UNIVERSITA' DEGLI STUDI DI MILANO

Department of Health, Animal Science and Food Safety

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OVARIAN TISSUE CRYOPRESERVATION FOR THE SAFEGUARD OF FEMALE FERTILITY

THESIS BY:
Dr. Sara Maffei
Registration Nr. R08995

TUTOR: Chiar.mo Prof. Fulvio Gandolfi
DOCTORAL COORDINATOR: Chiar.mo Prof. Fulvio Gandolfi

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ABSTRACT

Ovarian tissue cryobanking is considered an available method for preserving female fertility and represents the best option in young pre-pubertal cancer patients. Cryopreservation can be performed on ovarian cortical fragments or on the entire organ. To move forward, the cryopreservation of both avascular ovarian fragments and of whole ovaries must be improved. To this purpose, we performed a detailed comparison between conventional slow freezing and directional freezing on both cortical fragments and whole ovary.

We provide that directional freezing improves the viability of cryopreserved ovarian tissue both in whole ovaries and cortical fragments, but we observed a better preservation of follicles when the samples were frozen as entire organ.

We also developed a perfusion system for *ex vivo* culture of whole ovary demonstrating that it is possible to maintain live sheep ovary outside the body for up 4 days. This perfusion culture system could provide a future alternative for women at risk of ovarian involvement due to the threat of reintroducing malignant cells.

1 INTRODUCTION

1.1 CANCER AND FERTILITY

1.1.1 Cancer prevalence

Cancer is a major public health problem in the Europe ,United States and many other parts of the world. In these states, one in 4 deaths is due to cancer. The estimated total number of cancer deaths in Europe in 2012 was 1.75 milion, of which 56% (976,000) were in men and 44% (779,000) in women [1]. A total of 2,437,163 death were recorded in the United States in 2009, 567,628 of these from cancer [2]. Cancer is the second leading cause of death, following heart desease, accounting for 23% of all deaths. However, within 20-years age groups, cancer is the leading cause of death among both men and women aged 40 to 79 years (Figure 1) [2].

	ALL AGES		AGES 1 TO 19		AGES 20 TO 39		AGES 40 TO 59		AGES 60 TO 79		AGES ≥80	
	MALE All Causes 1,217,379	FEMALE All Causes 1,219,784	MALE All Causes 13,919	FEMALE All Causes 7,702	MALE All Causes 62,116	FEMALE All Causes 28,792	MALE All Causes 227,801	FEMALE All Causes 142,628	MALE All Causes 467,962	FEMALE All Causes 373,658	MALE All Causes 430,581	FEMALE All Causes 655,337
1	Heart diseases 307,225	Heart diseases 292,188	Accidents (unintentional injuries) 5,317	Accidents (unintentional injuries) 2,645	Accidents (unintentional injuries) 21,388	Accidents (unintentional injuries) 7,228	Cancer 54,483	Cancer 50,579	Cancer 154,168	Cancer 127,506	Heart diseases 130,332	Heart diseases 193,676
2	Cancer 296,763	Cancer 270,865	Assault (homicide) 2,031	Cancer 848	Intentional self-harm (suicide) 8,977	Cancer 4,629	Heart diseases 52,826	Heart diseases 21,353	Heart diseases 118,163	Heart diseases 74,294	Cancer 82,765	Cancer 87,264
3	Accidents (unintentional injuries) 75,022	Cerebro-vascular disease 76,769	Intentional self-harm (suicide) 1,500	Assault (homicide) 569	Assault (homicide) 7,214	Heart diseases 2,393	Accidents (unintentional injuries) 24,265	Accidents (unintentional injuries) 11,333	Chronic lower respiratory diseases 31,425	Chronic lower respiratory diseases 31,457	Chronic lower respiratory diseases 27,930	Cerebro-vascular disease 51,445
4	Chronic lower respiratory diseases 65,119	Chronic lower respiratory diseases 72,234	Cancer 1,042	Congenital anomalies 495	Heart diseases 5,256	Intentional self-harm (suicide) 2,140	Intentional self-harm (suicide) 11,858	Cerebro-vascular disease 5,283	Cerebro-vascular disease 19,751	Cerebro-vascular disease 19,317	Cerebro-vascular disease 24,649	Alzheimer disease 47,856
5	Cerebro-vascular disease 52,073	Alzheimer disease 55,103	Congenital anomalies 563	Intentional self-harm (suicide) 434	Cancer 4,256	Assault (homicide) 1,443	Chronic liver disease & cirrhosis 10,562	Chronic lower respiratory diseases 5,134	Diabetes mellitus 16,646	Diabetes mellitus 13,572	Alzheimer disease 18,689	Chronic lower respiratory diseases 35,212

Figure 1. The first five leading causes of death by age and sex in United States in 2009 (from Siegel et al., 2013).

There are more than 200 different types of cancer. The four most common cancer sites are breast cancer (464,000 cases, 13.5% of all cancer cases), followed by colorectal cancer (447,000, 13.0%), prostate cancer (417,000, 12.1%) and lung cancer (410,000, 11.9%) [1] (Figure 2).

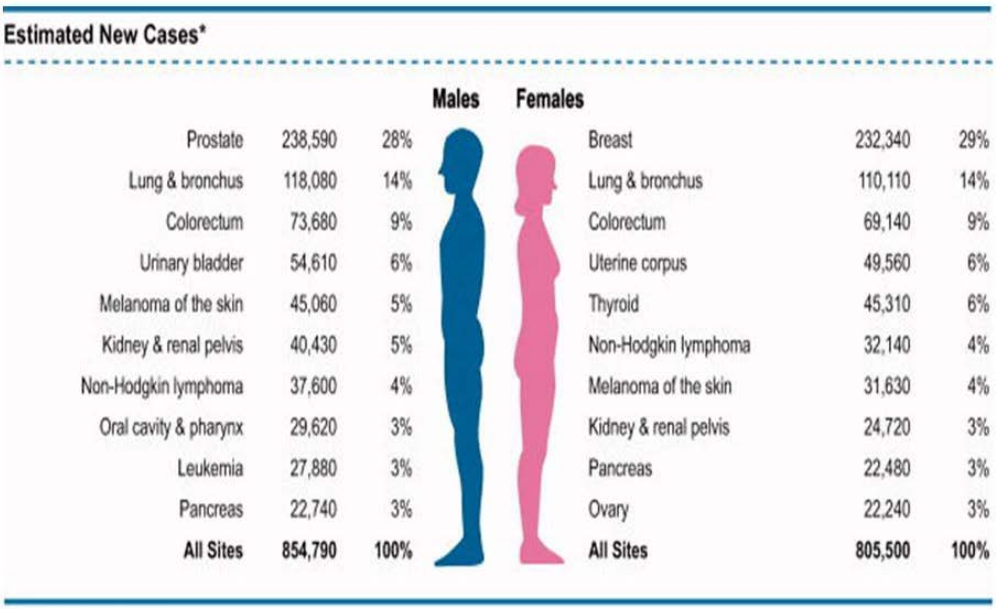


Figure 2. Ten leading cancer types for the estimated new cancer cases in United States in 2012 (from Siegel et al., 2013).

Cancer death rates decreased by 1.8% per year in males and by 1.5% per year in females during the most recent 5 years of data (2005-2009) [2].

1.1.2 Cancer prevalence in women

There were just over 1.6 million new cases of cancer (excluding non-melanoma skin cancer) in Europe in 2013. Breast cancer is the most common invasive cancer in women, affecting > 464,000 women annually in the Europe and accounting for 28.8% of all cancer diagnosed in women [1]. In women this cancer was by far the most frequently diagnosed neoplasm, followed by colorectal (205,000,

12.7%), lung (119,000, 7.4%) and corpus uteri (99,000, 6.1%) cancers (Figure 3 and Table 1).

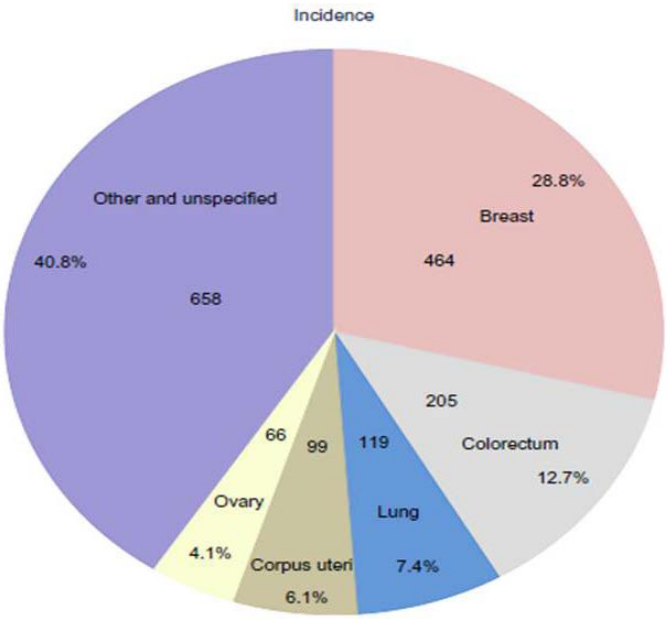


Figure 3. Distribution of the expected cases for the 5 most common cancer in Europe in 2012 in females (from Ferlay et al, 2013).

ESTIMATED NEW CASES	
SITE	CASES
Oral cavity & pharynx	25.8
Oesophagus	10.8
Stomach	55.4
Colon and rectum	205.2
Liver	20.6
Gallbladder	17.9
Pancreas	51.8
Larynx	3.9
Lung	119.2
Melanoma of skin	53.1
Breast	463.8
Cervix uteri	58.3
Corpus uteri	98.9
Ovary	65.5
Kidney	43.3
Bladder	32.9
Brain, nervous syste,	26.4
Thyroid	40.7
Hodgkin lymphoma	8.3
Non-Hodgkin lymphoma	43.9
Leukemia	35.9
All sites but non-melanoma skin cancers	1610.5

Table 1. Estimated numbers of new cancer cases (per 100,000) in female, by cancer site in Europe 2012.

1.1.3 Toxicity of cancer therapies

Although cancer incidence rates in women less than 50 years old continue to increase during recent years, mortality rates are decreasing due to modern advances in treatment. In 1990 the prevalence of cancer survivors was 1 in 1,000 for young adults (15-45 years of age). By the years 2010, as many as 1 in 250 patients in this age group will have survived cancer [3]. However, increasing numbers of survivors are now confronted with the long-term consequences of exposure to these treatments. Adverse effects of cancer treatment include disorders of the endocrine system, cardiac and pulmonary dysfunction, renal and hepatic impairment, secondary malignant disease and phychosocial difficulties.

Childhood and adolescence is a period of emotional and psychological instability, during which issues of sexuality, including fertility, are of particular importance. Many treatments for cancer, both in childhood and in later life, can impair future fertility. In fact, surgery, radiotherapy and chemotherapy, can have a profound impact of ovarian function, leading to premature menopause and loss of fertility. Treatment that disrupts fertility in later life can have a devasting effect, both at the time of therapy and whenever the patient wishes to start a family [4]. Therefore the effect of any proposed therapy is important to consider, as well as which strategies exist to protect or restore fertility in later life.

1.1.4 Gonadal toxicity after cancer treatment

Gonadal damage in female patients treated for cancer can result from either systemic chemotherapy or radiotherapy affecting the spinal pelvic area including whole-body irradiation. Fertility can also be impaired as a result of cranial irradiation by disruption to the hypothalamic-pituitary-gonal axis.

The risk of subfertility can be classified according to the type of malignant disease and its associated treatment (Table 2).

Acute ovarian failure can occur during or shortly after completion of irradiation or chemotherapy [5, 6] and may be transient or permanent. In contrast, premature ovarian failure (POF) or premature menopause typically manifests after a post-treatment return of regular menses with subsequent loss of ovarian function before the age of 40 years. As expected, surgical ablation of the ovaries leads to immediate and permanent loss of function.

Low risk (<20%)	**Acute lymphoblastic leukemia** **Wilms' tumor** **Soft-tissue sarcoma: stage I** **Germ-cell tumors** **Retinoblastoma** **Brain tumour: surgery only, cranial irradiation <24Gy**
Medium risk	Acute myeloblastic leukemia Hepatoblastoma Osteosarcoma Ewing's sarcoma: non-metastatic Soft-tissue sarcoma: stage II or III Neuroblastoma Non-Hodgkin lymphoma Hodgkin's disease: alternating treatment Brain tumor: craniospinal radiotherapy, cranial irradiation >24Gy
High Risk (>80%)	Whole-body irradiation Localised radiotherapy: pelvic Chemotherapy conditioning for bone-marrow transplantation Hodgkin's disease: treatment with alkylating-drugs Soft tissue sarcoma: stage IV (metastatic)

Table 2. Best assessment of risk of subfertility after current treatment for common cancers in childhood and adloscence (from Wallace et al., 2005).

Unlike men, women only have a finite number of germ cells at birth, and the total diminishes with age. The human ovary has a fixed number of primodial follicles, at a maximum at 5 months of gestational age. These follicles are progressively lost with increasing age in a biexponential fashion, culminating in menopause at about age of 50 years. The rate of oocyte decline rises at about age 37 years when there about 25000 primordial ooyctes, and precedes the menopause by 12-14 years when roughly 1000 oocytes remain. Younger patients have more oocytes, and thus gonadal damage could seem to be less severe than that in older patients because the ovary can still support regular ovulatory cycles even with small numbers of follicles (Figure 4).

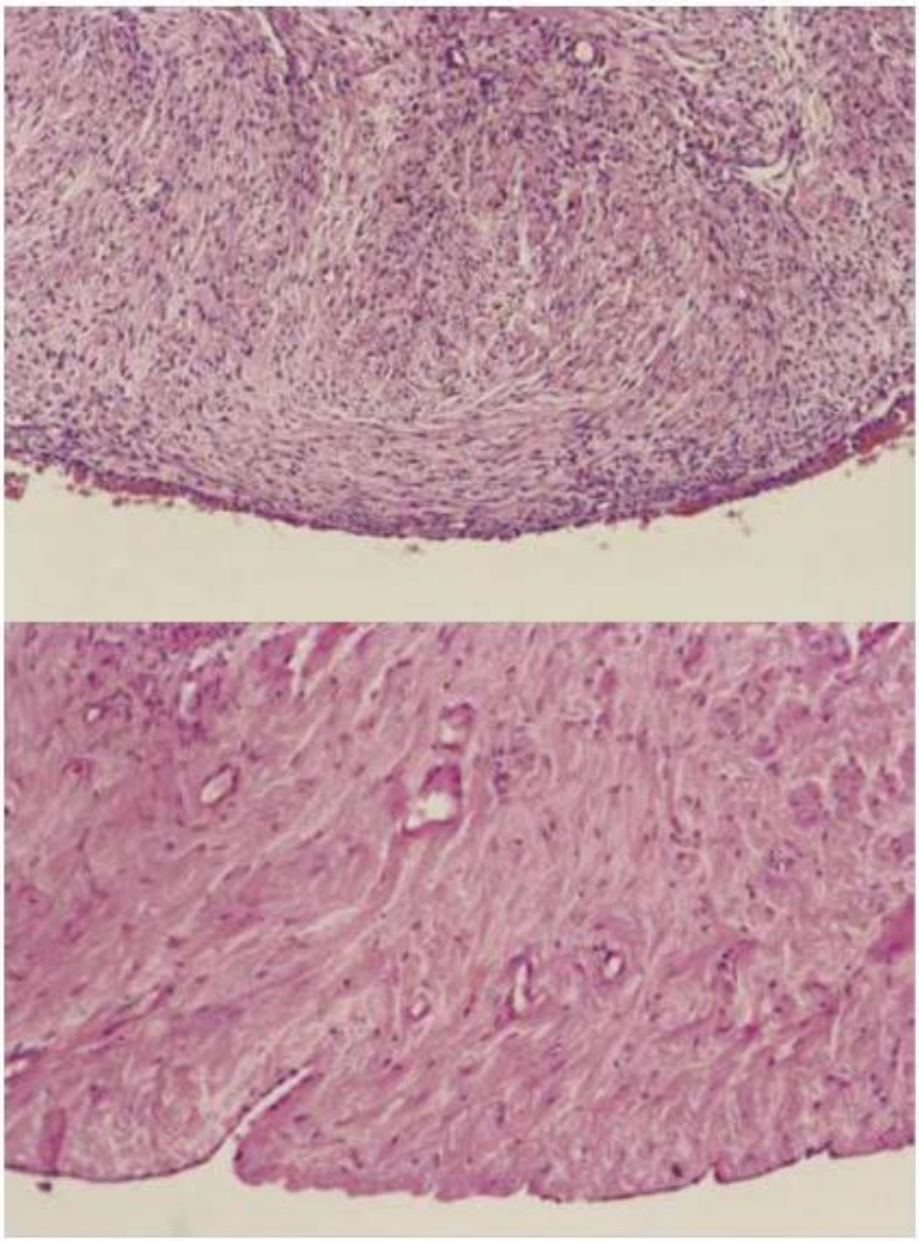


Figure 4 . Ovarian tissue after chemiotherapy. See the absence of primordial follicles, and the tissue fibrosis. Ematossilin-Eosin preparation, 10X magnification (from Lo Presti et al., 2004).

1.1.4.1 Effect of chemotherapy

Chemotherapy has an adverse effect on ovarian tissue. The damage will depend on the agent used [7, 8], the dose [7, 9], and the age of the patient [9-11] (Table 3). Many drugs are gonadotoxic, including procarbazine, cisplatin, and the alkylating drugs such as cyclophosphamide, melphalan, and chlorambucil [12-17] (Table 4).

AGE	POF
<20 years	13%
20-30 years	50%
> 30 years	100%

Table 3. Percentage of premature ovarian failure by age following chemotherapy.

HIGH	MEDIUM	LOW
Cyclophosphamide	Cisplatin	Vincristine
Ifosfamide	Carboplatin	Methotrexate
Chlormethine	Doxorubicin	Dactinomycin
Busulfan		Mercaptopurine
Melphalan		Vinblastine
Procarbazine		
Chlorambucil		

Table 4. Estimated risk of gonadal dysfunction with cytotoxic drugs.

The mechanisms by which the chemotherapeutic agents cause ovarian toxicity vary depending on the specific actions of the drug. The toxic effect by cyclophosphamide seems to be mainly on the granulosa cells and the surrounding basement membrane [18], with the sensitivity being related to the size of the follicle as shown in a study in the mouse model [19]. A decrease in overall ovarian size, as a consequence of a loss of large number of follicles of various stages, including the pool of primordial follicles, is seen in the ovary after chemotherapy [20]. Chemotherapy can also provoke injury to the ovarian blood vessels and focal fibrosis of the ovarian cortex [21]. A histological study on human ovarian biopsies from women that had received chemotherapy demonstrated that the number of primordial follicle count was decreased in the group of chemotherapy-treated patients, with the lowest follicle count found in patients that had been treated with alkylating agents, such as cyclophosphamide [22]. It was also shown that there was a considerable fall in anti-Müllerian hormone (AMH) concentrations as well as partial decrease of inhibin B during the anti- cancer treatment, indicating damage of small and preantral follicles [23]. Moreover, combinations of chemotherapeutic drugs, regardless of whether they include alkylating agent, can also have detrimental effects of the ovary and its follicles.

A recent large study of post-treatment parenthood in patients that had been treated for Hodgkin's lymphoma [24] demonstrated that the 10-year probabilities of achieving parenthood in Hodgkin's

lymphoma patients were 55% after treatment with chemotherapy of low gonadotoxicity, 51% after use of chemotherapy considered to be of medium gonadotoxicity and 27% after use of chemotherapy regarded as high gonadotoxic treatment [24].

It has been demonstrated in studies in female mice exposed to cyclophosphamide that even if pregnancy is accomplished, the rate of spontaneous abortion and fetal malformations may be substantially increased [19]. In contrast, available human large registry studies have revealed that there is no higher risk of genetic abnormality, birth defects, or cancer (aside from hereditary syndromes) in the children of cancer survivors that had been treated with chemotherapy [25]. One retrospective study conducted on childhood cancer survivors treated by chemotherapy showed no significant differences in neonatal outcome between cancer survivors and the control group [26].

1.1.4.2 Effect of radiotherapy

Radiation therapy can disrupt the functioning of the hypothalamic-pituitary axis [27] or directly cause ovarian failure [6, 28, 29] (Figure 5). The damage will depend on the field of treatment, total dose, fractionation schedule and age at the time of treatment [6, 30]. Cranial irradiation may damage the central nervous system, including the hypothalamic-pituitary axis leading to precious puberty and gonadotropin deficiency. Abdominal, pelvic and spinal

irradiation may directly affect the gonads, leading to infertility and impaired sex steroid production [31].

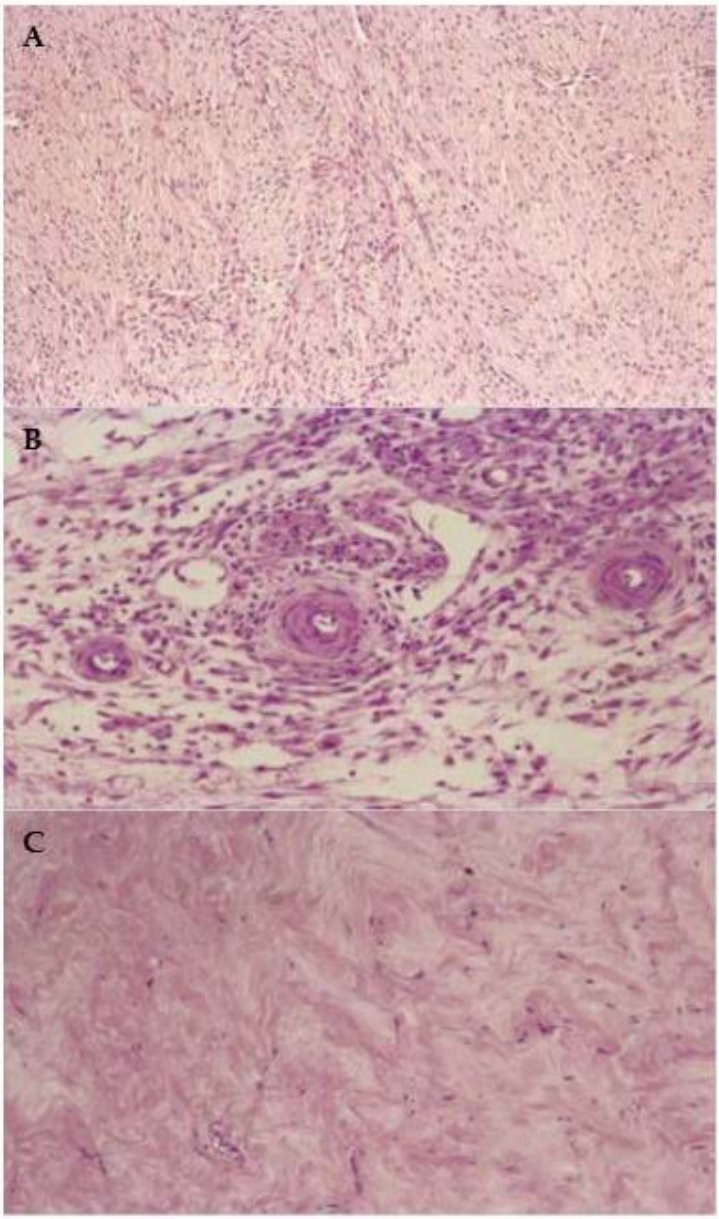


Figure 5. Ovarian tissue after radiotherapy. Absence of primordial follicles and tissue fibrousis (A); damage at oocyte structure (B); total damage of stromal and follicles structure (C). Ematossilin-Eosin preparation, 10X magnification (from Lo Presti et al., 2004).

Disruption of the hypothalamic-pituitay-ovarian axis can lead to amenorrhea and infertility. Radiation-induced damage is possible within the hypothalamus, pituitary gland, or both, and can lead to dysregulation of the hormonal milieu responsible for regulation of menstruation and fertility. This hormonal environment is predominantly balanced by the secretion of gonadotropin-release hormone (GnRH), follicle-stimulating hormone (FSH), luteinize hormone (LH), estradiol, progesterone, and prolactin.

Ionizing radiation can also cause direct DNA damage to ovarian follicles, leading to follicular atrophy and decreased ovarian follicular reserve, as shown by the Faddy-Gosden model (Figure 6). This can hasten the natural decline of follicles numbers, leading to impaired ovarian hormone production, uterine dysfunction due to inadequate estrogen exposure, and early menopause. Also the radiosensitivity of oocyte is thought to vary during the growth phase. In fact, the susceptibility to radiation-induced cell death depends on the developmental stage of the germ cell at the time of exposure. The oogonia in prenatal life that are undergoing mitosis are highly susceptible to radiation induced cell death. As the cells pass through the stages of meiotic division, resistance to radiation damage generally increases. Although the radiosensitivity of the oocyte varies during the growth phase , primordial follicles are thought to be more resistant than maturing follicles [31].

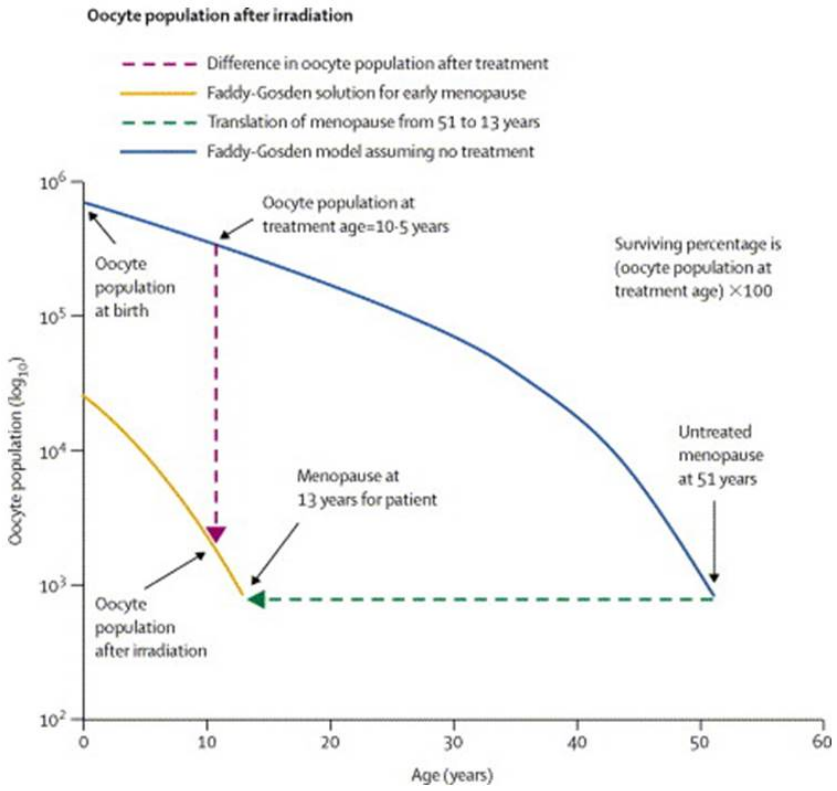


Figure 6. Faddy-Gosden model. The solution of the Faddy–Gosden equation enables the size of the oocyte pool to be determined for any given age from birth to menopause, at an estimated age of 51 years.Graph shows the calculation of an estimated surviving fraction for a patient treated at age 10•5 years with whole-body irradiation (14•4 Gy) who developed ovarian failure at age 13 years, which was 0•56% (from Wallace et al., 2005).

