed and stored prior to treatment. After recovery, the patient's cortical strips were thawed and grafted orthotopically, after which recovery of regular ovulatory cycles occurred. This patient later became pregnant, resulting in a live birth (Donnez, J et al., 2004). A second similar resumption of ovarian function using frozen/thawed autografts was seen in a patient treated with chemotherapy for sickle cell anemia (Donnez J et al., 2006). More recently, this technique was applied to cases of premature ovarian failure (POF) (Silber S et al., 2010). Nine women with POF received ovarian tissue donated by their monozygotic twin sisters whose ovaries were still functional. Ovarian function resumed in all nine women, and eight live births are obtained. Although the effectiveness of such grafts are unquestionable, there are situations where this approach would not be appropriate. If there is any chance of reintroducing cancer or another malignancy, ovarian cortex (or indeed, whole ovary) cryopreservation and grafting should be avoided (Shaw JM et al., 1996; Shaw J. et A. Trounson, 1997; Dolmans MM et

al., 2010). For such cases, the experimental strategy of maturing ovarian follicles *in vitro*, in hopes of producing mature eggs, offers promise.

3.8 In vitro culture techniques

In vitro mammalian ovarian follicle culture has been performed successfully for more than 15 years. Multiple groups have cultured mouse preantral follicles to an ovulatory state, produced mature eggs, and subsequently, embyros and offspring. Originally, follicles were isolated and cultured in plastic culture dishes (Cortvrindt R et al., 1996; Smitz J et al., 1996) (Fig. 4). To improve the morphological characteristics of cultured follicles and the efficiency of development to maturity, new approaches making use of bioengineered materials have been developed. Embedding follicles in a three-dimensional matrix can improve follicle growth performance *in vitro* (Fig. 4).

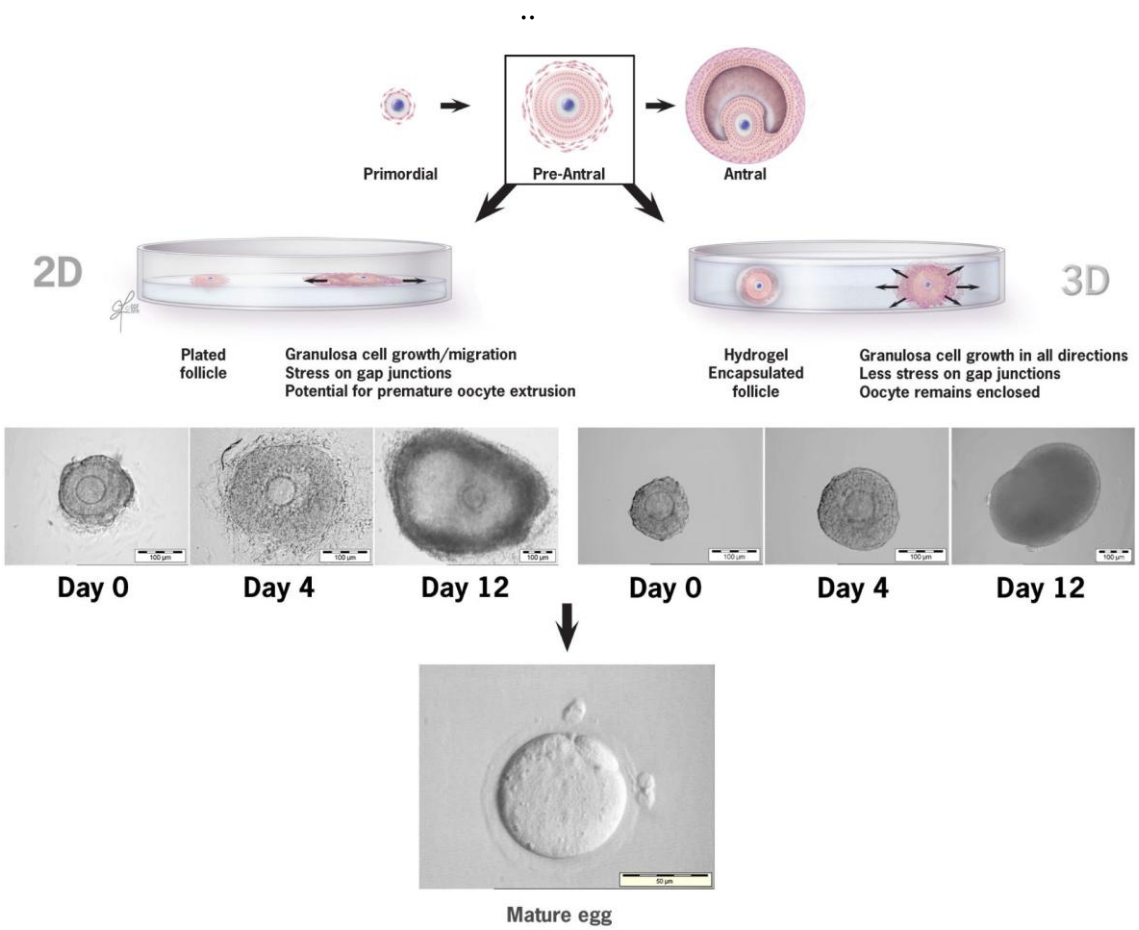


Fig. 4: 2D and 3D in vitro culture of follicles

Particularly, the use of alginate matrices (West ER et al., 2007) has resulted in promising follicle development *in vitro*, up to large antral stages. Such matrices are thought to provide an improved mechanical environment better approximating growth *in vivo*. In combination with cryopreservation, this technique holds striking promise for cancer survivors at risk for reintroduction of the disease (if orthotopic transplantation was performed) (Xu M et al., 2009). The growth of frozen/thawed follicles and their cytoskeletal properties approximated those seen in non-cryopreserved follicles (Barrett SL et al., 2010). One day, a patient might have the entire continuum of oocyte development, from the primordial oocyte to the mature MII egg, take place *in vitro*, with only an embryo transferred back for attempted pregnancy. An interesting technique where cortical strips are (optionally cryopreserved) cultured *in vitro* first, and then maturing follicles are isolated for further culture (Fig. 4), has also been developed. Telfer et al in 2008 have shown that a two-step procedure can result in the generation of large antral follicles. Cortical strips are first cultured intact, and then growing follicles are mechanically isolated and cultured singly potentially within a matrix as discussed earlier. This technique has the benefit of allowing follicles to grow surrounded by native ovarian tissue, potentially more like conditions *in vivo*. Further, it is likely that many of the growing follicles isolated will have started their growth in the primordial follicle stage. Primordial follicles are notoriously difficult to mechanically isolate, and their growth, first within the cortical strip, would help address this problem. Given the need for *in vitro* methodologies, we turn to recent advances in our understanding of the molecular control of follicle growth. If we can understand the mechanisms that control follicle growth to maturity, we will be able to optimize the above *in vitro* culture techniques.

3.9 Potential applications of cryopreserved ovarian tissue

3.9.1 Xenotransplantation

At present, xenotransplantation of the ovarian tissue is the only grafting option described for endangered species. It has often been combined with ovarian tissue

cryopreservation, and in all models tested so far, good post-thaw survival of the graft and its primordial follicles were reported (Paris et al., 2004; Paris and Schlatt, 2007). Independent of the species-specific differences, host environment can support, in most tested cases, the resumption of ovarian function and subsequent follicular development. In a wide range of species, including wallabies, wombats, elephants, cats, dogs, pigs, cows and marmoset monkeys, the formation of antral-sized follicles has been reported and the oocytes looked morphologically healthy (reviewed by Paris et al., 2004). What is unknown is whether the oocytes following development in such a different host environment are still normal from a functional point of view, and further studies are needed to answer this question. Another limiting factor is that, at present, it is possible to grow antral follicles from a certain species within an immunocompromised host but not mature and fertilize them in the in vivo host environment. The oocyte is thus rescued, and then IVM and IVF are still needed before an embryo is available for transfer. This is a problem, as for many endangered species, IVM, IVF and embryo transfer technologies have not been yet developed and significant hurdles are currently still envisioned. In species where IVM and IVF are well-established procedures, the next step is to obtain live offspring following ovarian tissue xenotransplantation. At present, this has only been done bridging the concordant xenogenetic barrier from rats to mice (Snow et al., 2002). Ovarian xenotransplantation has other limitations given the only successful recipients that have been immunocompromised are rats and mice (Snow et al., 2002), which have to be housed under sterile, pathogen free conditions: the overall procedure (until the harvest of an antral follicle) is expensive and technically demanding. Moreover, since follicles from larger animals do not develop up to their normal size in small hosts (Nottola et al., 2008), such as immunodeficient mice, we do not know if they could have a healthy oocyte capable of fertilization. Other available approaches for ovarian transplantation, such as allotransplantation, have so far received little consideration for animal conservation. However, this may change if developments in the area of tolerance induction take place. In practice, this would mean that if a genetically valuable animal dies, the ovaries could be rescued and grafted to several young recipients, and offspring from this specific female

germline could be indirectly produced. Thus, close collaborations between zoo workers and researchers are needed to bridge these gaps in time.

3.9.2 Fine control of human ovarian follicle development in vivo and in vitro

There are two key features of follicle development relevant to fertility preservation: primordial follicle growth activation and subsequent growth and survival. Ensuring that a subset of the many primordial follicles in a given piece of ovarian cortex productively grow, and that a subset of growing follicles go on to survive and reach the preovulatory stage, will be required to treat patients in need.

3.9.3 Follicular growth arrest

New information is available about the mechanisms that enforce growth arrest within primordial follicles. In 2003, Castrillon *et al.* made a striking initial observation that knockout of the FoxO3a gene in mice results in the simultaneous growth activation of all primordial follicles. Thus began several studies to determine how the process is controlled by signals upstream of FoxO3a. The mTOR signaling pathway (Hay N et al., 2005; Wullschleger S et al., 2006) was then implicated as a critical regulator of the growth activation of primordial follicles (Liu L et al., 2007; Adhikari D et al., 2009; Adhikari D et al., 2010; Reddy P et al., 2010; Li J et al., 2010) (Fig. 5). Increased mTOR activity is associated with protein translation, an active cell cycle, and tissue growth. When mTOR activity was increased specifically in the oocyte by using a tissue-specific knockout of its negative regulators Tsc1 or Tsc2, the entire pool of primordial follicles growth-activated around puberty. This resulted in nearly complete follicle loss by early adulthood, and thus POF. The importance of mTOR signaling in the control of primordial follicle growth activation was further confirmed by Li *et al*. Those investigators targeted the Phosphatase with TENsin homology deleted in chromosome 10 (PTEN) phosphatase, known to act as a negative regulator of mTOR signaling. Inhibition of PTEN, resulted in increased primordial follicle

growth activation in mouse and human ovarian grafts. This was consistent with the initial result with FoxO3a knockout mice (Castrillon DH et al., 2003), as PTEN inhibition was found to result in Fox03a export from the nucleus. Interestingly, the mTOR pathway has also been implicated in the control of the follicle growth and survival to preovulatory stages.

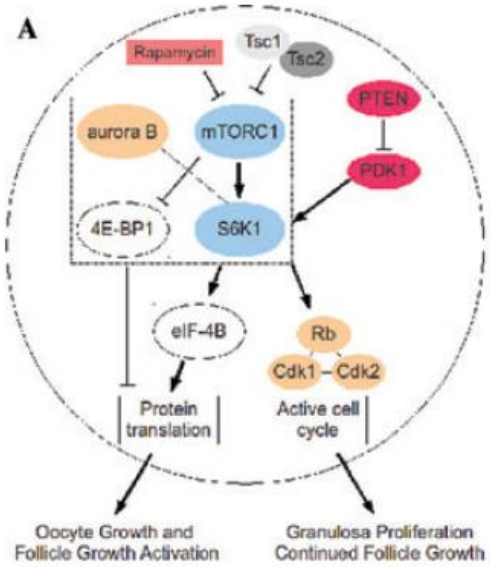


Fig. 5: mTORC1 signaling

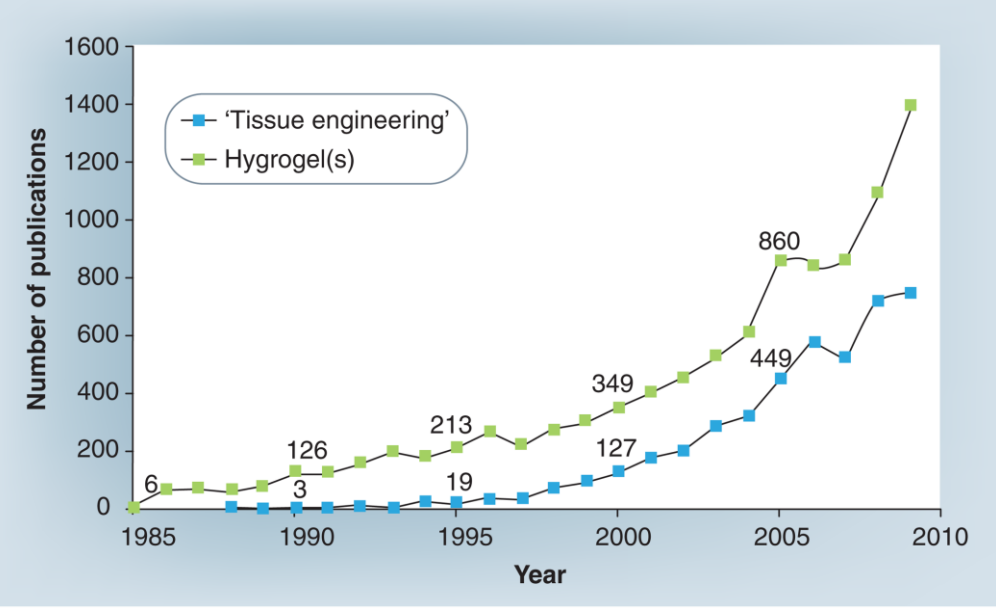
3.9.4 Follicle growth and granulosa cell proliferation

Signaling agents that control ovarian follicle growth, including follicle stimulating hormone (FSH) and members of the TGF- signaling family (Elvin JA et al., 2000; Chang H et al., 2001), are well known. Until recently, how the information from growth-stimulatory (and in some cases, growth inhibitory) factors is integrated within granulose cells was less clear. The mTOR pathway was an attractive candidate due to its function downstream of many growth factors, and the use of the mTOR specific inhibitor Rapamycin (red rectangle, Fig. 5) was found to antagonize mouse follicle growth *in vitro* at an accepted bioactive concentration, 100nM (Yaba A et al., 2008). As these follicles were cultured in the presence of FSH, this suggests that mTOR may act downstream of known follicle growth factors to foster growth and maturation. Because mTOR has been shown to be a positive regulator of the cell cycle (Fig. 5) *via* interactions with aurora B kinase

(Song J et al., 2007), granulosa cell cycle control by mTOR could underlie the control of follicle growth. Interestingly, culture in highly dilute (0.01pM) Rapamycin resulted in enhanced follicle growth. Whether such treatment can enhance human follicle growth *in vitro* remains to be seen.

4. Hydrogels: biomaterials mimicking the extracellular matrix

Cells and tissues are routinely cultured *in vitro* on 2D substrates (Ni Y et Chen R, 2009; Porro D et al., 2005; Zhang X et al., 2009). However, it has been demonstrated that cells or tissues cultured on 2D substrates (e.g., tissue culture plates or flasks) do not mimic cell growth *in vivo*, and fail to express certain tissue-specific genes and proteins at levels comparable to those found *in vivo*. For instance, it has been found that cell–drug interactions in a 2D culture system do not represent the actual working mechanism *in vivo*. Thus, 2D culture is not appropriate to be used in *in vitro* drug testing models. This is due to the fact that cells and tissues *in vivo* are immersed within a 3D network constituting a complex extracellular environment with a highly porous nanotopography, while a 2D culture system is too simple to mimic the native environment.



Graph. 5: development of tissue engineering and hydrogels

From a tissue engineering (TE) standpoint, constructing a culture environment that closely mimicks the native tissue, which is composed of the extracellular matrix (ECM), soluble bioactive factors, and products of homo- and hetero-typical cell–cell interactions, is desirable to replicate tissue functions *in vitro*. However, this remains as one of the major challenges in TE, given the complexity of cell–ECM interactions as well as multicellular architectural features such as repeating tissue units and proper vascular structure. Cells commit to their fate by deriving a vast amount of information from this environment. As a part of the cell environment, ECM has been the most emulated component in TE studies. In native tissue, ECM is mainly a mixture of two classes of macromolecules, glycosaminoglycans and fibrous proteins (e.g., collagen, elastin, fibronectin and laminin), which self-assemble into nanofibrillar supramolecular networks that fill the extracellular space between cells (Baker EL et al., 2009). ECM is a dynamic structure, which provides structural and anchoring support to the cells to improve tissue architecture. It also contributes to signaling, directing cell fate and function through cell–matrix interactions. In addition, the ECM is constantly remodeled by cells during development, homeostasis and wound healing by balancing its synthesis and degradation by a variety of enzymes (e.g., matrix metalloproteinases) (Hong H et al., 2007; Tibbitt MW et al., 2009). Significant advances in the design of artificial matrices have led to an evolution from a simple supporting scaffold to a more complex dynamic biomaterial environment. Ideally, the artificial matrices should: support cell growth and maintenance; provide appropriate mechanical, chemical and biological characteristics mimicking native ECM; and facilitate effective nutrient transfer, gas exchange (i.e., O2 and CO2), metabolic waste removal and signal transduction. Scaffolds in various forms, such as, hydrogel and nanofibers, have been studied and employed for different tissue regeneration purposes. A significant growth of interest in hydrogels started around the 1990s, partly due to the rapid emergence of the TE field, as hydrogels possess characteristics of native ECM (Du Y et al., 2008; Ling Y et al., 2007; Liu Tsang V et al., 2007; Khademhosseini A et al., 2007; Lutolf MP, 2009; Baroli B, 2007; Federovich NE et al., 2007; Jabbarzadeh E et al., 2008; Jay SM et Saltzman WM, 2009; Peppas NA et al., 2006), paving the way for functional tissues (graph. 5)

(Burdick JA et Vunjak-Novakovic G, 2009; Mano JF et al., 2007; Seunarine K et al., 2006). The biocompatibility of various hydrogels (e.g., collagen, agarose and polyethylene glycol) is well characterized and the state-of-the-art nano- and microfabrication technologies (e.g., lithography, nano- and micro-fluidics, micromolding and biopatterning) provide the techniques to engineer scaffolds with intricate structures (Kopecek J, 2007; Saunders BR et al., 2009; Lee WH et al., 2009; Seidlits SK et al., 2008, Rodriguez-Cabello JC et al., 2006; Madurantakam PA et al., 2009). However, challenges remain when it comes to engineering functional tissues. Hydrogel-based cell-encapsulating constructs with embedded microchannels have recently been investigated, and became a promising tool to generate active tissue mimics by improving nutrient and gas transport. Such cell-encapsulating hydrogel platforms could be employed for other applications, such as *in vitro* models for drug testing and toxicological assays. Given the intricate nature of the problem, the ultimate success of all these applications requires an interdisciplinary approach involving engineering, chemistry, materials science and cell biology.

4.1 Engineered hydrogel scaffolds as ECM mimics

The efforts to engineer a cell microenvironment that mimics the dynamic native ECM have been driven by the clinical demand for tissue (or organ) repair and replacement (Slaughter BV et al., 2009). Construction of functional tissues relies on the structural environment, cell–biomaterial interactions and incorporated biological signals (e.g., growth factors encapsulated in hydrogels) (Kim BS et al., 1998). Thus, the scaffolds must offer properties (i.e., mechanical and chemical) that lead to cellular function in a native manner. In this sense, hydrogels have advantages when utilized as scaffolds for TE as one can easily adjust their physico-chemical (electrical charge and pore size) (Schneider GB et al., 2004; Ford MC et al., 2006; Dadsetan M et al., 2008; Bryant SJ et al., 2007; Singh M et al., 2008), and mechanical (stiffness, tensile strength) (Anseth KS et al., 1996; Wenger MP et al., 2007) properties to levels that are desirable for tissue scaffolds (Choi NW et al., 2007; Tu C et al., 2003), cell encapsulation (Nicodemus GD et

Bryant SJ, 2008; Mcguigan AP et al., 2008; Tan W-H et Takeuchi S, 2007; Moon S et al., 2010), immobilization (Liu J et al., 2009) and drug delivery (Gao D et al., 2008; Qiu Y et Park K, 2001; Soppimath KS et al., 2002; Kretlow JD et al., 2007). Hydrogels are 3D cross-linked insoluble, hydrophilic networks of polymers that partially resemble the physical characteristics of native ECM. Polymers in hydrogel format can absorb a large amount of water or biological fluid (up to 99%) due to the presence of interconnected microscopic pores. Some hydrogels possess features of fluid transport and stimulus responsive characteristics (e.g., pH, temperature and light) (Bettinger CJ et al., 2005). Another appealing feature of hydrogels as scaffolds for TE is their biomechanical similarity to native ECM. The limitation of hydrogel mechanical properties is well known (Burdick JA, 2009). A hydrogel with the desired mechanical properties (in terms of stiffness and tensile strength) can be achieved by adjusting various parameters including the type of polymers used, their concentrations and the crosslinking density. Biocompatible hydrogel scaffolds can be obtained by selecting biocompatible synthetic or natural polymers and crosslinkers (Rivest C et al., 2007). A variety of natural and synthetic polymers have been used to fabricate hydrogels. Collagen (Gillette BM et al., 2008), hyaluronic acid (Sahoo S et al., 2008), chondroitin sulfate (Li Q et al., 2004), fibrin (Eyrich D et al., 2007), fibronectin (Fukuda J et al., 2006), alginate (Smidsrod O et Skjak-Braek G, 1990), agarose, chitosan (Azab AK et al., 2006) and silk (Kim HJ et al., 2008) have been the most commonly used natural polymers for TE and regenerative medicine applications. Among all these natural polymers, collagen has been the most widely investigated since it is the most abundant structural protein of ECM in multiple tissues (Black LD et al., 2008), including bladder (Bolland F et al., 2007), heart valve (Schenke-Layland K et al., 2003), blood vessel (Uchimura E et al., 2003), skin (Chen RN et al., 2004) and the liver (Lin P et al., 2004). Synthetic biodegradable polymers, such as poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA), poly(acrylic acid) (PAA), poly(propylene furmarate-co-ethylene glycol) (P(PF-co-EG)), poly(ethylene glycol) (PEG) (Park Y et al., 2004), poly(lactic acid) (PLA), poly(glycolic acid) (PGA) (Hiraoka Y et al., 2003), and a copolymer poly(lactic-glycolic) acid (Uematsu K et al., 2005) have also been used for engineered scaffolds. To

increase the biological (e.g., hydrophilicity, cell-adhesiveness, degradability), biophysical (e.g., porosity, branched vasculature) and mechanical (e.g., stiffness, viscoelasticity) properties of tissue scaffolds, combinations of natural or synthetic hydrogels (i.e., hybrid hydrogels) have also been utilized (Chen GP et al., 2004). Such 'bioartificial' scaffolds possess desirable mechanical properties and biocompatibility due to the coexistence of both synthetic and biological components. The biological properties of such scaffolds can further be improved by surface chemistry as the biomaterial composition makes them amenable to surface modification and biomimetic coatings (Savina IN et al., 2009; Teo WE et al., 2006; Hasirci V et al., 2006). Several approaches have been utilized to examine the mechanical (e.g., tension, compression, indentation, swelling) (Ahearne M et al., 2008; Ahearne M et al., 2005) and physicochemical (e.g., porosity, interconnectivity) properties of both natural and synthetic hydrogels, including extensiometry (Drury JL et Mooney DJ, 2003), compression test (Awad HA et al., 2004) and bulge test. However, these techniques are invasive and destructive. They are not appropriate to characterize mechanical properties of cell encapsulating hydrogels during culture. To overcome these specific problems, two techniques involving spherical indentation have been developed (Ahearne M et al., 2009); long focal microscopybased spherical microindentation and optical-coherence tomography-based spherical microindentation techniques. Both monitoring techniques can be utilized to determine the mechanical properties of cell-encapsulating hydrogels for *in vitro* engineering of soft tissues. While the former involves the central indention of a circumferentially suspended hydrogel using a sphere of a known weight and measurement of the resulting central deformation displacement, the latter is a noninvasive imaging technique based on Hertz contact theory, where the depth of indentation of a sphere into a hydrogel resting on a substrate can be used to calculate the mechanical properties of the hydrogel.

4.2 Properties of hydrogels

4.2.1 Synthetic materials

Synthetic hydrogels are appealing for tissue engineering because their chemistry and properties are controllable and reproducible. For example, synthetic polymers can be reproducibly produced with specific molecular weights, block structures, degradable linkages, and crosslinking modes. These properties in turn, determine gel formation dynamics, crosslinking density, and material mechanical and degradation properties. PEO is currently FDA approved for several medical applications and is one of the most commonly applied synthetic hydrogel polymers for tissue engineering. PEO and the chemically similar poly(ethylene glycol) (PEG) are hydrophilic polymers, that can be photocrosslinked by modifying each end of the polymer with either acrylates or methacrylates (Cruise GM et al., 1998; West JL et Hubbell JA, 1999; Mann BK et al., 2001). Hydrogels are then formed when the modified PEO or PEG is mixed with the appropriate photoinitiator and crosslinked via UV exposure (Bryant SJ et Anseth KS, 2001). Thermally reversible hydrogels have also been formed from block copolymers of PEO and poly(l-lactic acid) (PLLA) (Jeong B et al., 1997) and PEG and PLLA (Huh KM et Bae YH, 1999). In addition to the thermally reversible hydrogels, degradable PEO and PEG hydrogels have been formed by synthesizing block copolymers containing hydrolytically degradable poly(lactic acid) (PLA) (Metters AT et al., 2000) and enzyme specific cleavage sequences of oligopeptides. Another synthetic hydrophilic polymer widely explored for use in space filling and drug delivery applications is PVA. It can be physically crosslinked by repeated freeze-thawing cycles of aqueous polymer solutions (Cascone MG et al., 1995) or chemically crosslinked with glutaraldehyde (Nuttelman CR et al., 2001), succinyl chloride, adipoyl chloride, and sebacoyl chloride (Orienti I et al., 2001) to form hydrogels. It can also be blended with other water-soluble polymers and again crosslinked either physically or chemically (Cascone MG et al., 2001; Cauich-Rodriguez JV et al., 1996; Cauich-Rodriguez JV et al., 2001). It these forms, it is not dissolvable in aqueous solutions. A newer, synthetic hydrogel block copolymer, P(PFco- EG) has been created for use as an injectable carrier for

bone and blood vessel engineering (Suggs LJ et Mikos AG, 1999). The homopolymer poly(propylene fumarate) (PPF) is a hydrophobic, linear polyester which undergoes degradation by hydrolysis of the ester linkage. It can form hydrogels when synthesized as a block copolymer with hydrophilic PEG and crosslinked either chemically (Suggs LJ et al., 1999) or via UV exposure (He S et al., 2000).