A recent mathematical model employed by Wallace et al. suggested that the dose required to destroy 50% of the immature oocytes (LD50) is less than 2Gy [29]. Therefore, the authors demonstrated that it is possible to predict the age at which ovarian failure is likely to develop after radiation to a field that includes the ovary in women

treated for cancer. They have shown that the effective sterilizing dose (ESD), or dose of fractionated radiotherapy [Gy] at which premature ovarian failure takes place immediately after treatment in 97.5% of patients, falls with increasing age at treatment. The estimated ESD at birth was 20.3 Gy; at 10 years, 18.4 Gy; at 20 years, 16.5 Gy, and at 30 years, 14.3 Gy (Figure 7). The wide individual variability in ovarian follicular reserve at time of treatment can explain differences in onset of premature ovarian failure between patients treated at similar ages.

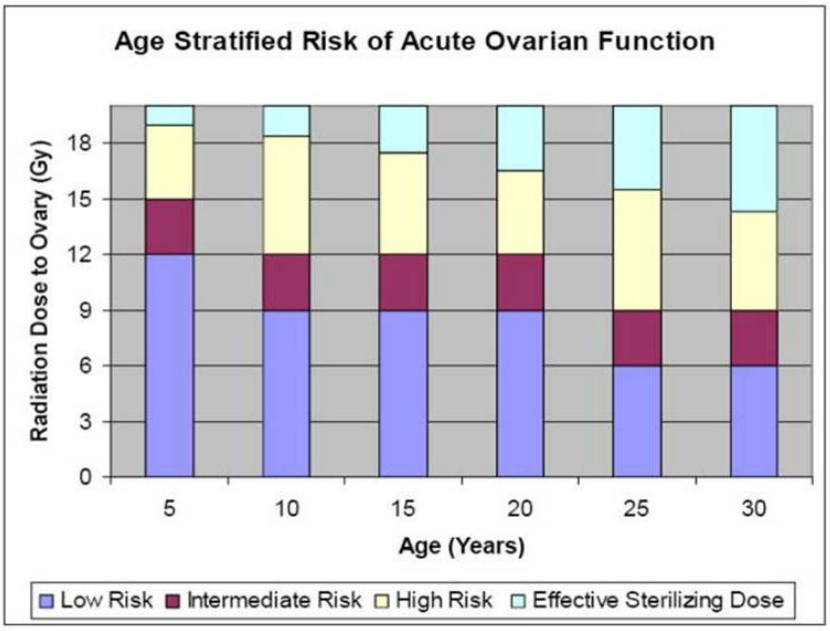


Figure 7. Risk of developing acute ovarian failure, defined as ovarian failure whitin 5 years, stratified by age and radiation dose to the ovary (from Wo et al., 2009).

1.1.5 Current fertility preservation options

A diagnosis of cancer is a life crisis for any person. Its impact varies with the type of cancer; treatment prospects; and the physical, emotional, and social resources of the patient. Younger persons face the additional potential loss of reproductive function and the opportunity to have children. Surveys of cancer patients reveal a very strong desire to be informed of available options for fertility preservation and future reproduction [32]. At the same time that patients (and their parents in cases of minors) receive a diagnosis of cancer, they must also consider possible effects on fertility. The options of fertility preservation in women are more than in men , but all are more intrusive, depend on the patient's age, type of treatment, diagnosis, whether she has a partner, the time available and the potential that cancer has metastasized to her ovaries [33]. Established fertility preservation methods recognized at this time for female patients include shielding to reduce radiation damage to reproductive organs, fertility-sparing surgery and embryo cryopreservation following ovarian stimulation with gonadotropins and *In Vitro* Fertilization (IVF) (Figure 8).

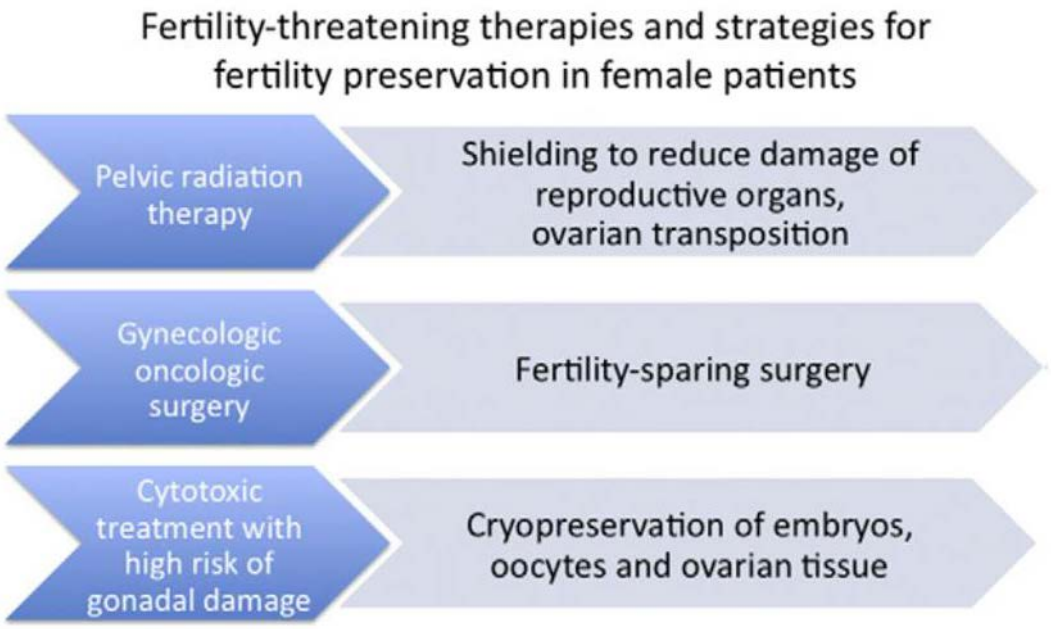


Figure 8. Strategies for fertility preservation in female patients (from Rodriguez-Walberg 2013).

All remaining options are still considered experimental. There have been technical improvements in methods for cryopreservation of oocytes after ovarian stimulation in the recent years resulting in an increasing number of pregnancies and children born worldwide after fertilization of frozen-thawed oocytes, however, overall pregnancy rates are still relatively lower than those with embryo freezing [34, 35]. There is also the option of retrieving immature oocytes without stimulation aiming at maturing them later *in vitro*, which has been developed in few centers worldwide [36]. The remaining option of freezing gonadal tissue is typically offered when there is insufficient time for ovarian stimulation and it is the

only option than can be offered to pre-pubertal girls. The ovarian tissue of whole ovary are usually retrieved through laparoscopy and it is aimed for future re-transplantation or for *in vitro* follicle culture, both techniques still in development (Figure 9).

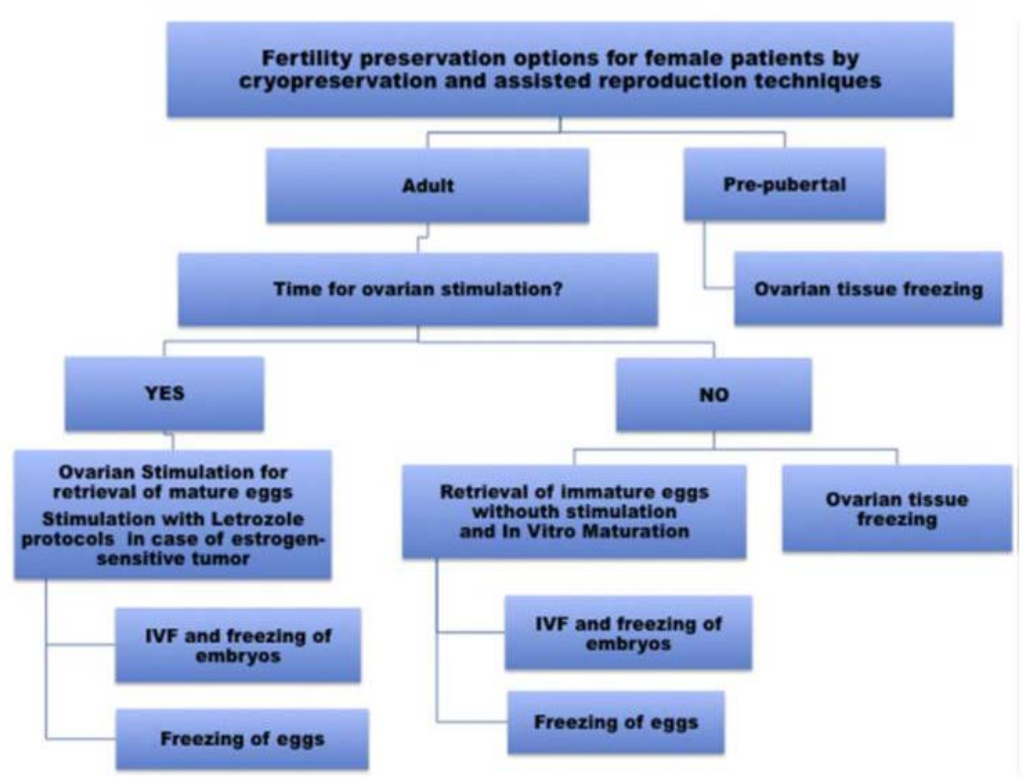


Figure 9. Current options for fertility preservation by cryopreservation and assisted reproductive techniques in adult and in prepubertal females (from Rodriguez-Walberg 2013).

1.1.5.1 Ovarian transposition

Shielding to reduce radiation to the reproductive organs when possible, is the standard medical procedure currently offered to female patients. When shielding of the gonadal area is not possible, the surgical procedure that moves the ovaries out of the radiation field, known as oophoropexy or ovarian transposition, may be considered. This procedure was initially described by laparotomy but it may be also carried out laparoscopically [37-40]. Briefly, the ovary and fallopian tube are dissected from the uterus, mobilized out of the pelvis, and ligated to the peritoneum as high and laterally as possible (Figure 10) [41].

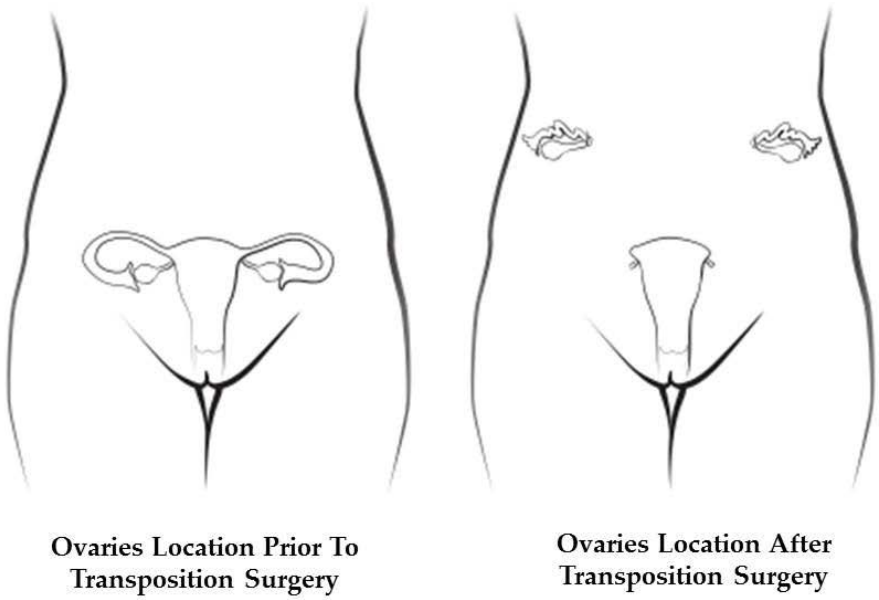


Figure 10. Ovarian transposition procedure

The most common indications for this procedure have been gynecological cancers such as cervical and vaginal cancer that include pelvic irradiation without oophorectomy in their treatment protocols. Pelvic irradiation may also be indicated in the treatment of pelvic and abdominal Hodgkin's disease or sarcomas and ovarian transposition may be a fertility preserving option in selected cases. There is a great variation in the reduction of risk of ovarian failure by ovarian transposition (16–90%) and about 50% of those patients may retain some menstrual function and fertility. Failure of this procedure is related to scattered radiation and damage of the blood vessels that supply the ovaries [25].

1.1.5.2 Hormonal Protection by Ovarian Activity Suppression

Ovarian protection from gonadotoxic treatment with GnRH agonists is still controversial. The idea of maintaining the ovarian metabolism quiescent to avoid any damage caused by oncologic treatment is still to be confirmed, and there are doubts about whether GnRH agonist administration has a potential beneficial effect on ovarian tissue because of the lack of FSH receptors on primordial follicles. The observation was made that prepubertal girls had lower rates of ovarian failure after chemotherapy and radiation therapy than post-pubertal patients. With this in mind, some postulate that continuous GnRH exposure leads to downregulation of the pituitary and induction of a prepubertal state. This ovarian quiescence during

cancer treatment might decrease susceptibility to gonadotoxic treatments. While some data in monkeys [42, 43] and in small, non-randomized clinical studies [44, 45] show benefit with GnRH therapy, one prospective randomized trial [46] showed no benefit. Primordial follicles in humans do not have follicle-stimulating hormone (FSH) receptors, so suppression of FSH with a GnRH agonist theoretically would not be protective. Younger patients may be less susceptible to chemotherapy because they have a greater number of oocytes, not because their ovaries are quiescent during chemotherapy.

1.1.5.3 Embryo cryopreservation

Embryo cryopreservation is currently the only highly successful method of preserving fertility for female cancer patients but this option requires the patient to be of pubertal age, have a partner or use donor sperm, and be able to undergo a cycle of ovarian stimulation, which is not possible when the chemotherapy has to be initiated immediately or when stimulation is contraindicated according to the type of cancer. This strategy requires that the patient undergos ovarian stimulation for the *in vivo* maturation of oocytes and subsequent retrieval of mature oocytes prior to initiation of cancer therapy. The oocytes are fertilized on the day of egg retrieval and the resultant embryos are cryopreserved. At the time of the patient's choosing, embryos can be thawed and transferred into

either the patient's own uterus, providing that her uterus is viable for pregnancy, or that of another woman (gestational surrogate). The basic technologies necessary for embryo freezing are in clinical use throughout the world on a daily basis and reported survival rates per thawed embryo range from 35% to 90%, implantation rates from 8% to 30%, and cumulative pregnancy rates of 30-40% [47, 48]. However, this procedure may not be an option for women with highly aggressive malignancies, including leukemia, some lymphomas, and sarcomas, which warrant immediate cancer treatment [49]. Successful ovarian stimulation requires several weeks to months depending on the response of the patient to the stimulation protocol, which may compromise the patient's long-term outcome. Many cancer patients do not respond well to the standard IVF stimulation protocols, which may result in decrease quality and/or quantity of oocyte collected during the cycle [50, 51]. In addition, ovarian stimulation is associated with high-serum estrogen concentrations. In fact, during conventional gonadotropin-stimulated ovulation induction for IVF, it is hoped that a minimum of around 4, and ideally about 10–15, dominant follicles develop. Generally, the actual number of mature follicles attainable decreases with declining ovarian reserve. During the process of ovulation induction, the development of multiple dominant follicles may give rise to substantial increases in ovarian estradiol production due to increased granulosa cell number. In these situations, circulating

estradiol concentrations may exceed 3,000 pg/ml . This is substantially greater than that of a natural, unstimulated ovulatory cycle with peak estradiol levels of about 300 pg/ml. This can be a concern for women with estrogen-dependent cancers such as certain breast cancers and benign diseases such as endometriosis [52]. A strategy to successfully induce ovulation for IVF in these women without producing high estradiol levels has been described [53, 54]. It involves adding an aromatase inhibitor to the usual gonadotropin-based ovarian stimulation protocol. Aromatase inhibitors prevent the formation of estrogen from androgen precursors and resultant serum estradiol levels are substantially reduced compared with conventional IVF stimulation and can actually be less than that seen in a natural cycle. Finally, ovarian stimulation is often not considered ethically appropriate for prepubertal females, and the requirement for a partner or sperm donor makes embryo cryopreservation undesirable for single women who prefer not to use sperm donor.

1.1.5.4 Oocyte cryopreservation

Oocyte cryopreservation is an alternative option for patients with the same characteristics as those described above for embryo cryopreservation but who are not with a partner and do not wish to use donated sperm. In this case, IVF of their oocytes to produce embryos to be frozen for future implantation is not possible. Thus,

the oocytes themselves must be cryopreserved either as mature or as immature oocytes. Human oocyte cryopreservation has been rapidly incorporated into clinical practice in several centers [55-57].

Mature oocyte freezing appears, at least in theory, to be the most logical way of storing female germ cells, comparable to the routinely performed sperm banking. It is an attractive option for women without a partner, if they have time to complete ovarian stimulation before cancer therapy. However, ovarian stimulation and oocyte collection are not applicable for children [53, 58, 59].

The first live birth with mature oocyte cryopreservation was reported in 1986 [60], but due to very low success rates (<2%) there were only five live births reported using this technique for over a decade [34]. Oktay et al., hypothesized that this low rate was derived from the fact that the metaphase II (MII) oocyte is a large and highly specialized cell that is extremely fragile [34] (Figure 23).

In 1997, intracytoplasmic sperm injection (ICSI) was first used to fertilize frozen-thawed oocytes [55], circumventing zona pellucida hardening caused by the premature exocytosis of the cortical fragments induced by cryopreservation process.

Although micromanipulation techniques helped improve fertilization of cryopreserved oocytes, further optimization of oocyte cryopreservation required another decade. In 1999, the first live birth with oocyte cryopreservation after vitrification was reported [61] followed by only a few case reports and clinical studies up until 2005

[34]. At that time, there were approximately 100 reported live births from oocyte cryopreservation; these were reviewed in a meta-analysis [34] which concluded that success rates with oocyte cryopreservation using slow freezing were lower than that of IVF with fresh oocytes.

A comparison between slow freezing and vitrification on oocyte freezing showed that that vitrification was more successful in terms of both embryological and clinical outcome [62] and although the efficiency of slow-freezing protocols has also been improved [63-69], the reported success rates remained lower compared with vitrification.

With the improvements in oocyte cryopreservation technology and associated clinical outcomes, its clinical applications widened, resulting in more than a thousand live births reported to date [70, 71]. Over the past 5 years, oocyte cryopreservation, especially with vitrification, has proven to be an efficient technique, resulting in pregnancy outcomes similar to that of IVF with fresh oocytes. As a results of this progress, the American Society of Reproductive Medicine has recently stated that mature oocyte cryopreservation should no longer be considered experimental for medical indications, outlying elective oocyte cryopreservation.

To circumventing the problem of hormonal stimulation in sensitive cancer and spindle damage in mature oocytes, cryopreservation of immature oocytes has been undertaken. Oocytes at the diplotene

stage of prophase I, or germinal vesicle (GV) stage, survive the cryopreservation procedure better than those frozen at the MII stage [72]. These cells have reached full size and complete meiotic competence but have not yet resumed their maturation process and initiated their second metaphase. Although the risk of hardening of the zona pellucida or damage to the cytoskeleton cannot be avoided, it is probable that the absence of a meiotic spindle and the presence of a nuclear membrane protecting the chromatin guarantees the absence of cytogenetic anomalies during further cellular divisions (Figure 11).

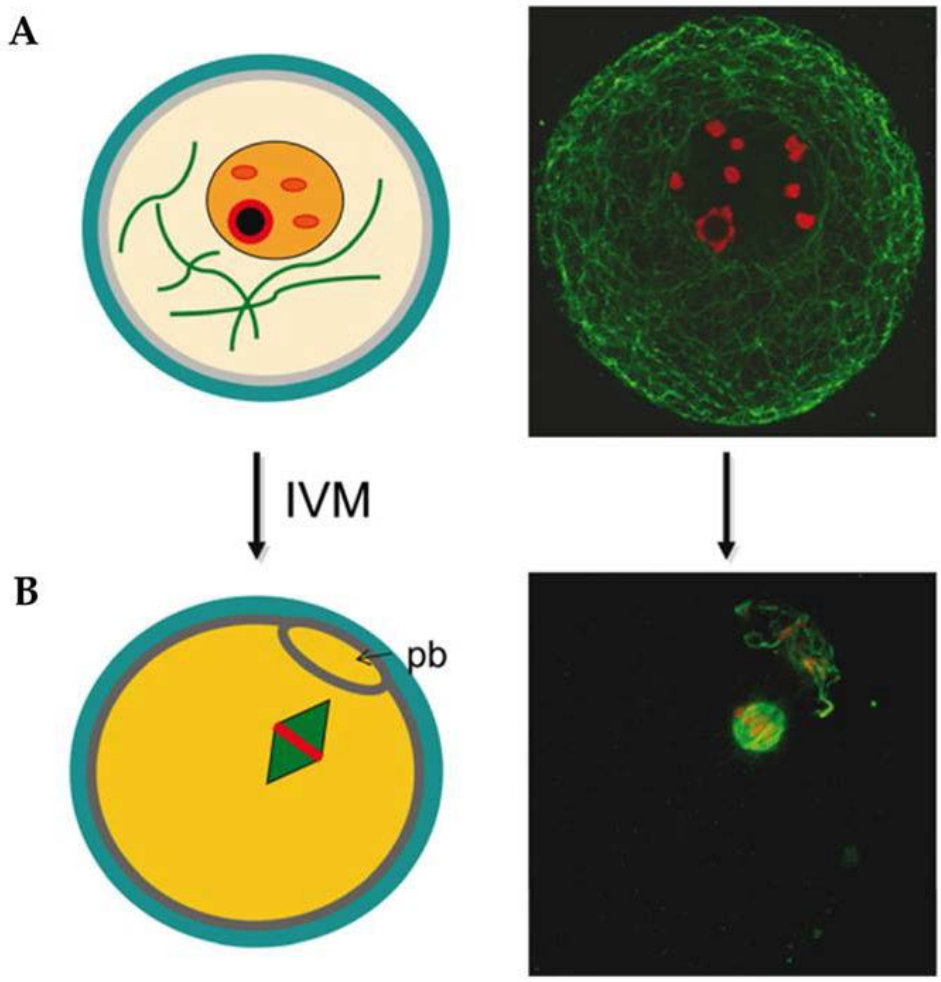


Figure 11. Germinal vesicle (A) and metaphase-II (B) human oocytes vary in critical cellular features, and thus perhaps in their ability to withstand cryoinjuries. At the germinal vesicle stage (prophase-I), oocytes have an intact nucleus with partially condensed chromatin and microtubules characteristic of interphase; in contrast, metaphase-II oocytes contain a meiotic spindle with dynamic microtubules and condensed chromosomes (IVM: *in vitro* maturation; pb: polar body) from Combelles et al., 2012).

The immature oocyte may be used in one of two ways: 1) the GV stage oocyte undergoes *in vitro* maturation (IVM) and is then frozen/cooled or 2) the GV stage oocyte is cryopreserved and upon thawing/warming it undergoes IVM (Figure 12).

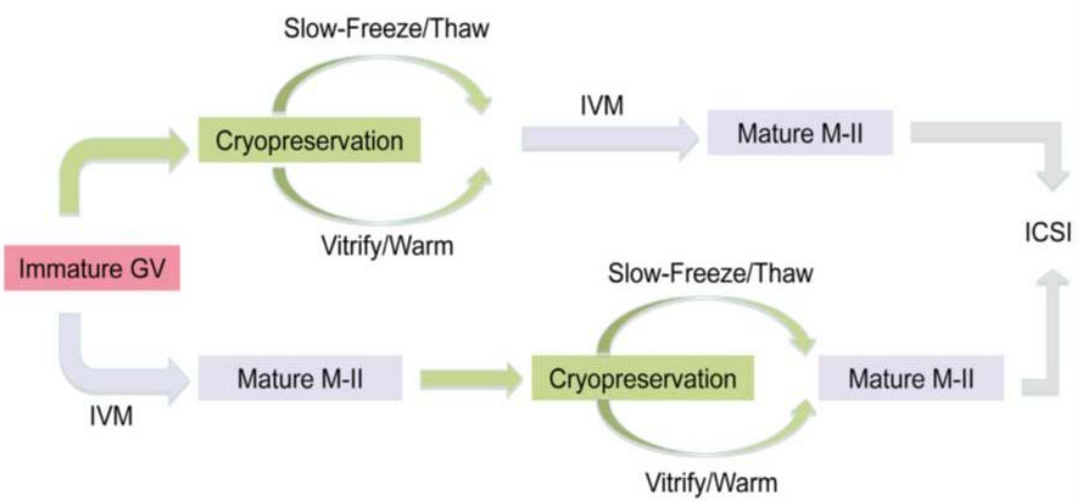


Figure 12. Diagram depicting how immature oocytes may be used for fertility preservation and restoration. Immature germinal vesicle (GV) stage oocytes can either be *in vitro* matured prior to cryopreservation or cryopreserved before *in vitro* maturation (IVM) to metaphase-II (M-II), followed by fertilization with intracytoplasmic sperm injection (ICSI) (from Combelles et al., 2012).

Freezing immature oocytes followed by *in vitro* maturation thus offers practical and theoretical advantages [73], but this method is still suboptimal. Frozen–thawed immature oocytes have to follow a process of *in vitro* maturation before they are ready to be fertilized.

Oocyte maturation is considered as the reinitiation and completion of the first meiotic division from the GV stage to the MII stage, and the accompanying cytoplasmic maturation phase for fertilization and early embryonic development [74]. The coordination of nuclear and cytoplasmic maturation *in vitro* has proved very difficult to achieve. Although there are several reports of pregnancies achieved after *in vitro* maturation of fresh GV-stage oocytes [75, 76], only one live birth has resulted from an immature oocyte cryopreserved at the GV stage, with subsequent *in vitro* maturation [77]. In conclusion, the cryopreservation of GV stage oocytes will not be a practicable strategy until *in vitro* maturation of these oocytes becomes more reliable [78].

1.1.5.5 Ovarian tissue banking

Ovarian tissue banking is the only viable option in prepubertal girls because sexual maturity is not required. As this procedure does not cause any significant delay to initiation of cancer treatment and it does not require ovarian stimulation [58, 79-82], some adult patients also prefer to preserve fertility by this method. In adults, however, this option is mainly indicated when there is a lack of time available for ovarian stimulation and whenever a hormonal stimulation treatment is contraindicated by medical reasons. Ovarian tissue banking has several theoretical advantages over other fertility-preserving strategies: 1) ovarian tissue may contain hundreds of

oocytes [83]. Thus, cryopreservation of ovarian tissue is a potentially more efficient method of storing reproductive potential; 2) unlike collection of oocytes and production of embryos, which require time-consuming hormonal stimulation, oophorectomy does not delay cancer treatment; 3) primordial follicles consist of immature oocytes surrounded by a single layer of flattened pre-granulosa cells. These oocytes are much smaller, metabolically less active, and are not arrested at a stage where the spindle is present. All of these characteristics may make them better suited for cryopreservation than mature metaphase II oocytes; 4) the immature oocytes within the ovarian tissue would be matured much later in life, thereby obviating the need for exogenous gonadotropin stimulation. Thus, ovarian tissue banking is appropriate for prepubertal girls.

Ovarian tissue banking has its disadvantages as well: 1) surgery is required to obtain the ovarian tissue; 2) ovarian tissue is theoretically difficult to freeze because of its heterogeneity. Each cell type that comprises ovarian tissue (oocytes, granulosa cells, interstitial cells) has unique biological characteristics that require different freezing protocols; 3) oocytes within ovarian tissue are immature, and require maturation before fertilization can occur. Follicles within ovarian tissue are arrested in early meiosis and cannot be fertilized.