4.2.2 Naturally derived materials

Naturally derived hydrogel forming polymers have frequently been used in tissue engineering applications because they are either components of or have macromolecular properties similar to the natural ECM. For example, collagens are the main protein of mammalian tissue ECM and comprise 25% of the total protein mass of most mammals (Alberts B et al., 1994; Lee CH et al., 2001) . Similarly, HA is found in varying amounts in all tissues of adult animals. Like HA, both alginate and chitosan are hydrophilic, linear polysaccharides (SmidsrØd O et Skjak-Bræk G, 1990; Suh J-KF et Matthew HWT, 2000). They have also been shown to interact in a favorable manner in vivo and thus have been utilized as hydrogel scaffold materials for tissue engineering.
Collagen is an attractive material for biomedical applications as it is the most abundant protein in mammalian tissues and is the main component of natural ECM. There are at least 19 different types of collagen, but the basic structure of all collagen is composed of three polypeptide chains, which wrap around one another to form a three-stranded rope structure. The strands are held together by both hydrogen and covalent bonds. Collagen strands can self aggregate to form stable fibers. In addition, collagen fibers and scaffolds can be created and their mechanical properties enhanced by introducing various chemical crosslinkers (i.e. glutaraldehyde, formaldehyde, carbodiimide) (Lee CR et al., 2001; Park S-N et al., 2002), by crosslinking with physical treatments (i.e. UV irradiation, freeze-drying, heating) (Schoof H et al., 2001), and by blending it with other polymers (i.e. HA, PLA, poly(glycolic acid) (PGA), poly(lactic-coglycolic acid) (PLGA), chitosan, PEO) (Tan W et al., 2001; Chen G et al., 2001; Huang L et al., 2001). Collagen is naturally degraded by metalloproteases, specifically collagenase, and

serine proteases, allowing for its degradation to be locally controlled by cells present in the engineered tissue. HA is the simplest glycosaminolglycan (GAG) and is found in nearly every mammalian tissue and fluid. It is especially prevalent during wound healing and in the synovial fluid of joints. It is a linear polysaccharide composed of a repeating disaccharide of (1–3) and (1–4)-linked b-d-glucuronic acid and N-acetyl-b-dglucosamine units. Hydrogels of HA are formed by covalent crosslinking with hydrazide derivatives (Vercruysse KP et al., 1997; Prestwich GD et al., 1998; Oerther S et al., 2000), by esterification (Mensitieri M et al., 1996, Borzacchiello A et Ambrosio L, 2001; Gamini A et al., 2002), and by annealing (Fujiwara J et al., 2000). Additionally, HA has been combined with both collagen and alginate to form composite hydrogels (Oerther S et al., 1999; Miralles G et al., 2001). HA is naturally degraded by hyaluronidase, again allowing cells in the body to regulate the clearance of the material in a localized manner. Alginate has been used in a variety of medical applications including cell encapsulation and drug stabilization and delivery, because it gels under gentle conditions, has low toxicity, and is readily available. It is a linear polysaccharide copolymer of (1–4)-linked b-d mannuronic acid (M) and a-l-guluronic acid (G) monomers, and is derived primarily from brown seaweed and bacteria (Johnson FA et al., 1997). Within the alginate polymer, the M and G monomers are sequentially distributed in either repeating or alternating blocks (Draget KI et al., 2000). The amount and distribution of each monomer depends on the species, location, and age of seaweed from which the alginate is isolated. Gels are formed when divalent cations such as Ca^{2+}, Ba^{2+}, or Sr^{2+} cooperatively interact with blocks of G monomers to form ionic bridges between different polymer chains. The crosslinking density and thus mechanical properties and pore size of the ionically crosslinked gels can be readily manipulated by varying the M to G ratio and molecular weight of the polymer chain. Gels can also be formed by covalently crosslinking alginate with adipic hydrazide and PEG using standard carbodiimide chemistry (Eiselt P et al., 1999; Lee KY et al., 2000). Ionically crosslinked alginate hydrogels do not specifically degrade but undergo slow, uncontrolled dissolution. Mass is lost through ion exchange of calcium followed by dissociation of individual chains, which results in loss of mechanical stiffness

over time (Le Roux MA et al., 1999). Hydrolytically degradable forms of alginate have been synthesized by partial oxidation of alginate and an alginate derivative, polyguluronate (Bouhadir KH et al., 1999), to form oxidized alginate (Bouhadir KH et al., 2001) and poly (aldehyde guluronate) (PAG) (Lee KY et al., 2000), respectively.

Chitosan has been investigated for a variety of tissue engineering applications because it is structurally similar to naturally occurring GAGs and is degradable by enzymes in humans. It is a linear polysaccharide of (1–4)-linked d-glucosamine and N-acetyl-d-glucosamine residues derived from chitin, which is found in arthropod exoskeletons (Zhang Y et Zhang M, 2001; VandeVord PJ et al., 2002). The degree of N-deacetylation usually varies from 50% to 90% and determines the crystallinity, which is greatest for 0% and 100% N-deacetylation. Chitosan is soluble in dilute acids which protonate the free amino groups (Chenite A et al., 2000). Once dissolved, chitosan can be gelled by increasing the pH or extruding the solution into a nonsolvent. Chitosan derivatives and blends have also been gelled via glutaraldehyde crosslinking (Mi F-L et al., 2000; Shen F et al., 2000), UV irradiation (Ono K et al., 2000), and thermal variations. Chitosan is degraded by lysozyme; the kinetics of degradation are inversely related to the degree of crystallinity (Lee KY et al., 1995; Varum KM et al., 1996; Tomihata K et al., 1997).

4.2.3 Scaffold design variables

Selection or synthesis of the appropriate hydrogel scaffold materials is governed by the physical property, the mass transport property, and the biological interaction requirements of each specific application. These properties or design variables are specified by the intended scaffold application and environment into which the scaffold will be placed. For example, scaffolds designed to encapsulate cells must be capable of being gelled without damaging the cells, must be nontoxic to the cells and the surrounding tissue after gelling, must allow appropriate diffusion of nutrients and metabolites to and from the encapsulated cells and surrounding tissue, and require sufficient mechanical integrity and

strength to withstand manipulations associated with implantation and in vivo existence (Lim F, 1984). Here, the defined physical properties include mechanical strength and gel formation dynamics, while diffusion requirements specify the mass transport properties. In addition, the biological properties are designated by the required non-toxicity. What follows are definitions and discussions of the physical, mass transport, and biological design variables relevant to the design of hydrogel scaffolds for tissue engineering.

4.3 Physical properties

Many scaffolds for tissue engineering initially fill a space otherwise occupied by natural tissue, and then provide a framework by which that tissue may be regenerated. In this capacity, the physical properties of the material are inherent to the success of the scaffold. Specific physical properties include gel formation mechanisms and dynamics, mechanical characteristics, and degradation behavior. In hydrogels, these properties are prescribed by the intrinsic properties of the main chain polymer and the crosslinking characteristics (i.e. amount, type, and size of crosslinking molecules), as well as environmental conditions. Gel formation mechanisms and dynamics dictate how molecules and cells are incorporated into a scaffold and how that scaffold is then delivered. Common fabrication processes and reagents such as temperature increases, pH changes, and various solvents can denature proteins (Creighton TE,1993) and cause cell damage or death. One approach to bypass this issue is to process the material and create a scaffold prior to incorporating bioactive molecules and cells. However, an exciting feature of many hydrogels is their ability to be mixed with cells and molecules prior to injection and in vivo gel formation. Injectable, in vivo gelling forms of alginate (Paige KT et al., 1995; Marler JJ et al., 2000; Alsberg E et al., 2001; Lee KY et al., 2001), PEO (Elisseeff J et al., 1999), chitosan, and P(PF-co-EG) have all been successfully combined with cells and/or bioactive molecules and delivered in a minimally invasive manner. The success of this approach depends on the ability to control both pre- and post-gel properties including gel formation rates and liquid flow properties. Once the scaffold is produced and placed, formation of tissues with desirable properties relies on scaffold material mechanical properties on both

the macroscopic and the microscopic level. Macroscopically, the scaffold must bear loads to provide stability to the tissues as it forms and to fulfill its volume maintenance function. On the microscopic level, evidence suggests that cell growth and differentiation and ultimate tissue formation are dependent on mechanical input to the cells (Kim B-S et al., 1999; Butler DL et al., 2000; Cowin SC, 2000; Sikavitsas VI et al., 2001). As a consequence, the scaffold must be able to both withstand specific loads and transmit them in an appropriate manner to the surrounding cells and tissues. Adequate mechanical performance of a scaffold depends on specifying, characterizing, and controlling the material mechanical properties including elasticity, compressibility, viscoelastic behavior, tensile strength, and failure strain. For hydrogels, these properties are affected by polymer and crosslinker characteristics, gelling conditions (e.g. temperature and pH), swelling, and degradation (Anseth KS et al., 1996). For example, the mechanical strength and compression modulus of alginate hydrogels increase with increasing ratios of G to M subunits as well as increasing lengths of G blocks. In addition, increasing the volume fraction of alginate from 1% to 3% results in an increase in both the compression modulus and equilibrium shear modulus. Similar increases in compression modulus were observed for PEG gels when the weight fraction of PEG was increased from 10% to 40% (Bryant SJ et Anseth KS, 2002) and for PVA hydrogels (Stammen JA et al., 2001). Hydrogel mechanical properties are also affected by the crosslinker type and density. The mechanical strength of ionically crosslinked alginate hydrogels increases when the ion concentration is increased and when divalent ions that have a higher affinity for alginate are used for crosslinking. Similarly, the mechanical shear modulus of covalently crosslinked alginate is dependent on the crosslinker density. In addition to the polymer and crosslinker characteristics, gel swelling usually results in a decrease in the mechanical strength of hydrogels. However, the mechanical properties and swelling have been independently controlled in covalently crosslinked alginate hydrogels by varying both the crosslinker type and density. Hydrogel degradation and dissolution usually lead to a weakening of the gels unless tissue in growth acts to strengthen them or these properties are decoupled. The desired kinetics for scaffold degradation depends on the tissue engineering

application. Degradation is essential in many small and large molecule release applications and in functional tissue regeneration applications. However, it may not be warranted if the application is related to cell encapsulation for immunoisolation. Ideally, the rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. Thus, it is important to understand and control both the mechanism and the rate by which each material is degraded. For hydrogels, there are three basic degradation mechanisms: hydrolysis, enzymatic cleavage, and dissolution. Most of the synthetic hydrogels are degraded through hydrolysis of ester linkages (Saito N et al., 2001). As hydrolysis occurs at a constant ratein vivo and in vitro, the degradation rate of hydrolytically labile gels (e.g. PEG-PLA copolymer) can be manipulated by the composition of the material but not the environment. As discussed in the materials section above, collagen, HA, and chitosan are all degraded by enzymatic action. Synthetic linkages have also been introduced into PEO to render it susceptible to enzymatic degradation. The rate of enzymatic degradation will depend both on the number of cleavage sites in the polymer and the amount of available enzymes in the scaffold environment (West JL et al., 1999; Mann BK et al., 2001). Ionically crosslinked alginate normally undergoes dissolution, but can also undergo controlled hydrolysis after partial oxidization. The rate of dissolution of ionically crosslinked alginate depends on the ionic environment in which the scaffold is placed (LeRoux MA et al., 1999).

4.4 Mass transport properties

The success of scaffolds for tissue engineering are typically coupled to the appropriate transport of gases, nutrients, proteins, cells, and waste products into, out of, and/or within the scaffold. Here, the primary mass transport property of interest, at least initially, is diffusion. In a scaffold, the rate and distance a molecule diffuses depend on both the material and molecule characteristics and interactions. Gel properties such as polymer fraction, polymer size, and crosslinker concentration determine the gels nanoporous structure (Lu S et Anseth KS Lu S, Anseth KS, 2000). As a consequence, diffusion rates will be affected by the molecular weight and size of the diffusion species (defined by Stokes radii)

compared to these pores. For example, molecules such as glucose, oxygen, and vitamin B12, with molecular weights less than 1300 Da and Stokes radii less than 1 nm, are able to freely diffuse into and from ionically crosslinked alginate microspheres (Tanaka H et al., 1984; Li RH et al., 1996). However, higher molecular weight molecules, including myoglobin, albumin, and fibrinogen are not able to freely diffuse, and their rate of diffusion is further decreased by increases in alginate concentration, in Ca^{2+} concentration, and/or in extent of gellation. The diffusion rates of molecules through glutaraldehyde crosslinked chitosan gels are also decreased when the crosslinker concentration is increased. For PEO hydrogels the size and molecular weight of molecules that are able to diffuse and the rate at which they diffuse both increase as functions of increasing polymer molecular weight and hydrolysable linkages. Interestingly, for alginate and likely other charged polymers, the diffusion rates of charged molecules are not solely size-dependent (Stewart WW et Swaisgood HE, 1993). Rather, they are also affected by charge interactions with the negatively charged alginate chains. Ultimately, diffusion requirements and subsequent material choice depend on the scaffold application. In the case of small and large molecule delivery, limiting free diffusion out of the scaffold may be a priority. In contrast, enhancing the supply of oxygen and nutrients and the removal of waste products is essential to the survival of implanted cells. In vivo, most cells exist within 100 mm of a capillary (Vander AJ et al., 1990), and diffusion is usually adequate for cell and tissue survival over this distance. However, for larger distances, other means of transport (e.g. simultaneous angiogenesis) must be incorporated.

4.5 Biological properties

Materials used to form gels engineered to exist in the body must simultaneously promote desirable cellular functions for a specific application (i.e. adherence, proliferation, differentiation) and tissue development, while not eliciting a severe and chronic inflammatory response. Hydrogel forming polymers are generally designed to be non toxic to the cells they are delivering and to the surrounding tissue. Both collagen and HA are major components of the native ECM and tissues (Alberts B et al., 1994; Lee CH et al., 2001). Both should theoretically

interact favorably with the body, provided that they have not been contaminated during processing and that there are no cross-species immunological issues (both are typically derived from bovine sources). PEO is currently FDA approved for many medical applications, while P(PF-co- EG) has been shown to be slightly toxic to cultured cells in vitro and to not induce a significant inflammatory response in vivo (Suggs LJ et al., 1999). Chitosan has also been shown to be nontoxic despite its chemotaxic effect on neutrophils (Lee KY et al., 1995; Ueno H et al., 2001). For alginate, it was thought that high M content induced an immune response, but recent data suggest that contaminants were the more likely cause of the response (Klock G et al., 1997). When purified, alginate is relatively immune quiescent; however, the purity of commercially available alginate continues to be a problem (Zhang WJ et al., 2001). While many hydrogels are non toxic and do not activate a chronic immune response, they also do not readily promote cellular adhesion and function. With the exception of collagen, which is a natural ECM protein, most cells do not have receptors to hydrogel forming polymers and thus cannot adhere. Furthermore, because of the hydrophilic nature of hydrogels, ECM proteins such as laminin, fibronectin, and vitronectin typically do not readily absorb to the gel surface (West JL et Hubbell JA, 1997). This fact has been exploited in the application of post-operative adhesion barriers (West JL et al., 1996) and in the design of specific adhesion surfaces (Hubbell JA, 1990). A common approach to design a highly specific adhesive surface is to covalently couple an entire ECM protein or peptide sequences capable of binding to cellular receptors (Rowley JA et al., 1999) to the polymer. The most common peptide used in this approach is the amino acid sequence arginine–glycine–aspartic acid (abbreviated Arg–Gly–Asp or more commonly, RGD), derived from numerous ECM proteins including fibronectin, laminin, vitronectin, and collagen. Other common peptides include arginine–glutamic acid–aspartic acid–valine (REDV) (from fibronectin), tyrosine–isoleucine–glycine–serine–arginine (YIGSR) (from laminin), and isoleucine–lysine–valine–alanine–valine (IKVAV) (from laminin). Most cell types are able to bind to RGD, thus both alginate and PEG (Hern DL et Hubbell JA, 1998; Mann BK et al., 2001) have been modified with this peptide to promote cellular adhesion. In an alternative approach, PVA was modified with the

complete fibronectin protein to promote cell adhesion. Growth factor tethering and incorporation are other avenues available by which hydrogels can be modified to regulate the functions of interacting cells. As with adhesion proteins and peptides, growth factors and growth factor derived peptides have been covalently attached to hydrogel polymers. For example, transforming growth factor-b (TGF-b) was tethered to PEG to enhance smooth muscle cell ECM production (Mann BK et al., 2001). Alternatively, an oligopeptide derived from bone morphogenetic protein-2 (BMP-2) was covalently attached to alginate to promote osteoblast migration into gels and subsequent calcification of the scaffolds (Suzuki Y et al., 2000). Multiple factors may also be incorporated into hydrogels to manipulate tissue formation (Elisseeff J et al., 2001).

5. In vitro culture of isolated follicles

5.1 Importance of maintaining of follicular architecture

Folliculogenesis within the ovary is a complex process requiring interaction between somatic cell components and the oocyte. At birth the human ovary contains 1-2 million primordial follicles, each containing an oocyte in meiotic arrest at the prophase stage (Baker TG, 1963). The oocyte is surrounded by a layer of somatic granulosa cells. Follicular growth from the primordial to the pre-ovulatory stage occurs in two distinct stages. The first growth phase occurs very slowly and is not directly dependent on gonadotrophin levels (Abir R, 2006). There is proliferation of the granaulosa cell layer surrounding the oocyte and an increase in both follicle and oocyte diameter. This stage can take weeks in rodents and months in larger animal species, including humans. In the human, follicles increase in size from 30-50 μm in primordial resting follicles, to 100-200 μm in pre-antral follicles (Gosden RG et al., 1993). The second phase of follicular growth is far more rapid and culminates with the ovulation of a mature oocyte. Follicles are now responsive to follicle stimulating hormone (FSH) and luteinizing hormone (LH). The formation of a fluid filled antrum and synthesis of steroid hormones marks the transition to the antral phase of follicle development. Human

follicles are over 18 mm when they reach the pre-ovulatory or Graafian stage and the oocyte is close to its final size, around 120 μM (Griffin J et al., 2006). The multi-layer follicle is now surrounded by a basement membrane that separates it from the underlying vascularized thecal cell layer. Oocyte growth and cytoplasmic meiotic competence are dependent on the gap junctions between the oocyte and the granulosa cells (Carabatsos MJ et al., 2000). Knock out mice lacking the gene encoding for gap junction protein connexin-37 have impaired folliculogenesis (Simon AM et al., 1997). The gap junctions connecting the granulosa cells and the oocyte enable sharing of secreted paracrine factors that promote the growth of both cell types (Eppig JJ et Schroeder AC, 1989; Herlands RL et Schultz RM, 1984; Murray A et Spears N, 2000; Diaz FJ et al., 2007) (reviewed in Buccione R et al., 1990; Su YQ et al., 2009). Evidence suggests that granulosa cell proliferation and certain metabolic processes are controlled by oocyte-derived secretions (Eppig JJ et al., 2005). The oocyte is unable to transport certain amino acids, carry out glycolysis and cholesterol biosynthesis without the cooperation of granulosa cells in providing necessary factors (Eppig JJ, 1991). The oocyte overcomes these metabolic deficiencies by stimulating expression of specific genes in the cumulus cells that control synthesis of enzymes and amino acids that it needs. Severing of the gap junction and intercellular communication during in vitro culture triggers premature ovulation and eventual degeneration of the released oocyte. Maintenance of the intricate 3-D architecture and granulosa-oocyte interaction may therefore be critical for successful in vitro maturation of follicles. In conventional 2-dimensional (2-D) tissue culture systems, the follicle tends to flatten and granulosa cells surrounding and nurturing the growing oocyte, migrate away, leaving it naked and unable to complete the maturation process (West ER et al., 2007). This is especially true when dealing with human primordial follicles, which may need as long as three months in culture (Gougeon A, 1986).

5.2 Culture systems for follicle growth 2-D versus 3-D

The majority of early and ground breaking work on in vitro follicle culture was undertaken using conventional 2-D culture methodology. Pre-antral follicle

growth in multi-well plates as well as in microdrop culture (Nation A et Selwood L, 2009; Cortvrindt R et al., 1996; Adam AA et al., 2004; Mousset-Simeon N et al., 2005) yields mature oocytes. Eppig and Schroeder were able to achieve live births after in vitro maturation of mouse pre-antral follicles on a collagen impregnated gel and in vitro fertilization of the IVM oocytes. By including eight days of in situ culture of the intact newborn mouse ovary, the same collagen culture methodology could also be used to successfully mature primordial follicles and produce live offspring (Eppig JJ et O'Brien MJ, 1996). Other 2-D systems used for follicle culture include membranes coated with extracellular matrix proteins (Oktem O et Oktay K, 2007; Hovatta O et al., 1997; Berkholtz CB et al., 2006; Figueiredo JR et al., 1995). Despite the successes achieved with these 2D systems, they have been sub-optimal for sustained culture of cow, sheep and human follicles (reviewed in West ER et al., 2007). Culture on treated membranes or tissue culture substrata, impedes preservation of the spatial arrangements of cells seen in vivo. Follicular flattening due to granulosa cell attachment to the tissue culture vessel is problematic making the follicle complex extremely vulnerable to disruption of gap junctions. With enzymatic follicle isolation techniques, perturbation of the basal lamina surrounding the follicle can lead to granulose cell migration away from the oocyte. Establishing an in vitro culture model that can more accurately mimic the in vivo ovarian growth environment has therefore been the focus of much research. To this end, a tissue bioengineering approach has attracted much interest. The recognition of the importance of spatial arrangements between cells has spurred research in to 3-D culture systems. Data from a variety of different cellular models indicate that 3-D culture modulates cell behavior, growth, secretions, response to stimuli and communication with surrounding cells. In a landmark study, investigators were able to block the cell surface receptor b-1 integrin and completely alter the behavior of breast cancer cells grown in 3-D culture, in a manner never observed during conventional 2-D culture (Weaver VM et al., 1996; Bissell MJ et al., 2003). Others have noted that the gene expression profile of cells grown in 3-D culture more closely resembles that seen in vivo (Hwa AJ et al., 2007) and distinctly differs from that found after conventional 2-D culture. In addition to the

spatial arrangement of the cells it is becoming increasingly evident that the extracellular matrix support structure (ECM) plays a defining role in organizing communication between cells, controlling cell differentiation and modulating response to biochemical signals from the cellular microenvironment (reviewed in Griffith LG et Swartz MA, 2006). Scaffolding, matrix proteins and 3-D culture systems to maintain follicular architecture are avenues of research currently being explored by numerous investigators to gain further insight on the growth requirements of follicles. These 3-D systems are characterized by their ability to maintain the spherical morphology of the ovarian follicle and preserve the critical cell-cell and cell-matrix interactions within the surrounding stromal tissue, thereby allowing follicles to successfully complete the maturation process. Figure 4 depicts growth of pre-antral follicles in a 2-D vs 3-D culture system. Encapsulation of follicles may protect them from gap junction disruption through shear stress (Heise M et al., 2005; Heise MK et al, 2009) and may preserve expression of the gene encoding for the gap-junction protein connexin (De Paola N et al., 1999). Contiguous assembly of granulosa cells around the oocyte also prevents the follicles from undergoing premature ovulation (Xu M et al., 2006). Another advantage may be that trophic factors released by granulosa cells remain in close proximity to the oocyte exerting a positive effect on oogenesis and possibly fostering new local gap junctions. This is an advantage not shared by 2-D culture vessels where the volume of medium in culture vessels and polarization of granulosa cells towards the culture substrata may result in a more diffuse and less uniform exposure to secreted factors. Despite the potential advantages with 3-D culture, there is still a good deal of controversy as to how best to achieve such a culture system. The questions revolve around the type of biomaterials available, their characteristics, permeability, toxicity and ability to be molded and handled with ease during follicle loading and harvest. In addition, their biologically usefulness ultimately depends on survival of the follicle and maturation of the oocyte in vitro. The animal species and the length of time needed for follicular culture also warrant consideration in determining whether 3-D culture will be beneficial. In humans, in vitro follicle maturation from primary to the antral stage can take more than 120 days (Eppig JJ, 1991), in contrast to 30 or so days for

follicle maturation in rodent species (reviewed in McGee EA et Hsueh AJ, 2000). Moreover, by the early antral stage human follicles measure 2-5 mm in diameter. Active perfusion systems may therefore prove necessary to assure sufficient nutrient supply to multilayered follicles if cultured in a 3-D environment.

5.3 Design parameters for biomaterials for 3-D culture

Chemical and physical properties of biomaterials present certain design limitations that must be meshed with the physiologic needs of the follicular unit. First and foremost, the chemical composition must be non-cytotoxic, allowing sustained cell viability for extended culture periods. Growth of follicular diameter during the course of in vitro maturation dictates materials with a certain amount of elasticity allowing expansion of the granulose cell layers, yet providing enough support to retain spherical shape and prevent inadvertent denudation of the oocyte. This is especially important in 3-D culture systems that physically encapsulate the follicle within a biomaterial. In addition to maintaining structural integrity, this biomaterial must allow adequate gas exchange, diffusion of nutrients and removal of cellular waste. Within the ovary there is an increase in vascularization as one moves deeper in to the ovarian cortex where secondary and pre-antral follicles grow (van Wezel IL et Rodgers RJ, 1996). This suggests a stronger need for oxygen diffusion during the final stages of follicle maturation. The need for oxygenation may also require an active perfusion system when dealing with longer in vitro maturation intervals. Diffusion across the biomaterial during 3-D culture is controlled by creating specific pore sizes (Eiselt P et al., 2000). The mechanical properties of the biomaterial, such as viscosity and its ability to be molded also contribute to its usefulness for follicle culture and are dependent on molecular weight (Wee S et Gombotz WR, 1998). Another important attribute is the biomaterial's rigidity, also referred to as shear modulus. The shear modulus of a biomaterial is a mathematical description of its elastic properties-that is its ability to resist deformation with the application of a force. Biomaterial rigidity and its effect on follicle diameter, theca formation, antrum formation, estradiol production, and rate of meiotic resumption (GVBD and metaphase II oocyte formation) can all be used to compare outcomes with follicle encapsulation in 3-D

culture models (Kong HJ et al., 2004; West ER et al., 2007; Xu M et al., 2006). The ideal biomaterial for in vitro follicle maturation would also be one that could mimic the extracellular matrix (ECM) found within the ovary (Abbott A, 2003). It has been suggested that since ovarian stromal composition can vary, the selected ECM for follicle culture should ideally share the inherent properties of the particular species being cultured (Xu M et al., 2009). A final consideration is whether to individually or coculture follicles. Culturing follicles in clusters allows sharing of autocrine/paracrine secretions, increasing follicle-to-follicle communication and possibly enhancing the culture environment (Hovatta O et al., 1999). However, some disadvantages of co-culture systems include the potential sharing of growth-inhibiting hormones, like AMH (anti-Mullerian hormone), amongst follicles (Durlinger AL et al., 1999). Also co-culture can interfere with the monitoring, tracking and harvesting of individual follicles during the maturation process.

5.4 Current 3-D culture models

5.4.1 In situ culture

In vitro culture of ovarian tissue pieces as a technique for in situ 3-D follicle maturation has not been very effective. While primordial follicle growth can be supported in this manner, development of follicles past the pre-antral stage is inhibited. To obtain complete in vitro maturation of follicles and the release of a metaphase II oocyte it is necessary to remove the follicle from the ovarian cortex (Abir R et al., 2001; Telfer EE et al., 2008). Two-step culture systems in which follicles are first grown from the primordial stage in situ and then mechanically or enzymatically isolated and grown in vitro have been investigated with mouse as well as human ovarian tissue. Models for human in vitro follicle maturation from primordial or even pre-antral stages to a mature oocyte are still in the early phase. Recently, Li and colleagues were able to induce maturation of primordial follicles in ovarian tissue fragments from cancer patients. Human ovarian tissue fragments were treated with PTEN gene inhibitor and transplanted to immunodeficient mice

(Li J et al., 2010). The PTEN (phosphatase and tensin homolog) gene plays a pivotal role in cell regulation and apoptosis. Interestingly, in this study, inhibition of the PTEN gene allowed primordial follicles to advance to the pre-ovulatory stage. The ability to activate dormant ovarian follicles may play a pivotal role in establishing a successful in vitro culture model for maturing primordial follicles. A much deeper understanding of factors regulating human folliculogenesis is still needed to successfully mature human follicles to the Graafian stage and to be able to assess fertilization potential. Progress in this arena may well depend on establishing adequate 3-D culture systems that preserve the normal follicular architecture and allow extended in vitro culture intervals.

5.4.2 Matrices for follicle culture

Synthetic and biologic matrices for the support of follicle growth and maturation have been studied in several animal models as well as in humans. Table 4 presents various 3-D systems and matrices that have been applied to the culture of rodent pre-antral follicles.

Adam et al. [25]	2004	Microdrops under oil Millicell-CM membrane insert	Mouse	6 days	150-174 µm 175-200 µm	Microdrops 77% Membrane 83%	NA	Membrane 79%	Membrane insert Fert rate 75%, blast rate 48%
Pangas et al. [70]	2003	Alginate	Mouse	10 days	82 µm	68%	NA	40%	TEM indicate follicles in ALG maintained ultrastructure
Gomes et al. [62]	1999	Collagen gel encapsulation	Mouse	6 days	135 µm	NA	NA	NA	Follicle volume and response to FSH increased with 3-D culture in collagen
Nayudu et al. [54]	1992	Millicell-CM membrane insert	Mouse	6-7 days 3-5 days	125-150 µm 150-180 µm	NA	NA	NA	FSH stimulated growth, antrum formation, E2 dose response to FSH levels
Torrance et al. [61]	1989	Collagen gel encapsulation	Mouse	14 days	20-95 µm	36%	NA	NA	Growth to multi-laminar stage but no antrum formation

NA: Not available.

Table 4: studies examining matrices for 3D culture of pre-antral ovarian follicles from rodents

Table 5 summarizes 3-D culture models for follicles from larger animals, primates and humans. All of the matrices adopted for 3-D culture essentially permit spherical growth of the follicle, preserving the physical integrity of granulosa cell

and oocyte's interaction. Nayadu et al in 1992 accomplished this using a Millicell hydrophobic insert.

Table 2 Summary of 3-D culture studies with follicles from human, primate and large domestic animal species

Author	Year	System Description	Species	Culture Period	Initial Follicle Diameter	Final Diameter	Survival Rate	Antrum Formation	Observations/Conclusions
Amorim et al. [72]	2009	Alginate (ALG) 1%	Human	7 days	34-52 μm	44–70 μm	90%	NA	Alginate culture system supported growth of isolated follicles from frozen-thawed ovary
Xu et al. [67]	2009	Alginate 0.5% Matrigel embedded	Human	30 days	~175 μm	715 μm	NA	75%	Both 3-D systems supported growth of isolated human follicles
Xu et al. [48]	2009	Alginate (ALG) 0.25% versus 0.5%	Rhesus monkey	30 days	100-300 μm	20 vs 78%	60 vs 78%	Yes	Higher ALG better survival and growth. LH addition with FSH negative effect on survival and P_4 secretion
Itoh et al. [104]	2002	Collagen gel	Cow	13 days	145-170 μm	304 μm	NA	Yes	Serum-free culture. Insulin, FSH and LH together induced earlier antrum formation
Abir et al. [51]	2001	Collagen gel	Human	24 hours	35-45 μm	70 μm	NA	NA	Collagen matrix supported growth of fully isolated follicles but not tissue slice with partially isolated follicles
Hovatta et al. [49]	1999	In situ and partially isolated follicles Millicell + Matrigel	Human	~28 days total	NA	NA	NA	No	Tissue slices better less oocyte extrusion than collagenese isolated. Four weeks to reach secondary stage
Yamamoto et al. [105]	1999	Collagen gel	Cow	14 days	500-700 μm	NA	37%	Yes	MII 27%, 42% fertilization, 4% blastocyst One live birth.
Hiraoi et al. [106]	1994	Collagen gel	Pig	16 days	220-300 μm	NA	NA	Yes	40% MII formation in oocytes ≥110 μm No MII from oocytes <110 μm. Oocytes capable of being fertilized
Roy and Treacy [107]	1993	Agar	Human	5 days	90-220 μm	NA	NA	Yes	FSH induced antrum formation, hormone secretion. No FSH, no E2 secretion

Table 5: summary of 3D culture studies with follicles from human, primate and large domestic animal species

The non-tissue culture treated surface prevented granulosa cell migration that could disrupt follicle architecture. A variety of optically clear gels have also been applied towards follicle culture in different animal models. Follicles have either been completely encapsulated to create a 3-D environment or grown on a gel membrane with medium bathing both surfaces to simulate 3-D culture. Gels that have been used for tissue engineering include hydrogels like agar/agarose, calcium alginate, and hyaluronan, all from naturally derived polymers, as well as synthetic polymers such as PEG and PVA (reviewed in Tibbitt MW et al., 2009). Gels containing collagen alone as well as compounds containing collagen in combination with ECM proteins have also been applied to in vitro follicle growth. The physical characteristics of each of these matrices permit physical expansion

of the follicular unit during growth. Hydrogels contain polymers that cross-link or self assemble into hydrophilic structures. The 3-D crosslinking is what gives the gel its stiffness. The temperature and conditions for this cross-linking can be a critical factor in determining subsequent development of the follicle. For instance agar, derived from seaweed, requires exposure to elevated non-physiologic temperatures for melting before the cross-linking or gelling step, potentially damaging the follicle (Sawhney AS et al., 1994). Higher rates of atresia were observed in follicles grown on agar as compared to those placed in microdrop culture or in 3-D culture on a hydrophobic membrane insert. In contrast, Huanmin et al. (2000) described active follicular growth and antrum formation with caprine follicles embedded within agar (Huanmin Z et Yong Z, 2000). Their data did however show that secondary follicles survived better than primary follicles in this 3-D agar culture system. Agar embedding has also been applied to human and hamster pre-antral follicles (Roy SK et Greenwald GS, 1989; Roy SK et Greenwald GS, 1996). Follicles were biologically competent, secreting steroids and synthesizing DNA. Low melting point agarose may be a better matrix for follicle embedding, permitting encapsulation at temperatures more conducive to continued cell growth. Collagen is rich in glycine and proline and can be hydrolyzed in to a gel by boiling. This biomaterial has been widely applied to follicular culture. Eppig and colleagues used collagen membrane inserts as substrata in an attempt to simulate 3-D follicle culture. The membrane inserts with follicles were suspended in wells, and follicles were exposed to culture medium from below as well as above (Eppig JJ et Telfer EE, 1993). The biomaterial was not tissue culture treated. It did however allow follicle attachment but minimized granulosa cell migration. In vitro follicle maturation resulted in the formation of metaphase II oocytes, with the capability of producing live young after in vitro fertilization, growth and transfer to foster mothers. Despite this achievement, follicle growth on collagen treated membranes had limited potential in terms of maintaining spheroid follicle structure and follicles were susceptible to flattening over time in culture and to premature oocyte ovulation. To create a more spatially uniform 3-D culture system, follicles have also been embedded in collagen gel (Torrance C et al., 1989). Spontaneous follicle disruption as a result of

discontinuous or distorted basal lamina and granulose cell migration was decreased in the 3-D collagen system compared to control 2-D culture systems (Gomes JE et al., 1999). Follicle growth rate has also been reported to be superior (Loret de Mola JR et al., 2004). Granulosa-cell oocyte complexes embedded in collagen matrix remained rounded and compacted with neuronal- like outgrowths towards the oocytes (Combelles CM et al., 2005). Two limitations of the collagen gel have however been noted. The collagen gel is susceptible to shrinkage over time, affecting the gel's natural properties as well as reducing visibility during microscopic assessment. Also, follicle extraction from the collagen requires enzymatic digestion of the gel, with the potential for subsequent damage to the oocyte (Telfer E, 1996). The natural scaffolding upon which cells are organized in vivo, known as the extracellular matrix (ECM), is composed of collagen, along with laminin and fibronectin. ECM has been shown to play an important role in regulating cell behavior, differentiation and secretory activity (reviewed in Berkholtz CB et al., 2006). One commercially available ECM tested for follicle growth is matrigel (Buyuk E, 2003; Xu M et al., 2009). This ECM product is derived from the Engelbreth- Holm-Swarm (EHS) mouse sarcoma. Matrigel is composed of collagen IV, laminin, fibronectin, entactin, heparin sulfate proteoglycans, and a variety of growth factors such as EGF, FGF, IGF-1, PDGF and TGF-b (Martin GR et Timpl R, 1987; Kleinman HK et Martin GR, 2005). Murine pre-antral follicles in 3-D culture in matrigel exhibited higher growth and survival rates than those in conventional culture. Hovatta et al demonstrated higher survival of follicles in frozen-thawed human ovarian tissue placed in culture on matrigel coated inserts. Autocrine and paracrine signaling by ECM molecules and associated growth factors likely affect folliculogenesis. The interactions between ECM proteins and follicles from different animal models needs to be further studied. The source and type of ECM could also play a role in regulating follicle growth during 3-D culture. The size of ECM molecules can present problems and an alternative solution has been to adsorb known sequences of matrix peptides, such as RGD (Arg-Gly-Asp) or laminin-derived peptide sequences on to synthetic matrices (reviewed in Berkholtz CB et al., 2006). To date the most widely applied system for follicle encapsulation and 3-D culture has

been alginate (Pangas SA et al., 2003; Kreeger PK et al., 2005; Amorim CA et al., 2009; Xu M et al., 2009), because of its possibility to form hydrogels in very mild conditions. This property facilitates encapsulation of follicles under physiologic conditions. Pangas et al. (2003) first applied this system to the 3-D culture of granulosa-cell oocyte complexes (GOC) from 12-day old mouse pre-antral follicles. GOCs were embedded in alginate beads ranging in size from 0.5 to 1 mm in diameter. Light microscopic and TEM ultra-structure studies suggested that the alginate did not interfere with oocyte or granulose cell growth development over a 10 day culture interval. Moreover, oocytes recovered from the encapsulated GOCs were able to resume meiosis, undergo fertilization and produce viable offspring. This 3-D system has also been applied to secondary follicles. Follicles embedded in alginate hydrogels responded to FSH stimulation in a dose-dependent fashion, secreting estradiol and progesterone. Alginate matrix stiffness and density can affect secondary follicle expansion, hormone production and oocyte maturation. Non-human primate follicles have also been successfully cultured in calcium alginate gels for up to 30 days. The encapsulated monkey pre-antral follicles secreted estrogen, progesterone and androstenedione and responded to FSH in the culture milieu. Interestingly, follicles cultured in 0.5% alginate performed better than those in 0.25% alginate, suggesting that primate follicles may require more physical support. One concern however is that denser matrices could potentially limit access to hormones and other nutrients. Heise et al. (2005) reported inhibited delivery of FSH to microencapsulated follicles. Follicle diameters increased with inclusion of FSH in the hydrogel but still did not reach that observed in un-encapsulated controls. Clearly, the physical attributes of the 3-D matrix selected for follicle culture needs to be tailored towards the species and follicle stage being cultured. In humans, pre-antral follicle growth in vitro offers an avenue through which cryopreserved ovarian tissue can be utilized without the need for transplantation. Human follicles isolated from fresh or cryopreserved ovarian tissue have been successfully cultivated in calcium alginate hydrogels but functionality needs to be further characterized. Initial data with frozen mouse ovarian tissue certainly suggests that meiotically competent oocytes can be recovered after in vitro maturation of isolated follicles in this 3-D culture

system. To further simulate the in vivo environment, ECM molecules have been combined with calcium alginate to construct synthetic ECM matrices for 3D culture (Kreeger PK et al., 2003). The adhesion peptide sequence arginine-glycine-aspartic acid (RGD) common to ECM proteins has been synthetically created and coupled to calcium alginate to construct such a synthetic matrix for follicle growth. Hormone secretion by follicles was directly related to adhesion peptide concentration and a three-fold increase in progesterone and estradiol secretion could be induced by adjusting matrix parameters. In a separate study, these investigators combined calcium alginate with additional ECM components such as collagen I, collagen IV, laminin and fibronectin (Kreeger PK et al., 2006). Matrix effect on growth from two-layered to multi-layered follicles as well as oocyte maturation to metaphase II was compared. Transition to the multi-layered, secondary follicle was enhanced in alginate matrices with RGD or collagen I. Final maturation of oocytes and resumption of meiosis was promoted by presence of fibronectin, laminin or RGD peptide.

5.4.3 Criteria for biomaterial evaluation

Increasing follicular diameter is typically used as a measure of follicle maturation. During in vitro growth, especially in traditional 2-D culture systems where there is granulosa cell expansion, an increase in horizontal diameter of the follicle does not necessarily correlate to overall follicular growth. With 3-D culture the biomaterial presents equal counter-forces in all directions, minimizing flattening and allowing equal growth along all axes. Follicle volume as well as diameter should therefore be taken into account when comparing different substrata. Another outcome measure indicative of follicle functionality and growth is antrum formation. This accumulation of fluid within the follicle complex has been shown to vary with 2- versus 3-D culture systems, as well as the biomaterial used for follicle encapsulation. The shear elastic modulus and diffusion characteristics of the biomaterial must be carefully balanced. Torrance et al. (1989) noted no antrum formation in follicles cultured in collagen, despite an apparent increase in follicular diameter over the 14 day culture interval. It was suggested that the double gelling of the collagen during follicle encapsulation allowed just enough

flexibility for some granulosa cell proliferation, but that the overall high shear elastic modulus (increased stiffness) inhibited antrum formation. Interestingly, this was not observed when follicles were individually cultured in collagen microbeads (Sharma GT et al., 2009). A relationship between decreased gel stiffness and greater antrum formation was also observed with calcium alginate hydrogel when tested at concentrations of 3%, 1.5% and 0.7%. The study of Xu et al. (2006) most clearly illustrates the opposing influences of the rigidity of the biomaterial at high gel concentration and its interference with diffusion and optimal growth. Oocytes obtained from follicles encapsulated in 0.25% alginate had a higher developmental capacity than those cultured in 1.5% alginate. In vitro maturation and fertilization of oocytes in 0.25% vs 1.5% calcium alginate were significantly higher (41% vs 5%, respectively). Moreover oocytes derived from the stiffer gel were clearly impaired and unable to undergo in vitro blastulation. Interestingly, follicles from primates showed the opposite relationship between gel rigidity and follicle growth. Follicle survival and diameter were increased with culture in 0.5% calcium alginate as compared to 0.25%. Ovarian stroma of primates is more rigid than that found in rodents and it has been suggested that perhaps primate as well as human follicles may require a stiffer biomaterial to optimize in vitro culture and growth. The 100% survival rate and 75% antrum formation observed with human secondary follicles grown in 3-D culture in 0.5% calcium alginate matrix further support this supposition.

5.4.4 Non-gel culture systems

Despite the aforementioned benefits of follicle encapsulation as a model for 3-D culture, there are also difficulties. The process of encapsulation as well as the removal of follicles from the gel can be problematic, sometimes resulting in loss of healthy follicles. Alternatives methods for 3-D culture of follicles that do not involve encapsulation have therefore also been explored. Suspension culture of follicles in orbiting test tubes, rotating-wall vessels, and roller bottle systems can maintain the 3-D morphology of the follicles without encapsulation. Unfortunately these systems have not been extremely effective. The rate of rotation necessary to keep the follicles from descending to the bottom of the

vessels imposes shear stress on the follicles causing follicle degeneration (Rowghani NM et al., 2004). Moreover, the only way to negate this effect was to encapsulate the follicles before subjecting them to suspension culture with rotation. Suspension culture in rotating systems with its accompanying shear stress resulted in more follicle loss than that observed with embedding and removal of follicles from gels. Follicle survival with culture in a rotating wall culture vessel was only 9% as compared to the 15% observed after embedding and removal from collagen gel culture. With marsupial follicles, survival rate in the roller culture system was higher; nearly 49%, but follicles exhibited no antrum formation. Other non-gel approaches have included serial culture of follicles in new wells each day to prevent attachment (Boland NI et al., 1993) and flattening, or culture in simple microdrop under an oil overlay (Bishonga C et al., 2001). Inverted microdrop suspension culture has also been tested as a means to maintain the 3-D architecture of follicles (Wycherley G et al., 2004). Follicles are placed in microdrops under oil on the bottom of a tissue culture plate and then hung upside down during culture. Oil is ideally suited as a biomaterial for microculture environments, allowing maintenance of pH and temperature around the follicle and free gas exchange (Tae JC et al., 2006). However, its hydrophobic properties could potentially allow the escape of lipid soluble follicle secretions and growth factors in to the oil layer, ultimately hindering growth (Miller KF et Pursel VG, 1987). It should however be noted that while inverted suspension culture yielded survival rates similar to that observed with alginate gels, the meiotic maturation rate was only 10%, far less than that what has been achieved with gel encapsulation of follicles. Handling large numbers of follicles in inverted suspension culture would also be a delicate and labor intensive process. This method would be especially unsuitable for follicles from the human ovary, which might require as long as three months of culture.

5.4.5 Microfluidic culture

The final aspect of follicle culture that needs some attention is the development of culture vessels or systems that maximize diffusion of nutrients and gases through

the selected biomaterial while allowing retention of the delicate micro-environment of the follicle and the concentration of essential trophic factors around the oocyte. To accurately mimic the in vivo ovarian environment, fluid flow across the encapsulated follicle is vital. Also, within the ovarian environment follicles are grown in close proximity of each other, allowing sharing and concentration of secreted factors. The logistics of co-culturing numerous encapsulated follicles can perhaps be aided by the use of microfluidics that allow precise control and manipulation of fluids using microchannels. Microchannels increase the surface areato-volume (SAV) ratio, implementing laminar fluid flow (Beebe D et al., 2002) (reviewed in Suh RS et al., 2003). Diffusion across biomaterials has been shown to be influenced by not only the biomaterial and its concentration but also by its shape or presentation. Encapsulating in microbeads of gel may allow more uniform diffusion across all surfaces as compared to culture with follicles embedded in a single continuous layer of gel. Survival and antrum formation by cultured pre-antral buffalo follicles was demonstrated to be better after culture in collagen microbeads as compared to a continuous layer of collagen matrix. Tiny microbeads containing follicles in a biomatrix, combined with a system of microchannels could be used to create a network of individual follicles sharing nutrients. A dynamic medium exchange could therefore be applied to follicle culture in a manner that avoids the shear stress observed with rotating culture systems and preserves a “coculture” atmosphere. A variety of microfluidic culture systems have been described. Cell immobilization with continuous media flow is the common goal. This can be accomplished with microposts on the culture surface to entrap cells and create a matrix support while still allowing laminar flow of fluid to pass by (Chen X, 2009; Hashimoto, 2008) or by entrapping cells between walls of PDMS with continuous flow of culture medium above the cells. Microwells can also be used as architectural supports in microfluidic systems and act as nests for cells to culture in while fluid is exchanged above or below (Khademhosseini A et al., 2005; Moeller HC et al., 2008). Microfluidics in combination with valves and micro-scale pumps provide the option of continuous media flow in ways similar to that seen in vivo (Heo YS et al., 2007; Lee PJ et al., 2007). Microfluidics thus permits dynamic culture

conditions and medium flow without disturbing the cell itself. Application of microfluidics to the field of reproductive biology has gained much attention. It has been applied to sperm sorting (Schuster TG et al., 2003; Chung Y et al., 2006), oocyte handling and fertilization (Sadani Z et al., 2005; Hogan B et al., 1994; Suh RS et al., 2006; Clark SG et al., 2005) and embryo culture (Glasgow IK et al., 2001; Raty S et al., 2004; Walters EM et al., 2004; Walters E, 2007; Bormann C et al., 2008). Follicle culture in microfluidic devices needs to be explored. This type of system may be ideal for providing the 3-D environment necessary for maintaining follicle architecture over long intervals in culture, allowing adequate oxygenation and nutrient exchange and at the same time permitting sequestration of autocrine/paracrine factors within the vicinity of the growing follicle. The ideal microfluidic model would allow monitoring and harvest of individual follicle but also a sharing of the microenvironment to attain the benefit of "coculture".

AIM OF RESEARCH

Aim of my PhD research project is development of innovative strategies of fertility preservation in cancer women. Currently, different biotechnologies have been developed for these patients as oocytes or embryos cryopreservation before chemo/radiotherapy dramatically reduces patients fertility. However, oocyte or embryo cryopreservation are not an option for pre-pubertal girls and for women who do not have enough time before beginning of cancer therapy. In order to give these patients the possibility to restore their fertility, I have focused my attention on development of two alternative strategies. The first one, more feasible, is ovarian tissue cryopreservation: it avoids the delay needed for obtaining mature oocytes, but the subsequent potential for establishing pregnancies is unknown However, cryopreservation methods have to be still investigated to improve their experimental efficiency. The second strategy is isolation of follicles from ovarian tissue and in vitro culture, allowing to avoid also the risk of transmitting residual malignant cells in cryopreserved tissue, but until now this biotechnology is still undeveloped and only a few studies have been performed. To this aim in these three years of my PhD thesis, I have focused my interest in development of:

1. new protocols for ovarian tissue cryopreservation;
2. new strategies for follicle isolation and in vitro follicle culture.

To improve cryopreservation efficiency the effect of choline chloride as substitute of sodium in freezing solutions will be studied. Sodium ion is one of the major cause of electrolyte accumulation within frozen cells, which induces cellular damages. Choline ion is, instead, larger and unable to across cell membrane. The effect of sodium-depleted choline supplemented cryopreservation protocols will be investigated through use of different imaging analysis, such as histological, immunohystochemical and ultrastructural analysis.

A different biotechnological approach will be used to develop an efficient method for follicle isolation and subsequent in vitro culture. To reproduce in vitro the architecture of follicle interactions the effects of alginate matrix on in vitro culture

of isolated follicles will be studied. Follicles will be firstly cultured for up to 10 days in alginates at different concentration to determine which one best supports follicle survival and growth. The efficiency of method will be investigated monitoring daily follicular growth through a NIS software imaging analysis and viability assays, using fluorescent dies. Since the physical properties of alginate hydrogels vary widely depending on their composition (e.g., the proportion of guluronic to mannuronic acid residues) and the sequential order of these residues, next step will be to investigate which factor could influence the growth characteristics of encapsulated follicles. Ovarian follicle culture systems provide an ideal tool to study the physical properties of a three-dimensional hydrogel matrix in follicle development. The hormonal regulation of folliculogenesis has been widely investigated, yet the role of the physical properties of the follicle microenvironment has not. Understanding the role of the physical environment on follicle development will be useful for the development of biomimetic matrices for the in vitro culture of follicles and other hydrogel-encapsulated cell culture systems. To this end, the following biomaterials will be tested: alginate SLG 20 1.5% (69% guluronic acid, 75kDa), alginate SLG 100 1.5% (68% guluronic acid, 200kDa), alginate 1.5% (50% guluronic acid, 50% mannuronic acid); As extracellular matrix (ECM) is essential for follicular development, to further simulate the in vivo environment, ECM molecules will be combined with calcium alginate to build synthetic ECM matrices for 3D culture. To this end, alginate 1.5% will be combined with collagen type IV 0.3mg/mL, as the latter is the main ECM component present during follicologenesis. Similarly, the efficiency of method will be studied through viability assays, growth monitoring and analysis at confocal microscope.

After, the development of follicles and their ability to survive to cryopreservation will be analysed. Particularly, two different protocols will be tested and validity of vitrification will be studied analyzing morphologically follicles after thawing. Moreover, viability assays will be applied.

Finally, to understand whether frozen thawed isolated follicles were able to undergo growth and development after transplantation, in the present study, in collaboration with the Research Laboratory on Human Reproduction in Brussels,

after in vitro culture and vitrification/thawing alginate encapsulated follicles will be xenografted to the kidney capsule or the back muscle in immunodeficient mice. Grafts will be removed after 3 months and analysed by histological assay in order to evaluate the physiological status of xenografted follicles.

MATERIALS AND METHODS

Tissue collection and dissection

Ovarian tissue has been collected as small biopsy pieces from 20 women donors with a median age of 31 years (range 22–40 years). All women have been informed about the ongoing project, and they signed an informed consent form. Ovarian tissue has been transferred in sterile 50 ml Falcon Tube containing 20 ml of pre-warmed gamete buffer (Cook Italy) and immediately transported at 37°C to the laboratory. The ovarian cortical tissue has been manually dissected from medullar tissue and divided into strips of about 1 mm^3. Two small pieces of fresh tissue have been fixed directly and subsequently used as non-frozen controls for light microscopy and transmission electron microscopic evaluation. The others have been randomly distributed into vitrification and slow-freezing groups.

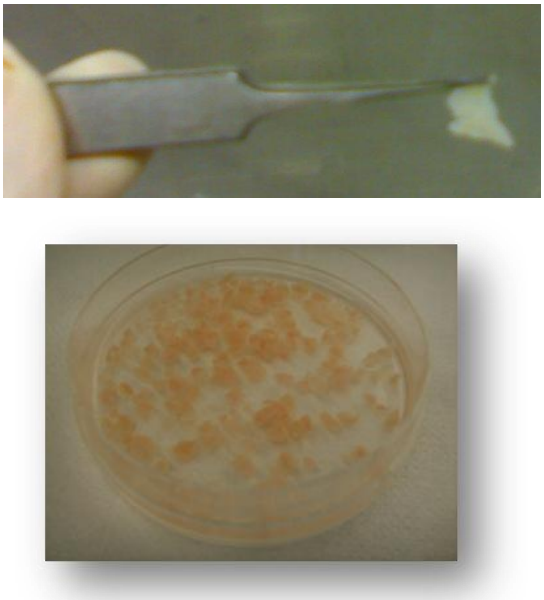


Fig. 6: ovarian dissection

Tissue cryopreservation

Freezing and thawing solutions have been dissolved in two different basal media: the first one was conventional Dulbecco Phosphate Buffered Saline (DPBS), the second one consisted of modified phosphate buffered saline in which chloride sodium has been replaced by choline chloride on an equimolar basis (CPBS).

Vitrification and warming

Basal media have been supplemented with 10 mg/ml human serum albumin (HSA).

The first vitrification protocol (VH and VHC) consisted of three incubation steps in solutions with increasing concentrations of DMSO (Sigma-Aldrich, Sweden), 1,2-propanediol (PrOH) and ethylene glycol (EG) dissolved in basal mediums. After washing for 5 min in basal media, pieces of ovarian cortex have been transferred for 5 min sequentially to 1 ml of vitrification incubation solutions VS1 (0.35 M DMSO, 0.38 M PrOH, 0.38 M EG), VS2 (0.7 M DMSO, 0.75 M PrOH, 0.75 M EG) at room temperature and VS3 (1.4 M DMSO, 1.5 M PrOH, 1.5 M EG) at 4°C. At the third step, VS3 has been supplemented (10% w/v) with polyvinylpyrrolidone (PVP; Sigma-Aldrich, Sweden) (Keros et al., 2009). The samples have been directly plunged into liquid nitrogen (-196°C) and placed in a pre-cooled 5.0 ml Nunc cryotube (Nunclon, Roskilde, Denmark), closed and stored in liquid nitrogen.

In the second vitrification protocol (VYB and VYBC) the strips have been dehydrated by using a two-step regimen: (1) 2.0 mol/L dimethyl sulfoxide (DMSO) + 0.1 mol/L sucrose for 5 minutes; (2) 2.0 mol/L DMSO + 2.0 mol/L propanediol (PROH) + 0.2 mol/L sucrose for 5 minutes. The samples have been directly plunged into liquid nitrogen as reported before (LI Yu Bin et al., 2007).

For warming, the vitrified pieces have been taken out of the cryovials in a container filled with liquid nitrogen, quickly immersed into a 38°C water bath, and gently agitated until the ice melted nearly completely. Afterwards, the tissues have been moved quickly into 0.5 mol/L sucrose in basal media for 5 minutes at room temperature, and then taken through 0.25 mol/L and 0.125 mol/L sucrose in basal media each for 5 minutes at room temperature. Finally, the warmed strips

have been rinsed three times in basal media and put into a 37°C, 5 % CO_2 humidified incubator for 15 minutes for following procedures.

Slow freezing and warming

Basal media have been supplemented with 25 mg/ml human serum albumin (HSA).

The first slow freezing cryopreservation protocol (Hovatta et al., 1996) (CLH and CLHC) consisted of three steps of incubation in cryopreservation solutions with PrOH as a permeating cryoprotectant and sucrose as non-permeating cryoprotectant. The tissue pieces have been first incubated for 5 min in 1 ml of basal media, then for 10 min in the first slow cooling solution (SC1) containing 1.5 M PrOH, and finally for 15 min in SC2 which contained 1.5 M PrOH and 0.1 M sucrose. The third incubation step has been performed in 1.8 ml Nunc cryovials (Nunclon, Roskilde, Denmark), which have been placed in a programmable freezer. Subsequently, the samples have been cooled from room temperature to -6.5°C at a rate of 2°C/min. Seeding has been performed by means of forceps pre-cooled in liquid nitrogen. After a 10-min holding period, the samples have been cooled to -35°C at a rate of -0.3°C/min, and after holding for 10 min they have been plunged directly into liquid nitrogen. For thawing, the cryovials have been first taken from liquid nitrogen and exposed for 30 s to room temperature. They have been then plunged for 2 min into a warm (37°C) water bath until the ice has been melted. Then pieces have been transferred to different thawing solutions (TS) in basal mediums. The first step has been 5 min in TS1 which contained 1.0 M PrOH and 0.2 M sucrose, then 5 min in TS2 containing 0.5 M PrOH and 0.2 M sucrose, then 10 min in TS3 with 0.2 M sucrose and finally 10 min in TS4 which consisted in pre-warmed basal media in 37°C, 5 % CO_2 air.

The second slow freezing cryopreservation protocol (CLYB and CLYBC) consisted of two incubation steps respectively in SC1 supplemented with 1.5 M DMSO for 5 min and in SC2 supplemented with 1.5 M DMSO and 0.1 M sucrose. This one has been performed in 1.8 ml Nunc cryovials (Nunclon, Roskilde, Denmark), which have been placed in a programmable freezer. The samples have been cooled as follows: from 4°C to -8°C at -2°C/min; soaked for 10 minutes at

-8°C, then seeded manually with prechilled forceps, continually held for 10 minutes; cooled to -40°C at -0.3°C/min and to -150°C at -50°C/min; finally samples have been plunged immediately into liquid nitrogen and stored until thawing. For warming, the pieces have been taken out of the cryovials in a container filled with liquid nitrogen, quickly immersed into a 38°C water bath, and gently agitated until the ice melted nearly completely. Afterwards, the tissues have been moved quickly into 0.5 mol/L sucrose in basal media for 5 minutes at room temperature, and then taken through 0.25 mol/L and 0.125 mol/L sucrose in basal media each for 5 minutes at room temperature. Finally, the warmed strips have been rinsed three times in basal media and put into a 37°C, 5 % CO_2 humidified incubator for 15 minutes for following procedures.

Histological analysis

For histological investigation, all tissue pieces have been fixed for 4h in Bouin's solution (15% picric acid, 5% formaldehyde, 1% acetic acid glacial) (Sigma). Next, pieces have been repeatedly washed in current water, in order to remove the exceeded fixative, and dehydrated through sequential passages in ethanol at increasing concentrations. Particularly pieces have been sequentially transferred in ethanole 75% for 24h, ethanole 95% for 24h and finally three times in ethanole 100%, 10' each one. Pieces have been then clarified by two sequential passages, of 30' each one, in Hystolemon, transferred in Hystolemon-paraffin 1:1 at 46°C for 45' and finally embedded in paraffin wax for 24h at 60°C. Embedded pieces have been serially sectioned at 5 μm.