1.2 CRYOBIOLOGY

1.2.1 Hystorical background

Cryopreservation originates from the Greek word “kryos”, which means “cold or frost”, it indicates storage of cells or tissue, usually in liquid nitrogen, at temperatures below -130°C. The lowest natural temperature on earth is -80°C. Under normal pressure, the inert gas nitrogen which is commonly used in cryopreservation becomes a liquid at -196°C.

A small number of species like various fish, frogs and insects can survive at low temperature using two different biological principles or a combination of these. The first process is cell dehydration, which reduces the freezing point secondary to a concentration of solutes. The other mechanism is by endogenous production of special antifreeze molecules, like sugars, which prevent formation of large ice crystal. However, recent studies provide the first demonstration that freeze tolerance can also occur amongst vertebrate species. A freeze tolerance vertebrate would make an excellent model system for studies of the medical cryopreservation of tissues and organs.

The main goal of the cryopreservation procedure is to minimize tissue injury from low, subzero temperatures [84]. The storage at a low temperature can continue for decades and the only theoretical limitation of storage time is influence by cosmic radiation, which

over several thousand years would degrade the genome of the cryopreserved cells. This factor can be neglected in any practical work of cryopreservation in our society. In other words, by usage of cryopreservation the biological clock can be halted for an unlimited time [85].

The term cryobiology refers to the knowledge and understanding of the effects of low temperature on cellular system and the utilization of this information to develop improved cryopreservation protocols. The science of cryobiology can be considered to have its starting point about 70 years ago [86]. At that time, Luyet tried to achieve cryopreservation by cooling epidermal plant cells quickly and published a monograph about his pioneer work in 1940 (Luyet, 1940). Three years later a scientist from England succeeded in cryopreservation of human and fowl spermatozoa, using glycerol as the agent that would protect against freezing injury (Polge et al. 1949).

The studies of cells and organ freezing have increased considerably since then and in particular in the clinical area of reproductive medicine [87]. The cryopreservation technique may also expand into transplantation surgery. The possibility of organ storage and future transplantation was early also recognized by the pioneering liver transplantation surgeon Thomas Starzl (Starzl 1970) and it may well be that the technique of cryoperservation may be routinely used in

the future to store organs that are retrieved and where a suitable recipient cannot be found at that moment.

1.2.2 Fundamental cryobiology

The cells are constituted of mostly water, which makes up 60-85% of the cell volume [88]. In addition to free water, biological systems also contain “bound “water molecules. The “bound“ water molecules are intimately hydrogen-bonded to the atoms within molecules are such as proteins, RNA, DNA or membrane phospholipids head groups. These water molecules form a substantial coat around the biological molecule and are essential to maintain cells structure and function [84] (Figure 13). The “bound” water molecules are incapable of freezing [89]. Thus, water plays an important role in cryobiology since at cooling to low subzero temperature, 90% of water in a cell will convert into ice [88] and the 10% of bound water will not freeze.

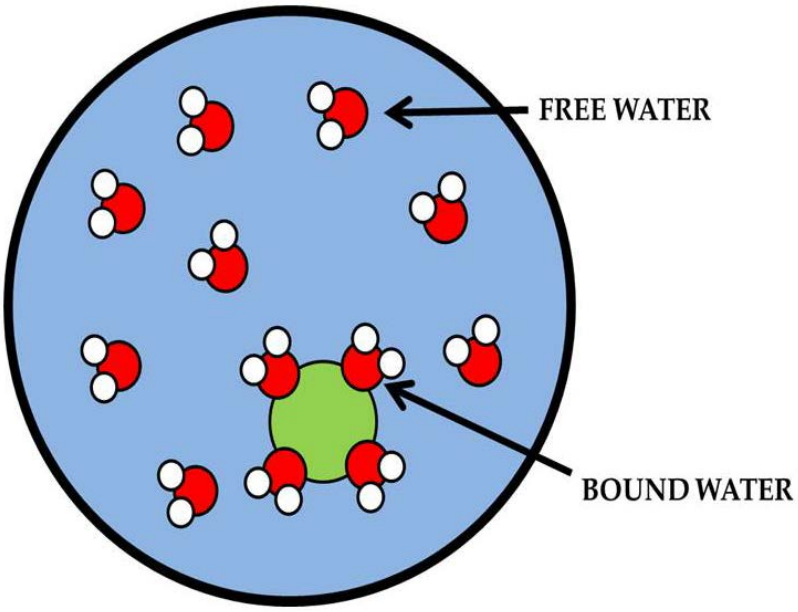


Figure 13. Figure illustrates how water molecules can be either free or bound to larger molecules of the cell.

Cell is in osmotic equilibrium, which means that concentration of any solution inside and outside the cell are the same, since the cell membrane is semipermeable. In the other words, water moves in or out of the cell depending on changes in solution concentration outside the cell and permeability of the cell membrane (Figure 14), which vary for each specific cell type.

In addition to passive diffusion through the membrane lipid bilayer [90], water moves through water transport pores known as aquaporins [91]. These active transport proteins can transport water up to 100 fold more efficient than passive diffusion. Also, permeability is temperature dependents [92], so that permeability is higher with increasing temperature.

Osmotic stress is defined as shrinkage and swelling of a cell due to osmotic differences between the inside and outside of the cell. When these changes are large it may lead to major damage of the cell and in the worst scenario to cell death.

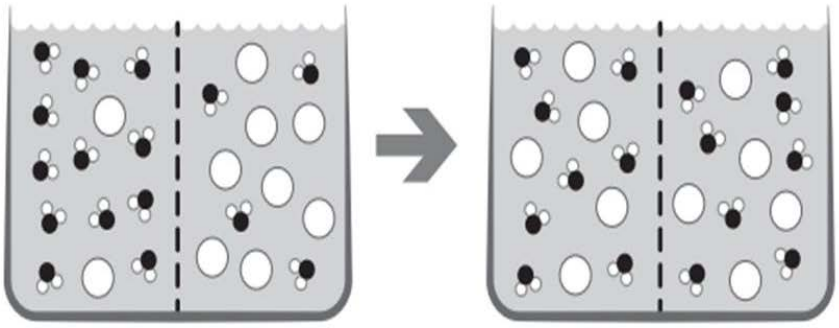


Figure 14. The figure illustrates the principles of osmotic equilibration. Water molecules move from compartment of lower concentration to a compartment of higher concentration, to equalize the concentrations.

In pure water, the highest temperature at which ice can form at normal pressure is 0°C, but ice nuclei form only very reluctantly at this temperature, with the result that nuclei do not usually form until the temperature falls below 0°C. In fact, ice in general forms at temperatures between -5 and -15°C; through spontaneous or induced (by seeding) ice nucleation. Once an ice nucleus has formed, further water molecules can very rapidly bond onto this frozen surface, allowing it to grow in size.

When the ice forms there is a release of heat (the latent heat of fusion or crystallization), as the change from liquid to crystal releases

energy. The amount of energy released may be considerable and commonly brings the temperature of the whole sample back up to 0°C. With pure water the temperature will remain at 0°C until the freezable water has formed ice, after which it will re-equilibrate with the ambient temperature. Following the crystallization of pure water, very little remains unfrozen even at temperatures as high as ±5°C (>80% ice) or ±10°C (>90% ice) state [84].

To thaw all this ice the temperature has to rise above the freezing point. Pure water therefore melts at 0°C, the same as the highest temperature at which ice crystals can first form. During the thawing process, the change from crystal to liquid requires energy energy input. The solution which remain free on ice in a temperature below freezing point is in a supercooled state [84].

It is well established that cell survival rate during cryopreservation depends mostly on the cooling rate [93]. In extensive studies of survival of yeast cells and human red blood cells, as a function of cooling rate, it was showed that a curve of survival versus cooling rate exhibited the shape of an inverted U (Figure 15). This curve can be interpreted as the resultant of two different mechanisms of which one damages living cells at high cooling rates and the other damages at low cooling rates. If the rate is too fast or too slow, the cells do not survive and balancing these two cryoinjuries is the key to success [93].

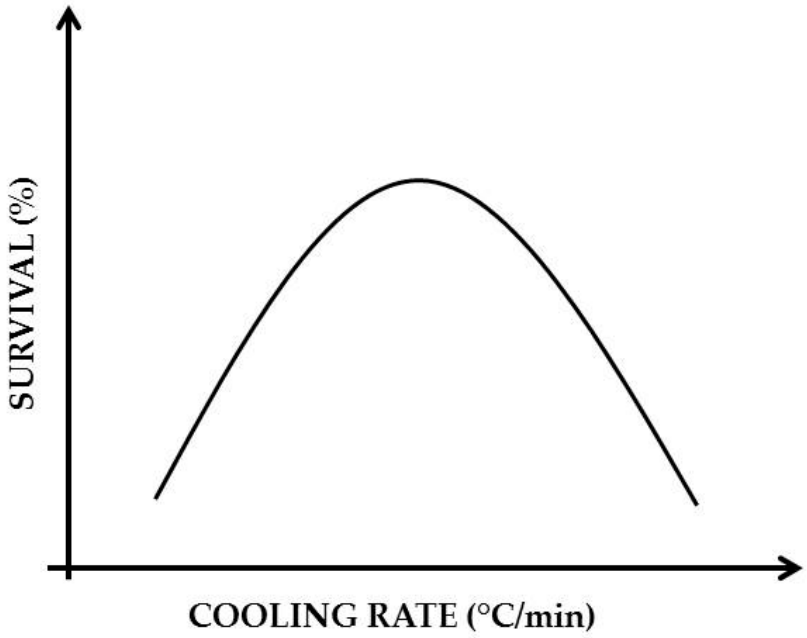


Figure 15. The U shape. The survival of frozen cells is a function of cooling rate

1.2.3 Cryoprotectant solutions

Adding salts and other solutes to pure water lowers the temperature which ice forms/melts. There is no limit to how low the freezing/melting point can be depressed by solutes, with the result that solutions with very high concentrations may never form ice, rather they will solidify into an ice-free (vitreous) state. Even when salts and solutes are present, they rarely become incorporated into the ice crystals themselves, because of the very specifc shape and bonding requirements of the growing ice crystals. Molecules other than water molecules are instead excluded from the growing ice

crystals, and tend to accumulate between crystals or are physically pushed ahead of an advancing ice front. The formation and growth of ice crystals can, however, be modifed by compounds such as antifreeze proteins [94], ice blocking agents [95] and cryoprotectants.

Many chemicals have been recognized as having a cryoprotective function and these are known as "cryoprotecting agents" (CPAs). During the freezing process, CPAs can serve several functions including lowering the freezing point, binding water to prevent it freezing at zero degree and decreasing membrane damages [96]. According to their ability to transport through cell membrane, CPAs can be divided into two categories, permeable and non-permeable. Permeable CPAs can diffuse trough cell membrane and non-permeable CPAs do not enter the cytoplasm.

Permeable CPAs are small, non-ionic molecules with low toxicity and high solubility in water. The rate of CPA permeation and dilution is determined by the species, cell type and stage of development, solution composition, temperature and hydrostatic pressure [84, 97]. The most commonly used permeable CPAs are DMSO, propylene glycol (PROH) and ethylene glycol (EG). The exact mechanism by which these permeable CPAs protect living cells from cryoinjury is not completely understood. However, their general mechanisms seem to be by lowering the freezing point by replacement of some of the bound water molecules in and around proteins, deoxyribonucleic acid and head groups of phospholipids

[84]. Moreover, the permeable CPAs stabilize cellular proteins in the cytoplasm as well as in the cell membrane [98]. At entrance into the cell, they also reduce the concentration of electrolytes by lowering the amount of ice formed at a given temperature [99].

Non-permeable CPAs are usually long-chain polymers that are soluble in water and increase the osmolality of the solution. The most frequently used non-permeable CPAs are disaccharides (sucrose, glucose, fructose, sorbitol, saccharose, trehalose), some macromolecules (polyvinilpyrrolidone, polyvinyl alcohol, Ficoll) and proteins (bovine serum albumin; BSA). They contribute to cell dehydration, counteract osmotic stress and reduce the toxicity of permeable CPAs [100].

1.2.4 Cryopreservation techniques

There are two principally different cryopreservation procedures: vitrification and slow freezing. Recently, a new directional freezing technology was introduced [101, 102] which provides identical cooling rates through the specimen being frozen and it is aimed to cryopreserve whole organs. All three procedures include four common steps: exposure of the samples to CPA, freezing/cooling to the storage temperature (-196°C), thawing/warming and CPA removal. The terms "freezing and thawing" relate to the slow and directional freezing procedures whereas "cooling and warming" are more correct in relation to the vitrification procedure.

1.2.4.1 Vitrification

The term vitrification originated from the Greek word “vitri”, which means “glass”. Vitrification is a procedure in which solution/specimen solidifies to form a glasslike, or vitreous, state without any ice crystal formation during cooling and remains in this state throughout the warming step [84].

The main idea of ultrarapid cooling is to pass rapidly through the critical temperature zone where the cells are most sensitive for chilling injury [97]. To achieve this glass-like solidification, high cooling rates are used in combination with high concentrations of CPA that interact strongly with water molecules to prevent ice formation , are used. In general, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are inversely related. Typically, the temperature is reduced directly from 0°C to -130°C by plunging the sample into LN. Cooling rate at vitrification varies in the range of 2,500 to 30,000°C/min or greater. However, one has to take into account that every cell seems to require its own optimal cooling rate. Also, the natural state of liquid water inside living cells is retained during vitrification [103], leading to minimal disturbance within the cryopreserved sample. A sample that will be vitrified is exposed to CPA at the same manner as during slow freezing, but the vitrification procedure requires higher concentration of CPAs to achieve high cooling rate. Warming after vitrification is usually also fast. A major potential drawback of

vitrification is the use of high concentration of cryoprotectant, and an unintentional negative impact of these cryoprotectants in turn can be their toxicity, which may affect the cell or tissue.

Vitrification has been recommended as the method of choice for oocytes or embryos. All developmental stages of human embryos cultured *in vitro* have been successfully vitrified and warmed, with resulting offspring. In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported [61]. Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in assisted reproductive technology (ART), oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice crystallization using vitrification.

Vitrification is very simple, requires no expensive programmable freezing equipment, and relies especially on the placement of the embryo in a very small volume of vitrification medium (refered also as "minimal volume approach") that must be cooled at extreme rates not obtainable in traditional enclosed cryo-storage devices such as straws and vials. The importance of the use of a small volume, also referred to "minimal volume approach" was described and published in 2005 [85, 104]. In addition, recent publications have shown the dominance of warming rate over cooling rates in the survival of oocytes subjected to a vitrification procedure [105, 106]. Vitrification has also been proposed for cryopreserve whole organs.

However, there is evidence that a large tissue or whole organ will require slow cooling rates [107]. In fact, fractures of the organ could be caused by vitrification and warming procedures, and devitrification if the storage temperature is above the glass transition temperature [108]. The problem is eliminating or sufficiently limiting ice formation throughout the large organs without inducing unacceptable toxicity caused by high doses of CPA [108, 109] (Figure 16).

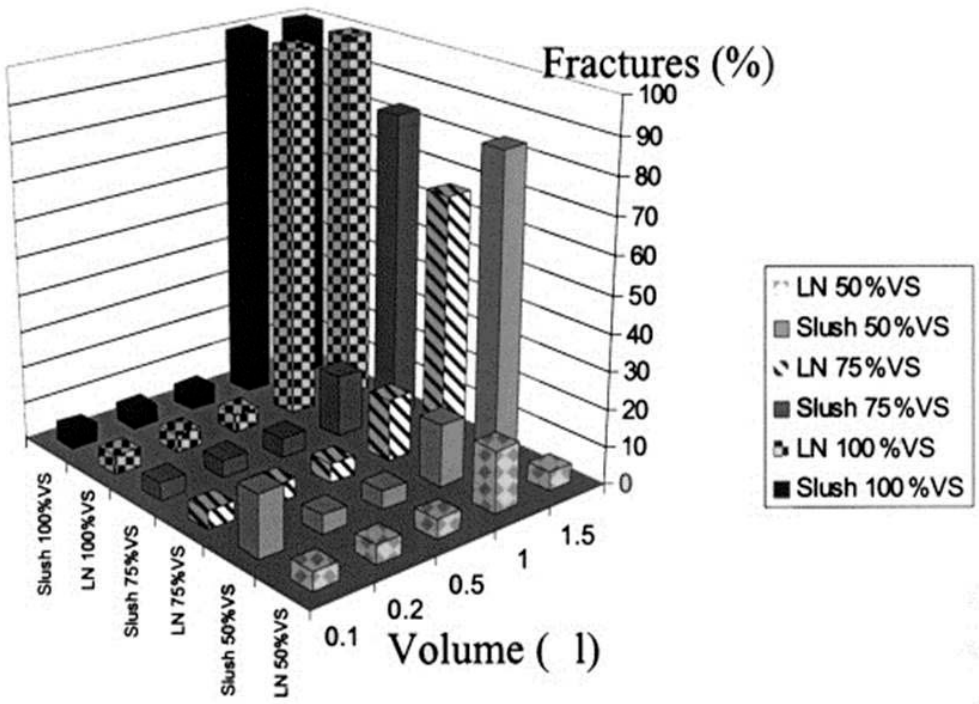


Figure 16. The effect of volume, cooling rate and CPA concentration on the probability of 'glass' fractures (form Arav et al., 2002).

1.2.4.2 Conventional freezing

Conventional slow freezing is a method which aims to have a protocol that is slow enough to dehydrate the cells in order to prevent intracellular crystallization and fast enough to minimize osmotic stress to the cells [110]. The conventional cooling procedure is usually ended when the temperature lies between -30°C and -80°C and after the sample can be plunged into LN [84]. When applied on cells the conventional freezing method reduces the likelihood of intracellular ice formation by initiation of extracellular ice crystal formation at a high subzero temperature. The freezing rate is then responsible for how fast the extracellular ice crystals will grow. The extracellular ice draws water out of the cell until little amount of free water remains and only small (non-lethal) ice crystal has been formed [111]. The best outcome of this cryopreservation method is obtained when the rate of freezing allows equilibrium between cell dehydration and the rate at which water is integrated into extracellular ice crystals.

The rate of dehydration, which is the movement of water outward across the cell membrane, depends on the cooling rate that is determined by temperature drop in relation to time. Thus, the movement of water through the cell membrane decreases as a consequence of lower temperature [112].

It is important to emphasize that in this procedure, the cooling rate is dependent on the size and permeability of the specific cells/tissue to

be cryopreserved. When trying to freeze a large tissue or a whole organ with conventional freezing, one of the major problems is that cooling rates will vary across the samples depending on the geometry (surface, depth, and volume) and heat conductivity. In fact, the conventional slow-freezing method involves lowering the temperature of the chamber in a controlled stepwise manner. This method is based on using multidirectional (equiaxial) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity and geometrical shape of the container and of the biological material within it [98, 113-115]. The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable. Furthermore, the ambient temperature gradients within the freezing chamber and the unreliability of temperature recording measurements add to the difficulty of achieving the optimal cooling rate.

One of the major problems that occurs during cryopreservation of large tissue using conventional freezing is the released of latent heat when crystallization occurs. There are two problems regarding latent heat release. The first problem is that the heat is transferred to colder areas in which the ice is just formed and so it could possibly re-warm the ice and cause local melting (Figure 17).

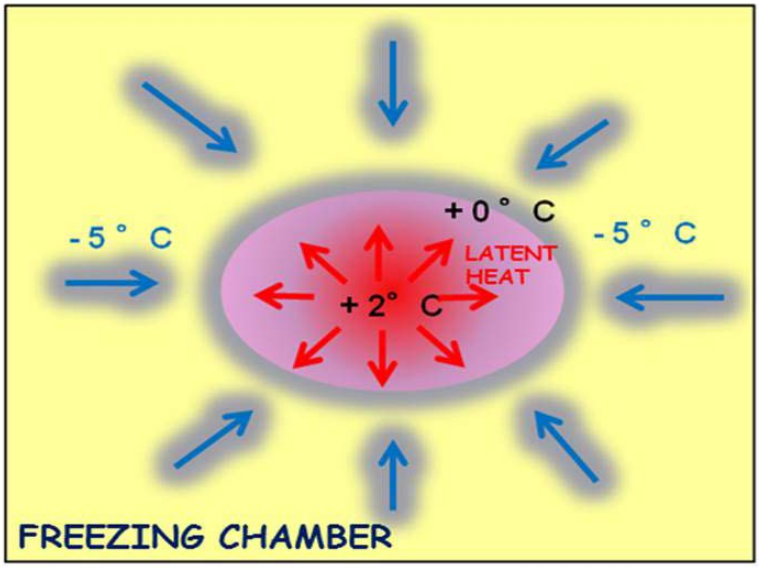


Figure 17. Release of latent heat. See the temperature differences between the core and the surface of the sample and between sample and the freezing chamber.

The second problem is that the isothermal period. When samples of large volume with relatively surface/volume are frozen, the release of latent heat may cause a long isothermal period in the material being frozen. At the same time, the temperature in the freezing chamber or on the surrounding medium is lowered, increasing the temperature different between the sample and its surrounding (Figure 17). Consequently, since the thermal conductivity of water (0.6) is lower than that of ice (1.6), when latent heat is no longer released, the temperature in the material being frozen will drop very quickly to a temperature close to the temperature of the surrounding environment (Figure 18). This might lead to a non-optimal cooling

rate and possibly to cellular damage trough intracellular crystallization [113-116].

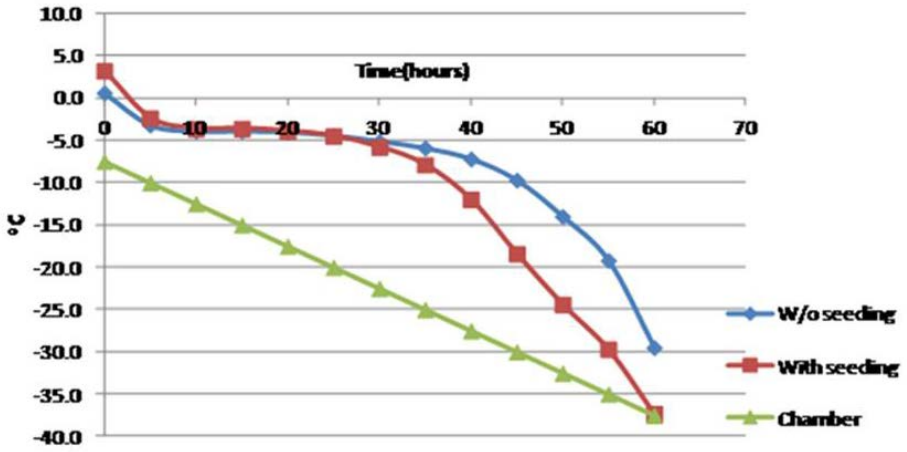


Figure 18. Different temperatures within samples (blue and red curves) and its surrounding (green curve) during the freezing process.

The current method to overcome the problem of cell damage due to release of latent heat is the removal of the excessive heat through adjustment of the cooling rate at specific time points and ensuring uniform freezing by maintaining a high ratio of surface to volume. In other words, the sample to be frozen is made as thin as possible, whereupon heat from the inner part of the sample, which is being released through the surface of the sample, will be removed faster due to the steep temperature gradient. In this way, it is possible to

apply the optimal cooling rates for each sample while providing a heat sink for rapid absorption of the released latent heat.

1.2.4.3 Directional freezing

Recently, a new directional freezing technology (MTG) was introduced [101, 102] which provides identical cooling rates through the specimen being frozen and it is aimed to cryopreserve whole organs (Figure 19).

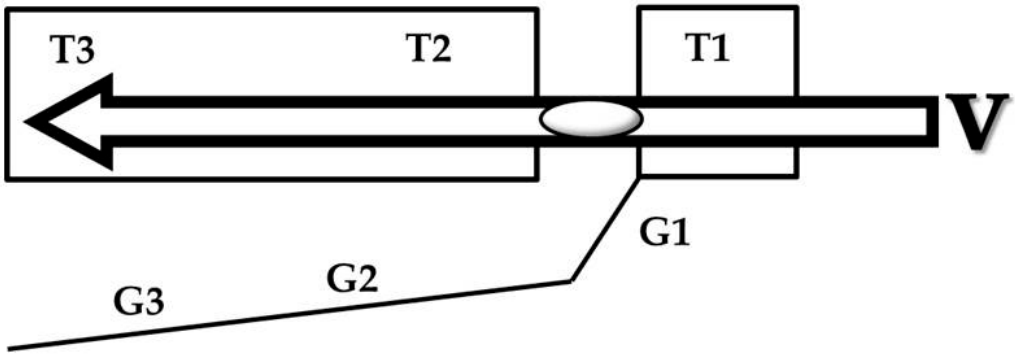


Figure 19. The Multi-Thermal-Gradient (MTG) freezing device. (A) Liquid nitrogen inlet; (B) cooling block; (C) entrance of the sample's glass tube; (D) metal rod, which advances the sample's glass tube forward into the cooling channel in the cooling block; and (E) electric motor (from Gavish et al., 2008).

The technology is based on a series of heat conductive block (usually built of brass or aluminum) arranged in a line, with a straight track running through the blocks. Along the blocks, different temperatures (T1, T2 and T3, Figure 8) can be set, in order to

obtained a temperature gradient along the blocks (G1, G2 and G3). The blocks are separated by gap and the temperature of the block on one side of the gap (T1) is above the freezing point temperature and on the other side of the gap (T2) is below the freezing point temperature, thereby imposing a temperature gradient across the gap (G1). Biological samples to be frozen are placed inside test tube and are moved along the track at a certain velocity (V). The samples are frozen at rates according to the specific protocols of the samples; the rate of freezing is given by the temperature gradient multiplied by the velocity (CR1,2,3=G1,2,3*V) (Figure 20).

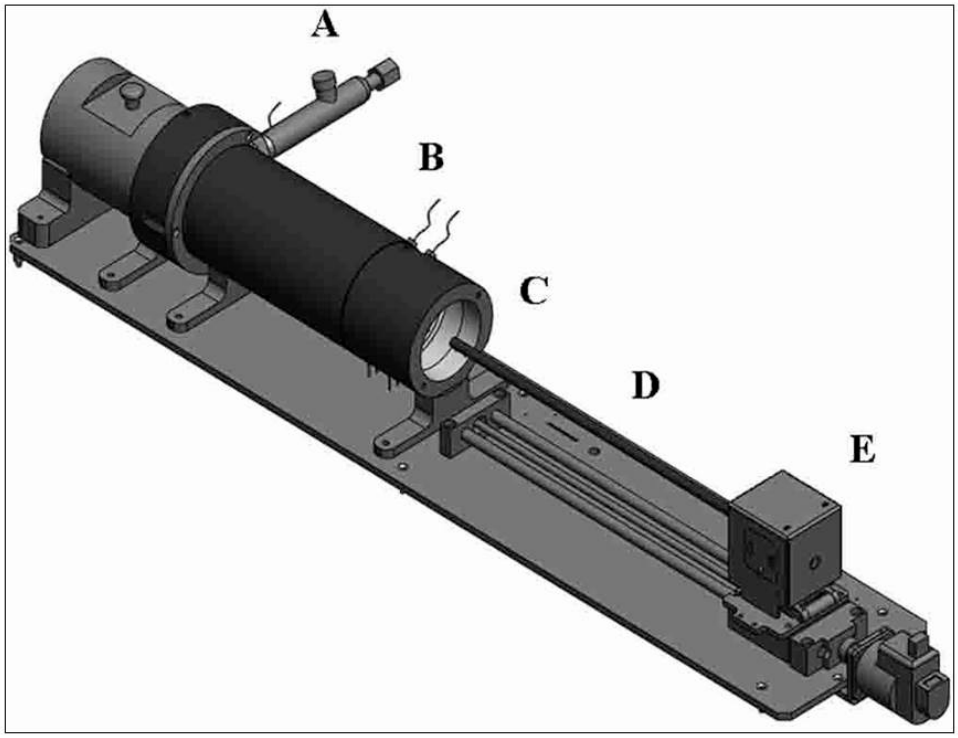


Figure 20. Schematic sketch of a directional freezing apparatus. G= gradient; T= temperature; V= velocity.

The MTG can generate a linear thermal gradient in an organ/tissue to be cooled. It is possible to change heat transfer in a directional way according to the heat transfer in the sample. The movement of the sample in the thermal gradient does not exceed this velocity in a way that heat will remain directional. In this way, heat transfer will propagate in the tissue in a linear way and the cooling rate for each point in the organ will propagated at the same (Figure 21).

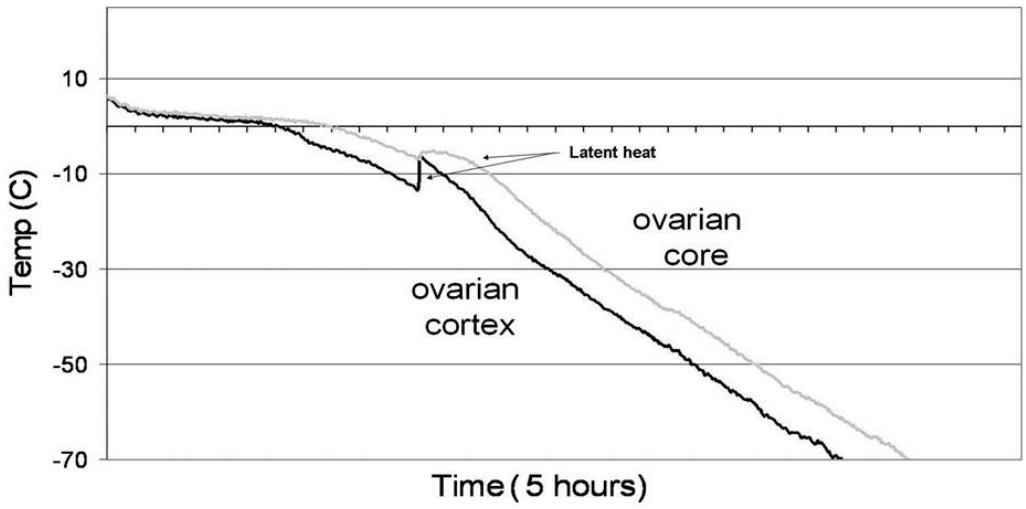


Figure 21. Multi-thermal gradient unidirectional freezing profile of a sheep ovary. See the same temperatures between the ovarian core and cortex. Arrows = latent heat release (from Arav et al., 2010).

Heat transfer in directional solidification is opposed to the direction of sample movement. This is when the velocity is shower than the

speed at which the heat is removed from the center of the sample toward the direction of the liquid and toward the conductive material of the device. In directional freezing ice formation occurs according to the solution's freezing point temperature. Therefore supercooling, a non-equilibrium thermodynamic state in which a solution is cooled below its freezing point without crystallization, is avoided completely.

The freezing technology is based on directional freezing in which the biological material is transferred through a linear temperature gradient so that the cooling rate and ice front propagation is precisely controlled. Thus maximizing the survival rate of cells subjected to freezing and thawing requires the careful control of the freezing process. Although this method is rather new, results showed that directional freezing is a promising method for cell, tissue and entire organ cryopreservation [101, 117, 118].

1.1.4.4 Thawing

The thawing process and the removal of cryoprotectant are also important step for a successful procedure. There is an opinion that the ice formed during thawing is less dangerous [119], although some authors recognize slow thawing as a prime destructive factor [102]. The longer the duration of the thawing period, the greater the damage occurring to the cells. The increase of the solute effects and the maximal growth of ice crystals are responsible for this damage.

The large crystals have an abrasive action producing a mechanical disruption of cells. Crystal growth is maximal at the range of 0°C and -40°C. The disruptive ultrastructural change in cells increasing with recrystallization. The thawing is most destructive if it is not done completely, so that all of the frozen tissue is thawed, which takes full advantage of recrystallization. For this author [102] basically two types of damage occur during slow thawing. The first is recrystallization, which is the growth of ice that was formed during the freezing process, and the second is the refreezing of the solution that occurs after complete thawing.

1.3 CRYOPRESERVATION OF OVARIAN CORTEX

1.3.1 Cryopreservation of ovarian cortex

One on the option available in cryopreservation of ovarian tissue is the preservation of ovarian cortical fragments. Ovarian cortical tissue is typically removed by laparoscopy, a minimally invasive procedure lasting approximately 1 h and requiring general anesthesia. The superficial cortical tissue must be sharply dissected from the underlying medullary portion of the ovary [120]. The cortex can be shaved to a thickness of approximately 1 mm to promote early revascularization once the thawed tissue is transplanted [121]. If the whole ovary or a large portion is removed, the cortex can be further sectioned into 5 × 5 mm segments and cryopreserved. Once

the patient has survived her cancer and its treatment, the potential uses for the thawed ovarian tissue include *in vitro* maturation and fertilization of the immature follicles or transplantation of the tissue (Figure 22).

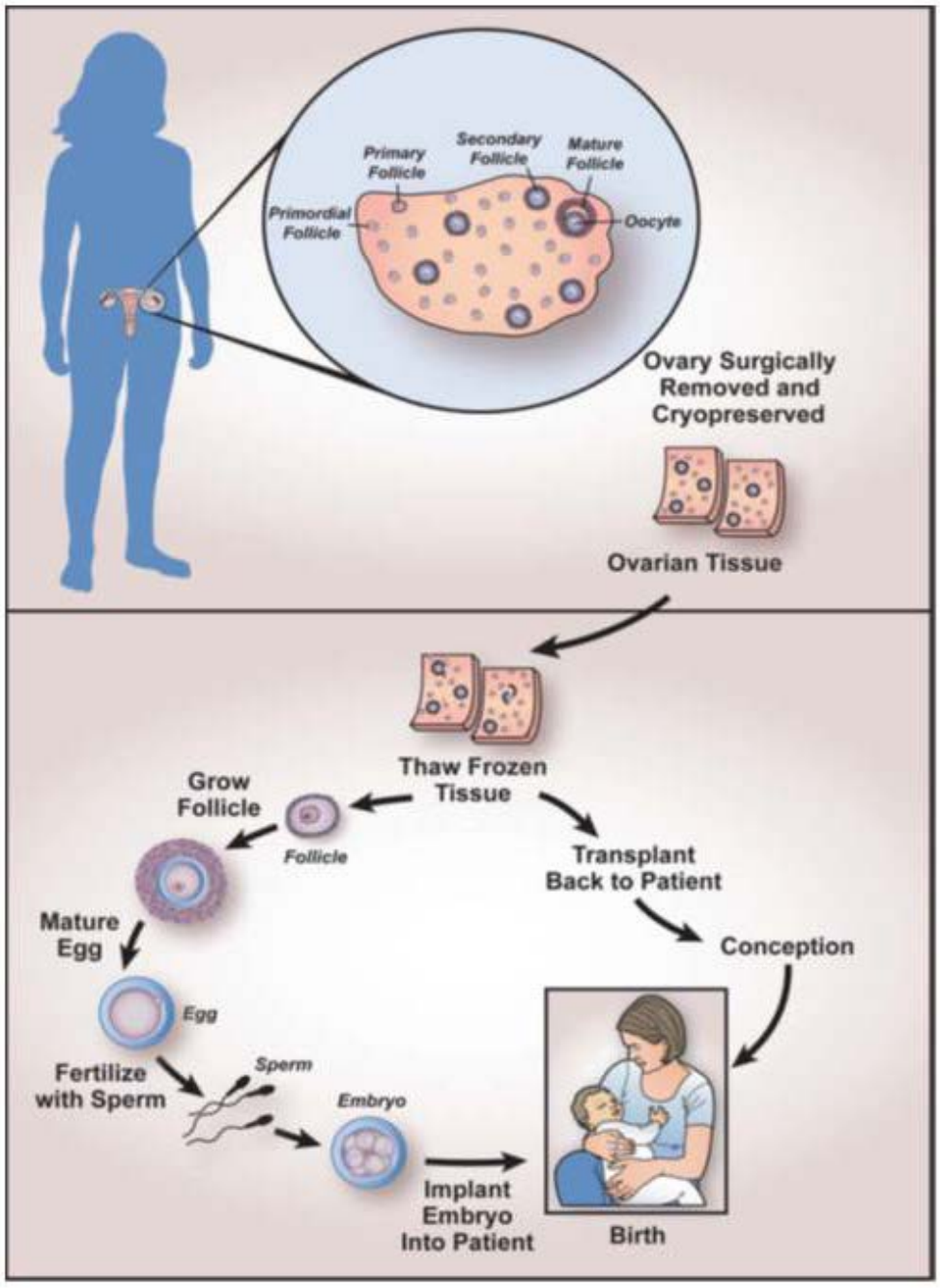


Figure 22. Uses of cryopreserved ovarian cortical fragments include transplantation and *in vitro* maturation of immature follicles and fertilization (from Oncofertility Medical Practice: Clinical Issues and Implementation, 2012).

The initial work on ovarian tissue cryopreservation, during the 1950s, made use of the mouse model. In this species viability of frozen-thawed ovarian tissue was shown for the first time in any species [122]. In a later study, Parrot [123] showed that restoration of fertility is possible in mice after orthotopic grafting of frozen-thawed ovarian tissue. In 1994, Gosden et al. [79] reported the first live births after ovarian cortex cryopreservation and transplantation in sheep. Similar reports have been published in rats [124] and rabbits [125].

The first case of cryopreservation of human tissue was described in 1996. Hovatta et al. [126] showed that cryopreservation of human ovarian tissue is feasible. They cryopreserved ovarian tissue from 19 patients aged 19–44 years using two different cryoprotectant protocols and histological investigations of fresh and frozen-thawed ovarian tissues showed similar follicular atresia rates and the morphology of follicles and oocytes in non-frozen and frozen-thawed ovarian tissues was not different. *In vivo* restoration of ovarian function after cryopreservation and autologous transplantation of ovarian cortex in human beings was first documented by Oktay and Karilkaya [127]. This orthotopic transplantation resulted in follicular development in ovarian tissue pieces grafted laparascopically beneath the left peritoneum in response to menopausal gonadotropin stimulation. Subsequently, restoration of normal endocrine function and follicular growth, after

heterotopic transplantation of the ovarian cortex, was reported by the same group [128, 129].

The first live birth from frozen-thawed ovarian cortex after autologous orthotopic transplantation was reported by Donnez et al. [130]. Doubts on the source of pregnancy was raised by Oktay and Tilly as to whether it arose from transplanted tissue or from the inactive ovary, which could have resumed its function [131]. Subsequently a series of live births after orthotopic transplantation of previously frozen cortical tissue have been reported by different groups [132-139]. Furthermore, very recently Stern et al. reported the first clinical pregnant after heterotopic ovarian transplantation in human [140].

Ovarian cryopreservation and transplantation of ovarian fragments appears to hold promise for fertility preservation in cancer patients, but there are numerous issues that need to be resolved. One of the major problems, is related to the freezing process. In fact, unlike single-cell freezing, it is much more difficult to optimize conditions for tissue freezing because tissue is composed of many different cell types. The main danger is intracellular ice crystal formation and salt concentration [141]. Currently, the best follicular survival rate obtained in frozen-thawed human ovarian tissue is approximately 70%–80%, with the majority of follicles morphologically normal by light microscopy [142, 143]. Furthermore, distinctive ultrastructural changes has been detected in frozen-thawed tissue by electron

microscopy [144, 145]. The other main obstacle to the wide application of ovarian cortex cryopreservation is that the majority of transplanted follicles are lost by ischaemic damage by the time sufficient neoangiogenesis has supplied the graft. This happens because transplantation of fragments of ovarian cortex is performed without vascular re-anastomosis, perfusion of the tissue depends on the growth invasion of new blood vessels. The ischemic injury occurring directly after transplantation without vascular anastomosis is involved in the dramatic follicular depletion observed in grafted ovarian tissue. This follicular depletion is a main concern, especially in humans and large animal species that have a dense ovarian cortex. At least 25% of the primordial follicles are lost as a result of cryopreserved xenografts of human ovarian tissue into mice [142, 144]. Others estimated that ischemic injury during autograft processes induces the depletion of 60–95% of the follicular reserve, including the loss of virtually the entire population of growing follicles [146, 147]. This phenomenon is associated with a dramatic reduction of the graft size and a significant fibrosis in most grafts [148]. It appears that ovarian slices can convey only short-term function due to loss of most follicles by ischaemia.

1.3.2 Cryopreservation of whole ovary

An alternative strategies to preserve fertility is the transplantation of the cryopreserved whole intact ovary. Whole ovary transplantation with vascular anastomosis was proposed as a mechanism to reduce ischemic damage and, in theory, prolong the longevity of the graft [149]. In this technique, the whole ovary with its vascular pedicle, is removed, cryopreserved, thawed, and then transplanted using a microvascular anastomosis into a heterotopic or orthotopic site. These procedure reduces the ischemic interval between transplantation and revascularization by allowing immediate revascularization of the transplanted tissue [150]. Vascular anastomosis of fresh ovary was successfully performed using the ovarian artery, inferior epigastric vessels, carotids vessels or iliac artery in various animal models [124, 151-154]. A limited number of human studies with transplantation of fresh intact ovaries in orthotopic [155, 156] and heterotopic [157, 158] sites have been attempted with some success. The first full-term pregnancy obtained using orhotopic whole fresh ovary transplantation between monozygotic twins who are discordant for POF was reported in 2008 by Silber et al. [155]. A fresh ovary from the fertile twin was implanted in her monozygotic twin with POF.

Whole frozen ovary transplantation with microvascular anastomosis was first described in rats by Wang et al. [124]. They described successful vascular transplantation of frozen–thawed rat ovaries and

reproductive tract in four of seven (57%) transplants, which survived for ≥60 days, were ovulatory and resulted in one pregnancy. Chen et al. [159] showed that frozen–thawed rabbit ovaries remained functional for at least 7 months after microvascular transplantation in 13 of 15 (86.7%) animals.

The challenge of whole ovary cryopreservation and transplantation technology is not only the surgical technique but also the cryopreservation protocol for an entire organ. It appears that, in large mammals and humans, cryopreserving an intact ovary may prove more problematic than in small animals, largely due to the physical constraints that limit an appropriate heat transfer between the core and the periphery of the organ [102]. In addition, the large volume of the whole organ poses some limitation to the perfusion and diffusion of cryoprotectants [160, 161] (see chapter 1.1.4.2). Both are essential for preventing intravascular ice formation which would irreversibly compromise a rapid and efficient resumption of the blood supply [162]. Nevertheless, data obtained from experiments mostly performed in sheep show some positive results [163] and pregnancies [164] but also highlight stromal and vascular damage following either slow freezing [165, 166] or vitrification [167, 168]. Imhof et al. reported that 18 months after transplantation of whole ovary frozen with slow freezing, the follicular survival rate was less than an 8% [169]. Other authors reported an even lower follicular survival rate (6%) and the depletion of the entire follicular

population after whole ovary vitrification and transplantation [168]. Bedaiwy et al. [170] reported the restoration of ovarian function after autotransplantation of intact frozen–thawed sheep ovaries with microvascular anastomosis, but it should be noted that 8 of 11 ovaries were lost due to thrombotic events in the reanastomosed vascular pedicle. 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. Recently, Martinez-Madrid et al. [171] described a cryopreservation protocol for intact human ovary with its vascular pedicle and observed high survival rates of follicles (75.1%), small vessels and stroma, and a normal histological structure in all the ovarian components after thawing. Recently, a multigradient freezing device was used with promising results (see chapter 1.2.4.3). In fact, this freezing method combined with microvascular anastomosis have improved outcome, and long lasting ovarian function has thus been obtained in sheep. Arav et al. [117] reported progesterone activity 36 months after vascular transplantation of frozen–thawed sheep ovaries in three of eight transplants, and retrieval of six oocytes, resulting in embryonic development up to the 8-cell stage after parthenogenic activation. Even after 6 years the cryopreserved and transplanted tissue appeared functional as was indicated by histological examination. Normal tissue architecture, healthy blood vessels and follicles at different stages were all

observed in these ovaries. Even after 6 years the cryopreserved and transplanted tissue appeared functional as was indicated by histological examination. Normal tissue architecture, healthy blood vessels and follicles at different stages were all observed in these ovaries [172]. These results are promising in finding an optimal method for the cryopreservation of whole ovaries, so that in the future it will be possible to restore fertility long-term and to prevent premature menopause in young female cancer patients.

1.3.3 Safety concerns with ovarian tissue transplantation

Autotransplantation of frozen and thawed ovarian tissue is only possible if absence of cancer cells in the graft is confirmed and there is a legitimate concern for the reseeding of malignant cells when carrying out ovarian transplantation. Theoretically, ovarian tissue could carry micro-metastases that could "re-infect" a patient who had been previously cured of her cancer. The risk of ovarian mestastasis was summarized by Sonmezer and Oktay [47] (Table 5).

Low risk	**Squamous cell carcinoma of the cervix** **Ewing's sarcoma** **Breast cancer stage I-III** **Wilm's tumore** **Non-hodgkin's lymphoma** **Hodgkin's lymphoma** **Osteogenic sarcoma** **Non-genital rhabdomyosarcoma**
Moderate risk	Breast cancer stage IV Colon cancer Adeno/adenosquamous carcinoma of the cervix Upper gastrointestinal system malignancies
High risk	Leukemia Burkitt lymphoma Neuroblastoma Genital rhabdomyosarcoma

Table 5. The risk of ovarian metastatis (from Sonmezer and Oktay 2004).

Ovarian transplantation might be particularly concerning with blood-born malignancies such as leukemia, where the cancer cells are already in the blood, and therefore presumably within the cryopreserved ovarian tissue. The transmission of lymphoma via graft of ovarian tissue from diseased donor mice to healthy recipients was reported by Shaw et al. [173]. This study showed that fresh and cryopreserved ovarian biopsy samples taken from mice with lymphoma transmitted cancer into recipients. The transplant of human ovarian tissue, however, from patient with Hodgkin's lymphoma in SCID mice did not transfer the disease to the recipients [174]. Hence, it is necessary to develop a method in the ovarian tissue to eliminate the risk of transmission of these cells with transplantation, or to consider xenografting, or *in vitro* maturation which would minimize re-introduction of cancer cells of women having suffered from heamatological malignancies are under development [175].

1.4 COMPARATIVE ANATOMY OF SHEEP AND HUMAN OVARY.

All experiments described in this thesis were performed on sheep ovary. The sheep is a traditional experimental animal in reproductive physiology research. There exist a large knowledge base concerning physiological changes of the ovine ovary and the similarities between the human and ovine ovary in regards to tissue

architecture make it suitable as a research tool towards optimization of procedures for human use.

The main ovarian functions are differentiation and release of mature oocyte competent for fertilization and production of steroid hormones necessary for secondary sexual characteristics and subsequent achievement of pregnancy.

Sheep and human ovaries are oval-shaped female gonads. In sheep the ovaries are approximately 15-20 mm in length, 10-15 mm width and weigh approximately 2.0 g (about 1/6th of the size of a human premenopausal ovary). In human ovaries are about 4 cm long, 1.5 cm wide, 1 cm thick and weight 2-4 g.

In both species, the ovarian surface is irregular because the follicles and corpora lutea protrude markedly. The tissue architecture of ovine and human ovaries is similar with a well-defined tunica albuginea and a collagen-dense cortical stroma containing the primordial follicles [117].

In sheep, ovaries are located in the antero-lateral abdominal wall of the female pelvis whereas in human they are located in the lateral wall of the female pelvis beneath the external iliac artery. The ovaries are suspended from the broad ligament near the end of the oviduct and lie near the tips.

The ovarian artery in sheep is in close apposition to the wall of the utero-ovarian vein. The contact area between vein and artery increases by the tortuous path of the artery over the surface of the

vein. The walls of the artery and vein are thin in the contact area between the two vessels (Figure 23).

The human ovarian artery, which ordinarily arises from the aorta, passes along the ovary, dividing into a number of branches. At several places in the broad ligament there are anastomotic connections between the tubal branch of the uterine artery and the ovarian artery. A branch of the uterine artery nourishes the round ligament. The veins generally accompany the arteries (Figure 24).

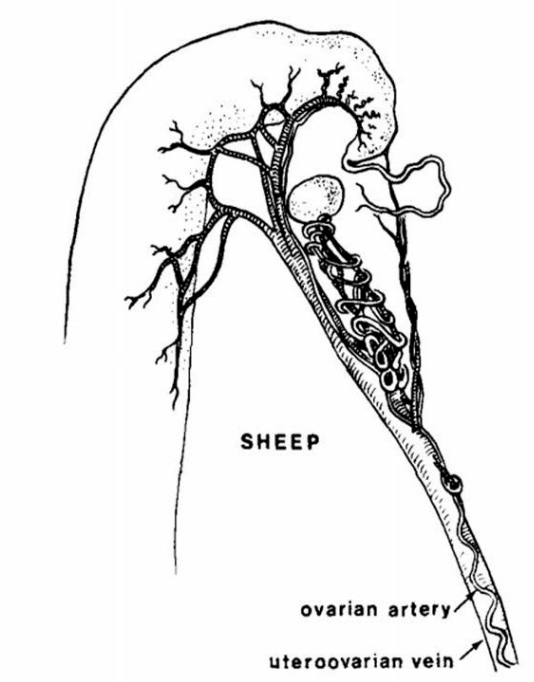


Figure 23. Diagrammatic presentation of arteries (clear) and veins (cross-bar) of a uterine horn and the adjacent ovary from sheep. The ovarian artery is tortuous and is closely applied to the utero-ovarian vein which drains most of the uterine horn (from Ginther ,1974).

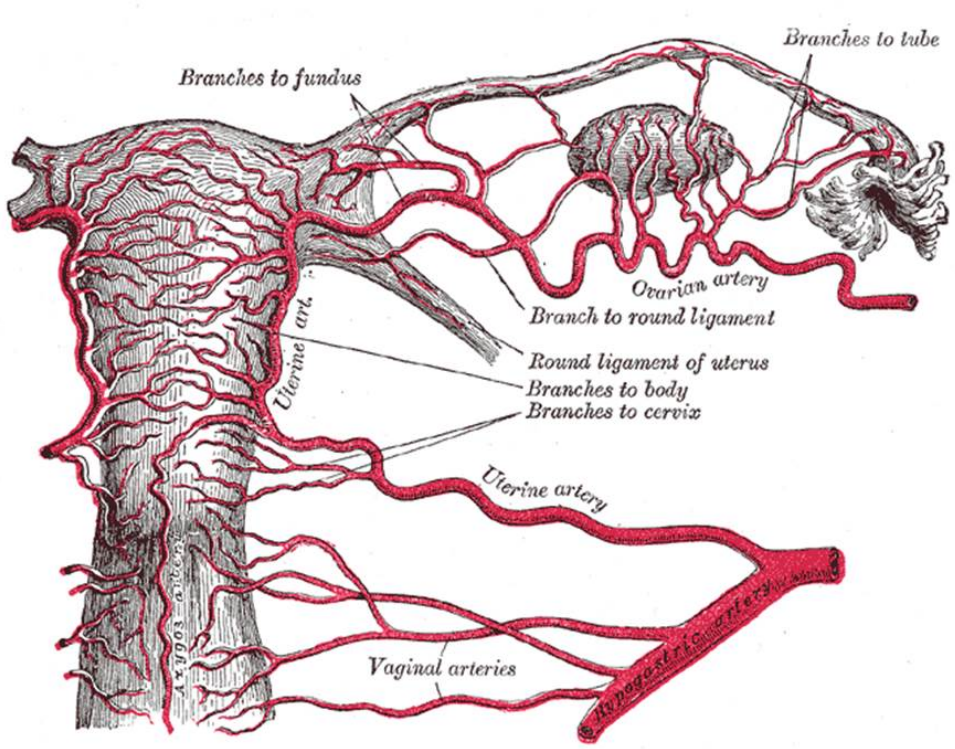


Figure 24. Blood supply of the human female reproductive organs. (from the 20th U.S. edition of Gray's Anatomy of the Human Body).

The ovary consists of a medulla and cortex (Figure 28). The medulla is the central part, which contains loose connective tissue, nerves and blood and lymphatic vessels. The ovarian cortex is the outer part of the ovary surrounding the medulla and is composed of the ovarian follicles that embedded in specialized stroma. The small ovarian follicles lie at a depth of approximately 0.03 mm in the ovarian cortex. Follicles are the functional units of the ovary and are composed of an oocyte and surrounding somatic granulosa cells (Figure 25) with or without theca cells, depending on the

developmental stage of the follicle. Granulosa cells and basal membrane separates the granulosa cells from the stromal/thecal tissue. Intercellular contacts between granulosa cells are provided by gap junctions, forming a compact functional syncytium of cells that allows metabolic exchange and transportation of molecules.

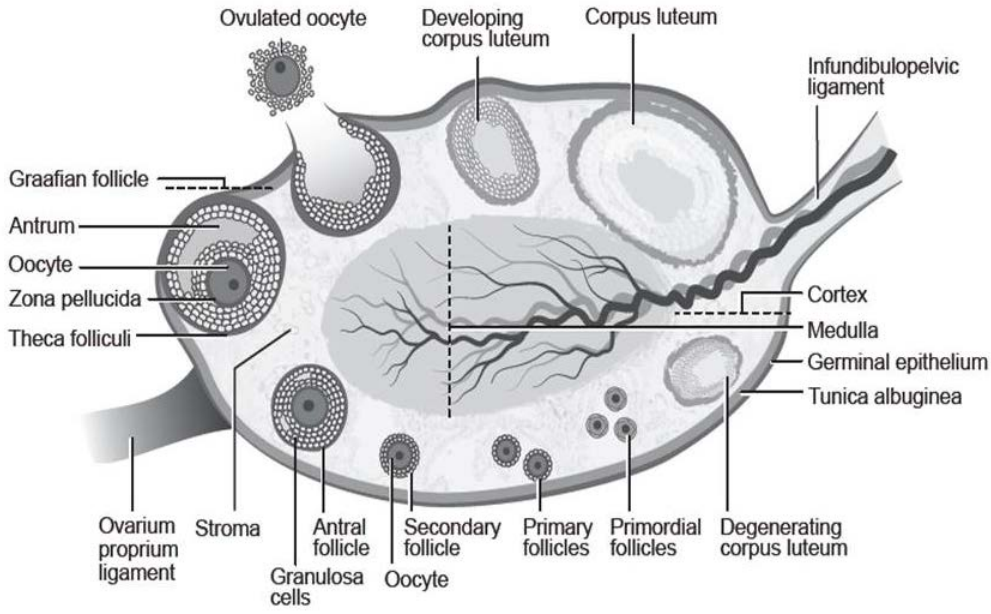


Figure 25. Schematic drawing of the cyclic changes of the ovary (form Mileknovic et al. 2011).