Sections have been then stained with hematoxylin/eosin, and analyzed under a microscope. For staining, sections have been deparaffinized, by two passages of 5' each one in Xylene (blot excess xylene before going into ethanol), rehydrated through sequential passages, of 2' each one, in ethanole 100%, 95%, 75% and 50% and then transferred in deionised water (blot excess water before going into hematoxylin). At this point, sections have been stained for 7' in hemalum, rinsed in tap water, stained for 7" in eosin 0.5%, dehydrated and mounted with

Hystovitrex (Carlo Erba). Hemalum is a complex formed from aluminium ions and oxidized haematoxylin. This colors nuclei of cells (and a few other objects, such as keratohyalin granules) blue. Instead, eosin colors eosinophilic structures in various shades of red, pink and orange.

The staining of nuclei by hemalum does not require the presence of DNA and is probably due to binding of the dye-metal complex to arginine-rich basic nucleoproteins such as histones. The mechanism is different from that of nuclear staining by basic (cationic) dyes such as thionine or toluidine blue. Staining by basic dyes is prevented by chemical or enzymatic extraction of nucleic acids. Such extractions do not prevent staining of nuclei by hemalum. The eosinophilic structures are generally composed of intracellular or extracellular protein. Most of the cytoplasm is eosinophilic.

The number of follicles of different developmental stages has been evaluated. Follicles with an oocyte surrounded by a single layer of flat granulosa cells have been defined as primordial. Follicles with the oocyte surrounded by one layer of only cuboidal granulosa cells have been defined as primary. Follicles with the oocyte surrounded by two or more layers of cuboidal granulosa cells have been classified as secondary. Eosinophilia of the ooplasm, contraction and clumping of the chromatin material, and wrinkling of nuclear membrane of the oocytes have been regarded as the signs of atresia (Gougeon A., 1986). The qualities of follicles have been graded from one to three. A follicle of grade 1 is spherical and is randomly distributed around oocytes granulosa cells, with homogenous cytoplasm and slightly granulated nucleus, in the center of which condensed chromatin in the form of dense spherical structure is detected. A follicle of grade 2 has the same peculiarities, but the granulosa cells cover irregular oocytes; these cells can be flat and condensed chromatin is not detected in cytoplasm. A follicle of grade 3 has partly or fully disrupted granulosa or cytoplasm and picnotic nucleus. Follicles of grades 1 and 2 have been denoted as normal and those of grade 3 have been denoted as degenerated. To illustrate the changes caused by different cryopreservation protocols, also oocyte germinative vescicle diameter and undamaged and pyknotic stromal cells have been considered.

Transmission electron microscopic analysis

For TEM, small pieces of all samples were fixed in 2.5% glutharaldehyde (SIC, Rome, Italy) in 0.1 M sodium cacodylate at pH 7.3 for 1.5 hour, washed three times for 10 minutes in the same buffer, postfixed in 1% osmium tetroxide (SIC) in 0.1 M sodium cacodylate at pH 7.3 on ice and washed three times for 10 minutes in the same buffer. Glutharaldehyde kills cells quickly by crosslinking their proteins and is usually employed alone or mixed with formaldehyde as the first of two fixative processes to stabilize specimens such as bacteria, plant material, and human cells. Osmium tetroxide is used in order to crosslink and stabilize cell and organelle membrane lipids. Samples were then treated in 0.1% tannic acid in cacodylate for 10 minutes and dehydrated in ascending series of ethanol (Carlo Erba Reagenti, Milan, Italy) on ice. All samples were treated twice for 5 minutes with propylene oxide (FLUKA, Milan, Italy), infiltrated in 1:1 propylene oxide/Epon 812 (Agar Scientific, Stansted, United Kingdom) overnight, and individually embedded in fresh resin. Thick (0.5–1 mm) and thin sections (60–80 nm) were cut with a diamond knife (Diatome, Biel, Switzerland) at a Reichert-Jung Ultracut E ultramicrotome and collected on glass slides or 200-mesh thin bar copper grids (SIC). Thick sections were stained with 0.1% toluidine blue in sodium borax, examined by light microscopy (LM) and photographed using a Nikon DS-cooled camera head DS-5Mc connected to a Nikon DS camera control unit DS-L1. Thin sections were stained with saturated uranyl acetate in methanol and Reynold's lead citrate and observed and photographed with a Philips (Eindhoven, The Netherlands) EM 208 S electron microscope (EM) at 80 KV. Uranyl acetate is the acetate salt of uranium and is a yellow crystalline solid made up of yellow rhombic crystals and has a slight acetic odor. Uranyl acetate is slightly radioactive, the precise radioactivity depends on the isotopes of uranium present. This compound is a nuclear fuel derivative, and its use and possession are sanctioned by international law. Uranyl acetate is used as a positive stain for TEM. Uranyl ions react strongly with phosphate and amino groups, staining DNA and some proteins. Organelles composed of membranes are not stained well. Note that the starting material is radioactive. Lead citrate may also be employed as a positive stain. Reynolds lead citrate stain binds lead ions to negative ions,

producing a general increase in contrast. Lead is a cumulative toxin, so skin contact must be avoided.

In the oocytes we studied the chromatin structures, the integrity of nuclear membranes, the density and integrity of the mitochondrial cristae, density of the cytoplasm, the size and numbers of vesicles in the cytoplasm, integrity of the cytoplasmic membrane and attachment of the oocyte to the granulosa cells. The same parameters were evaluated for assessment of granulosa cells. The nuclear and cytoplasmic membranes, nuclear chromatin of stromal cells, the attachment between granulosa cells and attachment to the basement membrane were also evaluated.

Immunohistochemical analysis

For immunohistochemical investigation, all procedures were carried out at room temperature unless otherwise stated. The paraffin-embedded ovary sections of all samples were deparaffinizated, rehydrated and then incubated for 5 minutes with 3% hydrogen peroxide in methanol (Sigma Aldrich), in order to inhibit the activity of endogen peroxidases. Samples are washed three times for 10 minutes in PBS supplemented with 0.1 % TRITON X100 (TPBS), incubated for 1 hour in blocking solution, containing 0.5% normal goat serum in TPBS and then washed three times for 10 minutes in TPBS. Triton X100 is used to permeabilize eukaryotic cell membranes and reduce the surface tension of aqueous solutions during immunostaining. Ovary sections were incubated with the following primary antibodies at 37 °C for 1 hour: mouse monoclonal p53 and p63 (8µg/mL); rabbit polyclonal p21 (8µg/mL) and rabbit polyclonal Apaf-1 (5µg/mL) (Santa Cruz Biotechnology). P53 plays a role in apoptosis, genomic stability, and inhibition of angiogenesis. In its anti-cancer role, p53 works through several mechanisms: 1) It can activate DNA repair proteins when DNA has sustained damage; 2) It can induce growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition (if it holds the cell here for long enough, the DNA repair proteins will have time to fix the damage and the cell will be allowed to continue the cell cycle); 3) It can initiate apoptosis, the programmed

cell death, if DNA damage proves to be irreparable. Activated p53 binds DNA and activates expression of several genes including WAF1/CIP1 encoding for p21. p21 (WAF1) binds to the G1-S/CDK (CDK2) and S/CDK complexes (molecules important for the G1/S transition in the cell cycle) inhibiting their activity. When p21(WAF1) is complexed with CDK2 the cell cannot continue to the next stage of cell division. Apaf 1 is an apoptotic protease activating factor 1. This gene encodes a cytoplasmic protein that forms one the central hubs in the apoptosis regulatory network.

Control sample was incubated with TBS alone. All sections are then washed three times for 10 minutes in TPBS. Bound antibodies were detected by incubating the sections with horseradish peroxidase-labeled secondary antibodies at 37°C for 2 hours. Sections are then washed three times for 10 minutes in TPBS and for 5 minutes in TRIS HCl 50mM pH7.4; reaction was then revealed with 0.7 mg/mL of 3,3' –diaminobenzidine tetrahydrocloride in TRIS HCl 50mM pH 7.4 for 20 seconds. After color development, the sections were washed three times in water, dehydrated and finally mounted for light microscopy. Sections were then dehydrated and mounted with Hystovitrex.

Ovarian follicle isolation

Ovary was transported to the lab in 0.9% NaCl solution at 4°C. Medullar tissue was removed using sterile blades and cortical portion was dissected in small pieces of about $1mm^3$. Ovarian fragments obtained were transferred to 50 ml conical tubes containing 10 ml of Leibovitz medium L15 (Sigma) supplemented with 1% fetal calf serum (Sigma) (FBS), 1 mg/ml collagenase type IA (Sigma), 0.25mg/ml Dnase I (Roche) and incubated in a water bath at 37°C for 30 min with gentle agitation. The ovarian digest was periodically (every 15 min) shaken with a pipette to mechanically disrupt the digested tissue. Digestion was completed by the addition of an equal volume of L15 medium supplemented with 10% FBS. The solution was transferred to Petri dishes and investigated for follicles under a stereomicroscope (Leica, Van Hopplynus Instruments, Brussels, Belgium). The follicles were picked up using a 135-µm-diameter flexipet (COOK) and washed

three times in L15 medium supplemented with 10% FBS in order to avoid introduction of stromal cells into the alginate matrix. Follicles were then transferred in culture medium, consisting of α- MEM supplemented with 20% FBS, 0.47mM pyruvic acid (Sigma), 1% insulin-transferrin-selenium (GIBCO) and 1% penicillin-streptomicin (Sigma), for 30' at 38°C. Follicle diameter was measured using NIS elements advanced software (Nikon). Two to five follicles were subsequently processed for live/dead assays in order to evaluate follicular viability after isolation. The remaining follicles were embedded in an alginate matrix (2-3 follicles/group).

Follicle viability

Follicles were incubated in 0.5ml of culture medium containing 50μg/ml propidium iodide (Sigma) for 30' at 38°C in the dark. Propidium iodide enters cells with damaged membranes and then binds to DNA with high affinity, resulting in a red fluorescence in dead cells. After exposure to propidium iodide (Excitation at 488 nm, emission at 617 nm), 10μg/ml Hoechst 33342 was added to the same medium and follicles were incubated for other 10' in the dark. Hoechst 33342 nucleic acid stain (excitation/ emission maxima ~350/461 nm) is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA.

At the end of incubation with fluorescent dyes, follicles were washed in fresh culture medium for three times, of 10' each one and observed under an inverted fluorescence microscope (Nikon).

3D confocal staining and imaging

Follicles for confocal microscopy studies were fixed in 4% PFA at 37°C for 1 h, followed by 1 h in wash buffer, consisted in PBS supplemented with 2% normal goat serum, 1% BSA, 0.1 M glycine, and 0.1% Triton X-100 (Xu et al., 2009; Barrett and Albertini, 2007). They were then stained overnight at 4°C with

Rhodamine-Phalloidin (1:50 Molecular Probes, Invitrogen, Eugene, Oregon), that labels F-actin in connections between somatic cells and the oocyte called transzonal projections (TZPs), and 1 μg/ml Hoechst 33342 (Sigma) for chromatin/DNA. Follicles were mounted in 5–10 ml of a 50% glycerol/PBS solution. The coverglass was placed on glass shards, to prevent the compression of the follicle. Follicles were imaged at a Leica TCS SP5 laser scanning confocal microscope, using a 40x or 63x oil objective. Overlapping 1–3 μm sections were taken throughout each follicle imaged.

Follicle Encapsulation

Materials

Alginate is a linear polysaccharide consisting of (1,4)-linked b-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G). The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks) or randomly organized blocks. Chemical composition, primary structure and average block lengths are conveniently determined by NMR spectroscopy. Commercial alginates are extracted from brown algae, and the relative amount of each block type varies with the origin of the alginate. Physical-chemical and biological properties of alginate vary widely with chemical composition. G-blocks form stable cross-linked junctions with divalent cations (e.g. Ca2+, Ba2+, Sr2+, among others) leading to a three-dimensional gel network. Alginate can also form gels under acidic conditions without cross-linking agents. Thickening properties are mainly dependent upon the average molecular weight, which can be determined by size exclusion chromatography combined with light scattering detection (Fig. 7)

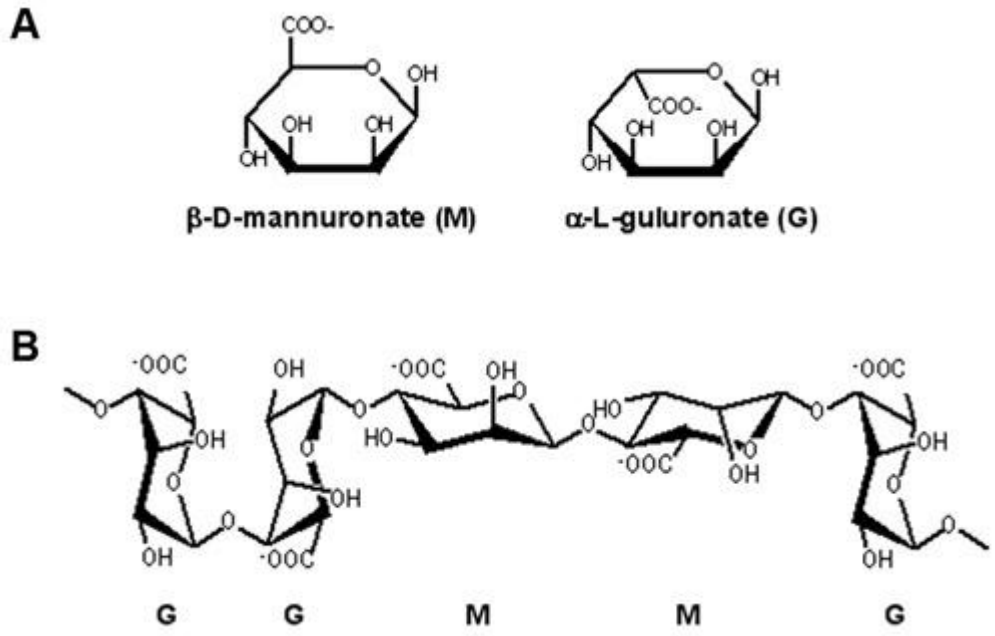


Fig. 7: chemical structure of alginate

Encapsulation

Morphologically normal follicles with centrally located spherical oocytes surrounded by a layer of granulose cells were used for further in vitro culture. The isolated follicles were transferred into 1.5% of sodium alginate solution. Droplets of alginate (~2–3 μL) were suspended on a cover of a 6 cm Petri dish. 2-3 follicles were pipetted into each droplet in a minimal amount of media, using a flexipet (Cook). Different types of alginate were used. Particularly, four different matrices were tested:

1. Alginic acid sodium salt from brown algae (Sigma), viscosity 4-12 cP (ALG). It is a straight-chain, hydrophilic, colloidal, polyuronic acid composed of 50 % glucuronic and 50% mannuronic acid residues. Alginate is an anionic polysaccharide distributed widely in the cell walls of brown algae. Alginate is biocompatible and gels making it useful for cell encapsulation and immobilization. A 3% (w/v) solution of sodium alginate was stripped using activated charcoal (0.5 g charcoal/g alginate) to remove organic impurities, sterile filtered using 0.22 μm filters. Sodium alginate was reconstituted to 1%, 2% (w/v) alginate in PBS1x for use. Alginate solutions were stored at 4°C.

2. ALG modified with collagen type IV (Sigma) (ALG-COLL). Particularly, collagen type IV was choose because it is the only component of extracellular matrix to be preserved during follicular development (RJ Rodgers et al., 2003). Lyophilized human placental collagen type IV (5mg) (Sigma) was dissolved in 1mL of glacial acetic acid (0.5%) and stored at -20°C. Aliquots of charcoal-stripped and sterilized sodium alginate were diluted to either 1% in 0.3 mg/mL collagen type IV solution, and vortexed well to mix. ALG-COLL solution was stored at 4°C.
3. Alginate SLG 100 (PRONOVA FMC Biopolymer) is made from an alginate where over 60% of the monomer units are guluronate and is a highly purified and well-characterized sodium alginate. One of the functional properties of alginate is viscosity. The viscosity of an alginate solution can be manipulated by changing the concentration of the alginate or by using materials with varying chain length (i.e. molecular weight). Typically, the molecular weight for PRONOVA SLG100 is in the 200000 – 300000 g/mol range. The ultra low levels of endotoxins, proteins and product sterility allows for a big variety of in vitro and in vivo applications. PRONOVA sterile alginate products are manufactured and documented in compliance with ISO 9001:2008 and ISO 13485:2003 (medical device directive) and in accordance with ICH Q7 guidelines. The products are characterized using validated analytical methods.
4. Alginate SLG20 (PRONOVA FMC Biopolymer) is also made from an alginate where over 60% of the monomer units are guluronate but in this case the molecular weight for PRONOVA SLG20 is in the 75000 – 220000 g/mol range.

After, all droplets had been filled, the cover was immersed in a 6 cm Petri dish, containing sterile 50 mM $CaCl_2$ and 140 mM NaCl encapsulation solution for 2 minutes to cross-link the alginate, and then rinsed in culture media. The composition of medium is reported before. The beads were transferred into four-well multidish for 10 days at 38°C in 5% CO_2. Every two days, half of the media

volume was exchanged and follicles were examined for survival and size measurements.

Follicle Vitrification And Thawing

At day 2 of culture period, encapsulated follicles were vitrified as follows:

Protocol 1

Encapsulated follicles were vitrified using a modified ethylene glycol (EG)-sucrose-based protocol, as reported by Desay et al. 2011. The basal media for the preparation of equilibration and vitrification solutions was L15 + 20% FCS. Follicles were equilibrated for 5 min in 2M EG followed by a 30–60-s incubation in vitrification solution containing 6M EG + 0.3M sucrose. All equilibration and vitrification steps were performed at room temperature. Follicles were then pipetted on the surface of a cryoleaf, held for 2 min in the vapor phase, just above the surface of the LN2, before immersion into LN2. Vitrified follicles were warmed by immersion in basal medium containing 1M sucrose. Follicles were left in the warming solution for 10 min at room temperature and then washed at 37°C. After 5 min, follicles were pipetted into fresh basal medium at 37°C for another 5–10 min before processing for viability assays, as reported before.

Protocol 2

Second vitrification and thawing protocol was modified from the method described by Xing et al., 2010. Briefly, encapsulated follicles were initially exposed to the first vitrification solution (4% EG in DPBS + 10% FBS) for 15 min. Subsequently, they were rinsed three times in the second vitrification solution (35% EG and 0.5 M sucrose in DPBS + 10% FBS) and equilibrated at room temperature for 20-30 sec. Then follicles were directly dropped onto the surface of a cryoleaf. Droplets containing the vitrification solution and follicles

were instantaneously vitrified into transparent spherical droplets, by direct immersion in LN_2. Cryoleafs were closed and stored in LN_2.
Warming was achieved by direct transfer into warming solution containing 0.25 M sucrose and 10% FBS in DPBS at 37°C for 5-10 min followed by three washes in DPBS. Then, follicles were transferred in 0.5 mL of culture medium and incubated for 15-20', before processing for follicular viability, as reported before.

Xenotransplantation of alginate beads into SCID mice

Xenotransplantation of encapsulated follicles was performed in collaboration with the Lab of Research in Human Reproduction, in Brussels (ULB: universitè libre de Bruxelles).

Animals

All animals were obtained by Harlan laboratories. The scid (severe combined immunodeficiency) mutation was discovered in a C.B-17/Icr congenic strain in 1980 by Dr. M.J. Bosma at the Fox Chase Cancer Center (Philadelphia, PA). Harlan SCID® models are licensed under agreement with the Fox Chase Cancer Center, produced within flexible-film isolators and monitored for microbiologic integrity. SCID mice accept xenografts, making them a useful model for oncology, immunology, HIV pathology, and other fields of biomedical research. A total of ? nude 6-week-old female mice were used. The animals were housed in ventilation cabinets at a positive pressure of 6 cm H_2O. They had unlimited access to gamma-irradiated food pellets and sterile water, and were inspected daily. The oophorectomy and transplantation procedure were executed in a laminar flow cabinet to provide a sterile environment for the immunocompromised animals.

Fig. 8: SCID mouse

Subcutaneous grafting

The recipient animals were anesthetized by intraperitoneal (IP) injection of ketamine (75 mg/kg; ketamine 1000; CEVA Sant e Animale, Brussels, Belgium) and xylazine HCl (10 mg/kg; Rompun; Bayer Animal Health, Brussels, Belgium). Analgesia was obtained by IP administration of buprenorphine (0.1 mg/kg; Temgesic; Schering Plough, Brussels, Belgium). A single dorsal transverse skin incision of 0.5 cm was made, allowing sufficient access to both left and right abdominal cavity, and the abdominal wall was incised in the lumbar fossa. Before the actual transplantation procedure, the animals were bilaterally oophorectomized. Each ovary was exteriorized, the top of the cornus uteri was ligated with polyglactin (Vicryl 6/0, Ethicon, Somerville,
NJ), and the ovary was excised. The transplantation procedure was performed with the aid of a binocular microscope (Motic SMZ168; Motic China Group Co. Ltd., Xiamen, People's Republic of China) at ×7.5 magnification. The left kidney was exteriorized, and a small slit was made in the kidney capsule with a 27-gauge needle. Encapsulated follicles were pipetted under the kidney capsule. The kidney was returned to its normal anatomical position, the abdominal wall was sutured with polyglactin (Vicryl 6/0), and the skin defect closed with polypropylene (Prolene 6/0). Similarly, encapsulated follicles were grafted under the back muscle. All the procedure was performed at 37°C. Recovery was unproblematic, and no signs of abdominal discomfort or infection were noted. After 3 months

from xenografting, mice were sacrified and xenografts were collected and analysed by histological assays, as reported before.

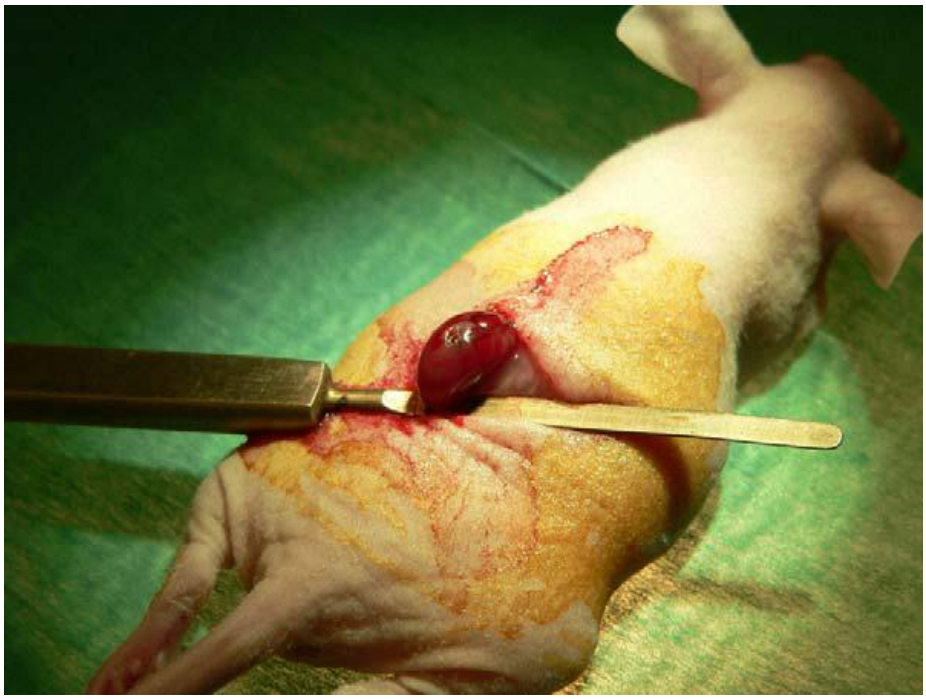


Fig. 9: xenografting

Statistical analysis

The data are presented as mean±SD. Overall analysis was performed by the estimate model of analysis of variance (ANOVA) followed by the Tukey's honestly significant difference test for pairwise comparisons when overall significance was detected. Percentage data were compared by χ^2 or Fisher's exact test. Statistical significance was defined as $P<.05$.

RESULTS

Light microscopy

A total of 892 follicles was analysed by LM to evaluate the viability and the developmental stages in non-frozen, slowly cryopreserved and vitrified tissues. Particularly, 406 follicles were slow cooled and 247 were vitrified. Most of the follicles were at primordial, intermediary and primary stages in all samples. Difference between the total numbers of follicles in different samples is not attributed to different protocols, since large variation in the distribution of the follicles between patients and pieces from the same ovary were observed (table-graph 6).

K	34.9±7.3
SCH	28.7±14.7
SCHC	48.8±19.5
SCYB	50.5±2.1
SCYBC	54,5±14.8
VH	33±11.3
VHC	24.5±3.5
VYB	9.5±0.7
VYBC	13±0.6

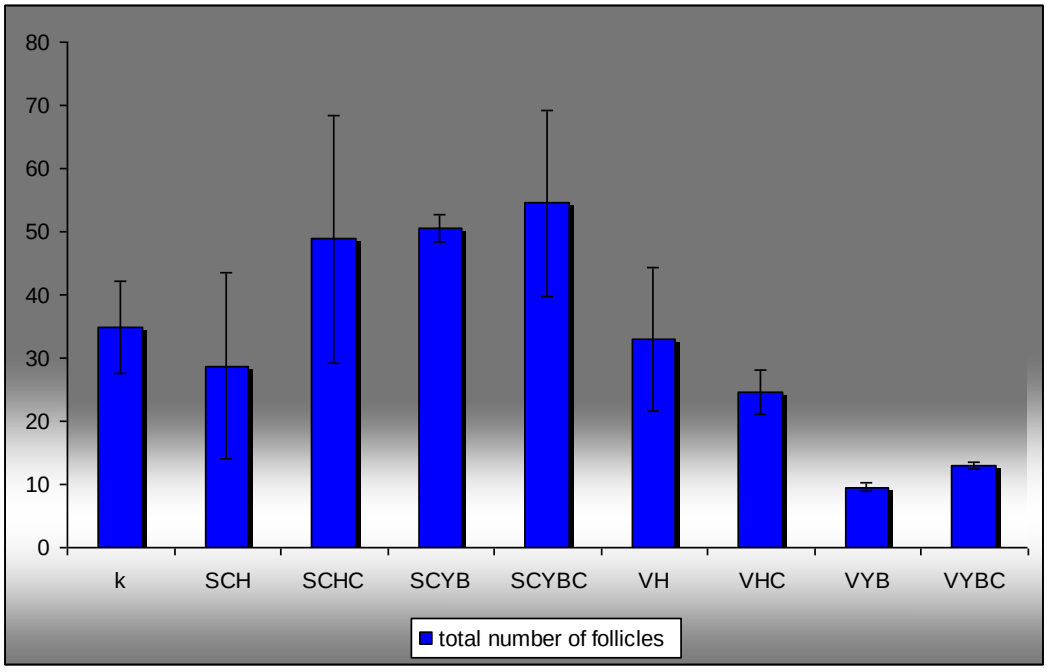


Table-graph 6: total number of analyzed follicles in different samples

As reported previously, eosinophilia of the ooplasm, contraction and clumping of the chromatin material, and wrinkling of nuclear membrane of the oocytes were regarded as the signs of atresia (Gougeon A., 1986). The qualities of follicles were graded from one to three. A follicle of grade 1 is spherical and is randomly distributed around oocytes granulosa cells, with homogenous cytoplasm and slightly granulated nucleus, in the center of which condensed chromatin in the form of dense spherical structure is detected (Fig. 10). A follicle of grade 2 has the same peculiarities, but the granulosa cells cover irregular oocytes; these cells can be flat and condensed chromatin is not detected in cytoplasm (Fig. 11A). A follicle of grade 3 has partly or fully disrupted granulosa or cytoplasm and picnotic nucleus (Fig. 11B). Follicles of grades 1 and 2 were denoted as normal and those of grade 3 were denoted as degenerated.

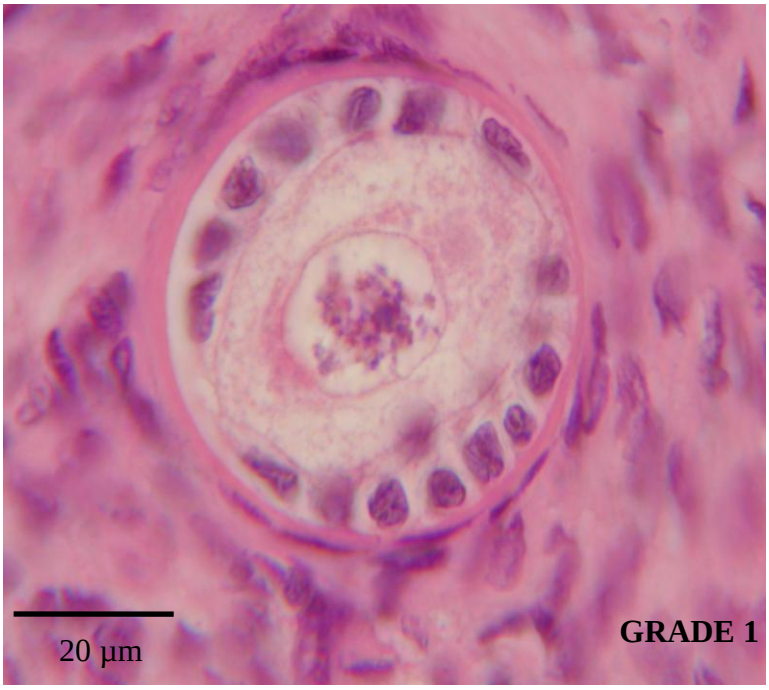


Fig. 10: follicle of grade 1

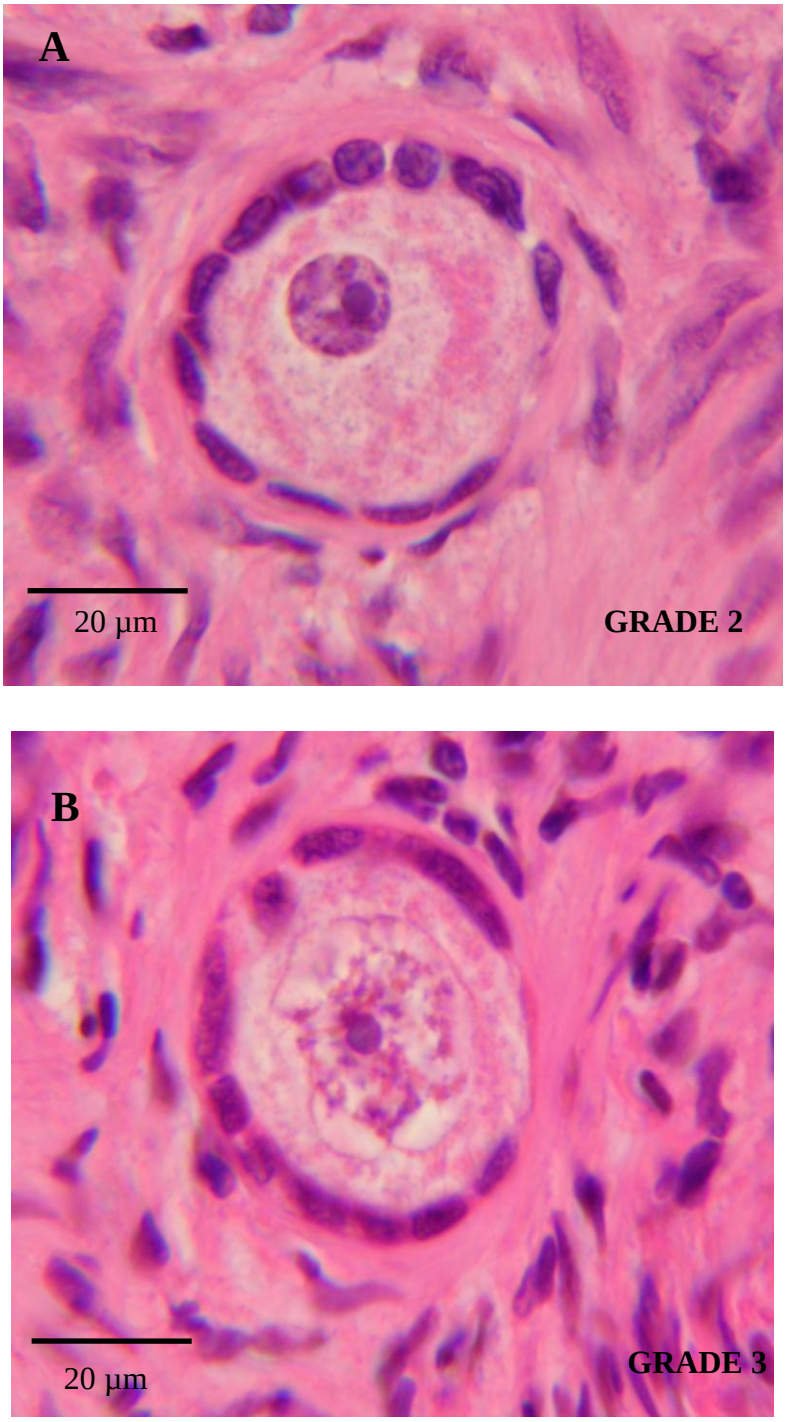


Fig. 11: (A) follicle of grade 2; (B) follicle of grade 3

Microscopic visualization of haematoxylin/eosin-stained sections reveal clear differences in the structures of the follicles cryopreserved using the different protocols. Particularly, in SCYB follicles oocyte is separated from surrounding picnotic granulosa cells, and ooplasm is highly eosinophile; in contrast, SCYBC follicles are better preserved. However, germinative vescicle is not well condensed. SCH and SCHC follicles show a more regular morphology, characterised by a more homogeneous cytoplasm and follicular cells well distributed around the oocyte, but only in SCHC follicles, no intercellular spaces are present in stromal tissue and germinative vescicle shows a well condensed chromatine (Fig. 12).

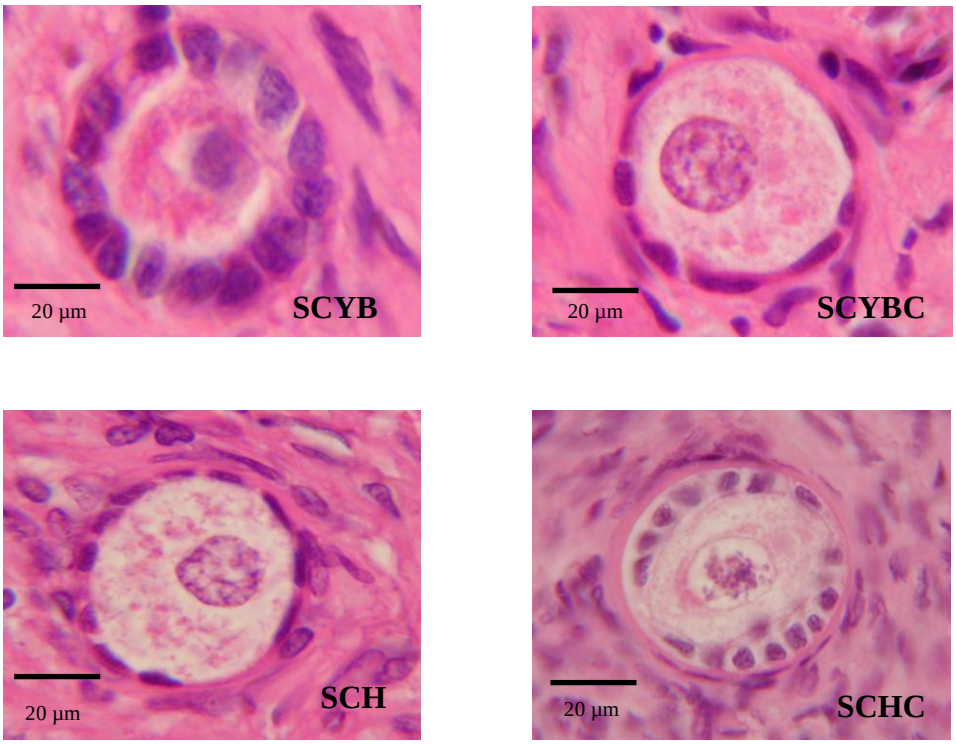


Fig. 12: frozen/thawed follicles. Different superscripts indicate slow freezing protocol

The percentages of different grade follicles in slowly cooled samples are showed in table 7. In fresh tissue, 51.86±12.85 of total follicles were of grade1. In slow freezing groups percentage of morphologically normal follicles were significantly higher in choline modified protocols than in conventional ones (graph. 7) Particularly, for both first and second slow cooling protocols, percentages of

follicles of grade 1 were 50.5±13.43 and 51.3±12.75 vs 21.17±15.69 and 30.25±14.72.

	% follicles of grade 1	% follicles of grade 2	% follicles of grade 3
K	51.86±12.85	30±15.95	18.14±13.51
SCH	21.17±15.69** (vs K)	26.67±1.63	50.33±16.44* (vs K)
SCHC	50.5±13.43** (vs SCH)	17.5±5.2	32±16.06
SCYB	30.25±14.72* (vs K)	15.05±2.56* (vs K)	54.7±15.52* (vs K)
SCYBC	51.3±12.75	15.35±6.33* (vs K)	33.35±14.56

* $p<0.05$

** $p<0.01$

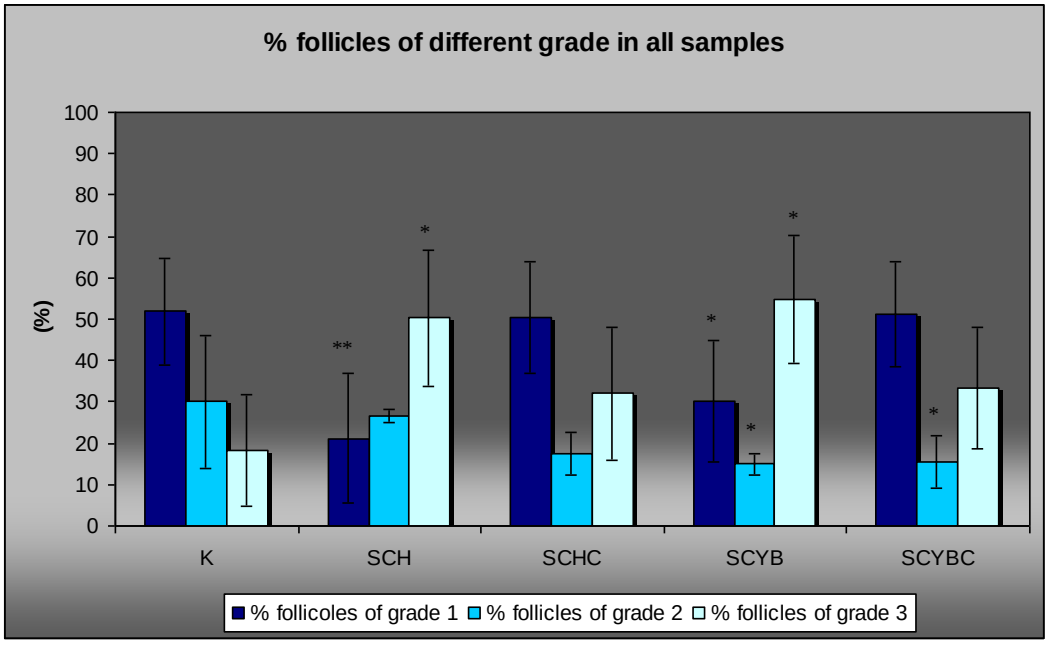


Table-graph 7: percentages of follicles of different grade in all slowly cooled samples

In vitrification groups percentage of morphologically normal follicles were also higher in choline modified protocols than in conventional ones, but follicular morphology is not well preserved (Fig. 13). In fact, all follicle are characterised by vacuolization and intercellular spaces. Moreover, nuclei of granulosa cells are picnotic and germinative vescicle is highly damaged.

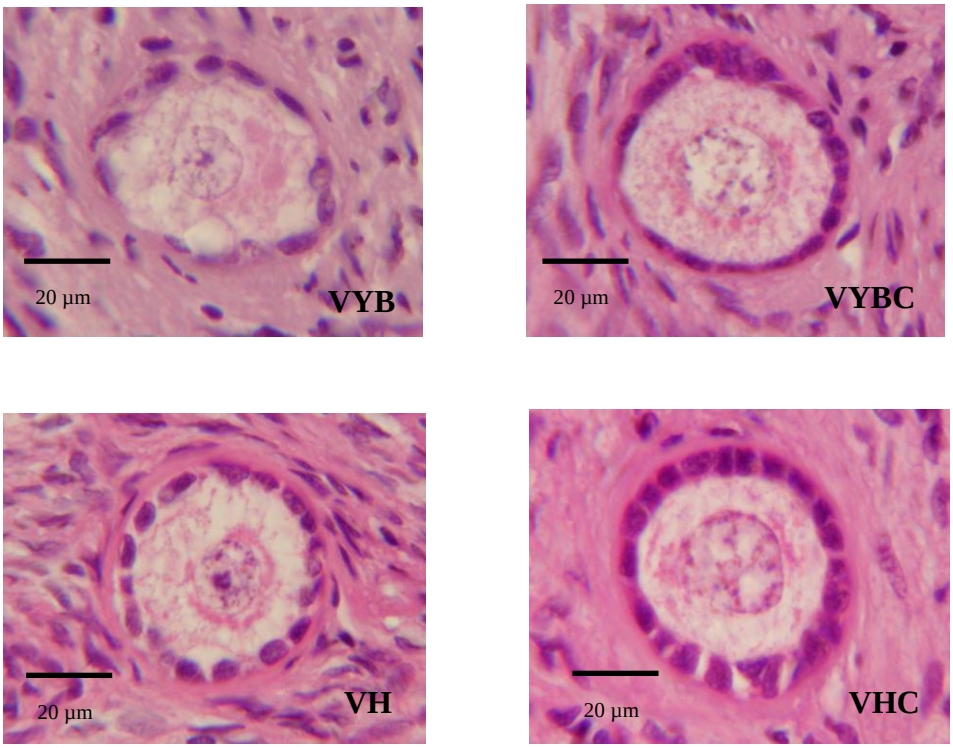


Fig. 12: frozen/thawed follicles. Different superscripts indicate vitrification protocol

Particularly as showed in table 8, percentages of follicles of grade 1 were 3.5±4.95 and 24±9.26 vs 28±7.07 and 29.95±5.52. After thawing, the proportion of the morphologically intact follicles were significantly reduced in the two vitrification groups.

	% follicoles of grade 1	% follicles of grade2	% follicles of grade3
K	61±11.31	15±9.9	24±1.41
HV	3.5±2.95** (vs K)	26±8.49	70.5±3.54** (vs K)
HVC	28±7.07* (vs K)	23.5±12.02	48.5±4.95
YBV	24±9.26** (vs K)	23.6±1.98	42.4±7.28* (vs HV)
YBVC	29.95±5.52* (vs K)	15.4±2.55	49.2±12.44

* $p<0.05$

** $p<0.01$

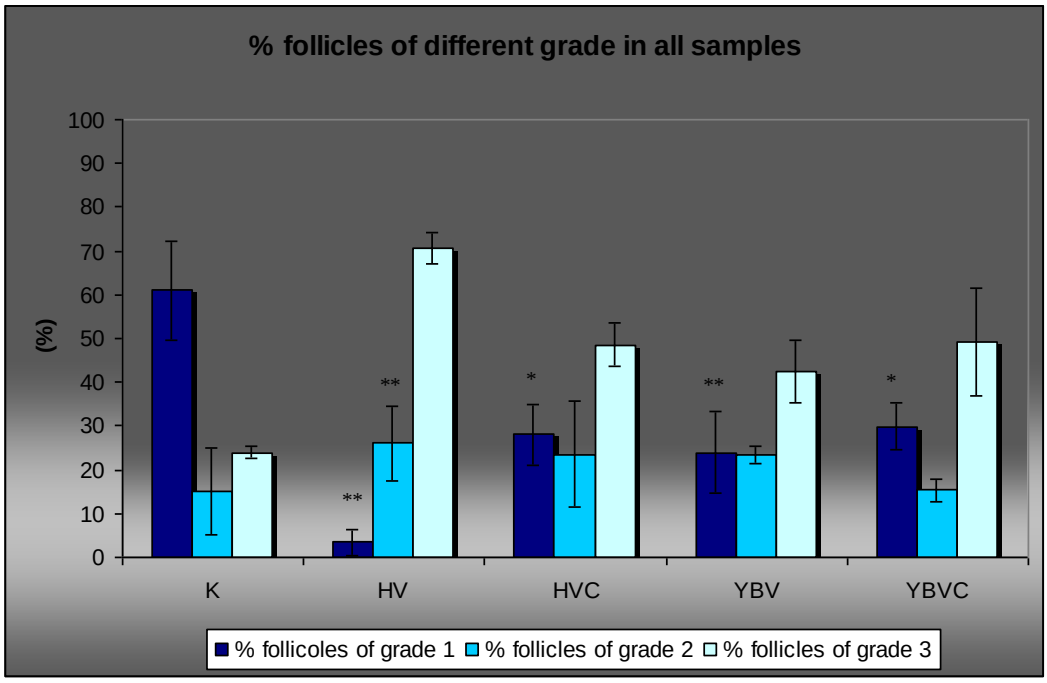


Table-graph 8: percentages of follicles of different grade in all vitrified samples

Moreover, diameter of fresh and cryopreserved follicles germinative vescicle was measured and there are no significative differences between all samples, as shown in Table 9.

K	22.7±2.6
SCH	20.4±2.4
SCHC	20.5±2.6
SCYB	21.3±2.8
SCYBC	21±3.1
VH	17.9±2.9
VHC	18.3±2.7
VYB	17.1±2.8
VYBC	18.5±1.4

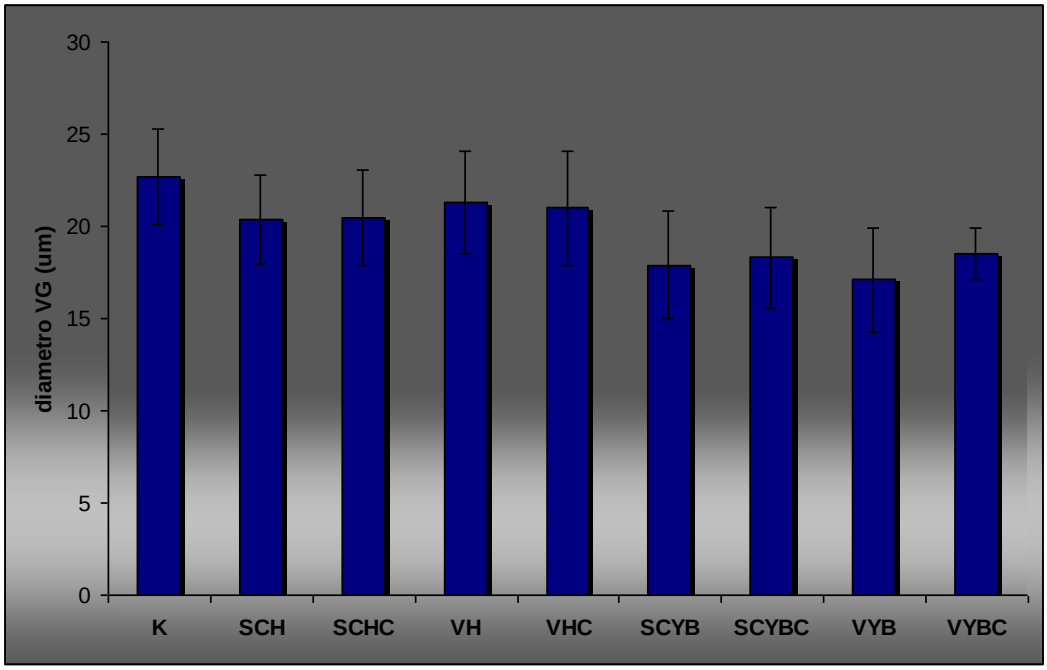


Table-graph 9: diameter of germinative vescicle in all samples

Immunohistochemical analysis

In an immunohistochemical study, three different situations were retrieved. Follicles of grade 1 are negative for p53, p21 and Apaf1 (Fig. 14).

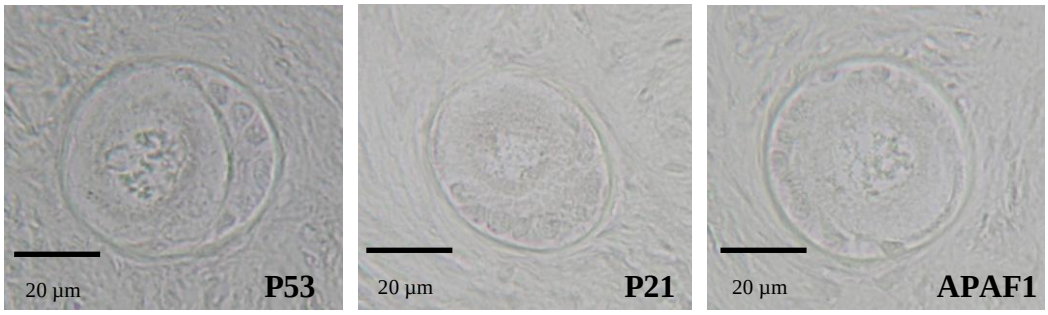


Fig. 14: Immunohistochemistry of follicles of grade 1

Follicles of grade 2 are immunoreactive for p53, and p21 but staining for Apaf-1 is revealed only in 50±24.04% of them (Fig. 15).

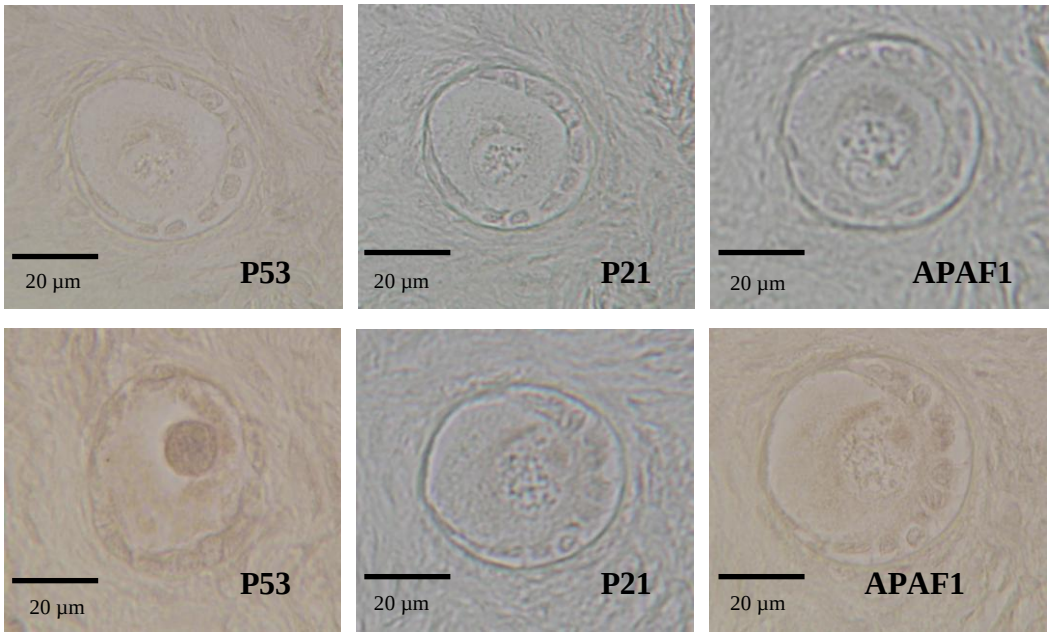


Fig. 15: Immunohistochemistry of follicles of grade 2

Follicles of grade 3 are immunoreactive for all tested markers (Fig. 16).

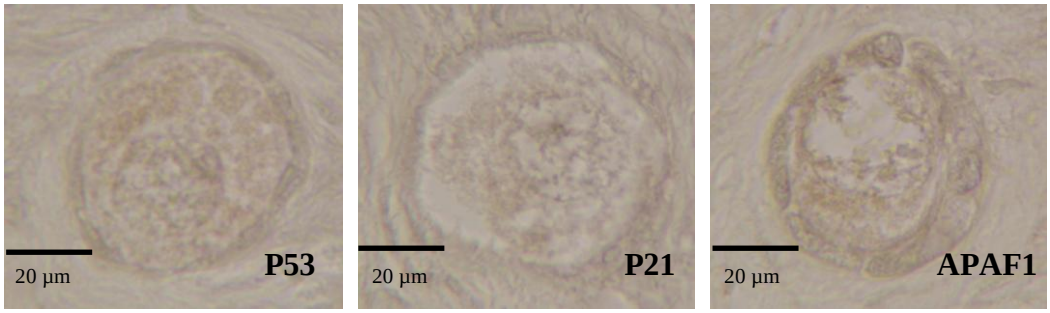


Fig. 16: Immunohistochemistry of follicles of grade 3

In the control sections, replacing the primary antibody with only phosphate buffered saline, resultes in the complete inhibition of staining in oocyte and follicular cells, indicating the specificity of immunoreaction.

Ultrastructural analysis

All follicles analyzed by TEM are at a primordial stage. Follicles at a different developmental stage are not revealed.

In fresh human ovarian biopsies, the oocyte and the follicular structures generally are well preserved. Follicles are characterised by an intact and well defined oolemma, an homogeneous cytoplasm and nucleus with well condensed chromatine. There are no lipid droplets, vesicles or lysosomes found in these follicles (Fig. 17). Particularly, mitochondria had the typical morphological features described in human oocytes (Sathananthan AH et al., 2000; Motta PM et al., 2000). They occur as spherical/oval elements, approximately 0.5 mm in diameter, and typically contain a few short cristae rarely crossing a highly electron dense matrix

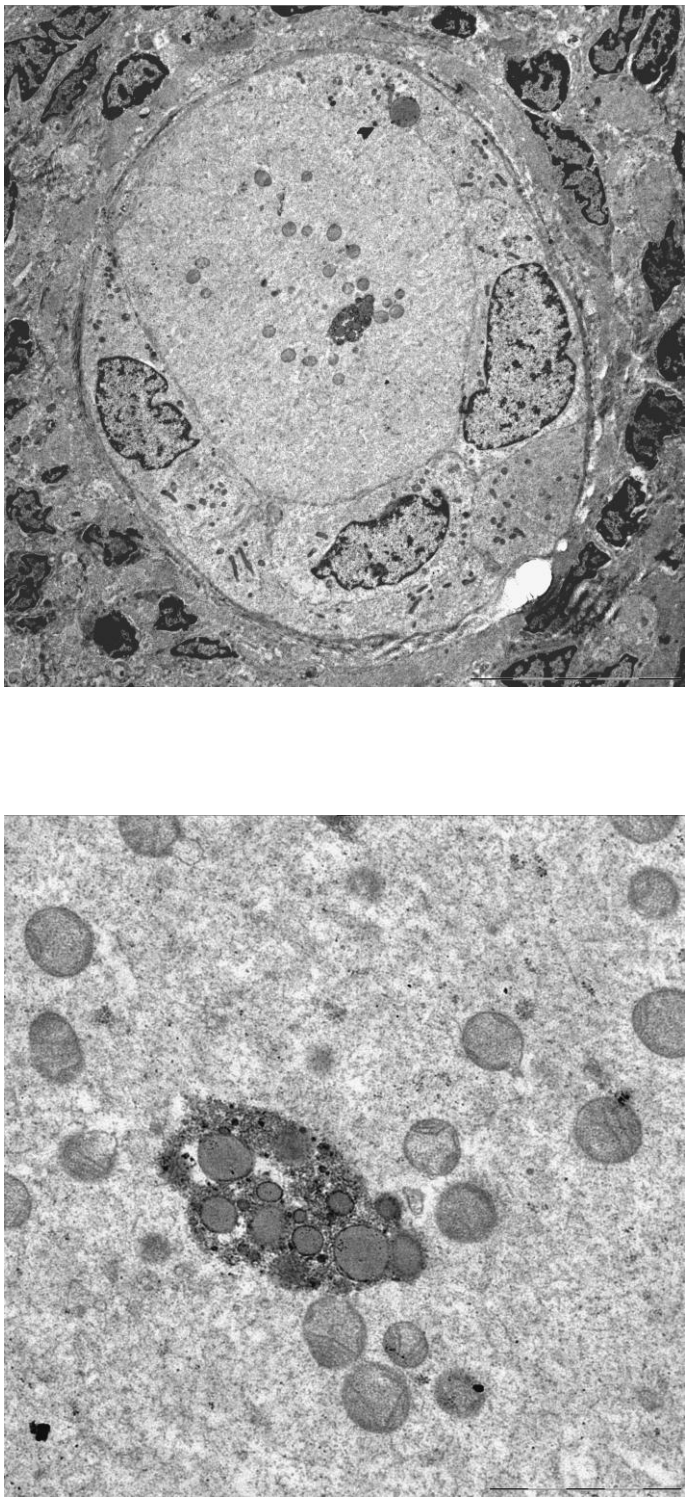
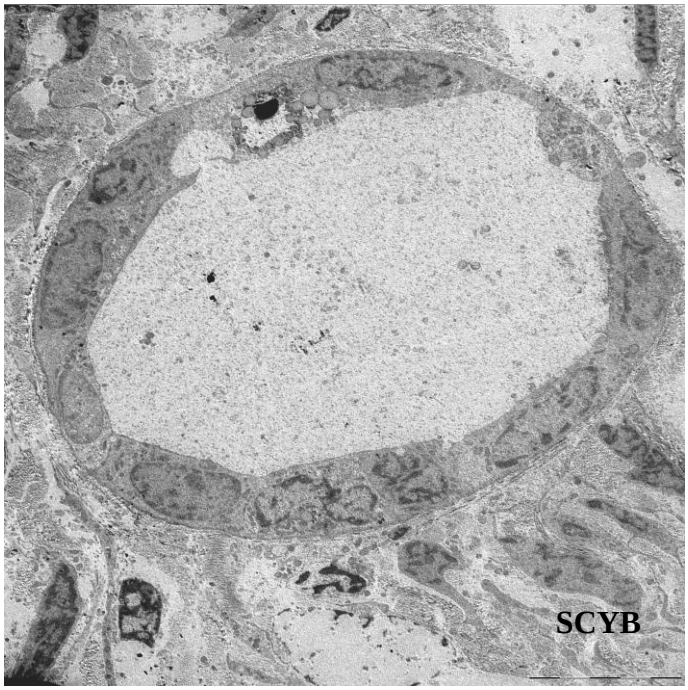


Fig. 17: Control follicle

Compared to fresh follicles, in cryopreserved follicles, mitochondria have a decreased electron density of the matrix or irregular shape. However, choline slowly freezed follicles, for both protocols, are characterised by a more

homogeneous cytoplasm both in oocyte and in follicular cells, well defined oolemma and nuclear membrane, mitochondria of regular shape and at a cytoplasmatic level typical structures called anulatae lamellae are well preserved (Fig. 18).