It has been estimated that there are about 100,000–200,000 follicles in lambs at birth [176]. Follicles are classified as: primordial (the earliest stage) containing an oocyte surrounded by a single layer of flattened granulosa cells; primary surrounded by at least one cuboidal layer of granulosa cells; secondary with more than one layer of cuboidal

granulosa cells and theca externa and interna cells which originate from ovarian stroma; tertiary when a fluid filled antrum inside the granulosa cells is become evident. During follicular development also the oocyte increases in diameter and becomes surrounded by a zona pellucida (Figure 26), which is a thick extracellular coat of glycoproteins. The zona pellucida plays an important role during oogenesis, fertilization and preimplantation development.

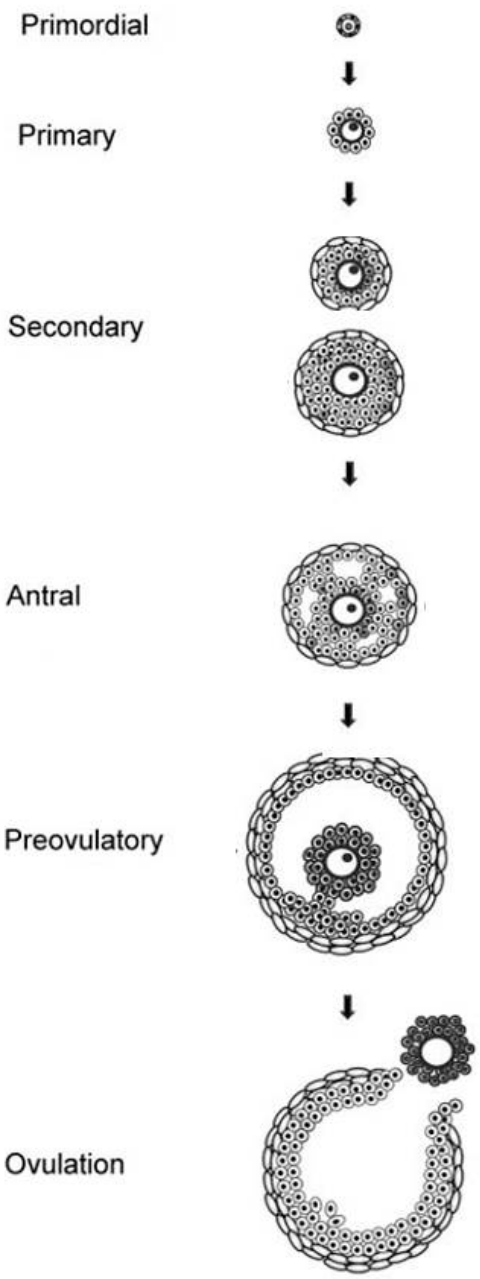


Figure 26. Classification of ovarian follicles during folliculogenesis.

During follicular development, the follicle grows from an initial size of 30 μm to up 7 mm in sheep and from 40 μm to up 15-22 mm in human (Figure 30). A large pool of resting primordial follicles is laid down during fetal development, with the first follicles being formed at about 70 days of gestation in sheep and about 100 days in human. This pool is nonrenewable and during the lifetime of the animal, follicles develop to primary, secondary and tertiary stages before ovulating. Very few follicles progress to ovulation and most die by atresia at any stage of development. The duration of the transition from a primordial into pre-ovulatory follicles is similar between sheep (180 days) [177] and human (200 days) [178] (Figure 27).

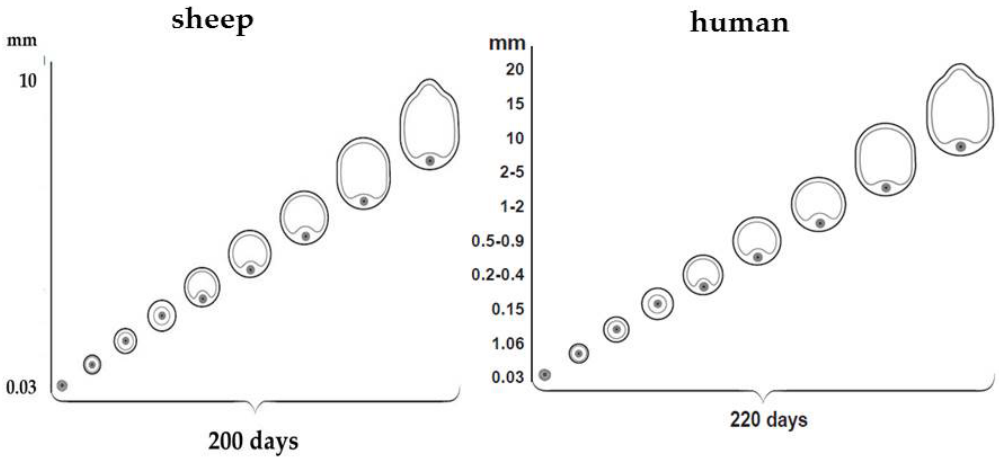


Figure 27. Schematic diagram of follicle growth in sheep and in human from the primordial stage to the preovulatory stage (modified from Mileknovic et al, 2011).

2 AIM

Ovarian tissue cryobanking is considered a viable method for preserving female fertility and represents the best option in young pre-pubertal cancer patients. Cryopreservation can be performed on ovarian cortical fragments or on the entire organ. However, both procedures are still considered to be experimental by the American Society for Reproductive Medicine and require further research to reach a reliable clinical application and provide a concrete improvement to the quality of life of these patients.

To move forward, the cryopreservation of both avascular ovarian fragments and of whole ovaries must be improved. To this purpose, we performed a detailed comparison between two different methods of cryopreservation on both cortical fragments and whole ovary.

Based on our results [179] and those in literature [102, 180], in this thesis the sheep ovary was chosen as a model because its size and texture are comparable to young human ovaries.

Although re-implantation of frozen-thawed ovarian cortical fragments is the most common approach to ovarian banking the main problem associated with this method is the loss of over 50% of the primordial follicles due to cryopreservation and tissue ischemia that take places after transplantation. Current research is investigating the potential of whole ovary transplantation with vascular anastomosis as a mechanism to reduce ischemic time.

However, cryopreservation of whole ovary is more complicated than cortical fragments, due to the large volume of the sample to be frozen.

In the first part of this thesis, we perform a direct comparison between conventional and directional freezing, cryopreserving sheep whole ovaries. This study was carried out in order to analyse if the new directional freezing method allows a better preservation of ovaries, reducing the problem associated with conventional method, including stromal and vascular damage.

In the second set of experiments, ovarian cortical strips were frozen with both freezing methods, in order to determine whether directional freezing could also improve the viability of ovarian fragments. The obtained results were compared with whole ovaries frozen with the same method.

In both set of experiments, we analysed ovarian tissue morphology after freezing/thawing, including the determination of stromal cells density and blood vessels integrity. Follicle viability after freezing/thawing, was investigated by *in vitro* culture evaluating the capacity of primordial follicles to growth into primary stage. Furthermore, we investigated the ability to respond to thermal stress and the dynamics of DNA repair mechanisms.

Finally, in the last part of this thesis, we developed a perfusion system for *ex vivo* culture of whole ovaries, which could provide appealing alternative to the re-trasplantation of frozen-thawed

ovarian tissue, with the purpose to eliminate the risk of transmission of cancer cells in women who have systemic hematologic malignancies.

3 MATERIAL AND METHODS

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (Italy).

3.1 Ovaries collection

One hundred and forty-one sheep ovaries with ovarian pedicle were collected at the local abattoir and transported to the laboratory in physiological saline (9.6 g/l NaCl). All the selected ovaries were in follicular phase and the ovarian vessels were cut so as to be a long as possible.

Ovaries were randomly assigned to fresh control group (CTR) and experimental groups. The experimental groups included:

1. Directional freezing of whole ovary;
2. Directional freezing of ovarian fragments;
3. Conventional freezing of whole ovary;
4. Conventional freezing of ovarian fragments .

3.2 Sample preparation

Samples to be frozen as whole organs were perfused via the ovarian artery with Ringer's solution and 10 IU/l heparin for 10 minutes, followed by perfusion with cryoprotectant solution containing Leibovitz L-15 medium (Life Technologies), 10% fetal bovine serum (v/v; Life Technologies) and 1.5 M dimethylsulfoxide for 10 minutes at a flow rate of 1.3 ml/min [163] at room temperature.

Ovarian fragments (10 x 5 x 1 mm) were sliced from the cortical region and immersed in the same cryoprotectant solution.

Each ovary and all fragments (8-10) from a single ovary were inserted into a 16 mm diameter and 80 mm long glass test tube (Manara) containing 10 ml of the same solution. The tube wall is 1 mm thick and made of quartz. Tubes were closed with specifically designed silicon plugs. All samples were equilibrated at +4°C before the freezing process.

3.3 Cryopreservation procedures

3.3.1 Conventional freezing

Conventional slow freezing was performed in a Kryo 560M apparatus (Planer) with the following program [163]:

a) from +4° to –40°C using a cooling rate of -0.5°C/min (seeding was induced at -7°C);

b) from –40° to –100°C at -5°C/min;

c) immersion in liquid nitrogen.

3.3.2 Directional freezing

Directional freezing was performed with a Multi-Thermal-Gradient (MTG, IMT Ltd., Ness Ziona). The 3 thermal blocks were set at +4°, -10° and -70°C respectively, thereby imposing a temperature gradient around the tubes. Freezing tubes were pushed lengthwise along the thermal gradient and the speed was set at 0.01 mm/s resulting in a

cooling rate of -0.3°C/min down to -70°C, after which samples were plunged into liquid nitrogen [117, 172].

3.4 Thawing

After minimum two weeks, the glass test tube were removes from the liquid nitrogen tank. Samples were thawed by plunging the test tubes into a 68°C water bath for 20 seconds and then into a 37°C water bath for 2 minutes. The content of the tubes was quickly emptied into a petri dish (Sarstedt) containing Leibovitz L-15 medium at 37°C. The cryoprotectant solution was rinsed out of the whole ovaries by perfusion via ovarian artery with 10 ml of Leibovitz L-15 medium at 37°C supplemented with decreasing sucrose concentrations (0.25 M, 0.125 M and 0 M) at 3 ml/min for a total of 30 minutes. Perfusion times were 10 minutes for each step [149, 170].

After thawing, ovarian fragments were washed three times for 10 minutes each in Leibovitz L-15 supplemented with the same sucrose concentrations (0.25 M, 0.125 M and 0 M). The material was then randomly allocated for further analysis. Each experiment was performed on a minimum of three independent biological replicates and all samples were blinded for analysis.

3.5 Follicle culture

The ability of primordial follicles to resume development after cryopreservation was studied culturing cortical fragments for 7 days [181]. After thawing, several ovarian fragments of approximately 2 x 2 x 1 mm were derived from whole ovaries and isolated cortical pieces. Ovarian cortical fragments derived from fresh ovaries were used as control. Three randomly selected fragments from each ovary were immediately fixed in 10% formaldehyde solution for histological studies (time 0, control) as described in paragraph 3.6. Other randomly selected fragments were placed into 24-well culture dishes (Sarstedt) and individually cultured in 1ml of medium (see Table 6) for 7 days at 39°C under an atmosphere of 5% CO_2 in air. The medium was replaced every 2 days. At the end of culture, samples were processed for further analysis.

FOLLICLE COLTURE MEDIUM	
αMEM	
Insulin	6.25 µg/ml
Transferrin	6.25 µg/ml
Selenium	6.25 ng/mL
Glutamine	2 mM
Pyruvate	0.23 mM
Hypoxantine	2 mM
Bovine Serum Albumin	1.25mg/ml
Penicillin G	100 µg/ml
Streptomycin	100 µg/ml
Sheep FSH	200 mIU/ml
Human recombinant bFGF	50 ng/ml

Table 6. Composition of medium used for follicle culture

3.6 Histology procedures

3.6.1 Tissue fixation

To improve the fixation, whole ovaries were cut in half through its major axis with scalpel and forceps and immediately fixed in 10% (v/v) formalin from 48 hours. Cortical fragments were directly immersed in 10% (v/v) formalin for 24 hours.

3.6.2 Paraffin embedding

After fixation, samples were dehydrated by bathing them successively in a gradually increasing grade of mixtures of ethanol and water (50%, 70%, 90% and 100%). The samples were immersed in xylene and then passed through paraffin: xylene (50:50) solutions overnight. On the following day, samples were washed for 3 times, each for 1 hour with melted paraffin (60°C). They were then isolated and embedded in the plastic blocks. Finally, the paraffin embedded blocks were preserved at room temperature.

3.6.3 Preparation of histological sections

Serial sections (thickness, 5 μm) were cut from each ovary at a regular interval of 100 μm using the microtome (Microm, Bio-Optica) at cooling. They were then transferred on slides and put on the hot rack for 24 hours

3.6.4 Hematoxylin and eosin staining

Slides were deparaffinazed in xylene, and rehydrated through a decreasing grade of alcohols (100%, 90%, 70% and 50%).

Slides were then stained with hematoxylin (Emallueme Carazzi, Bio-Optica) combined with an acid dye eosin (Eosin 1% aqueous solution, Bio-Optica) according to well-established procedures. Excess dyes were removed by re-passing the slides through an increasing grade of alcohols (50%, 70%, 90% and 100%) until return to absolute Xylene. The samples still wet with xylene were mounted

with synthetic based mounting media, Biomount® (Bio-Optica, Milan, Italy) and covered with microscopic cover-slip.

3.6.5 Immunohistochemistry

The paraffin-embedded ovarian sections (thickness, 5μm) were mounted on poly-L-lysine coated slides (Microscope slides Polysine™, Bio-Optica), deparaffinazed (xilene, xilene:ethanol, absolute ethanol, 95% ethanol, 70% ethanol) and washed in phosphate-buffered saline (PBS). To unmask the antigen, section were immersed in a citrate-based antigen unmasking solutions (Vector Laboratories) and boiled for 5 minutes, using pressure cooker. Non-specific site were blocked with a solution of PBS containing 5% BSA and 10% non-immuneserum. Sections were incubated overnight with the primary antibodies listed in Table 7. Sections were washed three times with PBS and incubated with suitable secondary antibodies (Alexafluor; Life Technologies) for 45 minutes. Nuclei were stained with 4'-6'-diamino-2-phenylindole (DAPI). Sections were washed three times in PBS, mounted with VECTASHIELD mounting medium to prevent photobleaching (Vector Laboratories) and covered with microscopic cover-slip. Blinded samples were observed under Eclipse E600 microscope (Nikon), pictures were acquired wit Nis Elements Software (Version 4.0) and the images were analyzed using Image J software as described in paragraph 3.11.

ANTIBODY	Description	Host Species	Company	Catalog Number	IHC Working Solution
Ki67	Proliferation marker	rabbit	Abcam	ab15580	1:500
γH2AX	DNA damage marker	mouse	Abcam	ab26350	1:200
RAD51	DNA repair marker	rabbit	Santa Cruz	sc-8349	1:200

Table 7. List of antibodies used for immunohistochemistry.

3.7 Morphologically analysis

After hematoxylin and eosin staining, slides were observed under an Eclipse E600 microscope (Nikon) and pictured were acquired with Nis Elements Software (Version 4.0). For each measure, a minimum of four sections, 200 µm apart, form each sections were examined.

Follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte), intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte) or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Follicles were further classified as normal, when a spherical oocyte with a non-pyknotic nucleus was surrounded by granulosa cells organized in discrete layers, or degenerated, when a misshapen oocyte with or without vacuolation with a pyknotic

nucleus was surrounded by disorganized granulosa cells detached from the basement membrane [182] (Figures 28). Stromal cell density was quantified by counting nuclei with ImageJ software as described in paragraph 3.11 (Figure 29).

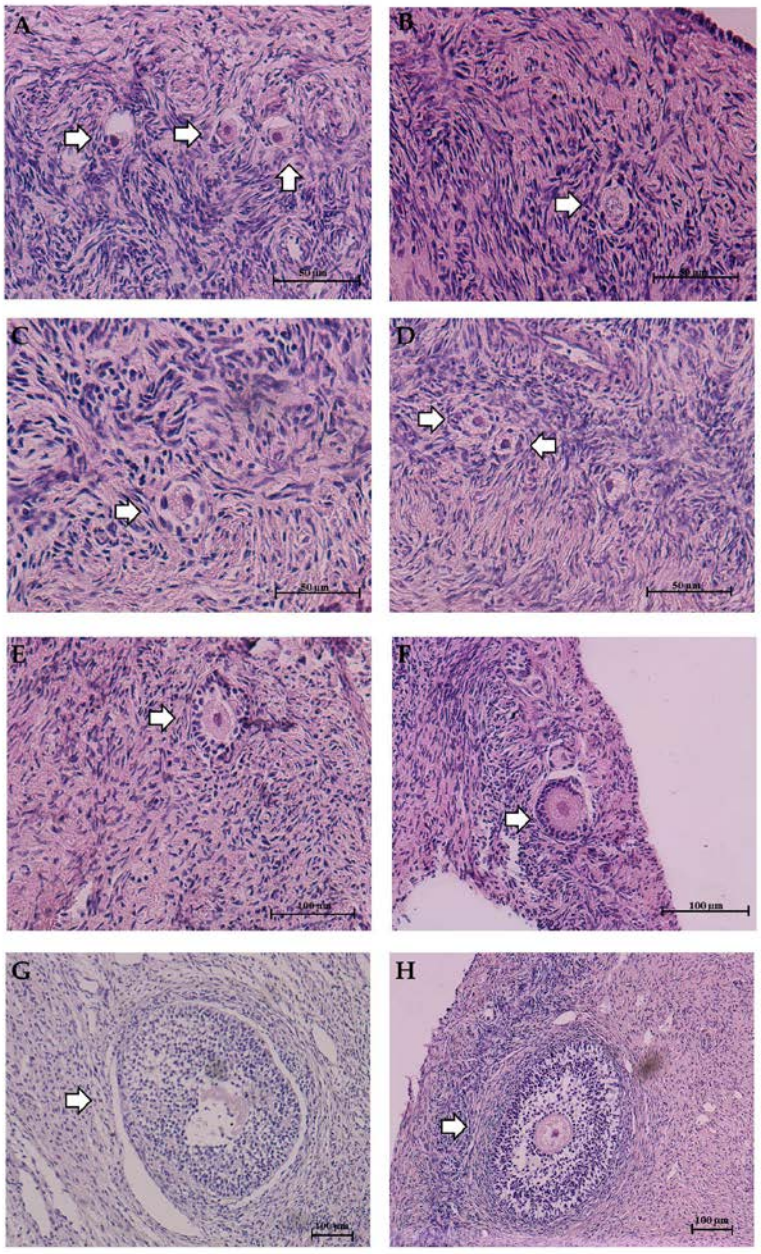


Figure 28. Representative pictures of ovarian follicles in the cortical region. A= intact primordial follicles; B= degenerate primordial follicles; C= intact intermediate follicles; D= degenerated intermediate follicles; E= intact primary follicles; F= degenerated primordial follicles; G= intact secondary follicles; H= degenerated secondary follicles.

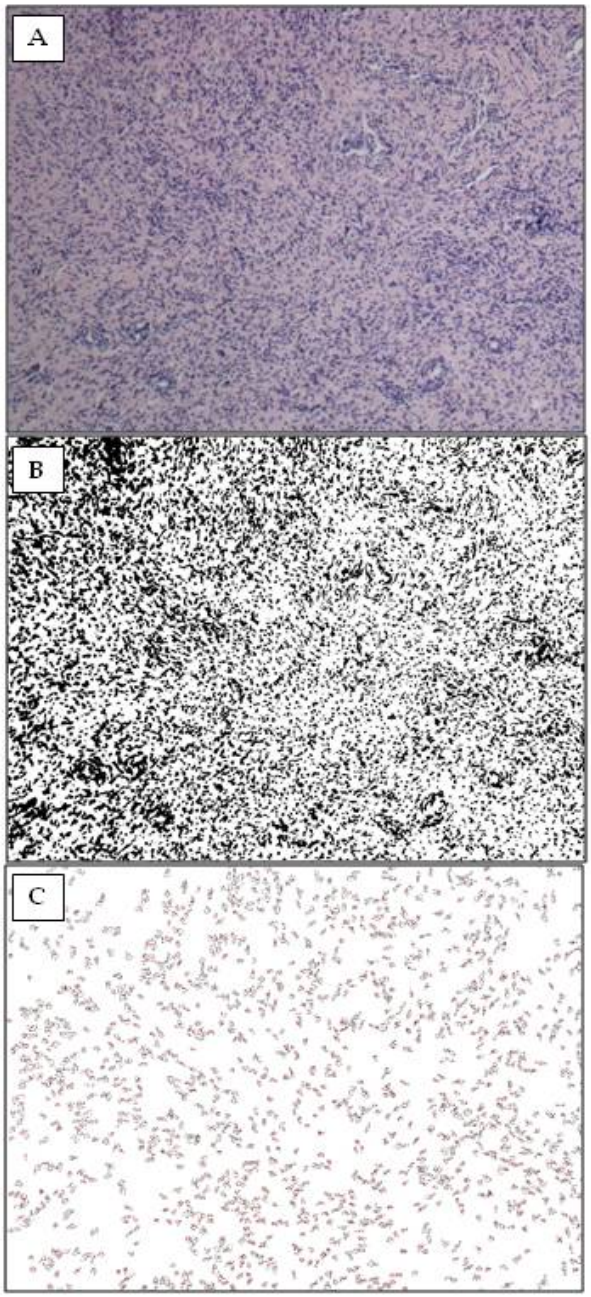


Figure 29. Representative figure of analysis of stroma cells densisty. (A) Original hematoxylin and eosin staining image; (B) The same image after conversion in 8-bit; (c) Output that show nuclear outlines.

3.8 Blood vessel integrity

Vessels integrity was assessed using Indian Ink. Fresh ovaries were perfused with 10 ml Ringer's solution supplemented with 10 IU/ml heparin, whereas thawed ovaries were perfused as describe in paragraph 3.4. Thereafter, six ovaries from each groups (CTR, DFct and CFct) were perfused via the ovarian artery with a solution of 25% Indian Ink in 0.9% NaCl supplemented with 167 IU heparin/ml for 5 minutes [183], with a flow rate of 1.3ml/min. A perfusion was considered successful if Indian Ink was macroscopically seen to perfuse the whole ovary (Figure 30). After perfusion ovaries were fixed, embedded in paraffin for hematoxylin and eosin staining as described in paragraph 3.6.

Perfused vessels were classified as intact or damaged, according to the presence or absence of Indian Ink in the lumen, respectively. Vessels were divided into three categories according to their size: small (Ø ≤ 75 µm), medium (Ø 21 µm-74 µm), and large (Ø ≥ 75 µm). Vessels number was determined in six randomly selected fields per section of both medulla and cortical tissue of each ovary. For each experimental groups a minimum of 200 vessels were counted.

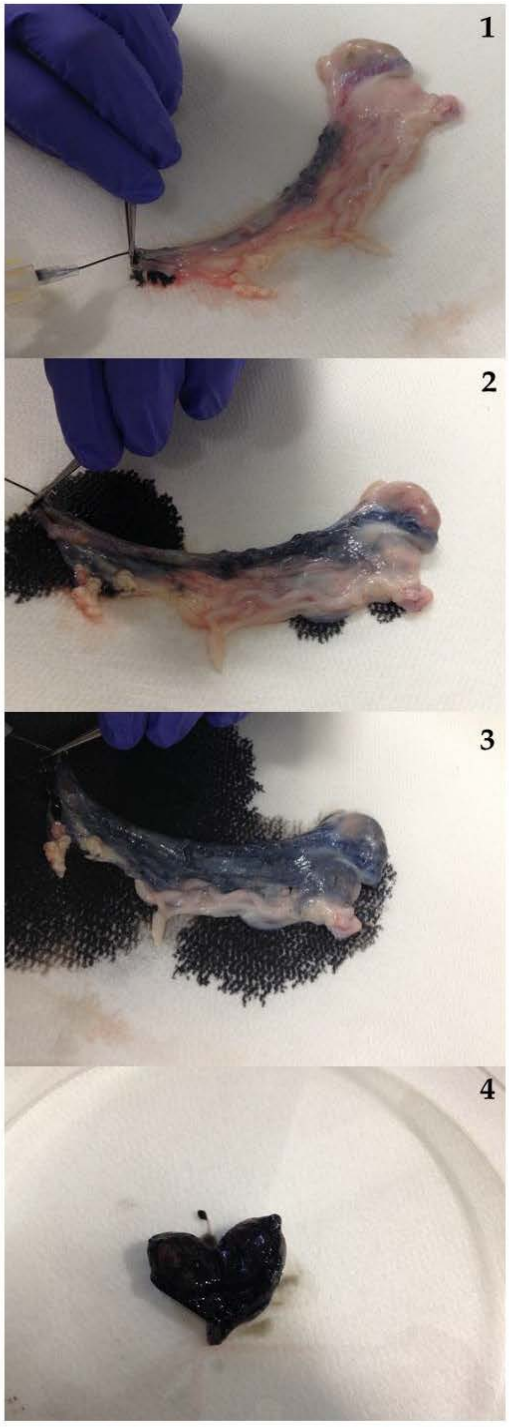


Figure 30. Perfusion of sheep ovary with a Indian Ink/heparin solution. (1) Beginning of perfusion; (2-3) during perfusion; (4) after perfusion: ovary after transversal dissection.

3.9 Western Blotting

Ovarian fragments (~2 mm^3) derived from five different experimental groups at Days 0 and 7 of follicles culture were homogenized individually, lysed and constitutive proteins were extracted using ReadyPrep™ Protein extraction Kit (Bio-Rad), following the manufacture instruction. Protein concentration was assessed with DC Protein Assay (Bio-Rad). Aliquots of 50 μg were prepared and resuspended in (1:1) 2x Laemmli sample buffer consisting of 4% (w/v) sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% (w/v) glycerol, g on the 0.004% bromophenol blue and 0.125 M Tris–HCl (pH 6.8). Samples were loaded and electrophoresed on 8 or 10 % SDS-polyacrilamide gels depending on the molecular weight of the target molecule. Proteins were then transferred onto 0.45-μm pore size nitrocellulose filters (Life Technologies) according to Towbin et al [184] using 0.5 A/cm^2. Equal sample loading and transfer efficiency were confirmed by staining of the membrane with Ponceau Red. The membrane was probed with the primary antibodies listed in Table 8, as described by Pennarossa et al. [185] and previously validated in sheep. Monoclonal anti-β-actin was used as a loading control. Protein bands were visualized by Western Breeze® chemiluminescent kit (Life Technologies).

ANTIBODY	Host species	Company	Catalog number	WB working dilution
HSF1	Goat	Santa Cruz	sc-8061	1:1000
HSP40	Rabbit	Abcam	ab69402	1:10000
HSP70	Mouse	Abcam	ab5439	1:5000
HSP90	Mouse	Abcam	ab13492	1:1000
β-Actin	Mouse	Sigma	a5441	1:1000

Table 8. List of antibodies used for western blot analysis.

3.10 TUNEL assay

DNA fragmentation was detected by *In situ* Cell Death Detection Kit, TMR red (Roche). Sections were placed on silanized slides (Bio-Optica), deparaffinazed (xilene 10 min, xilene:ethanol 5 min, absolute ethanol 10 min, 95% ethanol 5 min, 70% ethanol) and washed with PBS. Unmask of antigens was carried out using Proteinase K solution for 30 minutes at 37°C in humid chamber. Samples were than incubate with TDT TUNEL reaction mixture for 1 hour at 37°C in an humidified atmosphere in the dark. Positive controls were treated with DNAse I recombinant (50 U/ml; Roche) in 50 mM Tris-Hcl (pH 7.5), 1 mg/ml BSA for 10 minutes at 25°C before DNA end labeling. For negative control, TDT was omitted from the reaction mixture. Sections were washed three times in PBS, mounted

with VECTASHIELD mounting medium to prevent photobleaching (Vector Laboratories) and covered with microscopic cover-slip. TUNEL-positive cells were calculated as cells per area of ovarian tissue using ImageJ software as detailed in paragraph 3.11.

3.11 Image analysis

To quantify fluorescent signal, picture were taken with constant exposure parameters in order to be analyzed with the image analysis software Image J (http://rsb.info.nih.gov/ij/). Threshold adjustments were applied to generate a black and white image. In each case, marker expression was normalized by DAPI fluorescent. To study the stromal cells density, cell proliferation and TUNEL positive cells, a specific plugins for counting the number cells within an image was used (ITC Image-based Tool for Counting Nuclei). The macro initially removes the image background with a Gaussian blur. The image is then segmented with a thresholding algorithm to highlight areas occupied by the nuclei and remove regions in the background (black nuclei). After conversion in 8-bit, the nuclei diameters and the minimum distance between nuclei centers were set. The black and white mask image is then automatically processed to identify each individual nucleus and the output is a count.

3.12 Semiquantitative Polymerase Chain Reaction

Total RNA was extracted from the ovarian cortical tissue region using the TRIzol Reagent (Life Technologies) and reverse transcribed into cDNA in a total volume of 20 μl reaction mixture containing 1μl oligo(dT)12-18 (500 ng/μl). RT was performed with 200U Superscript II reverse transcriptase (Life Technologies) for 1hour at 42°C. cDNA amplifications were carried out in an automated thermal cycler (iCycler, Bio-Rad), using the appropriate conditions for Ki67 specific primers (forward 5'-atggggagtgagaaggaggt-3'; reverse 5'-tccaagttccccttgcatac-3'; Tm 59°C). Sheep DNA was used as positive control. The optimal cycle number at which the transcript was amplified exponentially was established running a linear cycle series, and the number of PCR cycles was kept within this range. An aliquot of each cDNA sample was amplified by PCR with β-actin gene-specific primers (forward primer: 5'-ccaaggccaaccgtgagaag-3'; reverse primer 5'-ccatctcctgcttcgaagtcc-3'; Tm 57°C). RT-PCR products were subjected to electrophoresis on a 2% agarose gel and the intensity of each band was assessed by densitometric analysis performed with the Quantity One software (Bio-Rad). The relative amount of Ki67 transcript was calculated by dividing the intensity of the Ki67 band by the intensity of the β-actin band.

3.13 *Ex vivo* culture of whole ovary

Fresh ovaries and ovaries frozen as entire organ with directional freezing, were cultured *ex vivo* for up to 4 days using a perfusion system (Figure 31).

This was a closed system where 100 ml of recirculating medium (Table 9) was pumped into the ovarian artery. Culture medium was pumped into the ovarian artery using a peristaltic pump and the flow rate through the ovary was maintained between 1 and 1.5 ml/min. Whole ovaries were cultured at 38.5°C for 2 or 4 days. Medium was refresh every day. After culture, ovaries were fixed with 10% formaldehyde.

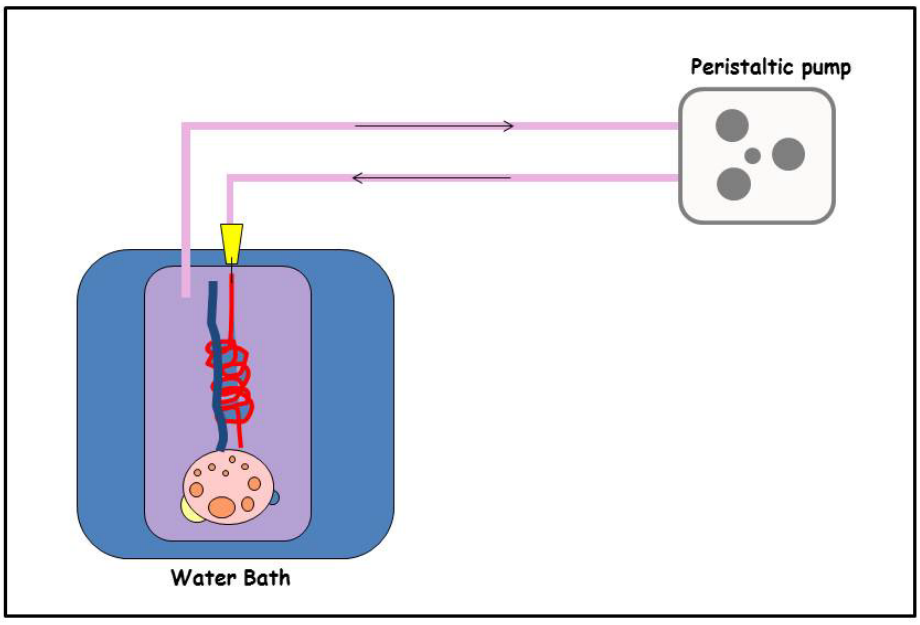


Figure 31. Schematic drawing of the *in vitro* perfusion apparatus.

Perfusion medium	
M199	
Bovine serum albumin	40 mg/ml
L- Glutamine	2 mM
Penicillin G	100 μg/ml
Streptomycin	100 μg/ml
Hepes	25 mM

Table 9. Composition of medium used for ovary *ex vivo* culture.

3.14 Statistical analysis

All experiments were repeated a minimum of three independent biological replicates. All data are reported as mean ± SEM. Mann-Whitney U (Statistical Package for the Social Sciences, SPSS 20; IBM) test was used to analyze vessel number and one-way analysis of variance followed by Turkey's post hoc tests (SPSS 20; IBM) were used for all the remaining experiments. Differences were considered statistically significant if $P<0.05$.

4. RESULTS

PART I: COMPARISON OF CONVENTIONAL AND DIRECTIONAL FREEZING ON SHEEP WHOLE OVARY

Morphologic assessment immediately after thawing

Immediately after thawing we analyzed ovarian follicles morphology and stomal cells density. The rate of intact follicles in the DF group (89%± 1.7%) was significantly higher than that in the CF group (61%±3.4%), and it was not different from that in fresh control tissue (96%± 1.5%) (Figure 32).

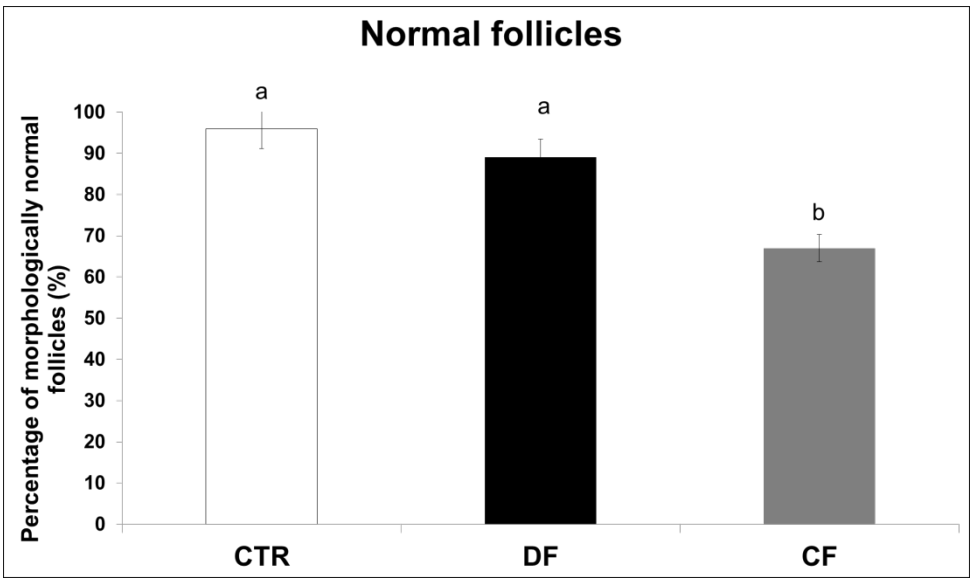


Figure 32. Overall distribution of morphologically normal follicles in control (CTR) and in thawed whole ovaries frozen with directional (DF) or with conventional freezing (CF). Values with different indices (a-b) are significantly different (P<0.05)

Quantitative assessment of stromal cell density indicated that DF ovaries had values (17,041 nuclei per mm^2) similar to those of fresh organs (17,833 nuclei per mm^2) but significantly higher than in CF ones (10,875 nuclei per mm^2) (Figures 33 and 34).

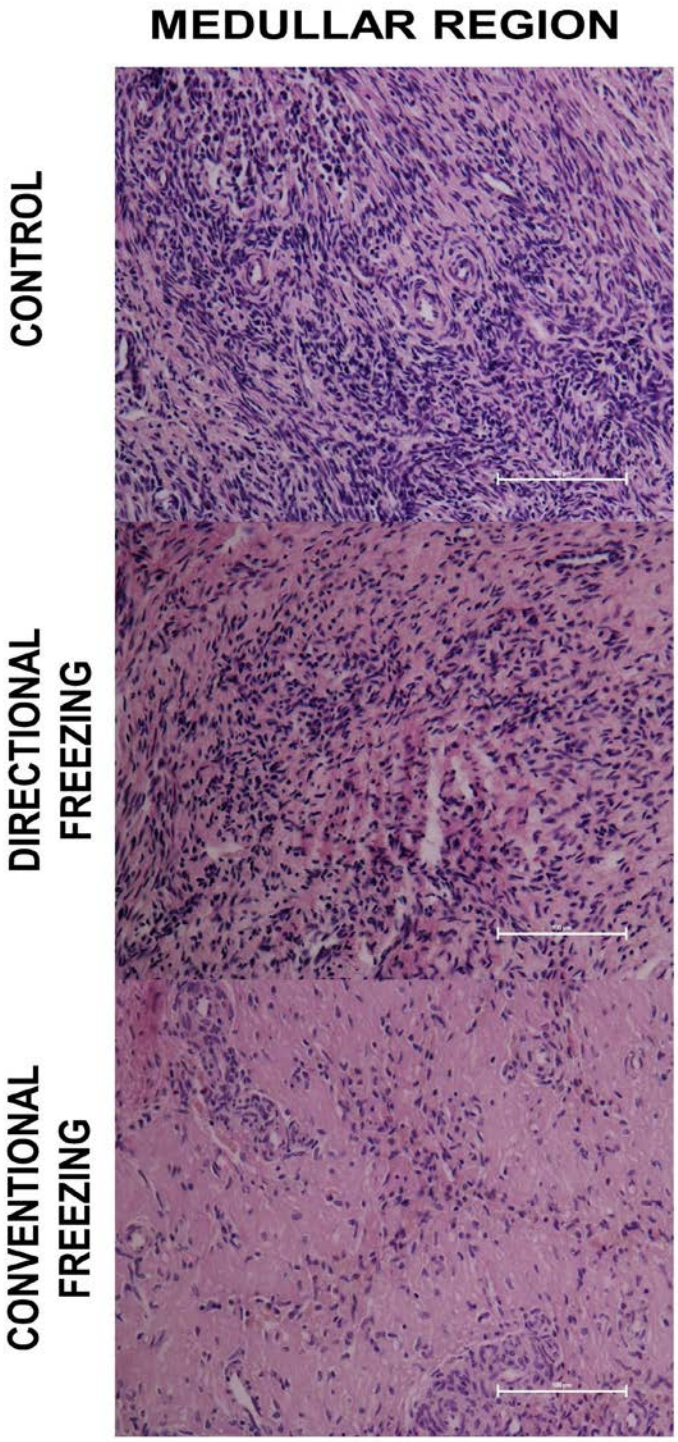


Figure 33. Representative pictures of the medullar region of the different experimental groups. Scale bars 100 μm.

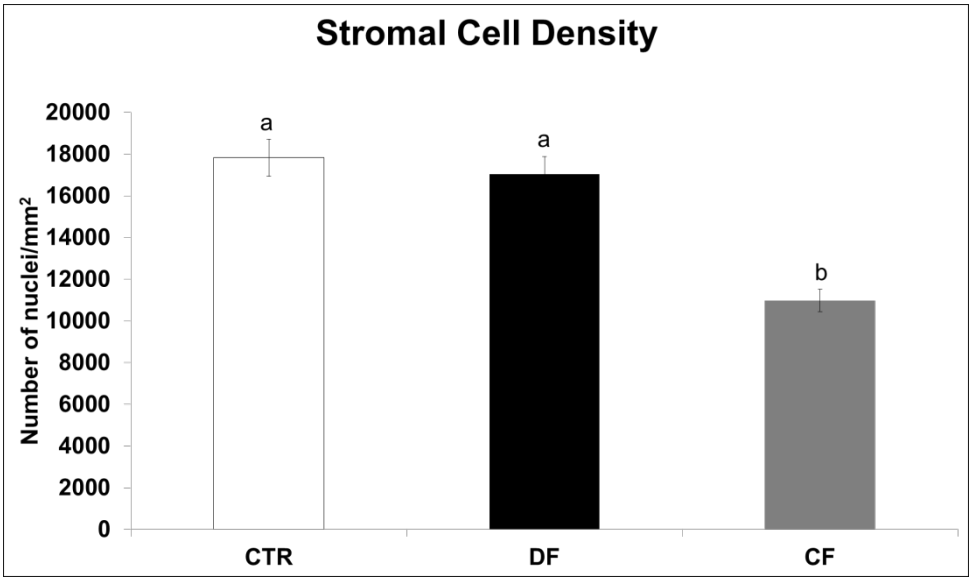


Figure 34. Quantitative assessment of density of stromal cells by image analysis. Values with different indices (a-b) are significantly different (P<0.05).

Vessels integrity was studied using Indian Ink. Indian Ink only was observed only in blood vessels and not in the surrounding tissue, indicating an optimal perfusion (Figure 35A). Whole ovaries frozen with directional freezing and fresh ovaries displayed the same rate of intact vessels in ovaries, both in medulla (stromal vessels) and in the cortex (perifollicular vessels) (Figure 35B). Conversely, ovaries frozen with conventional freezing displayed a significantly lower number of intact vessels (P=0.009). No significant differences between ovarian medulla and cortex vessels were observed, either in fresh or in cryopreserved samples (Figure 35B).

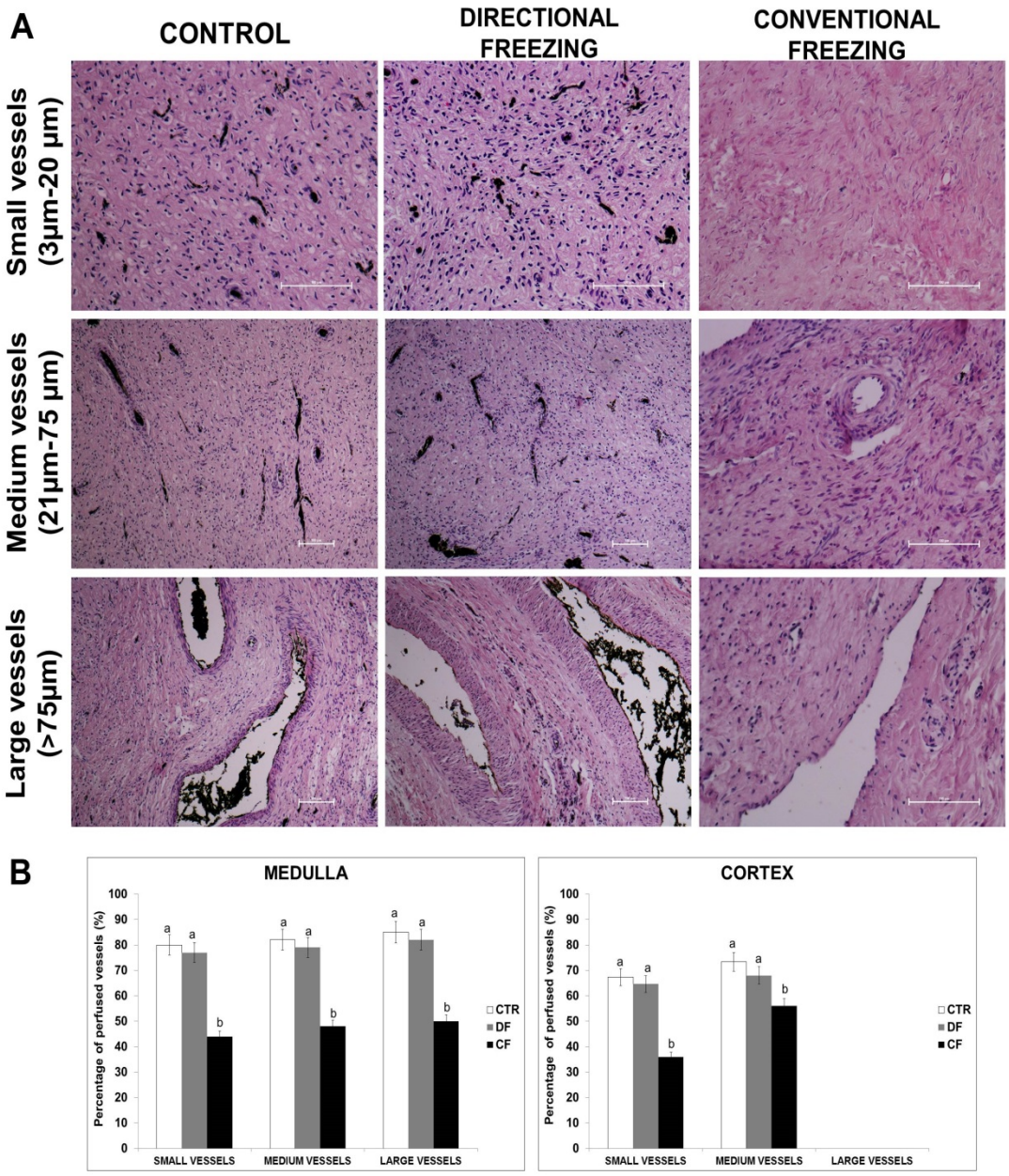


Figure 35. Representative pictures of blood vessels in the different experimental groups classified in three different categories (A) and their distribution in the medulla and cortex regions (B). Values with different indices (a-b) are significantly different (P<0.05). Scale bars =100 µm.

Functional assessment

After 7 days of *in vitro* culture the rate of primordial follicles that developed into the primary stage was higher in whole ovaries frozen with directional freezing than in ovaries cryopreserved with conventional freezing. The developmental competence of DF ovarian fragments was the same as in fresh control tissue, whereas CF fragments showed a significant decrease of the overall follicle number (P=0.038) (Figure 36).

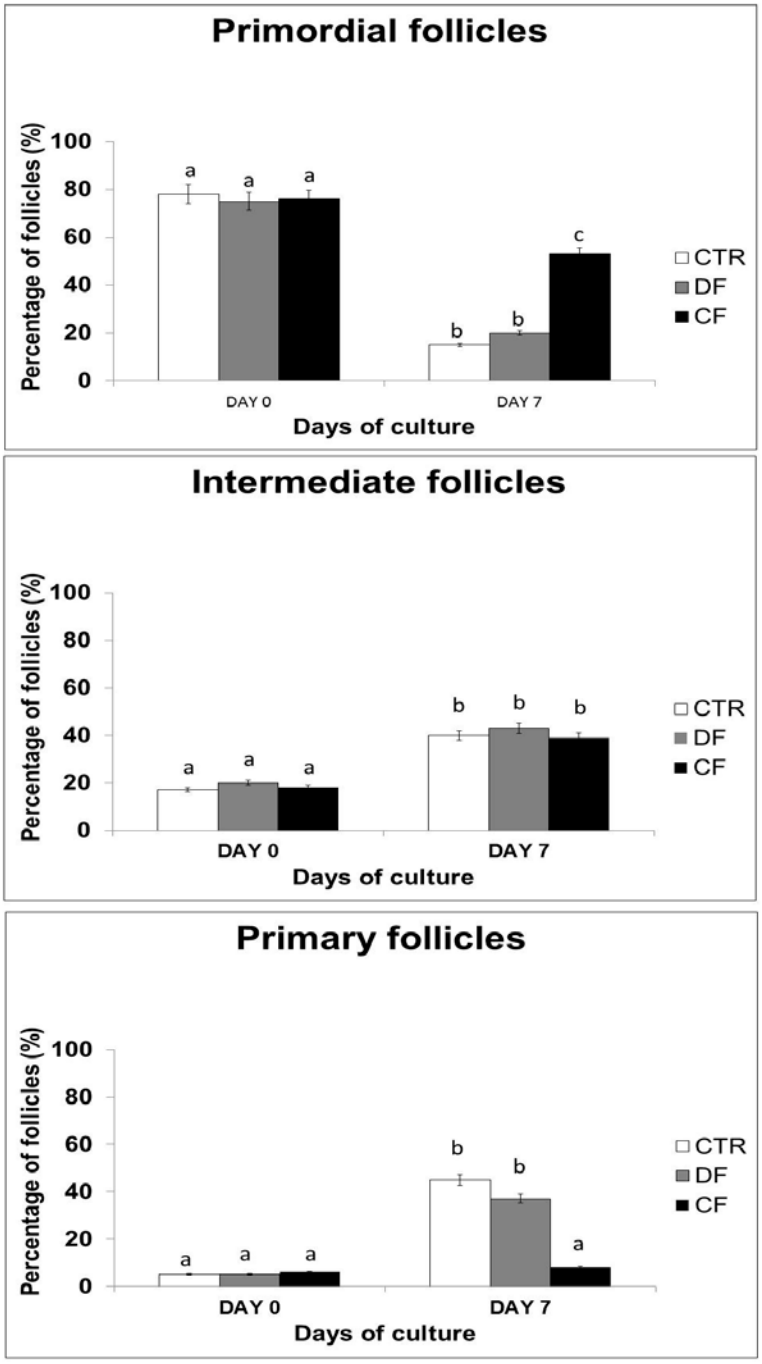


Figure 36. Distribution of primordial, intermediate and primary follicles at the beginning (DAY 0) and the end (DAY 7) of the culture period. Values with different indices (a-c) are significantly different ($p<0.05$).

Consistent with their developmental capacity, the rate of morphologically normal primordial, intermediate and primary follicles in the directional freezing group was not different from that in the fresh group upon thawing. By contrast, the conventional freezing group displayed a higher rate ($P<0.05$) of degenerated follicles and oocyte with pyknotic nuclei. Seven days later a significant reduction in the percentage of histologic normal follicles was found in the conventional freezing group (24% ± 5.7%) compared with directional freezing (77% ± 2.8%) and fresh groups (85% ± 2.43%) (Figure 37).

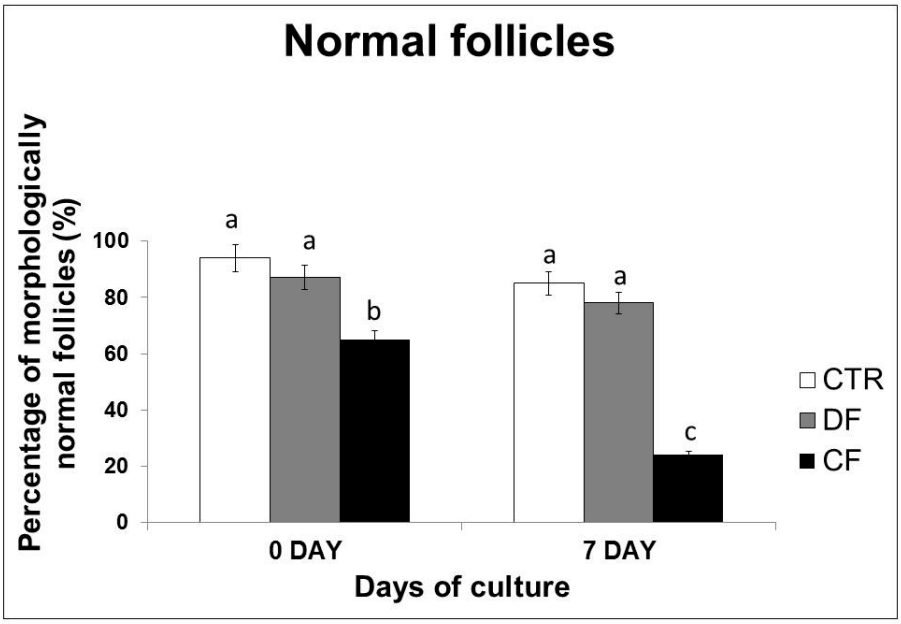


Figure 37. Overall distribution of morphologically intact follicles during culture in the three experimental groups. Values with different indices (a-c) are significantly different ($P<0.05$).

Cell proliferation

Differences in follicles viability were consistent with differences in cell proliferation rate, obtained through the analysis of the expression of Ki67. After 7 days of culture abundance of Ki67 transcripts significantly increased in directional freezing group and in fresh tissue ($P<0.05$). By contrast, a reduction in Ki67 messenger RNA level was detected in conventional freezing tissue ($P<0.05$) (Figure 38A). Ki67 immunofluorescence results were in agreement with the observed transcript levels (Figure 38B). The percentage of proliferation was comparable among the different groups at the beginning of the culture (DAY 0). At the end of the culture (DAY 7) an average of only 9% ± 0.85% of cells expressed the Ki67 antigen in conventional freezing tissue, compared with 26% ± 0.64% and 30% ± 0.73% for directional freezing and fresh groups respectively (Figure 38B).

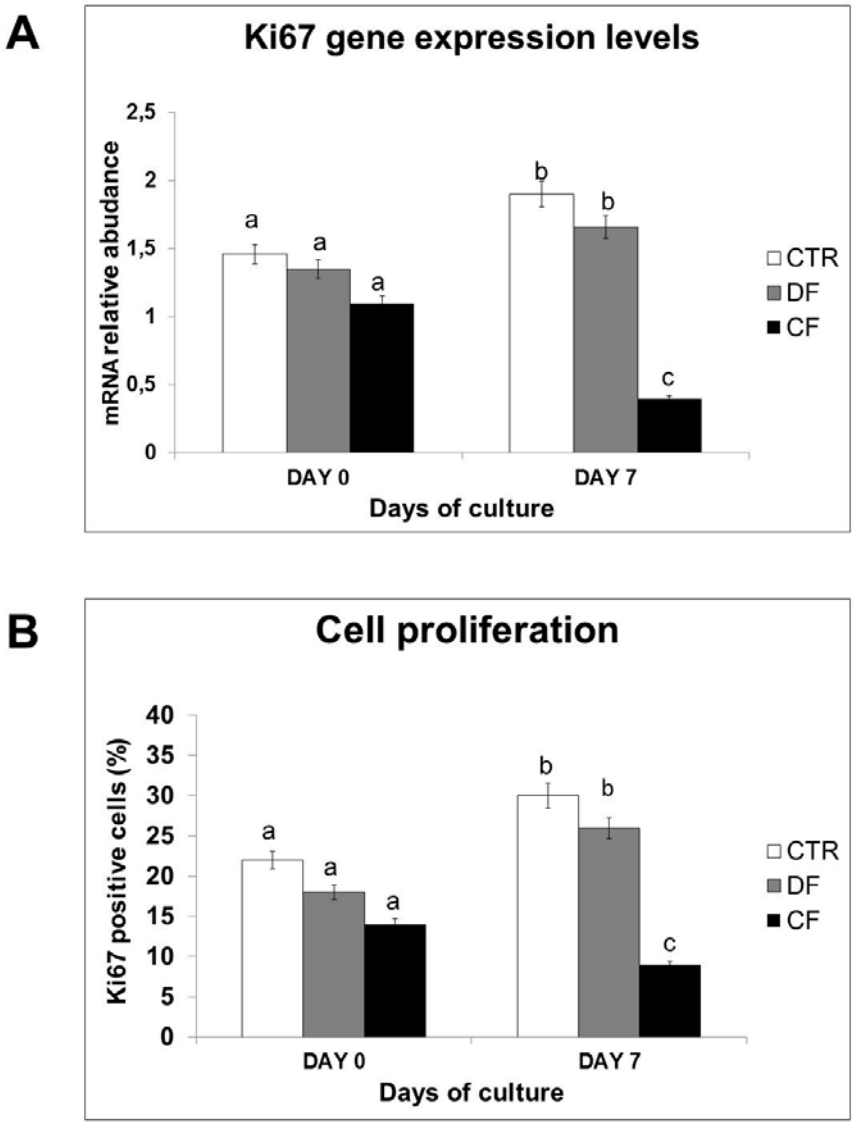


Figure 38. Quantitative evaluation of cell proliferation assessed both as Ki67 messenger RNA level (A) and as percentage of immunopositive cells for encoded protein (B). Values with differences (a-c) are significantly different (P<0.05).