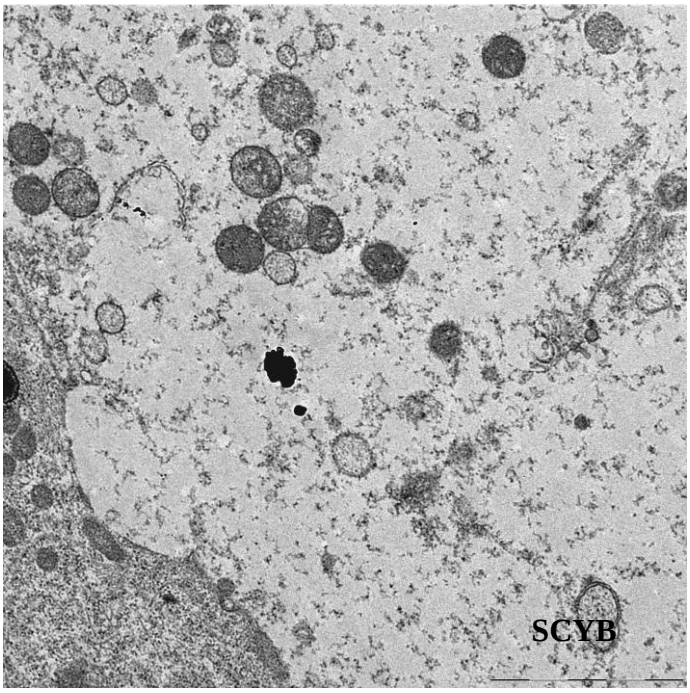
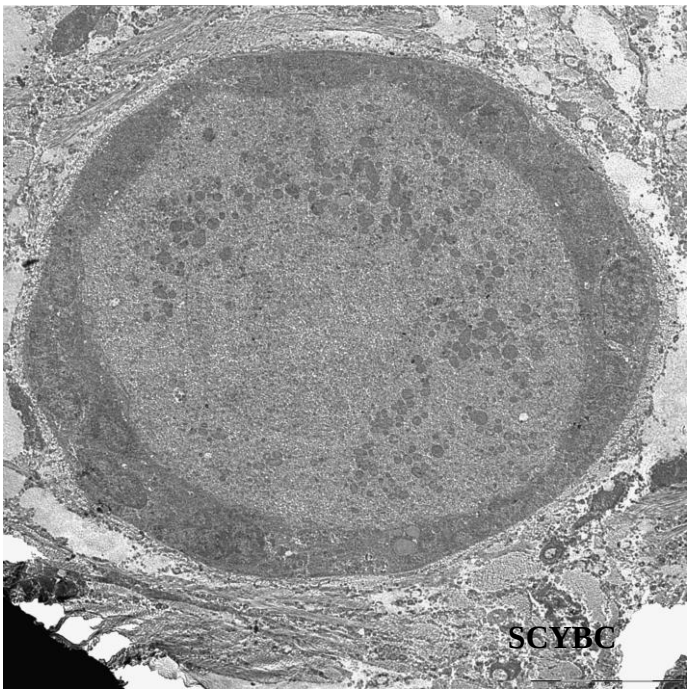


Fig. 18A : SCYB follicle



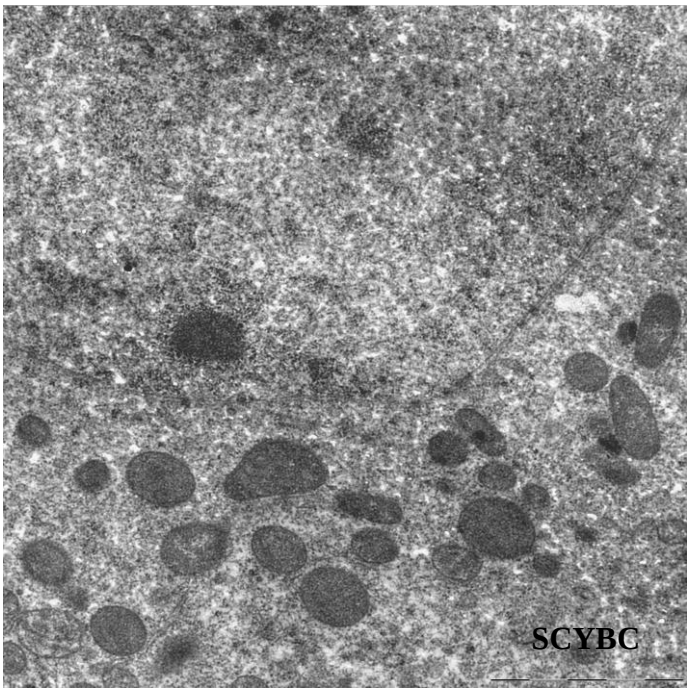
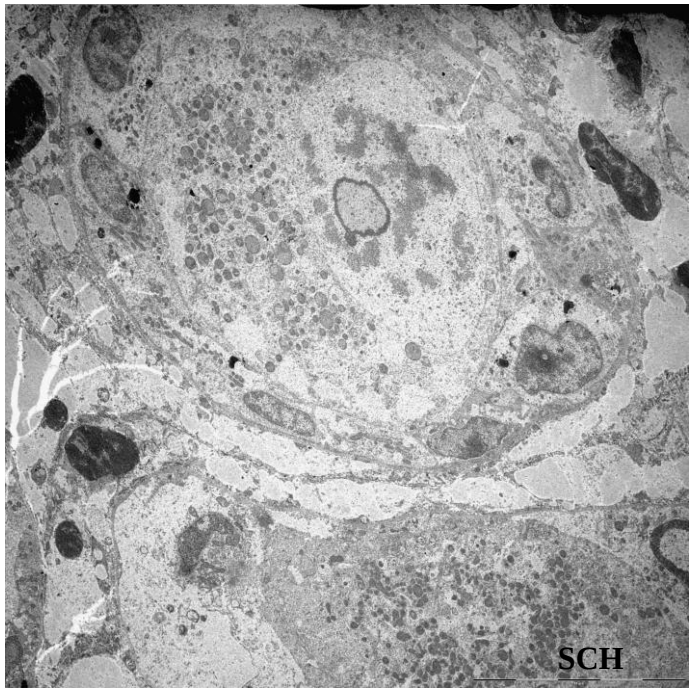


Fig. 18B : SCYBC follicle



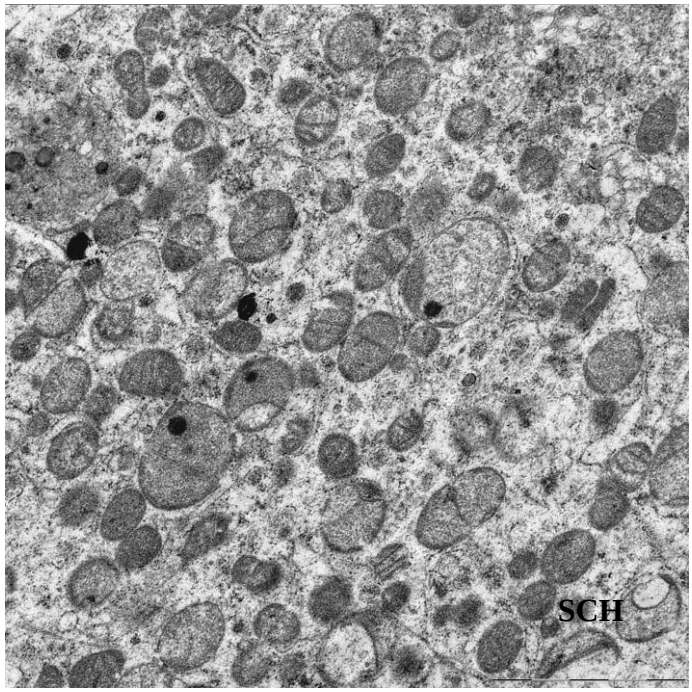
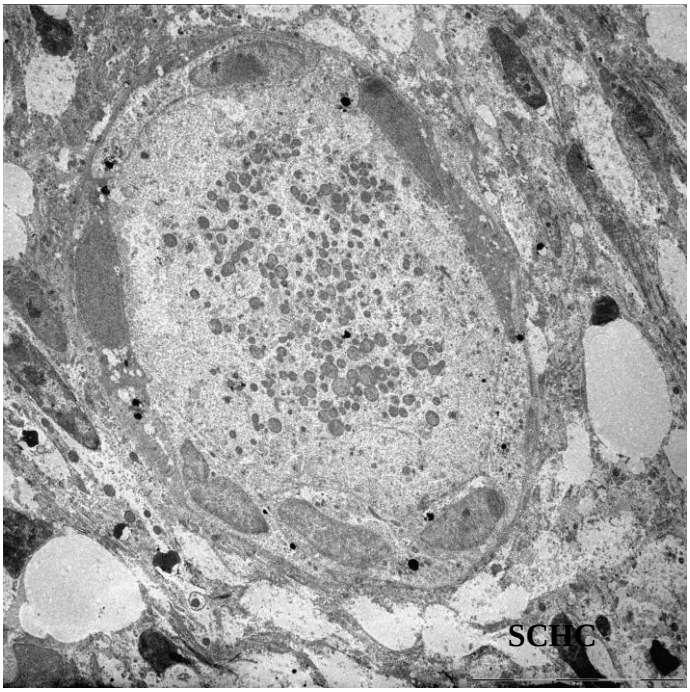


Fig. 18C : SCH follicle



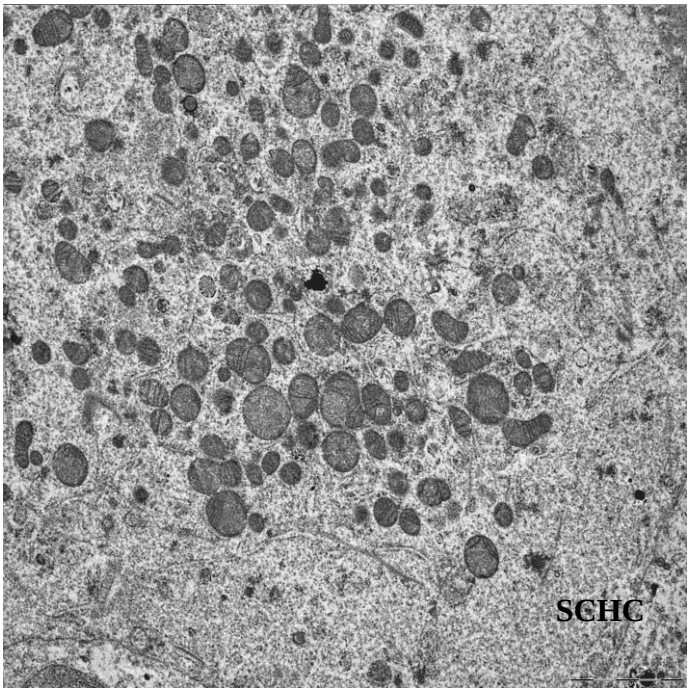


Fig. 18D : SCHC follicle

Follicles from vitrified tissue are characterised by lipid droplets, vesicles or lysosomes not revealed in control samples. However, choline vitrified follicles have no intercellular spaces between follicular cells, mitochondria are better preserved and at a cytoplasmatic level typical structures called anulatae lamellae are well visible (Fig. 19).

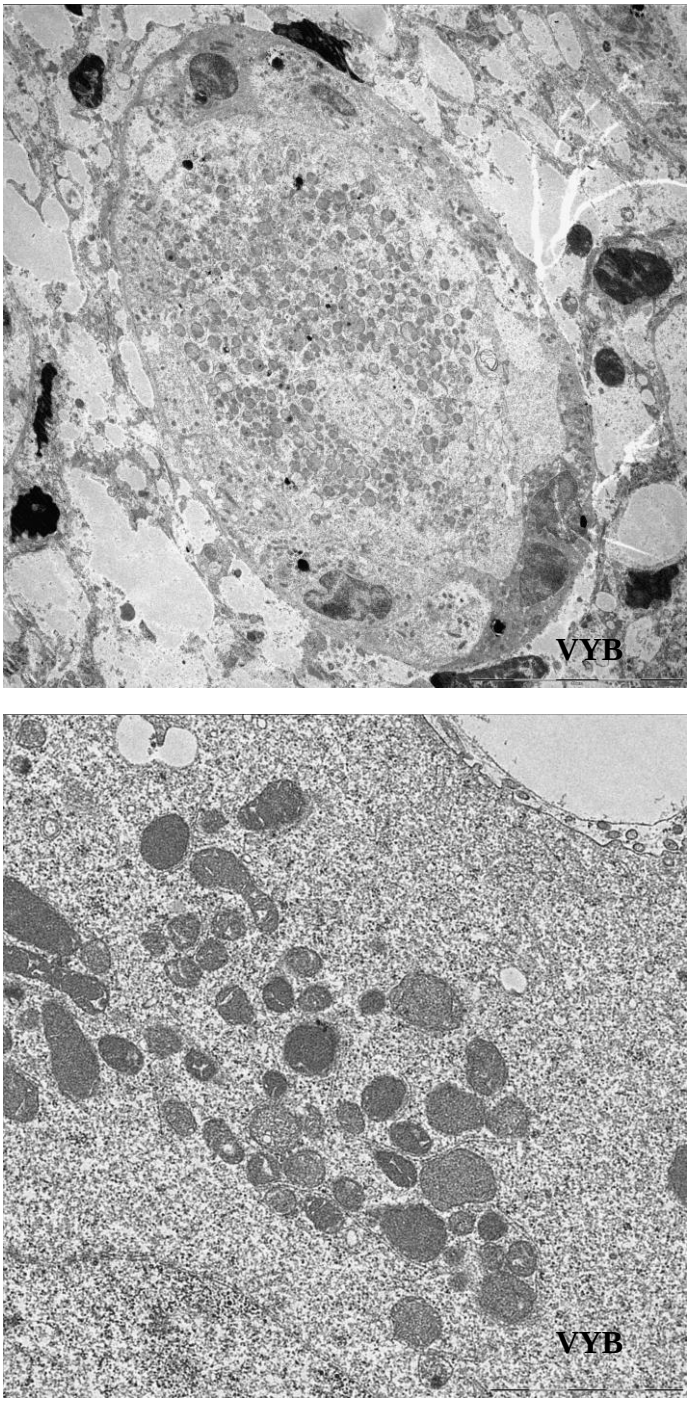


Fig. 19A : VYB follicle

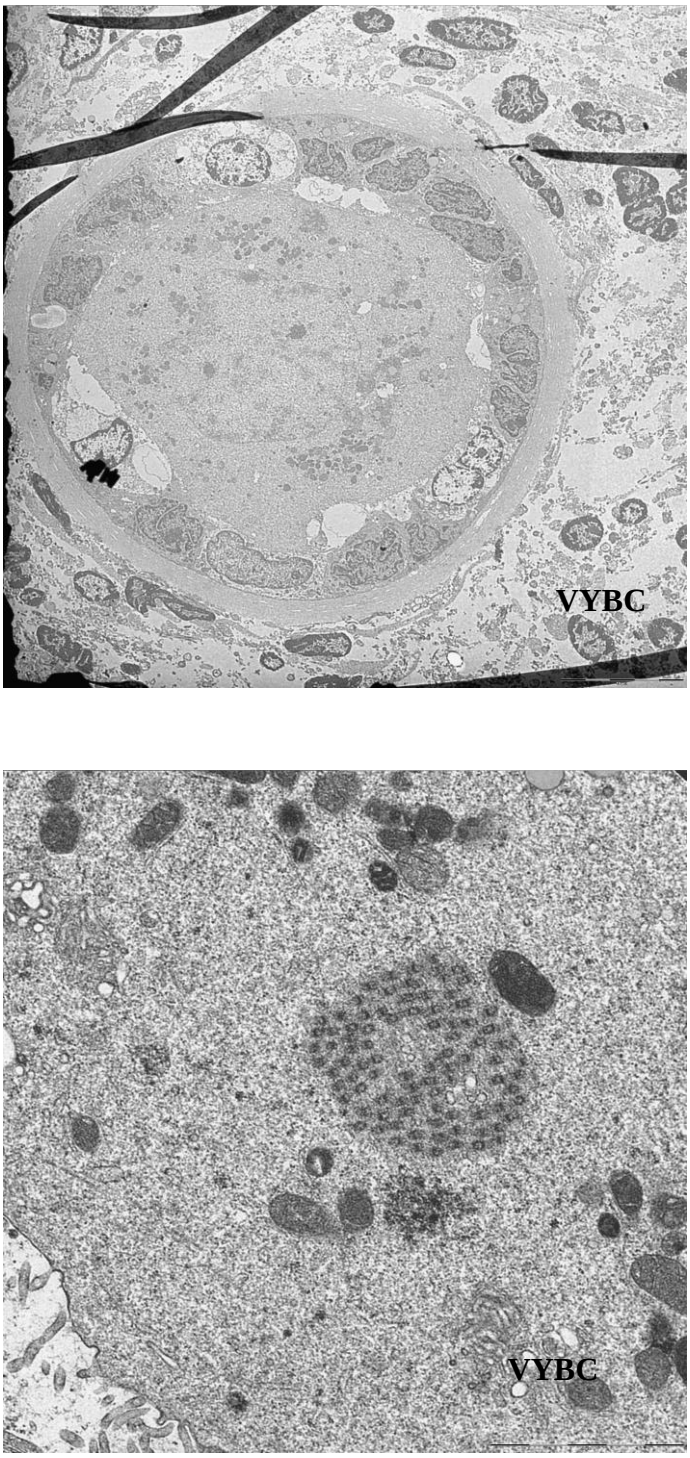
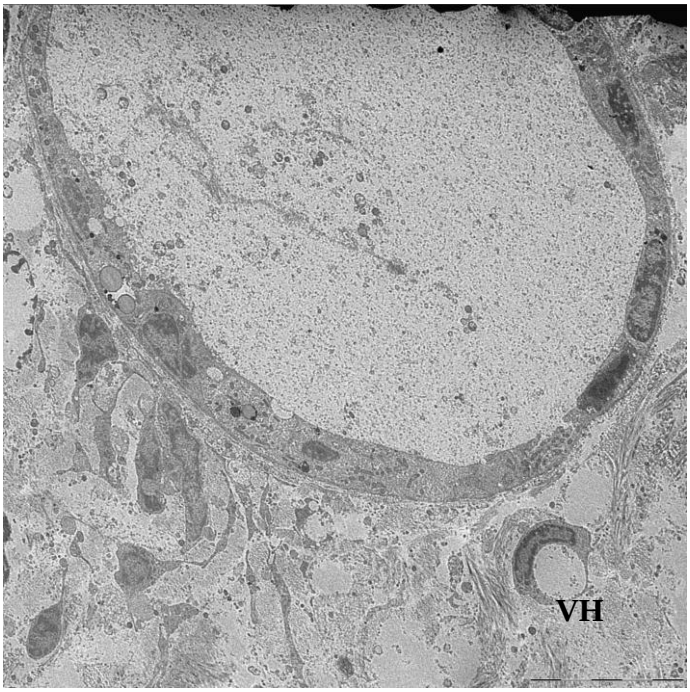


Fig. 19B : VYBC follicle



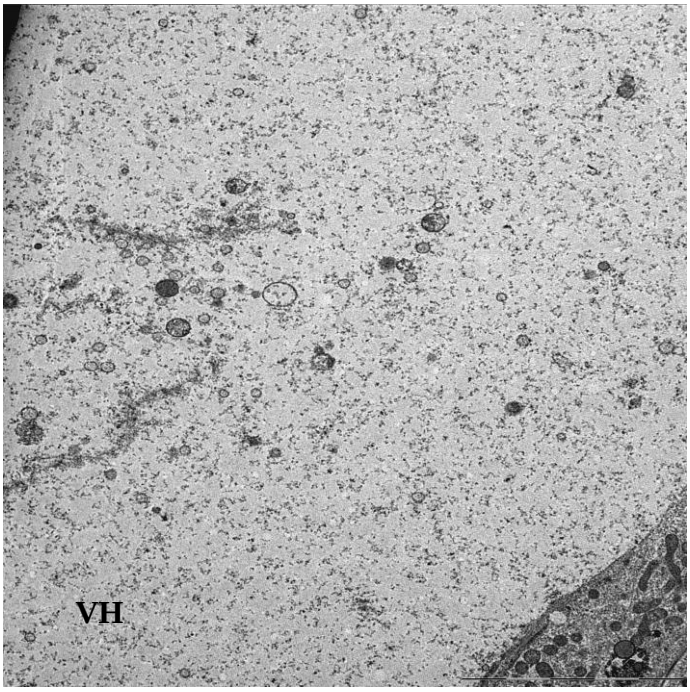
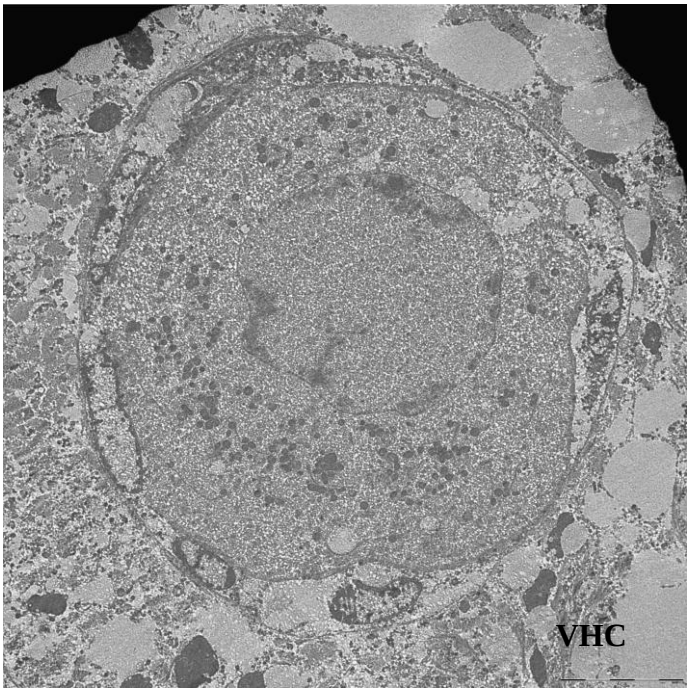


Fig. 19C : VH follicle



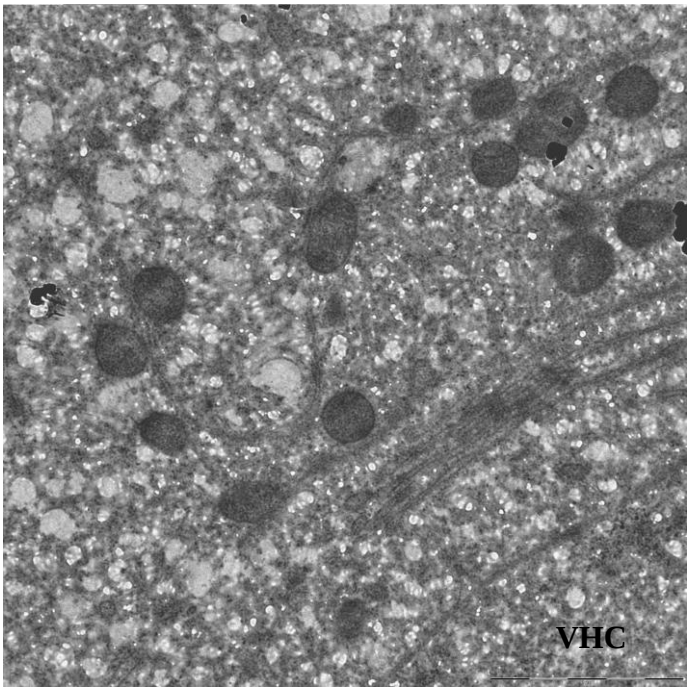


Fig. 19D : VHC follicle

Mitochondrial analysis

Density of mitochondria has been evaluated by counting number of mitochondria inside an area of 50µm^2. Results demonstrated no significant difference between control and slowly cooled samples. Instead, in all vitrified a significant decrease of mitochondrial density has been reported. (Table-Graph 10)

K	72±3.5
SCH	52±13.2
SCHC	58±9.3
SCYB	54±12.5
SCYBC	53±8.2
VH	32±10.3
VHC	35±7.5
VYB	49±7.2
VYBC	36±5.6

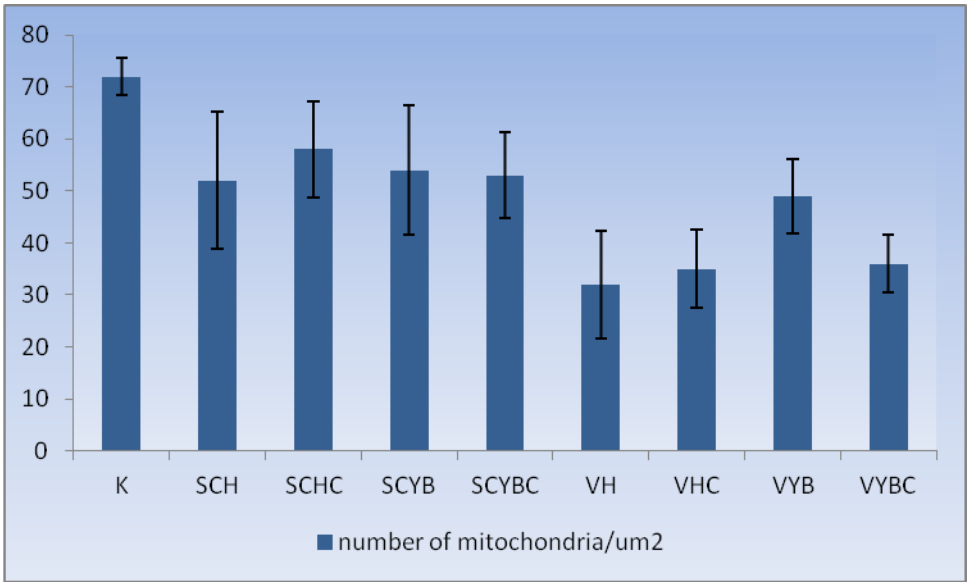
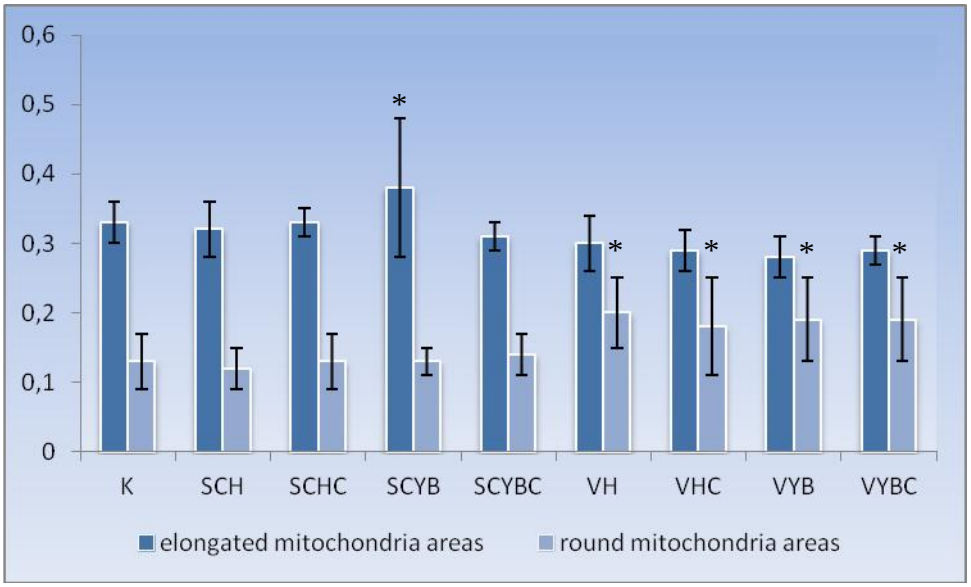


Table-Graph 10: Number of mitochondria / µm^2 in all samples

Mitochondrial areas have been classified in two categories:

1. Elongated mitochondria, characterised by an extended shape and numerous mitochondrial cristae;
2. Round mitochondria, characterised by a circular shape and few mitochondrial cristae.

Results demonstrated that, in all analysed follicles, no significant differences between elongated mitochondria areas have been found, except for SCYB follicles, in which case an increase has been recovered. However, round mitochondria areas have been significantly decreased after vitrification/thawing, for both protocols. (Graph 11)



Graph 11: mitochondria areas in all samples. * $p<0.05$

Isolation of ovarian follicles

In order to assess the ability of ovarian follicles to grow in 3D matrices, I isolated follicles from 30 different ovarian tissues The mean number of isolated follicles is 200±35 (Fig. 20).