DNA double-strand breaks

We investigated the occurrence of DNA double-strand breaks by analysis of the expression of γ-H2AX, a marker of DNA damage, and RAD51, a marker of DNA repair. As expected, virtually no γ-H2AX and RAD51 positive signals were detected in fresh tissue (Figures 39 and 42).

Clear signals of DNA damage were observed in directional freezing ovaries at the beginning of culture, together with a weak staining for DNA repair. However, the DNA damage significantly decreased after 7 days of culture concomitantly with a sharp increase of DNA repair, as indicated by a clear immune signal of RAD51 (Figures 40 and 42).

In contrast, a very strong immune staining for γ-H2AX was observed in conventional freezing ovaries immediately after thawing, indicating widespread by RAD51 labeling either at the beginning or at the end of culture, suggesting a lack of DNA repairing ability (Figures 41 and 42).

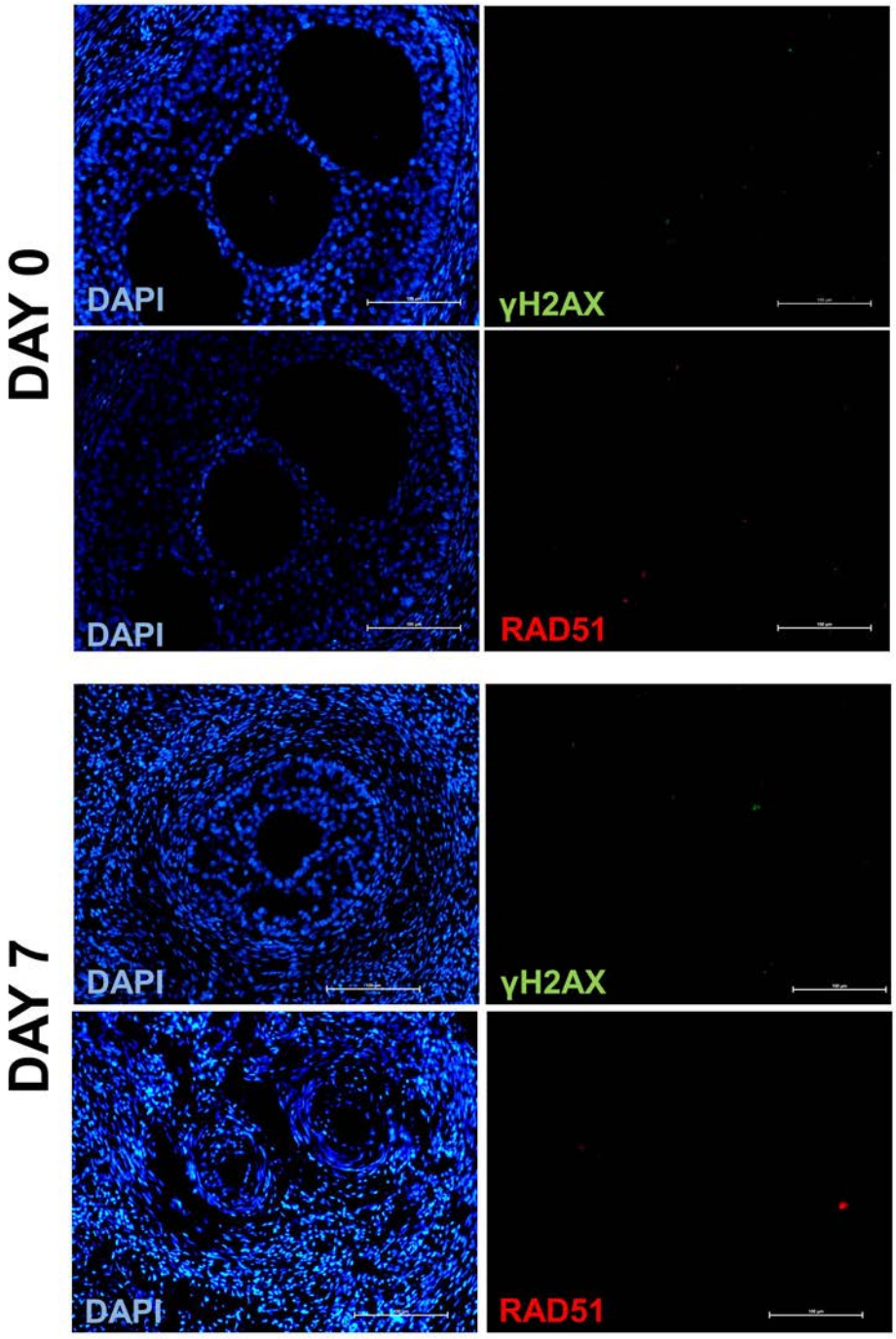


Figure 39. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in fresh tissue. Scale bars = 100μm.

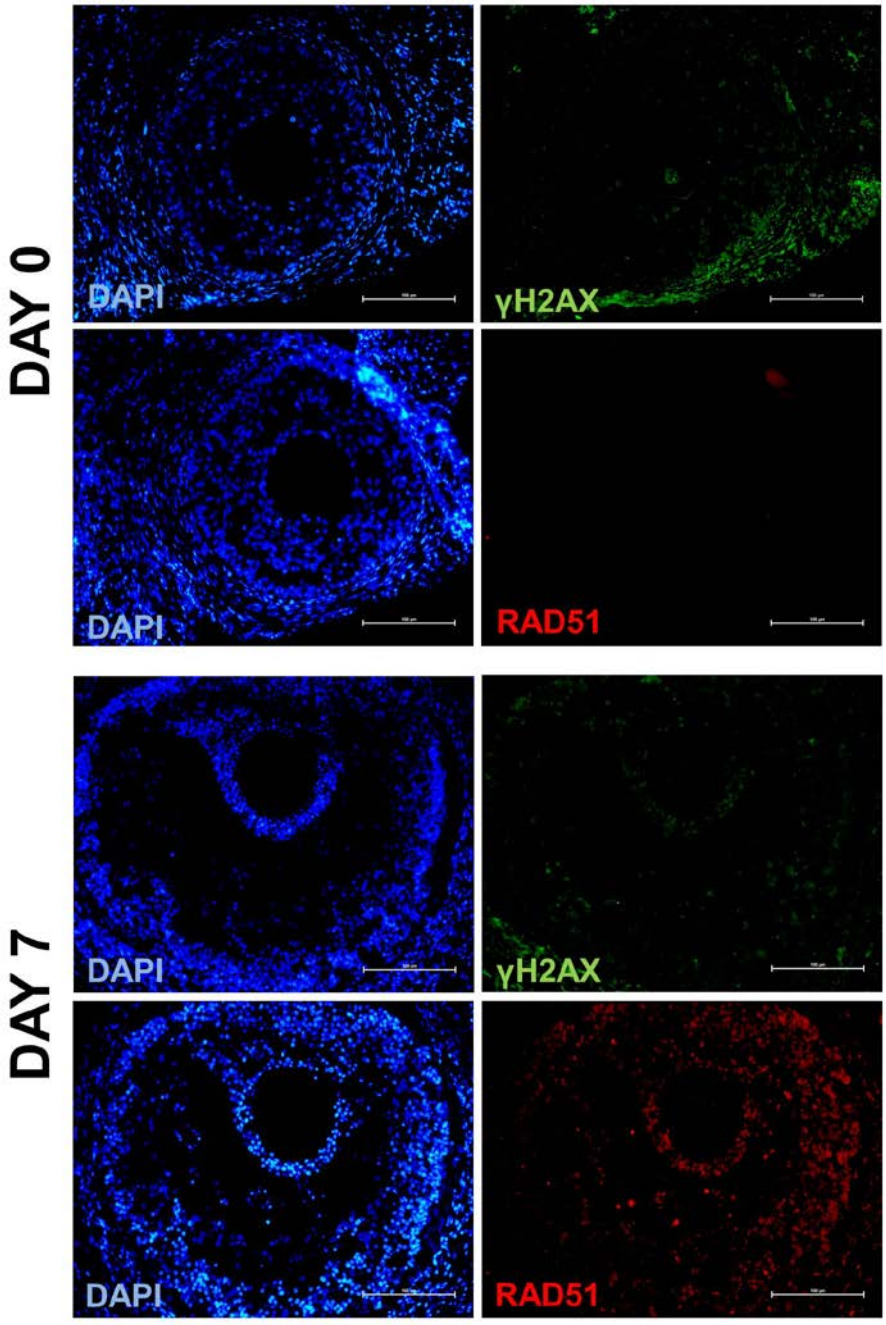


Figure 40. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in directional freezing tissue. Scale bars = 100μm.

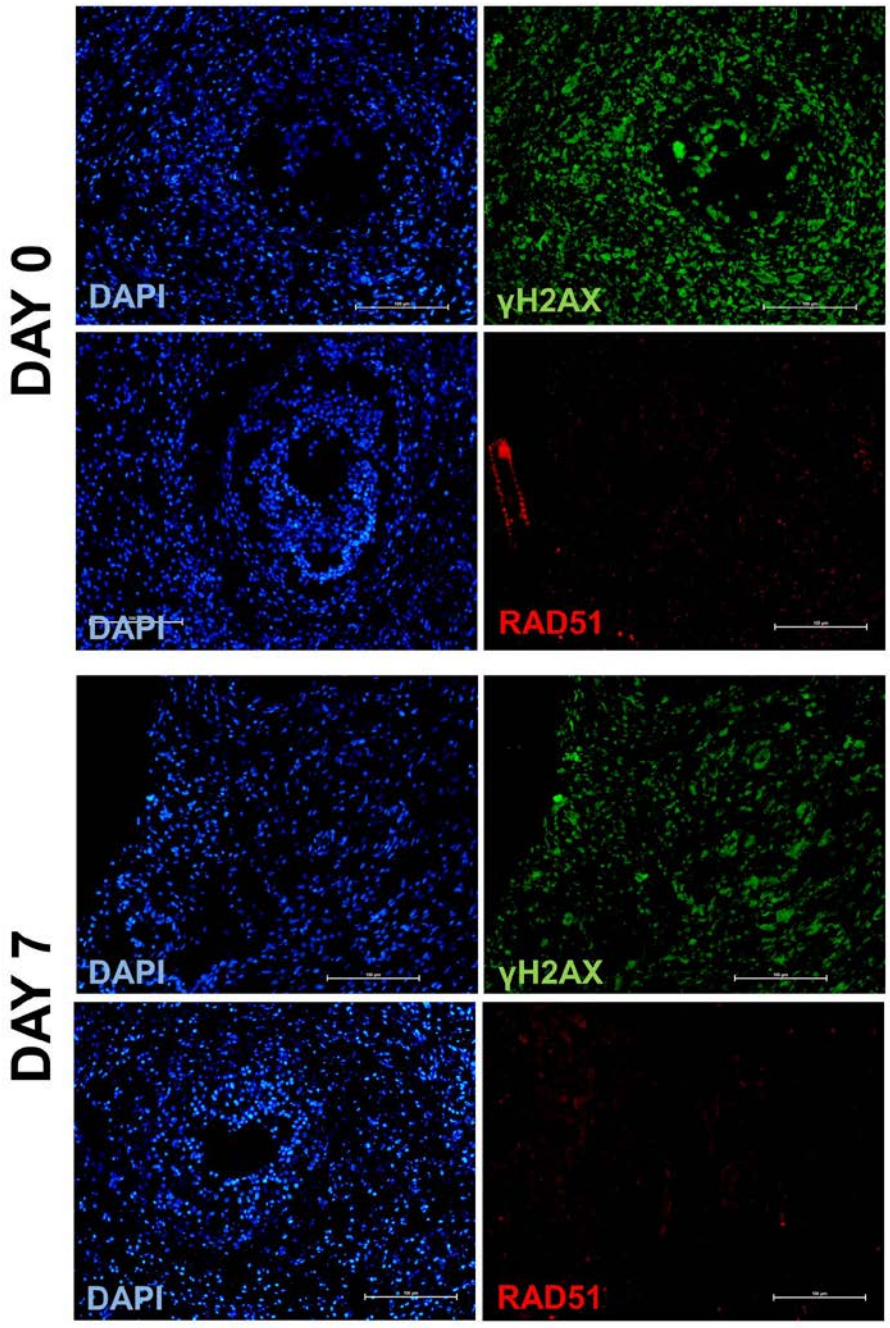


Figure 41. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in conventional freezing tissue. Scale bars = 100μm.

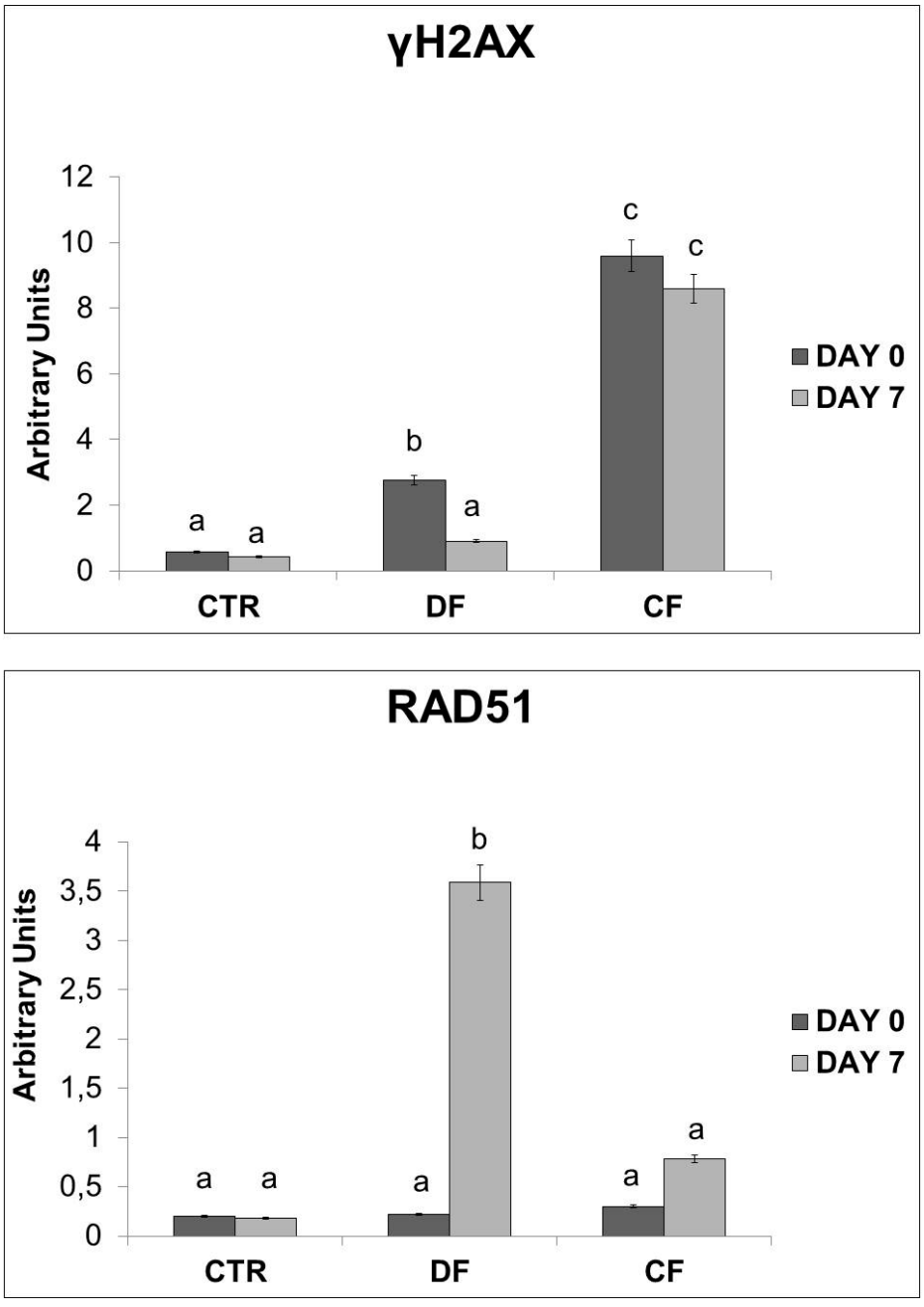


Figure 42. Quantitative assessment of γH2AX and RAD51 by image analysis. Values with different indices (a-c) are significantly different ($p<0.05$).

PART II: A 2X2 FACTORIAL DESIGN: COMPARISON BETWEEN OVARIAN CORTICAL FRAGMENTS AND WHOLE OVARIES FROZEN WITH CONVENTIONAL OR DIRECTIONAL FREEZING

All experimental groups (which included: 1. Directional freezing of whole ovary; 2. Directional freezing of ovarian fragments; 3. Conventional freezing of whole ovary; 4. Conventional freezing of ovarian fragments) were compared with each other and with a fresh control, shortly after thawing and after 7 days of *in vitro* culture.

Morphological assessment of cryopreserved samples

Follicle morphology was investigated immediately after thawing and cryoprotectant removal. The percentage of morphologically normal follicles was inversely related to the stage of development, in all groups including fresh control. Where compared with conventional freezing, there were more intact follicles of all developmental stages following directional freezing. Ovarian fragments showed a higher rate of normal morphology compared with whole ovaries when frozen with conventional freezing. Interestingly, the opposite situation was observed when whole ovaries and ovarian fragments were preserved with directional freezing (Table 10).

Morphologically normal follicles (%)					
	Follicle Type				
	Primordial	**Intermediate**	**Primary**	**Secondary**	**All follicles**
CTR	97.5± 4.5 [a] (n=600)*	91.2± 4.34[a] (n=350)*	88.40±4.12[a] (n=300)*	71.5±4.44 [a] (n= 60)*	92.54± 4.36 [a] (n= 1310)*
DFwo	94.8±4.15[a] (n=600)*	89.28± 4.29[a] (n=350)*	85.23±3.95[a] (n=300)*	68.62±3.34 [a] (n=60)*	89.93± 4.10 [a] (n= 1310)*
DFof	88.7±4.12[b] (n=300)*	80.17±4.30[b] (n=150)*	72.91±3.68[b] (n=100)*	53.25±3.21[b] (n= 20)*	82.24± 4.82 [b] (n= 570)*
CFwo	78.4±3.97[c] (n=600)*	58.10±3.32[c] (n=350)*	43.5±3.21[c] (n=300)*	23.4±1.92[c] (n=60)*	62.46± 3.52[c] (n=1310)*
CFof	84.40±4.03[d] (n=300)*	69.72±3.27[d] (n=150)*	64.20±3.34[d] (n=100)*	42.70±2.76[d] (n=20)*	75.53± 3.52[d] (n= 570)*

Table 10. Percentage (mean±s.e.m) of morphologically normal primordial, intermediate, primary and secondary follicles in fresh tissue (CTR) and in experimental groups immediately after thawing. *number of evaluated follicles. [a,b,c] Different superscripts in the same column indicate a significant difference amongst experimental groups for the same follicle type (P<0.05).

In vitro follicles development

At the end of the culture, the proportion of primordial follicles which developed to the primary stage both in whole ovaries (53% ± 1.7%) and in ovarian fragments (44% ± 1.8%) cryopreserved with directional freezing was significantly higher than with conventional frozen whole ovaries (6% ± 0.5%, P=0.001) or fragments (32% ± 1.5%, P=0.004).

The percentage of developing follicles in whole ovaries was higher than in ovarian fragments when frozen with directional freezing but lower when frozen with conventional freezing. Finally, whole ovaries frozen with directional freezing had a rate of growing follicles not significantly different from fresh ovaries (55% ± 1.9%, P=0.106) (Figure 43).

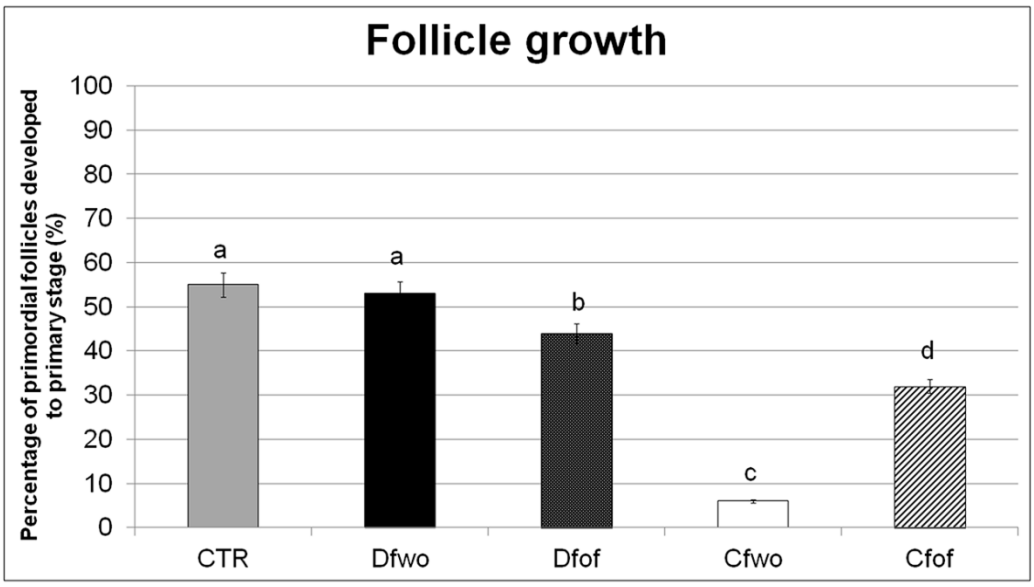


Figure 43. Percentage (mean ± s.e.m) of primordial follicles developed in the primary stage after 7 days of culture. Values with different indices (a-d) are significantly different (P<0.005). CTR= fresh control (n=280); DFwo= directional freezing whole ovary (n=280); DFof= directional freezing ovarian fragments (n=260); CFwo= conventional freezing whole ovary (n= 230); CFof= conventional freezing ovarian fragments (n=250).

Cell proliferation and apoptosis

Differences in developmental competence of primordial follicles were consistent with differences in cell proliferation rate as examined by the expression of Ki67. The percentage of proliferating cells was comparable among the different groups at the beginning of culture (DAY 0) (Figure 44). After 7 days of culture, Ki67-positive cells in fresh tissue (30% ± 0.78%) and in whole ovaries frozen with directional freezing (28% ± 0.73%) were the highest of all groups. The rate of cells expressing Ki67 in ovarian fragments frozen with directional freezing (23% ± 0.81%) was significantly higher than those in ovarian fragments frozen with conventional freezing (20% ± 0.79%, P=0.03). An average of only 9% ± 0.85% of Ki67-positive cells was found in whole ovaries frozen with conventional freezing (Figure 44).

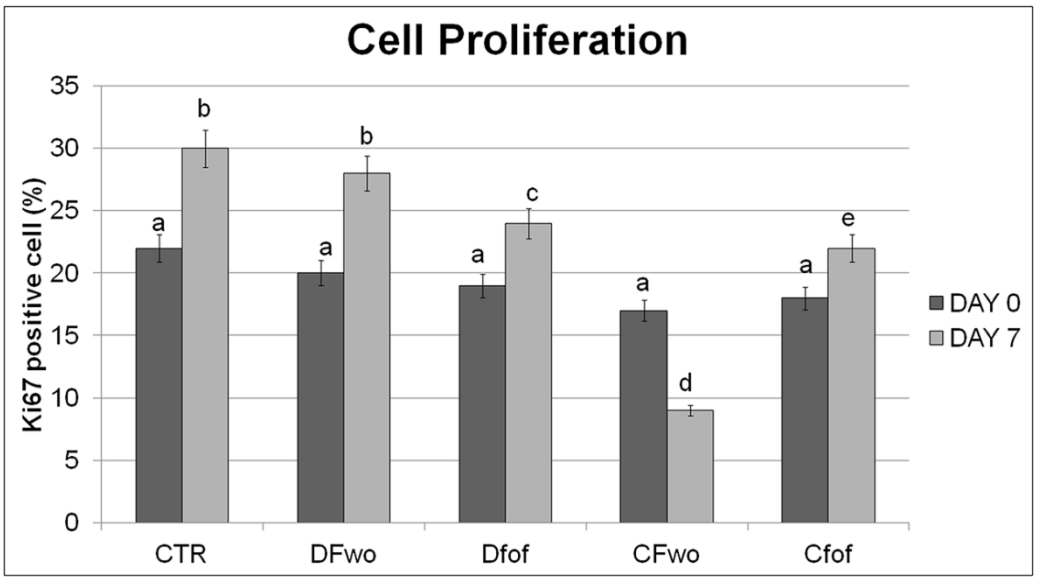


Figure 44. Percentage of immunofluorescence positive cells for Ki67 at the beginning (DAY 0) and the end (DAY 7) of *in vitro* culture. Values with different indices (a-e) are significantly different (P<0.05). n=2360 ± 340 nuclei counted for each section.

TUNEL-positive cells were detected both ad Days 0 and 7 of culture in all experimental groups. Interestingly, apoptotic level were not related to cell proliferation. Immediately after thawing and the removal of cryoprotectant, the number of apoptotic cells per mm^2 was low and comparable among all groups (Figure 45). The TUNEL assay showed that the number of positive cells after 7 days of culture was comparable for all experimental groups except in whole ovaries frozen with conventional freezing, where a significantly higher percentage of TUNEL- positive cells was visible after 7 days (29.34 ± 1.13, P=0.002) (Figure 45).