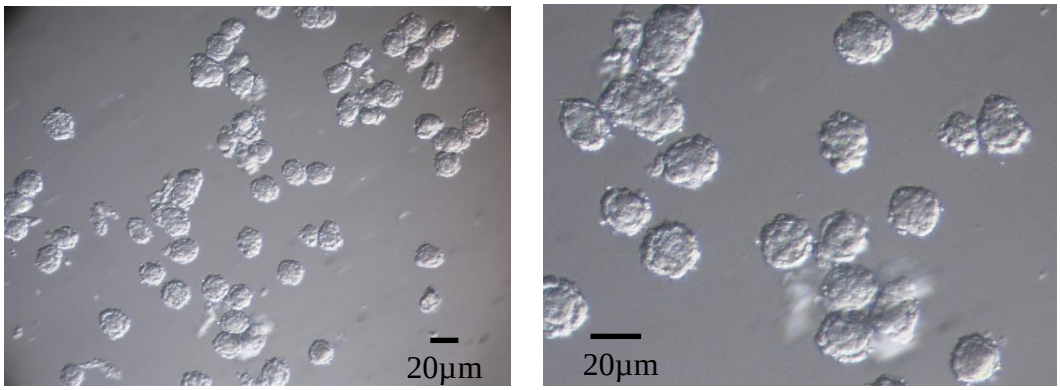


Fig. 20: Isolated follicles

The initial mean diameter was 41.9±5.20µm and fluorescent assays through staining with propidium iodide and Hoechst 33342 show that viable follicles are 87.7±5.4% (Fig. 21).

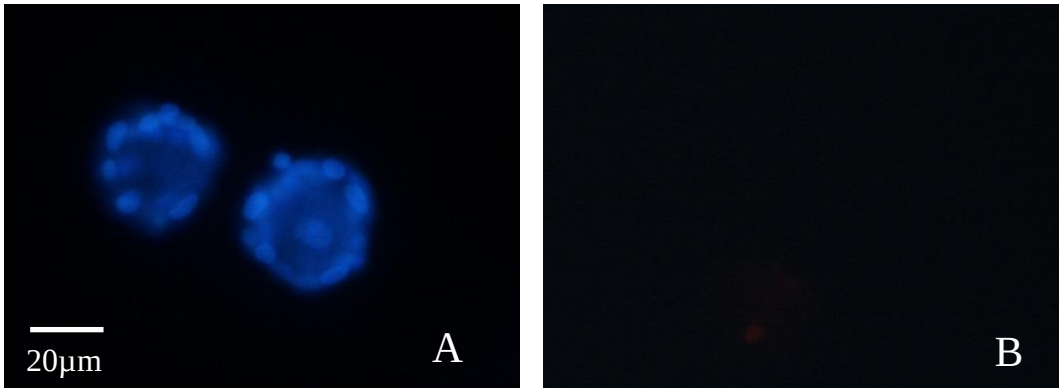
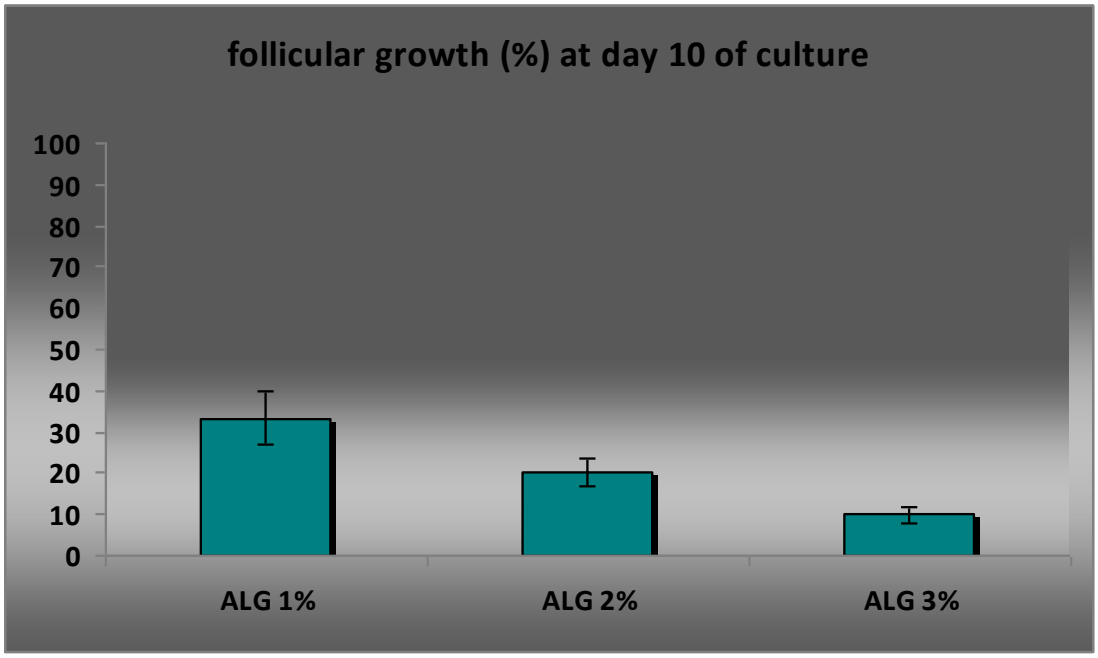


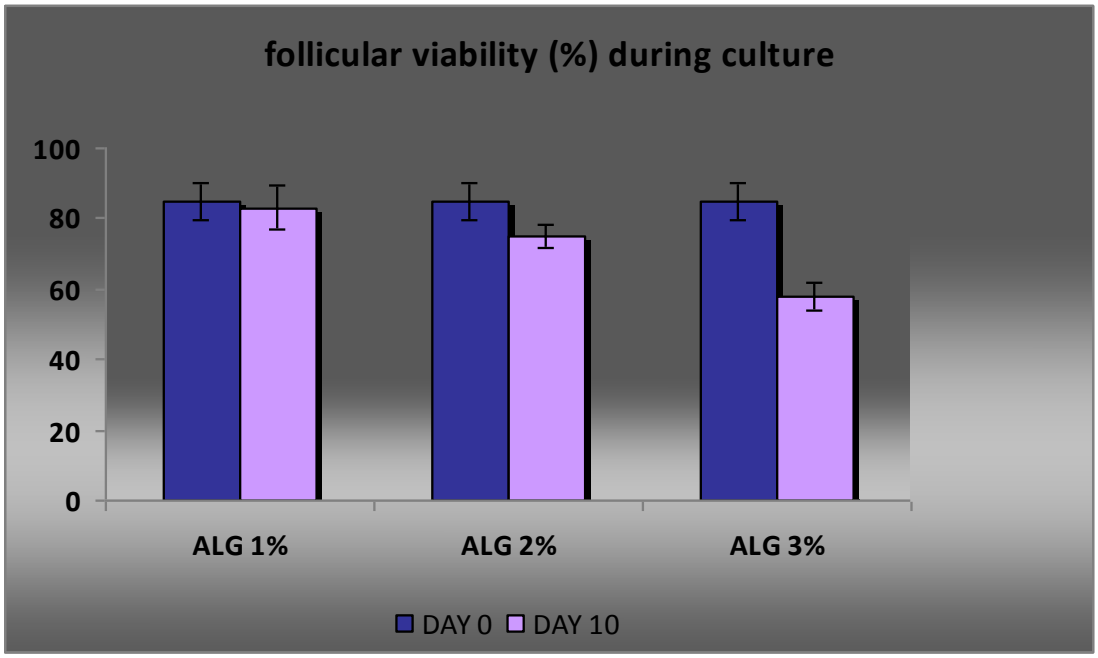
Fig. 21: Imaging of a viable follicle

Effect of Alginate Rigidity on Follicle Survival and Growth

Follicles were firstly cultured for up to 10 days in alginates at different concentration to determine which one best supports follicle survival and growth. Particularly follicles were encapsulated in alginate 1%, 2% and 3%. At the end of the culture, mean follicular growths are 33.2±6.5% for ALG1%, 20±3.4% for ALG2% and 10±2% for ALG3% (Graph 12). Furthermore, at day 10 of culture, fluorescent assays show that viable follicles are 83.2±6% after encapsulation in ALG1%, 75±3% for follicles encapsulated in ALG2% and 58±4% after encapsulation in ALG3% (Graph 13).



Graph 12: follicular growth at day 10 of culture in alginates at different concentration



Graph 13: follicular viability at day 10 of culture in alginates at different concentration

Effect of Alginate Physical properties on Follicle Survival and Growth

Next step was to study the effects of physical properties of alginate matrix on in vitro culture of isolated follicles. In order to improve the efficiency of culture system, four matrices were tested, which differ for percentage of guluronic acid, molecular weight, viscosity and combination with ECM molecules. Particularly, as reported previously, follicles were encapsulated in: SLG20 1%, SLG100 1%, ALG 1% and ALG-COLL 1%. All matrices were able to support follicular growth and able to preserve tridimensional structure of follicles. In fact, at day 10 of culture, follicles observed by brightfield microscopy significantly increase their initial diameter (Fig. 22-23). However, no antral cavity is revealed.

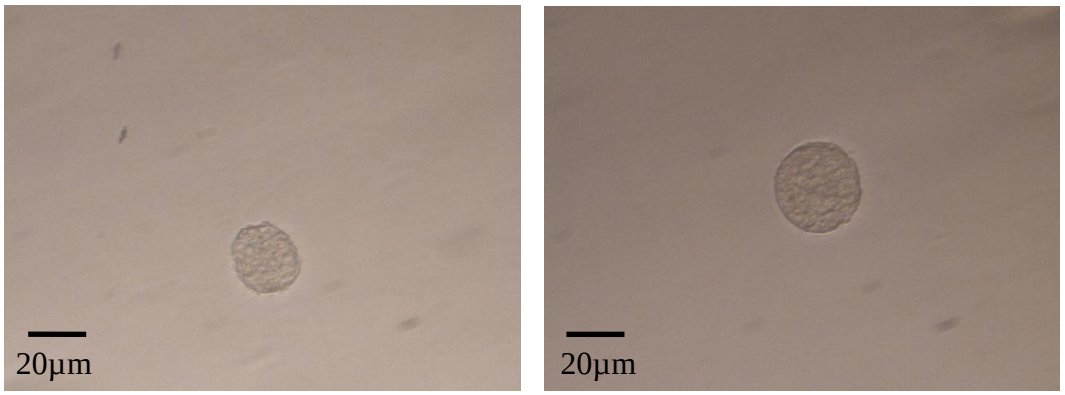


Fig. 22: follicle at day 0

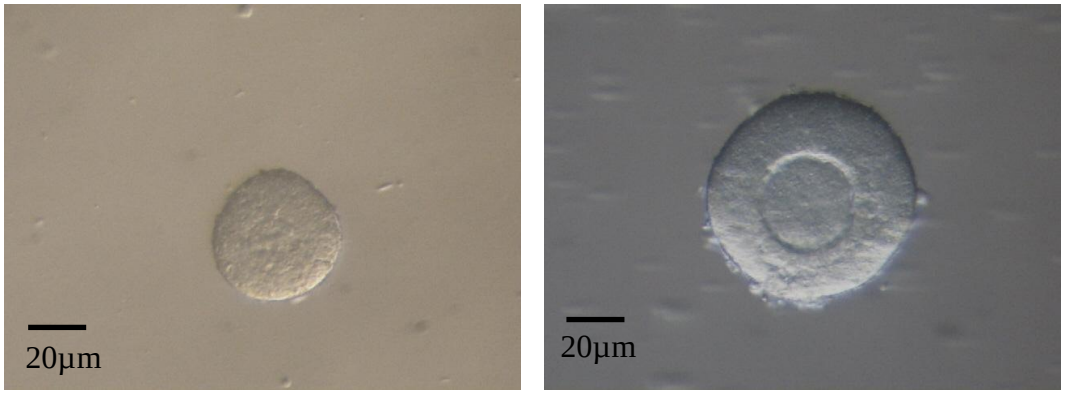
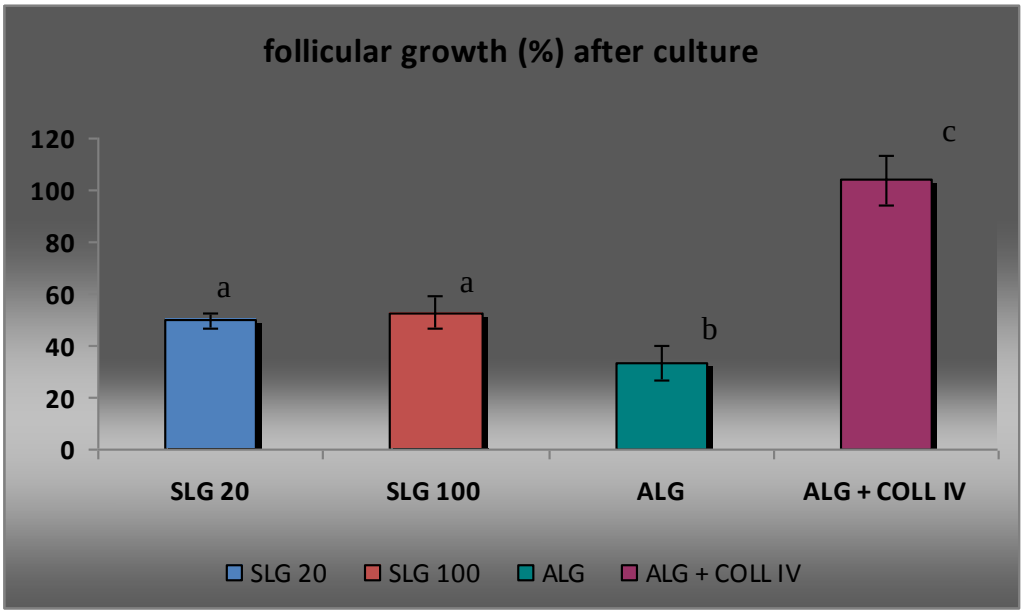


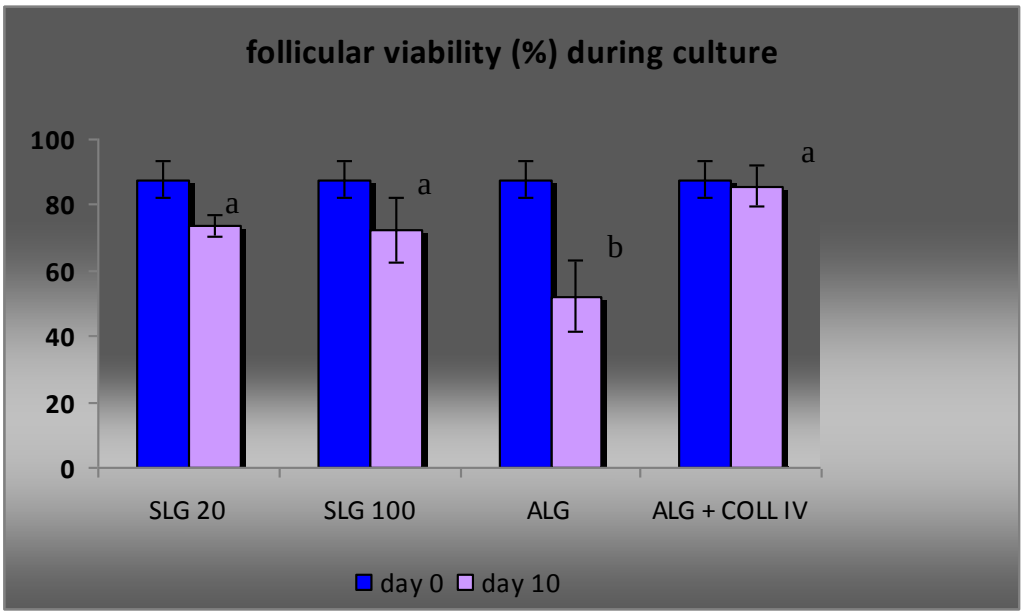
Fig. 23: follicle at day 10 of culture

Similarly, follicular growths and viabilities in different culture conditions were investigated. Results demonstrated that at the end of culture period, mean follicular growths were 49.7±2.8% for SLG 20 1.5%, 52.7±6.2% for SLG 100 1.5%, 33.2±6.8% for ALG 1.5% and 103.8±9.3% for ALG-COLL (graph 14).



Graph 14: follicular growths at day 10 of culture in different alginates. Different superscripts denote significant differences among treatment groups

Moreover, follicular viabilities, at day 10, were respectively 73.7±3.2% for SLG 20 1.5%, 72.3±10.1% for SLG 100 1.5%, 52.2±10.81% for ALG 1.5% and 85.7±6.2% for ALG-COLL (graph 15).



Graph 15: follicular viabilities at day 10 of culture in different alginates. Different superscripts denote significant differences among treatment groups

Analysis at confocal microscope

Follicles were also stained for F-actin with rhodamine-phalloidin and Hoechst 33342 for analysis at confocal microscope. Results demonstrated a marked and significant increase of follicular diameter, due to the presence, at the end of the culture period, of multilayered granulosa cells. In fact, after isolation, follicles display at most two cell layers of granulosa cells (Fig. 24). Oocyte is centred and show a clear germinative vescicle. Nuclei of granulosa cells are well visible and regularly distributed around the oocyte.

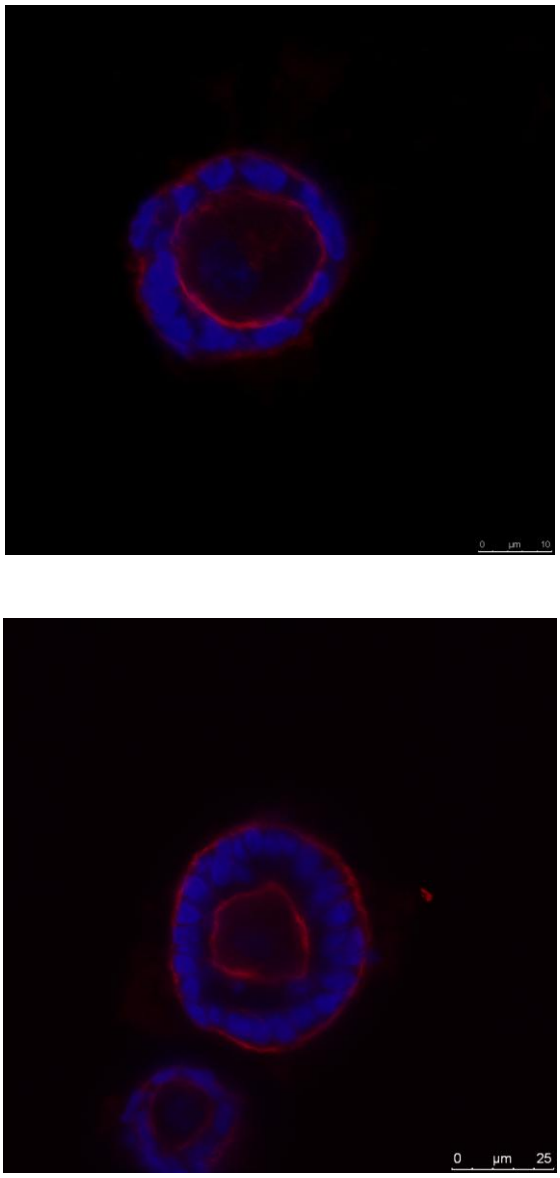


Fig. 24: follicles at day 0. In blue nuclei (Hoechst 33342); in red actin (Rhodaminate Phalloidin)

At day 10 of culture, follicles increase their size and a multilayered granulosa cells is well visible. Moreover, the oocyte size also increases in culture as the follicle grow three-dimensionally and preserve e regular morphology (Fig. 25).

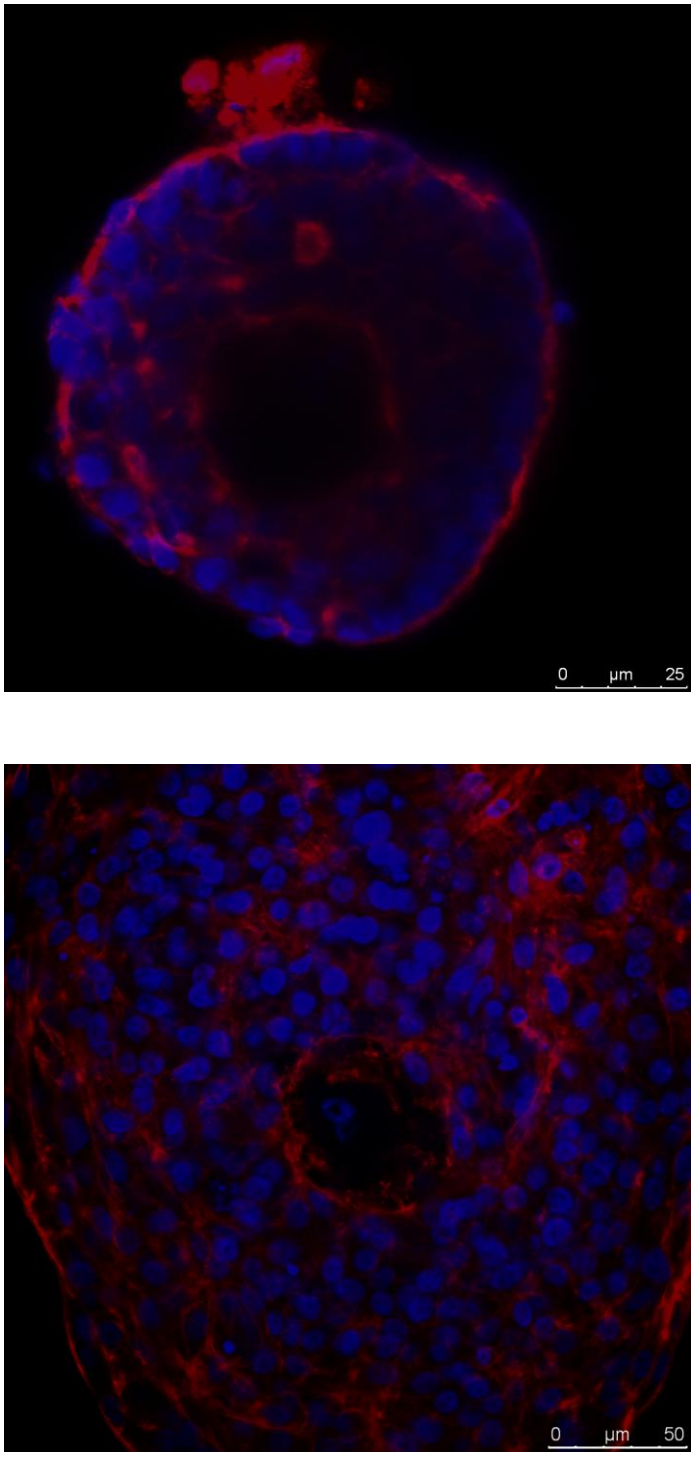


Fig. 25: follicle at day 10. In blue nuclei (Hoechst 33342); in red actin (Rhodaminate Phalloidin)

Vitrification of follicles

A total of 285 encapsulated follicles were vitrified. Particularly, 140 follicles were vitrified immediately after enzymatic digestion and 145 follicles were vitrified after 2 days of in vitro culture in ALG-COLL matrix. Morphological features and viability of follicles before (control) and after vitrification were investigated.
At day 0, after thawing, no viable follicles were recovered and 91.2±3.5% of follicles extruded their oocytes, with both vitrification protocols (Fig. 26-27).

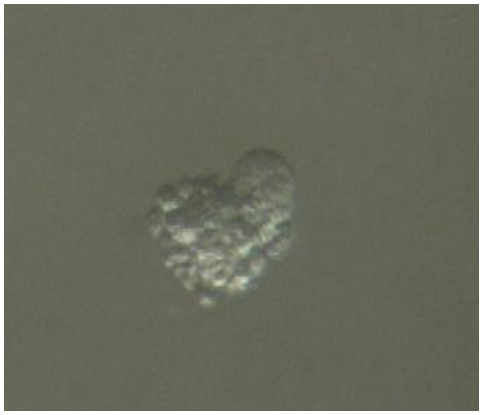


Fig. 26: frozen/thawed follicle after vitrification as reported by Xing et al. Oocyte has been extruded.

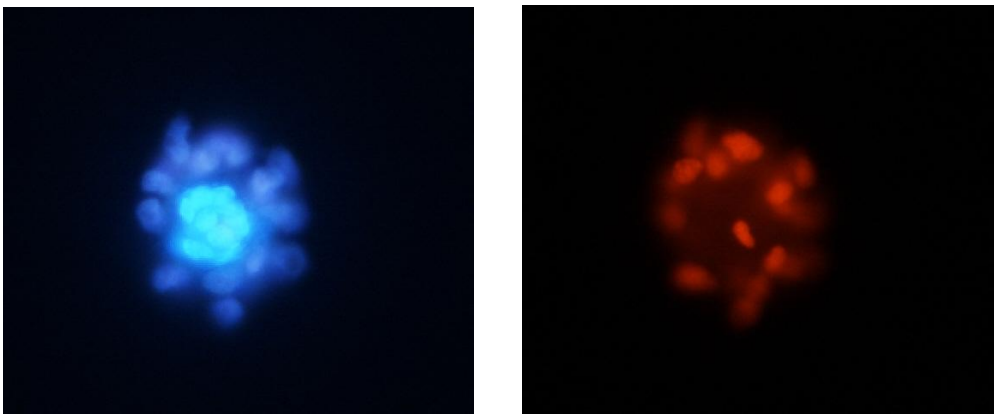


Fig. 26: frozen/thawed follicle after vitrification as reported by Xing et al. In blue Hoecsht 33342; in red propidium iodide.

At day 2 of in vitro culture, in the control group, 87.7±5.4% of encapsulated follicles were morphologically normal and viable. After thawing, there was a significative difference in terms of morphologically normal and viable follicles relative to the control in the two different tested protocols (Fig. 28).

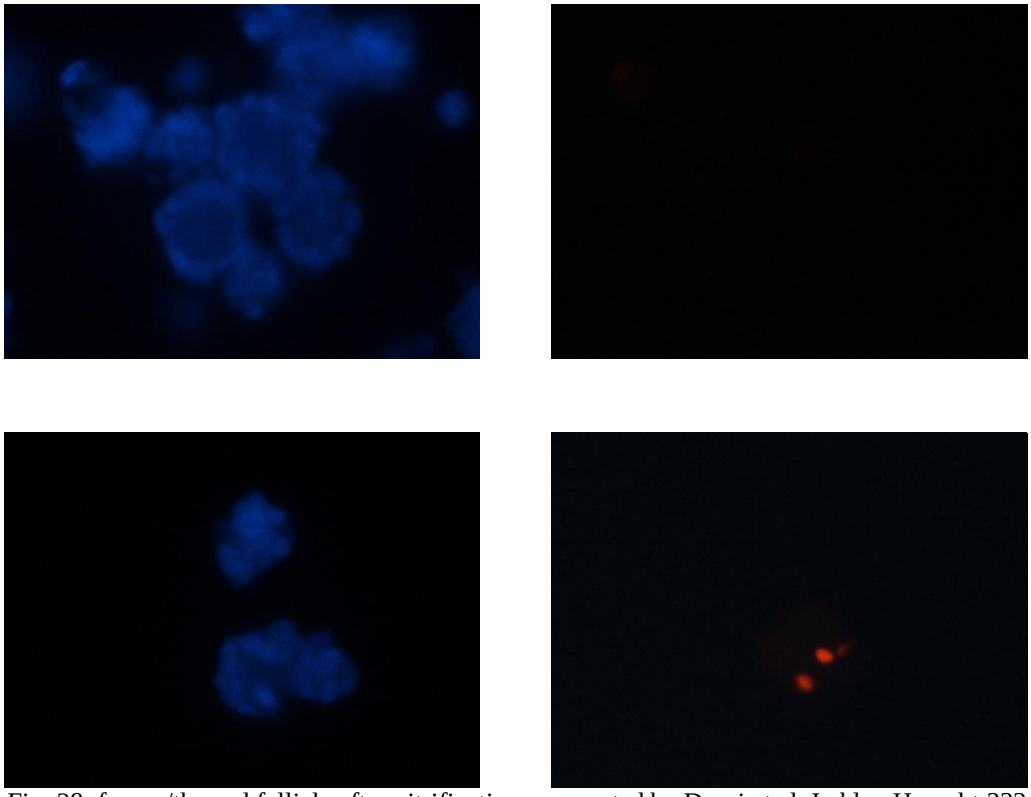
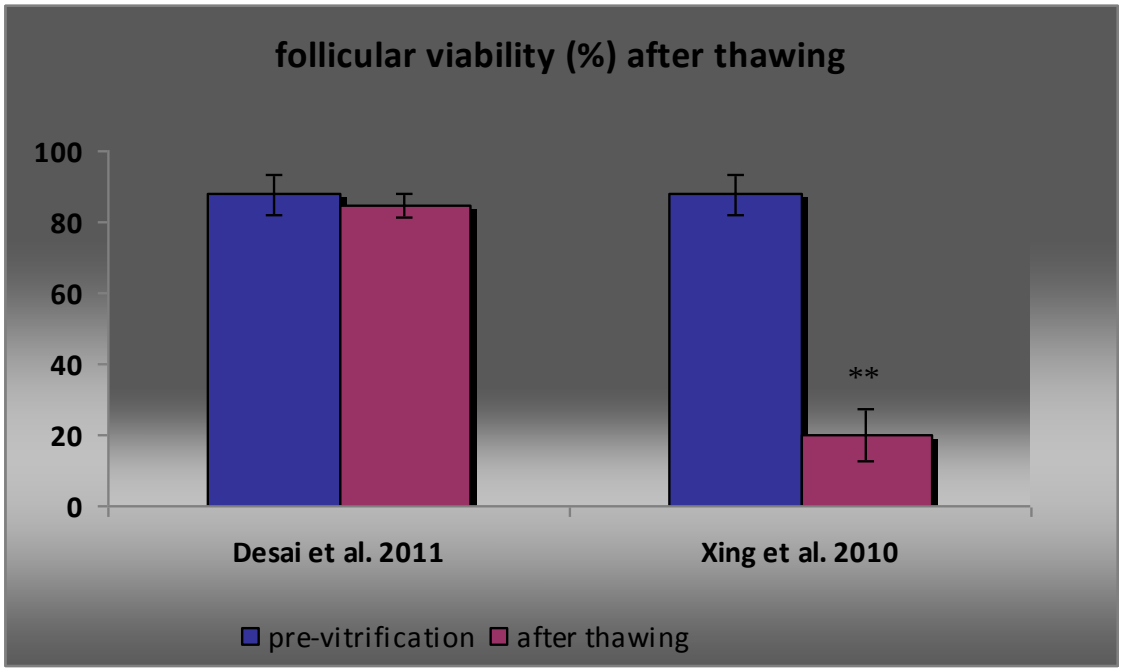


Fig. 28: frozen/thawed follicle after vitrification as reported by Desai et al. In blue Hoecsht 33342; in red propidium iodide.

Particularly, follicles vitrified as reported by Desai et al. 2011 show, after thawing, a viability of 84.53±3.51% and 80±2.4% of them are morphologically normal. Instead, there was a significantly lower proportion of morphologically normal follicles after vitrification as reported by Xing et al. 2010. Moreover, in this case viable follicles are 20±7.2% (Graph 15). Furthermore, follicles, after thawing, were able to restore their growth ability. In fact, thawed follicles, after 5 days of in vitro culture, show a growth of 10%.



Graph 15:follicular viabilities after vitrification/thawing. * $p<0.01$; ** $p<0.05$

Xenotransplantation of encapsulated follicles in immunodeficient mice

In collaboration with Research Laboratory on Human Reproduction in Brussels, at day 2 of in vitro culture, after monitoring viability and growth of encapsulated follicles, four different alginate beads (ten follicles/bead) were xenotransplanted to the kidney capsule or the back muscle of two immunodeficient mice. After three months, grafts were readily identifiable and removed. No follicles could be identified after histological analysis of kidney and back muscle.

DISCUSSION AND CONCLUSIONS

In recent years cancer incidence is constantly increasing as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets. (World Health Organization Databank WHO Statistical Information System, 2010). Moreover, recent data coming from AIRTUM Working group about children cancer incidence (Epidemiol Prev 2008; 32(2) Suppl 2: 1-112) indicate that there is a growth trend of both all new cases of cancer and mean tumor sites at 0-14 years. In contrast, early detection of cancer and progresses in diagnosis and enhanced effectiveness of chemotherapy and radiotherapy has increased the survival rate of young female cancer patients (von Wolff et al., 2009). Unfortunately these growing population of adolescent and adult long-term cancer survivors may experience infertility problems due to induced premature ovarian failure (POF) caused by cancer therapies, in a manner dependent on the age of the patient at the time of diagnosis and dependent on the type and quantity of drugs used for therapies. In fact, the ovaries, containing a definite number of follicles from the birth, are very sensitive to cytotoxic treatment, especially to alkylating agents, which are classified as high risk for gonadal dysfunction (e.g. cyclophosphamide, busulfan, melphalan, chlorambucil, dacarbazine, procarbazine, ifosfamide, thiotepa and nitrogen mustard) (Warne et al., 1973; Koyama et al., 1977; Fisher et al., 1979; Viviani et al., 1985; Mackie et al., 1996; Teinturier et al., 1998; Legault and Bonny, 1999; Meirow et al., 1999; Blumenfeld et al., 2000; Kenney et al., 2001; Tauchmanova et al., 2002). The follicular destruction following these treatments generally results in the loss of both endocrine and reproductive functions. Enthusiasm has therefore generated for young female patients, in which case the improvement of cancer therapies allows that young cancer patients can survive and undergo a normal reproductive life. In order to preserve their fertility, advances on biology of reproduction and Assisted Reproductive Technologies have opened a wide range of options. There are a few options available to preserve fertility in these patients. These include

cryopreservation of embryos, oocytes (Yang et al., 2007; Porcu et al., 2008) or ovarian tissue (Hovatta et al., 1996; Nugent et al., 1997; Donnez et al., 2000; Kim, 2006). Embryo cryopreservation is the most mature technology available for fertility preservation and it is the most effective strategy to date. Human embryos can survive the freezing and thawing process up to 95% of the time, and cumulative pregnancy rates can be greater than 60% if multiple embryos are available (Oktay et al., 2003, 2005; Rao et al., 2004; Juretzka et al., 2005; Lee et al., 2006; Seli et al., 2005). Recently, oocyte cryopreservation has improved significantly allowing better survival and fertilization rate (Fabbri et al., 2001; Oktay et al., 2006). However, clinical pregnancy and live birth rates are lower than those observed with unfrozen oocytes. On the other hand, both methods require a delay in cancer treatment and hormonal stimulation. Furthermore, there are ethical problems for embryos cryopreservation. Thus, these methods are not an option for patients who need to start immediately cytotoxic treatments, for patients who have an estrogen sensitive cancer, such as breast cancer and for pre-pubertal patients. Moreover, embryo cryopreservation couldn't be performed for single women. In order to preserve fertility in these patients, ovarian tissue cryopreservation followed by transplant is a promising fertility preservation approach. The advantages of the ovarian tissue cryopreservation are the possibility to: preserve a large number of primordial follicles present in the cortex of the ovarian tissue, preserve both steroidogenic and gametogenic functions, avoid postponement of cancer treatment, collect ovarian tissue independently of the stage of the menstrual cycle and in a non invasive manner, through laparoscopy, and finally restore the natural state, when the graft is transplanted in fully remised patients allowing conception *in vivo* with their own germ cells. Autotransplantation of ovarian cortical strips has resulted in viable offspring in animal models (Bordes et al. 2005) and human. Worldwide, 14 live births have been reported as a result of autotransplanting frozen/thawed ovarian tissues (Donnez et al., 2004, 2011; Meirow et al., 2005; Demeestere et al., 2007; Andersen et al., 2008; Silber et al., 2008; von Wolff et al., 2009; Piver P et al., 2009; Ernst et al., 2010; Sanchez-Serrano et al., 2010; Roux et al., 2010). Despite these promising findings, transplantation of cryopreserved tissue carries the risk

of re-introducing cancer cells into the patient (Shaw and Trounson, 1997; Meirow et al., 1998, 2008). In order to avoid this risk, the only possibility to preserve fertility is represented by the in vitro growth of the oocytes from the primordial stage. However, until now, only in a murine model, isolated follicle culture using a 3-dimensional alginate matrix has yielded mature oocytes capable of fertilization and delivery of healthy mouse pups (Abir et al., 2006). Thus, even though the risks of transmitting disease in grafted tissue carrying malignant cells related to ovarian tissue cryopreservation is still an open question up to now, and even if only a few studies about in vitro culture of human isolated follicle have been performed, these ones represent the only suitable technology for fertility preservation for pre-pubertal girls and women who cannot delay the start of chemotherapy. In this scientific contest, aim of my PhD research was improvement of these two alternative strategies. In fact, cryopreservation methods have to be still investigated in order to improve their experimental efficiency, and, to date, isolation of follicles from ovarian tissue and in vitro culture is still undeveloped.

As first step, in the present study I studied the development of new protocols for ovarian tissue cryopreservation in order to improve the efficiency of the method. Main results demonstrated a beneficial effect of replacement of sodium with choline in freezing media. Two studies have already reported the efficacy of sodium-depleted slow freezing for cryopreservation of human oocytes. In a series of 12 patients, a total of 144 oocytes were cryopreserved and thawed (Quintans C et al., 2002). The median survival was 63%. Nonetheless, based on the surviving oocytes, a fertilization rate of 59%, an implantation rate of 25%, six clinical pregnancies, and two live births were obtained. Boldt et al. (Boldt J et al., 2003) compared two slow freezing protocols: oocytes cryopreserved in the sodium-depleted freezing medium resulted in a significantly higher survival rate than oocytes in the sodium-based freezing group after thawing. In the sodium-depleted group, 59% fertilization rate was obtained, and out of 11 women who received embryo transfer four pregnancies and five live births were reported. Two potential explanations have been proposed (Stachecki et al., 1998, 2000, 2002, 2006). The first theory relates to the ‘‘solute effect’’, involving the transport of a large

quantity of sodium ions cross the cell membrane through the plasma membrane–associated sodium-potassium (Na-K) pump. During equilibration in PBS-based freezing media which contains a relative high concentration of Na, an excess Na may be pumped into the ooplasm. However, the functions of Na-K pumps may be impaired during freezing and thawing, resulting in an intracellular accumulation of Na and ultimately in cell death. An alternative explanation is that the choline may have a direct cryoprotective effect by stabilizing the cell membrane (Toner et al., 1993). Unlike the sodium ion, choline is thought not to cross the cell membrane and therefore would not be expected to contribute to the intracellular solute load.

In the present study, the improvement of ovarian tissue cryopreservation after replacing of sodium with choline has been demonstrated by histological, immunohystochemical and ultrastructural analysis. The ovarian cortex contains follicles mainly at primordial and primary stages, which are particularly resistant to cryopreservation procedures (Hovatta, 2005). At these stages, the follicles possess certain characteristics that make them less sensitive to cryoinjury, such as a low metabolic rate and number of granulosa cells, absence of a ZP and peripheral cortical granules, and a small immature oocyte (arrested in the prophase of the first meiotic division) with low amounts of intracytoplasmic lipids. In the present study, microscopic visualization of haematoxylin/eosin-stained sections revealed clear differences between non frozen, slowly cooled and vitrified follicles. Results demonstrated that in slow freezing groups percentage of morphologically normal follicles were higher in choline modified protocols than in conventional ones, according to criteria reported by Gougeon A in 1986. In vitrification groups percentage of morphologically normal follicles were also higher in choline modified protocols than in conventional ones, but follicular morphology is not well preserved and the proportion of the morphologically intact follicles were significantly reduced in the two vitrification groups.

To deepen these data, an ultrustructural study has been performed. Different studies have demonstrated that cryopreservation of ovarian tissue may injure follicular structure (Cortvrindt et al., 1996; Oktay et al., 1997; Gook et al., 1999; Nisolle et al., 2000; Abir et al., 2001; Hreinsson et al., 2003; Eyden et al., 2004;

Imhof et al., 2004; Lucci et al., 2004; Martinez-Madrid et al., 2004a; Rodrigues et al., 2004a,b; Camboni et al., 2005; Fabbri et al., 2006a,b; Santos et al., 2006; Nottola et al., 2007b; Fauque et al., 2007). In fact, the structure and functionality of the follicles present in the ovarian cortex might be altered by inadequate cryopreservation procedures. Microorganelles responsible for a specific biological event has a different susceptibility to be damaged from freezing, and follicles cryodamaged may be responsible of retard follicle growth and oocyte maturation after transplantation. Particularly, alterations in mitochondrial morphology, as well as in the association between mitochondria and smooth endoplasmic reticulum (SER) elements (membranes and vesicles), may influence the developmental competence of human oocytes (Van Blerkom, 2004). Decrease in temperature appears to have an influence on oocytes. Chilling has been implicated in the modification of membranes (Ghetler *et al.*, 2005), which may affect their integrity, while freezing has been shown to cause mitochondrial enlargement and alterations in the relationship of SER elements with mitochondria in bovine oocytes (Schmidt *et al.*, 1995). Sathananthan *et al.* (1988) also observed mitochondrial swelling and SER element damage after cooling human oocytes to 0°C. In the present study, electron microscopy investigations showed that, compared to fresh follicles, in cryopreserved follicles, mitochondria have a decreased electron density of the matrix or irregular shape. Particularly, mitochondrial area and number of mitochondria/μm^2 have been analyzed. Moreover, two categories of mitochondria have been investigated: elongated and round mitochondria, different for shape and respectively more or less mitochondrial cristae. Results demonstrated that all slowly freezed follicles, for both protocols, have been characterised by a similar density of mitochondria, if compared with non frozen samples, but elongated mitochondria areas significantly decreased in conventional freezing, while these ones have been well preserved in sodium depleted choline-supplemented slow freezing techniques. Furthermore, at an ultrastructural level, follicles slowly cooled with both choline modified protocols, have been characterised also by a more homogeneous cytoplasm both in oocyte and in follicular cells, well defined oolemma and nuclear membrane and, at a cytoplasmatic level, only in choline cryopreserved follicles, typical

structures called anulatae lamellae are well preserved. Instead, all follicles from vitrified tissue have been characterised by an inferior mitochondrial density and increased round mitochondria areas if compared with non frozen samples. Similarly, follicles vitrified with choline modified protocols, if compared with follicles vitrified with conventional protocols, have no intercellular spaces between follicular cells and at a cytoplasmatic level, also in this case, anulatae lamellae are well visible. However, in vitrified follicles, different injuries have been found, not revealed in control samples, such as lipid droplets, vesicles or lysosomes. Finally, I further investigated whether cryopreservation affects primordial follicle physiology and functionality, specifically the p53-p21-Apaf1 pathway, which has been shown to play an important role in regulating ovarian apoptosis. Several studies demonstrated that p53 expression was not altered after slow-freezing (Hussein et al. 2006) and no significant difference between the p53 mRNA level in vitrified and non-vitrified tissue (Mazoochi et al., 2009). However, expression of p53 is the result of DNA damage (Lu X and Lane DP, 1993) and the expression of p53 protein in the apoptotic granulosa cells of atretic follicles suggests its possible role in atresia (Kim et al., 1999). p21 is an important p53 target, which gene product associates with and inhibits cyclin-Cdk complex kinase and thereby blocks the transition from G1 to S in the cell cycle (EI-Deiry WS et al., 1993; Harper JW et al. 1993). Moreover, in the mitochondrial signalling pathway, it is generally believed that when the cells receive a stressful stimulus which leads to mitochondrial damage, cytochrome c is released from the mitochondria (Green and Reed, 1998; Wang, 2001) and binds with Apaf1 (Zou et al., 1997; Robles et al., 1999), and then the Apaf1–cytochrome c complex binds with procaspase- 9 (Cecconi et al., 1998; Qin et al., 1999). The Apaf1–cytochrome c–procaspase-9 complex plays a key role in mitochondrion-dependent apoptosis (Cecconi, 1999; Grutter, 2000;Wang, 2001). In this project research, expression of p53, p21 and Apaf1 have been investigated by immunohistochemical approach. Results showed that three different situations have been observed. Follicles of grade 1 are negative for p53, p21 and Apaf1. Follicles of grade 2 are immunoreactive for p53, and p21 but staining for Apaf-1 is revealed only in part of them. Finally, follicles of grade 3 are immunoreactive

for all tested markers. Thus, immunohistochemical analysis confirms histological and ultrustructural ones and demonstrated that morphology of follicles is highly related to their functionality. Overall, data herein indicated that sodium-depleted choline-supplemented cryopreservation preserves ovarian tissue better than conventional sodium based one.

A new biotechnological approach has been used to develop an efficient method for follicle isolation and subsequent in vitro culture. Conventional culture, as reported by different studies, is unable to support follicular growth and hormone production. In conventional 2-dimensional (2-D) tissue culture systems, the follicle tends to flatten and granulosa cells surrounding and nurturing the growing oocyte, migrate away, leaving it naked and unable to complete the maturation process (West ER et al., 2007). In the first stages of follicologenesis, interactions between oocyte and granulosa cells are necessary (Carabatsos MJ et al., 2000) and only a tridimensional culture system can preserve this condition. Encapsulation of follicles may protect them from gap junction disruption through shear stress (Heise M et al., 2005; Heise MK et al, 2009) and may preserve expression of the gene encoding for the gap-junction protein connexin (De Paola N et al., 1999). Contiguous assembly of granulosa cells around the oocyte also prevents the follicles from undergoing premature ovulation (Xu M et al., 2006). Another advantage may be that trophic factors released by granulosa cells remain in close proximity to the oocyte exerting a positive effect on oogenesis and possibly fostering new local gap junctions. To reproduce in vitro the architecture of follicle interactions, in this research project, the effects of alginate matrix on in vitro culture of isolated follicles has been studied. In the alginate system, follicles have been able to grow (Pangas et al., 2003), produce fluid-filled antral cavities (Xu et al., 2006), and produce meiotically competent oocytes (Kreeger et al., 2006; 2005), which were successfully fertilized and implanted to yield multiple live births of healthy mouse pups (Xu et al., 2006). Moreover, alginate is one of the most used biomaterials for microencapsulation, for its biocompatibility, high affinity to water and ability to form hydrogels in very mild conditions (Pangas SA et al., 2003; Kreeger PK et al., 2005; Amorim CA et al., 2009; Xu M et al., 2009). The properties of alginate hydrogels vary widely depending on different physical

and mechanical properties. Ovarian follicle culture systems provide an ideal tool to study the properties of a three-dimensional hydrogel matrix in follicle development. The hormonal regulation of folliculogenesis has been widely investigated, yet the role of the physical properties of the follicle microenvironment has not. Understanding the role of the environment on follicle development will be useful for the development of biomimetic matrices for the in vitro culture of follicles and other hydrogel-encapsulated cell culture systems.

The mechanical properties of alginate are dependent on several factors, such as the mass of polymer and the extent of cross-linking (Anseth KS et al., 1996). Decreasing the percentage of alginate reduces the extent of cross-linking within the gel, thereby decreasing the modulus for the material (Kong HJ et al., 2004). Less rigid hydrogels deform more readily, which would create space as the follicle increases in size. The other mechanism by which the alginate percentage may affect follicle growth is the extracellular transport of macromolecules (Wee S et al., 1998; Peters MC et al., 1998). The culture media contains macromolecules necessary for follicle growth, and cells within the follicle are producing and secreting factors that affect the maturation process. Decreasing the percentage of alginate increases the mean mesh size within the hydrogel, which may enhance macromolecular transport through the hydrogel. However, natural and synthetic matrices, such as the negative-charged polysaccharide alginate, can also bind macromolecules to serve as reservoirs for growth factors (Peters MC et al., 1998). Decreasing the percentage of alginate would reduce binding sites for these macromolecules and increase transport. Therefore, factors added to the culture media would be transported through the gel more easily with a reduction in the alginate percentage. In addition, factors produced by the follicle, which may affect growth positively and negatively, would more effectively escape from the follicle.

In this contest, different studies show that the strength of the alginate gel network is an important factor that influences the growth characteristics of encapsulated cells. Xu et al. in 2006 reported that follicles encapsulated in 0.5% and 0.25% alginate had increased to more than 300μm, significantly larger than those cultured in 1.5% alginate. Also, follicles encapsulated in 1.5% alginate were less likely to develop an antrum, did not develop laminar-like teca cells, and produced

lower androstenedione levels. Thus, alginate concentration not only affected theca cell proliferation and differentiation but also regulated granulosa cell proliferation and differentiation. Interestingly, different studies demonstrated, instead, that follicles cultured in 0.5% alginate performed better than those in 0.25% alginate, suggesting that primate follicles may require more physical support. One concern however is that denser matrices could potentially limit access to hormones and other nutrients. Heise et al. (2005) reported inhibited delivery of FSH to microencapsulated follicles.

Three different concentrations of alginate were chosen for the present study: primordial and primary follicles have been enzimatically isolated and cultured for up to 10 days in alginates 1%, 2% and 3% to determine which one best supports follicle survival and growth. Viability of follicles has been detected using a double staining with Hoechst 33342 and propidium iodide; follicular growth has been instead monitored daily through a NIS element imaging software. All alginates were able to support in vitro follicular culture. However, results demonstrated opposing influences of the rigidity of the biomaterial at high gel concentration and its interference with diffusion and optimal growth. In fact, best results have been obtained with encapsulation of follicles in ALG1%. At the end of the culture, mean follicular growths and follicular viability are higher for follicles encapsulated in ALG1% and they decrease at increasing of alginate concentration.

Next step, in this research project, has been studying the effects of physical properties of alginate matrix on in vitro culture of isolated follicles. The physical properties of alginate hydrogels vary widely depending on their composition (e.g., the proportion of guluronic to mannuronic acid residues) and the sequential order of these residues. Briefly, alginates possessing a high guluronic acid content develop stiffer, more porous gels which maintain their integrity for longer periods of time. During cationic cross-linking, they do not undergo excessive swelling and subsequent shrinking, thus they better maintain their form. Conversely, alginates rich in mannuronic acid residues develop softer, less porous gels that tend to disintegrate with time. Alginates with a high mannuronic acid content are also plagued by a high degree of swelling and shrinking during cationic crosslinking.

In the present study, different matrices have been tested, which differ for percentage of guluronic acid, molecular weight and viscosity. To date the most widely applied system for follicle encapsulation and 3-D culture has been alginate produced by brown algae (Xu M et al., 2006; West ER et al., 2007; Xu M et al., 2006, 2009a,b,c; Pangas SA et al., 2003; Kreeger PK et al., 2005; Amorim CA et al., 2009). To further simulate the in vivo environment, ECM molecules have been combined with calcium alginate to construct synthetic ECM matrices for 3D culture (Kreeger PK et al., 2003). In a separate study, these investigators combined calcium alginate with additional ECM components such as collagen I, collagen IV, laminin and fibronectin (Kreeger PK et al., 2006). Transition to the multi-layered, secondary follicle was enhanced in alginate matrices with RGD or collagen I and final maturation of oocytes and resumption of meiosis was promoted by presence of fibronectin, laminin or RGD peptide.

In the present study, the following biomaterials have been tested: alginate SLG 20 1% (69% guluronic acid, 75kDa), alginate SLG 100 1% (68% guluronic acid, 200kDa), alginate 1% (50% guluronic acid, 50% mannuronic acid). Moreover, alginate 1% has been combined with collagen type IV 0.3mg/mL, as the latter is the main ECM component present during follicologenesis. At day 10 of culture, all follicles observed by brightfield microscopy increase significantly their initial diameter but no antral cavity is revealed. However, at the end of culture period, improvement of mean follicular growths and viability were obtained for SLG 20 1% and for SLG 100 1%, showing a beneficial effect of a major percentage of guluronic acid in alginate molecule and, moreover, follicular growth and viability percentages have been significantly improved after encapsulation in ALG-COLL. In fact, although all follicles are able to grown, only follicles encapsulated in alginate combined to collagen IV double their initial diameter and viability is better preserved during culture period. Follicles were also stained for F-actin with rhodamine-phalloidin and Hoechst 33342 for analysis at confocal microscope. Results demonstrated a marked and significant increase of follicular diameter, due to the presence, at the end of the culture period, of multilayered granulosa cells. Under these conditions oocytes retained a normal morphology. Thus, although all matrices were able to support follicular growth, the presence of components of

ECM, such as collagen, improves the efficiency of in vitro culture of isolated follicles, better preserving follicular growth and viability. As a result, new prospectives in fertility preservation in cancer women could be opened, because follicles cultured in vitro could be cryopreserved and re-implanted at the pathology remission, in order to avoid also the risk of re-introduction of malignant cells associated to ovarian tissue cryopreservation. Isolated follicles can be cryopreserved by slow-freezing or by vitrification. Vitrification is a method with a high concentration cryoprotectant combined with a fast cooling rate, which avoids ice crystal formation during the cryopreservation process (Fahy GM et al., 1984). The recent advance of ultrarapid vitrification procedures has been attributed to new devices which enabled high cooling rates, such as electron microscopic copper grids (Martino A et al., 1996), OPS (Vajta G et al., 1997), cryoloop (Lane M et al., 1999), microdrops (Papis K et al., 2000), SSV (Dinnyes A et al., 2000) and nylon mesh (Matsumoto H et al., 2001). Xing et al. (2010) demonstrated, in rat, that the rate of totally viable follicles of the SSV group was slightly higher than that of the OPS group and significantly higher than that of the slow-rate freezing group. Moreover, after *in vitro* culture, the increase of follicle diameter of SSV group was significantly higher than that of the slow-rate freezing group. Desai et al. 2011 reported that EG-raffinose vitrification protocol resulted in excellent post-warming survival. About 95% of mouse follicles were morphologically intact immediately post warming and 65% survived to the end of the in vitro culture interval period, with 41% of oocytes maturing to the MII stage. However, after collection, isolated follicles could be stressed by the enzymatic digestion and 2-3 days of in vitro culture after encapsulation in a tridimensional matrix could allow the recovery of follicle physiological functions after enzymatic digestion. In this study, follicles were vitrified immediately after enzymatic digestion and after 2 days of in vitro culture in ALG-COLL matrix and their ability to survive to cryopreservation has been analysed by fluorescent assays. Particularly, two different vitrification methods have been tested (Desai et al. 2011; Xing et sl. 2010) and thawed follicles have been stained and imaged at the fluorescence microscope to detect eventual damages induced by the cryopreservation. At day 0, after thawing, no viable follicles have been recovered

and the most part of follicles extruded their oocytes, with both vitrification protocols. At day 2 of in vitro culture, after thawing, a significative difference in terms of morphologically normal and viable follicles relative to the control have been retrieved in the two different tested protocols. Results demonstrated that, after thawing, follicles vitrified according to Desai et al had a higher viability compared to the protocol of Xing et al. Morphological analysis of follicles confirmed these data. Furthermore, follicles, after thawing, have been able to restore their growth ability. In fact, thawed follicles, after 5 days of in vitro culture, showed a growth of 10%.

Thereafter, to understand whether frozen thawed isolated follicles were able to undergo growth and development after transplantation three potential possibilities exist: heterotopic autografting, orthotopic autografting and xenografting. In transplantation experiments with isolated follicles to the ovarian bursa of mice, Gosden (1990) had resorted to the plasma clot procedure to retain follicles in situ. Also, Dolmans et al. (2007) reported 58.3% recovery rate for isolated follicles xenografted to the murine ovarian bursa in a plasma clot. Ovarian tissue is commonly grafted to the kidney capsule or the back muscle of immunodeficient mice because these sites favour rapid revascularization due to its rich capillary bed. Recently (Aerts et al., 2010) a novel transplantation technique, consisted in microinjecting follicles underneath the kidney capsule to prevent follicle extrusion from the renal membrane into the abdomen, thus avoiding the need for a plasma clot as a vehicle for transplantation, resulted in 100% of grafted follicles recovered from the kidney capsule, indicating that the microinjection technique successfully retains the transplants in situ.

In the present study, after in vitro culture and vitrification/thawing alginate encapsulated follicles have been xenografted in immunodeficient mice. Thus, in collaboration with Research Laboratory on Human Reproduction in Brussels, after monitoring viability and growth of encapsulated follicles, four different alginate beads (ten follicles/bead) have been xenotransplanted to the kidney capsule or the back muscle of two immunodeficient mice. After three months, grafts were readily identifiable. The grafts have been removed after 3 months and analysed by light microscopy. Unfortunately, until now, no follicles could be identified after

histological analysis of kidney and back muscle. However, observations on the physiological status of xenografted follicles are currently under way. Thus, data obtained in this PhD thesis encourage the development of new strategies to preserve fertility in cancer women. Improvements to in vitro follicle culture systems, along with improved cryopreservation and follicle isolation techniques, may provide a viable avenue for the maturation of cryopreserved immature ovarian follicles in vitro for subsequent fertilization and implantation to preserve reproductive options for female cancer patients. In addition to germline preservation, follicle culture techniques may also revolutionize approaches to treat female infertility. In addition, ovulation induction requested for actual fertility preservation methods produces a limited number of oocytes, many of which may not produce viable embryos. In vitro culture systems may allow for the maturation of immature follicles for subsequent oocyte collection and fertilization. If a viable human in vitro follicle culture system is developed, a small portion of the ovary could be removed by laparoscopy, and follicles could be cultured and observed to yield fertilizable germ cells. This approach may also potentially allow women to delay childbearing until later in life due to lifestyle or other reasons. In vitro follicle culture systems have many potential clinical and research applications, but improvements to current technologies are needed to make these applications a reality. The application of tissue engineering techniques to follicle culture have already led to improvements to culture system technologies, and further improvements may one day produce culture systems capable of producing successfully maturing human ovarian follicles.

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