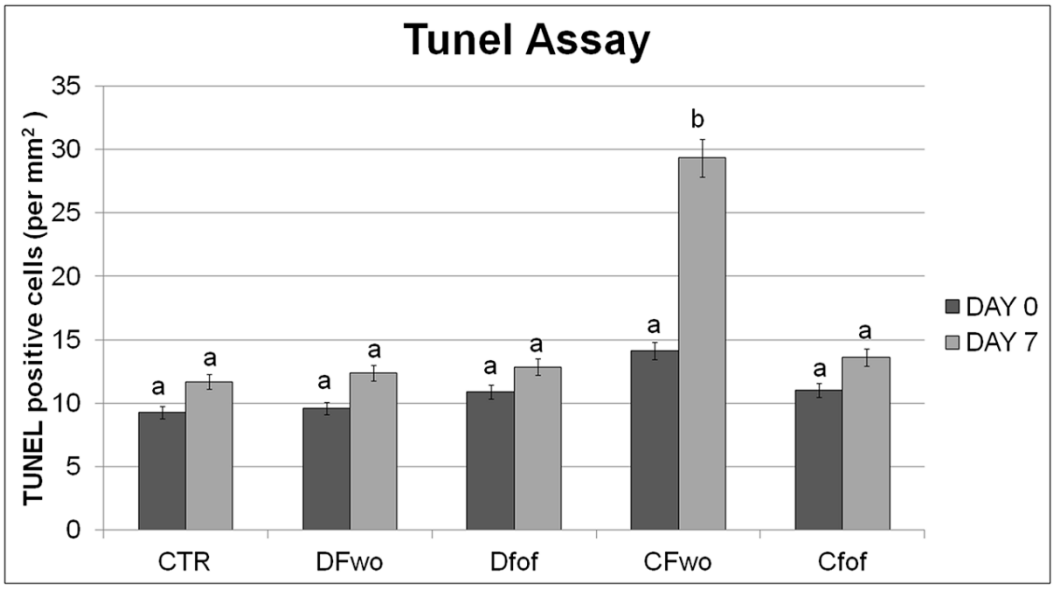


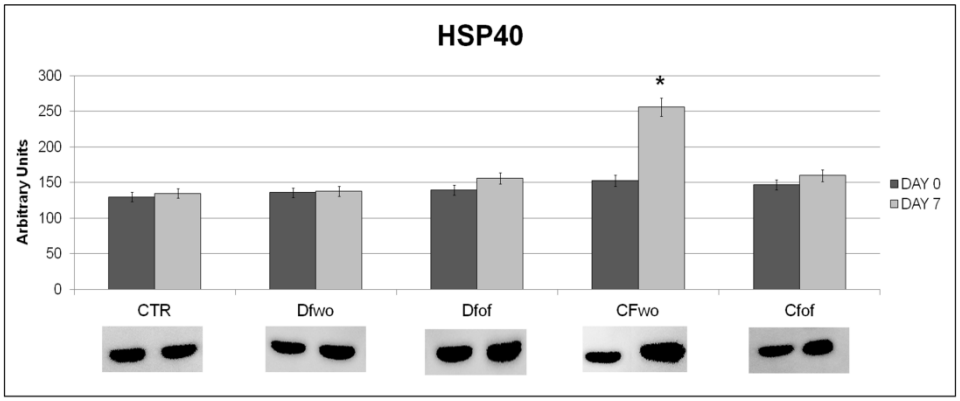
Figure 45. Number of TUNEL-positive cells per mm² at the beginning (DAY 0) and the end (DAY 7) of *in vitro* culture. Values with different indices (a-e) are significantly different (P<0.05). n=2360 ± 340 nuclei counted for each section.

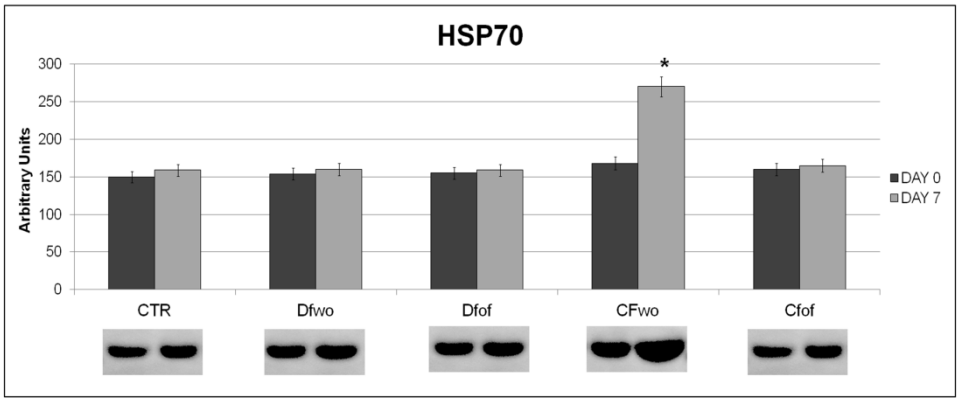
Expression level of Heat Shock Protein

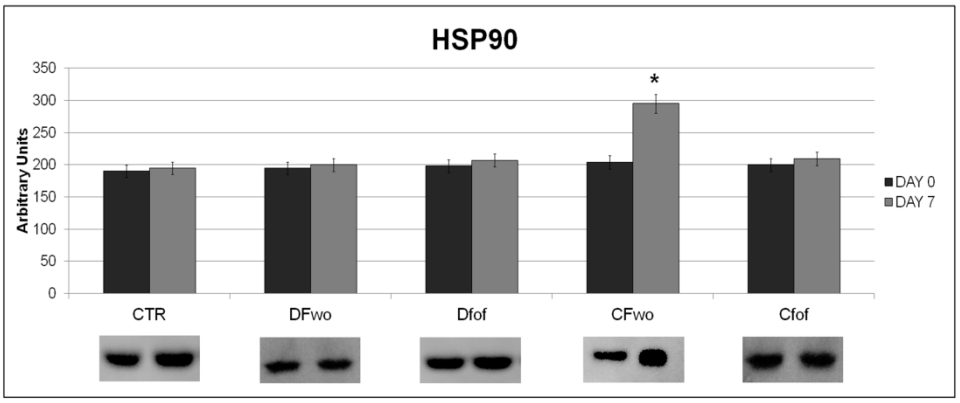
The effect of heat stress response after cryopreservation was examined by quantititative analysis of heat shock protein 40 (HSP40), heat shock protein 70 (HSP70), heat shock protein 90 (HSP90) and heat shock transcription factor 1 (HSF1) proteins at the end of the warming and cryoprotectant removal steps and after 7 days of *in vitro* culture.

Changes were only observed in whole ovaries frozen with conventional freezing after 7 days of culture, where the protein levels were significantly higher than on Day 0 (Figure 46), indicating

that conventional freezing applied to the whole ovary induced the activation of protein involved in stress-response pathways. There were no differences within and between the other groups (Figure 46).


HSP40
Arbitrary Units
300
250
200
150
100
50
0
*
CTR
Dfwo
Dfof
CFwo
Cfof
DAY 0
DAY 7


HSP70
Arbitrary Units
300
250
200
150
100
50
0
*
CTR
Dfwo
Dfof
CFwo
Cfof
DAY 0
DAY 7


HSP90
Arbitrary Units
350
300
250
200
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50
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CTR
DFwo
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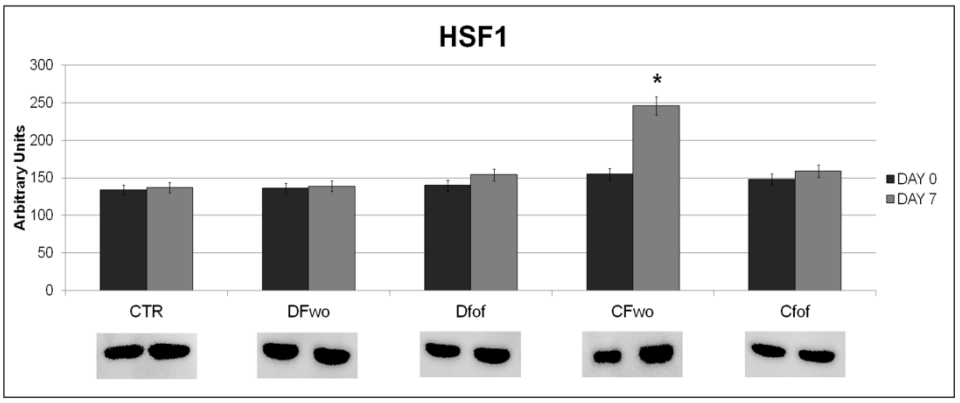
Cfof
DAY 0
DAY 7


HSF1
Arbitrary Units
300
250
200
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*
CTR
DFwo
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CFwo
Cfof
DAY 0
DAY 7

Figure 46. Western blots and densytometric analysis of heat shock proteins in sheep ovarian proteins extract from all experimental groups at the beginning (DAY 0) and the end (DAY 7) of culture. Values are expressed as mean ± s.e.m of three replicated measured in arbitrary units. * P<0.05. n= 10 fragments (2x2x1mm) for each replicate.

DNA damage and repair

The expression of a marker of DNA damage, γ-H2AX and a marker of DNA repair, RAD51, were analyzed immediately after thawing and after 7 days of culture. No γ-H2AX and RAD51 signals were detected in fresh tissue (Figures 47 and 52). A the beginning of culture (Day 0), a clear signals of DNA damage, in the form of γ-H2AX foci, were observed in whole ovaries and cortical fragments frozen with directional freezing (Figures 48 and 49) and in cortical fragments frozen with conventional freezing (Figure 51), together with a weak staining of DNA repair. After 7 days of culture, DNA damage was significantly decreased while DNA repair activity increased, as indicated by a clear RAD51 signal. A very strong staining for γ-H2AX was observed in conventional freezing whole ovary group at Day 0 (Figure 50) indicating widespread DNA damage but RAD51 labeling was low at that time, suggesting a lack of DNA repair.

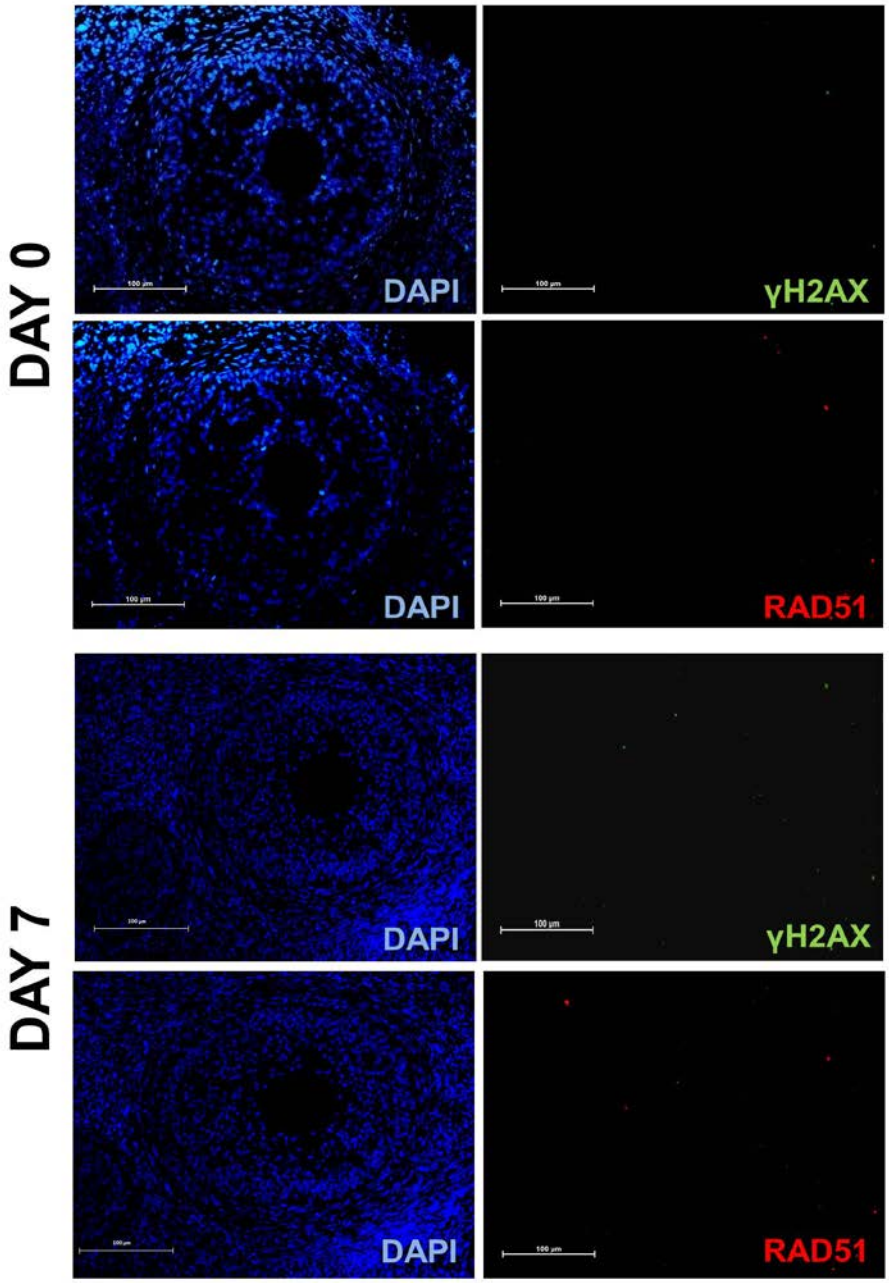

Figure 47. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in fresh ovaries. Scale bars = 100μm.

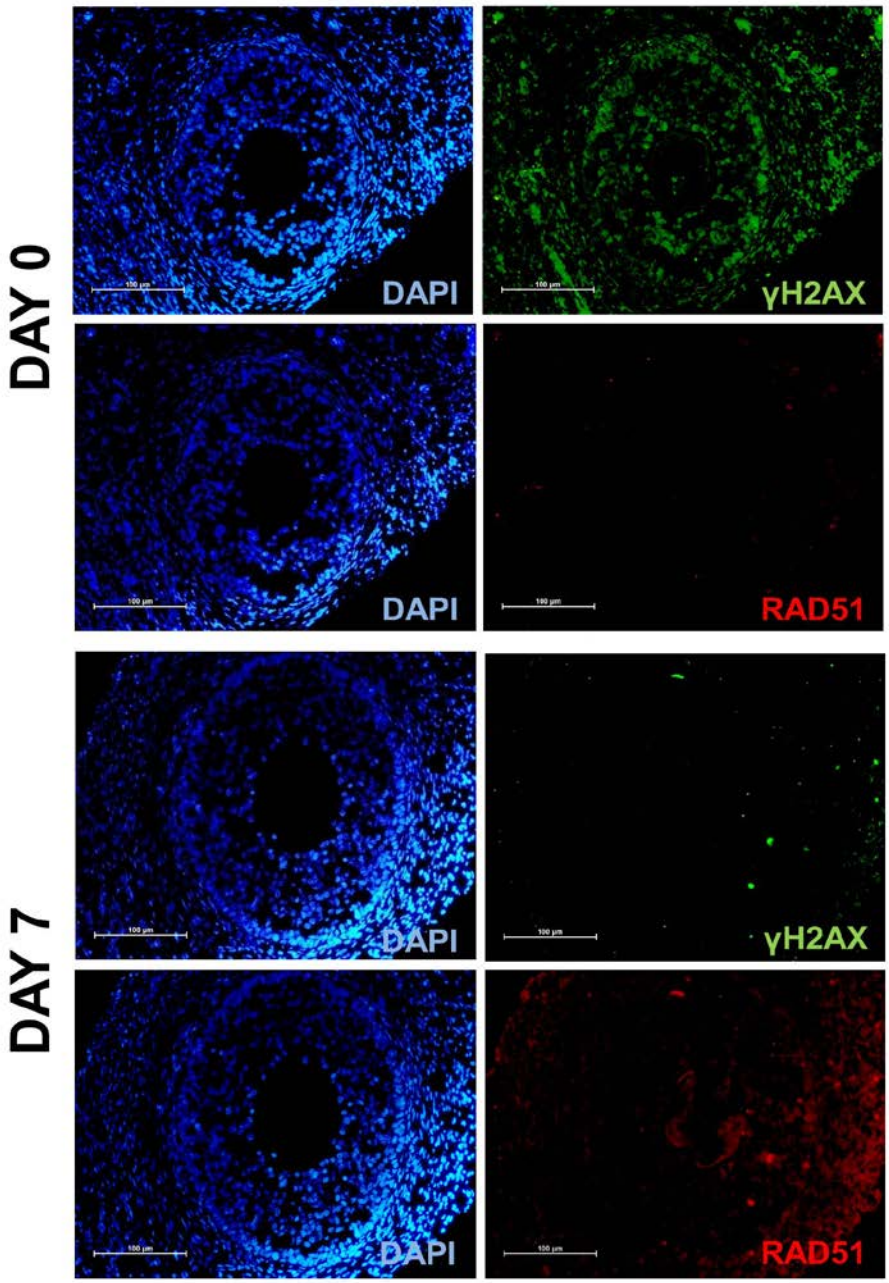

Figure 48. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in whole ovaries frozen with directional freezing. Scale bars = 100μm.

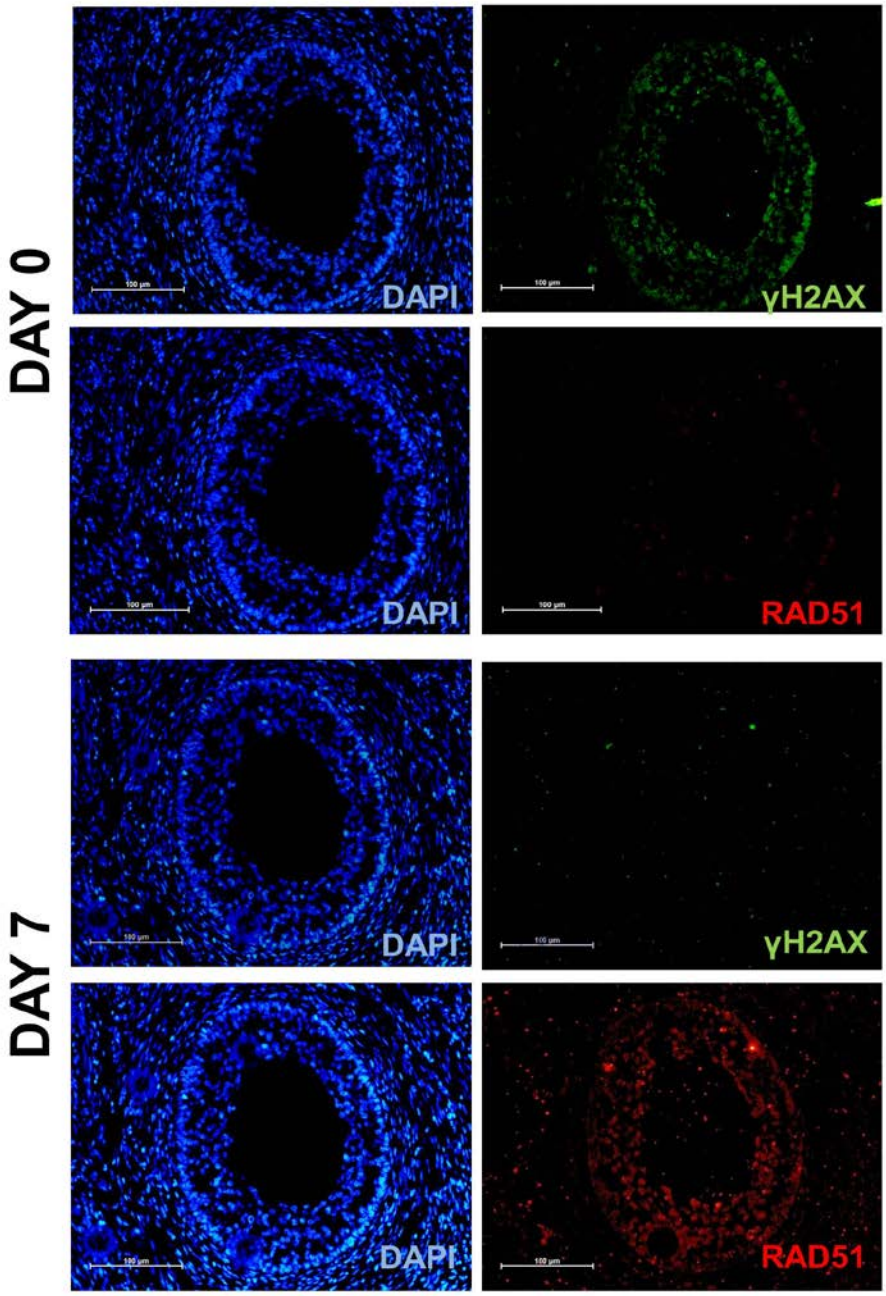

Figure 49. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in ovarian cortical fragments frozen with directional freezing. Scale bars = 100μm.

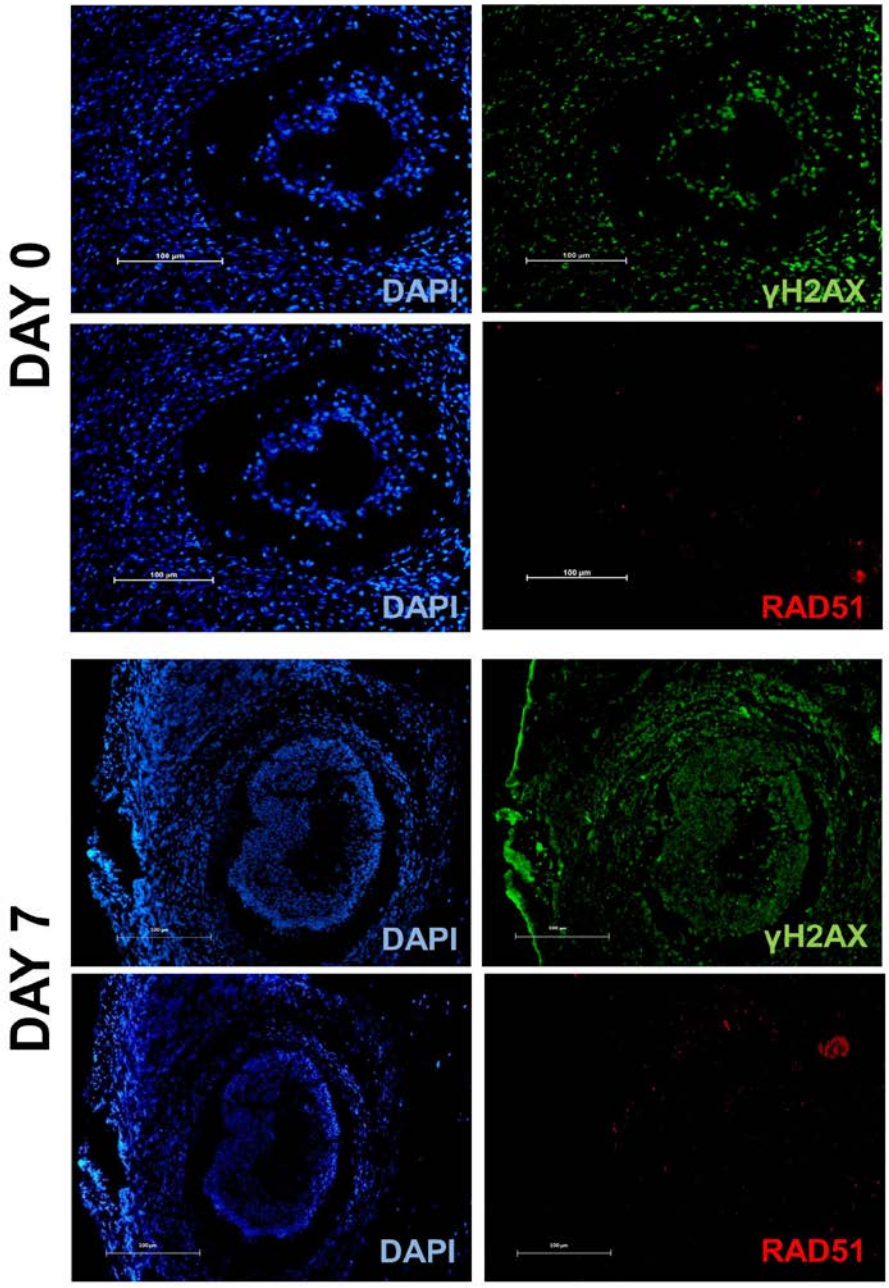

Figure 50. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in whole ovaries frozen with conventional freezing. Scale bars = 100μm.

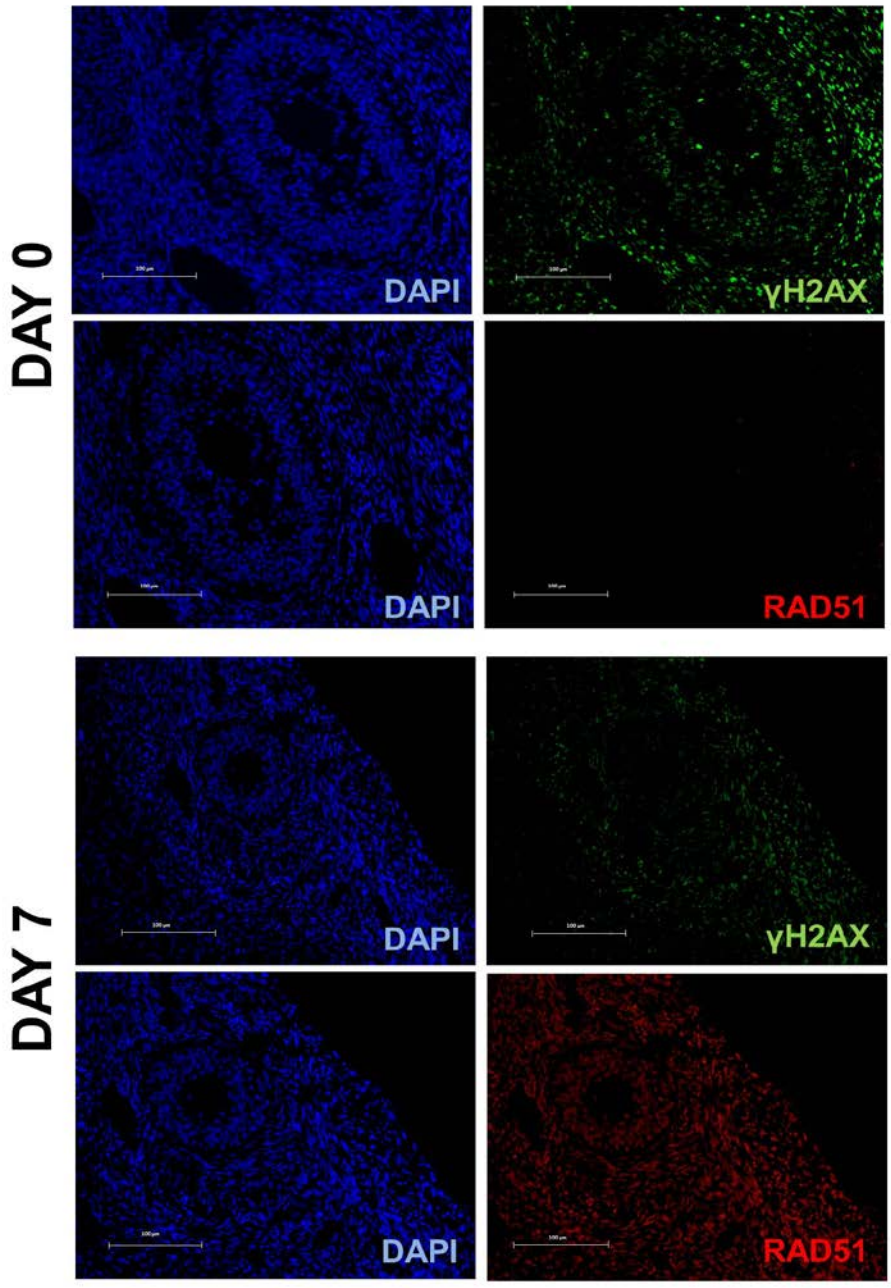

Figure 51. Representative pictures of positive cells for γ-H2AX (marker of DNA breaks; GREEN) and RAD51 (marker of repair ability; RED) in ovarian cortical fragments frozen with conventional freezing. Scale bars = 100μm.

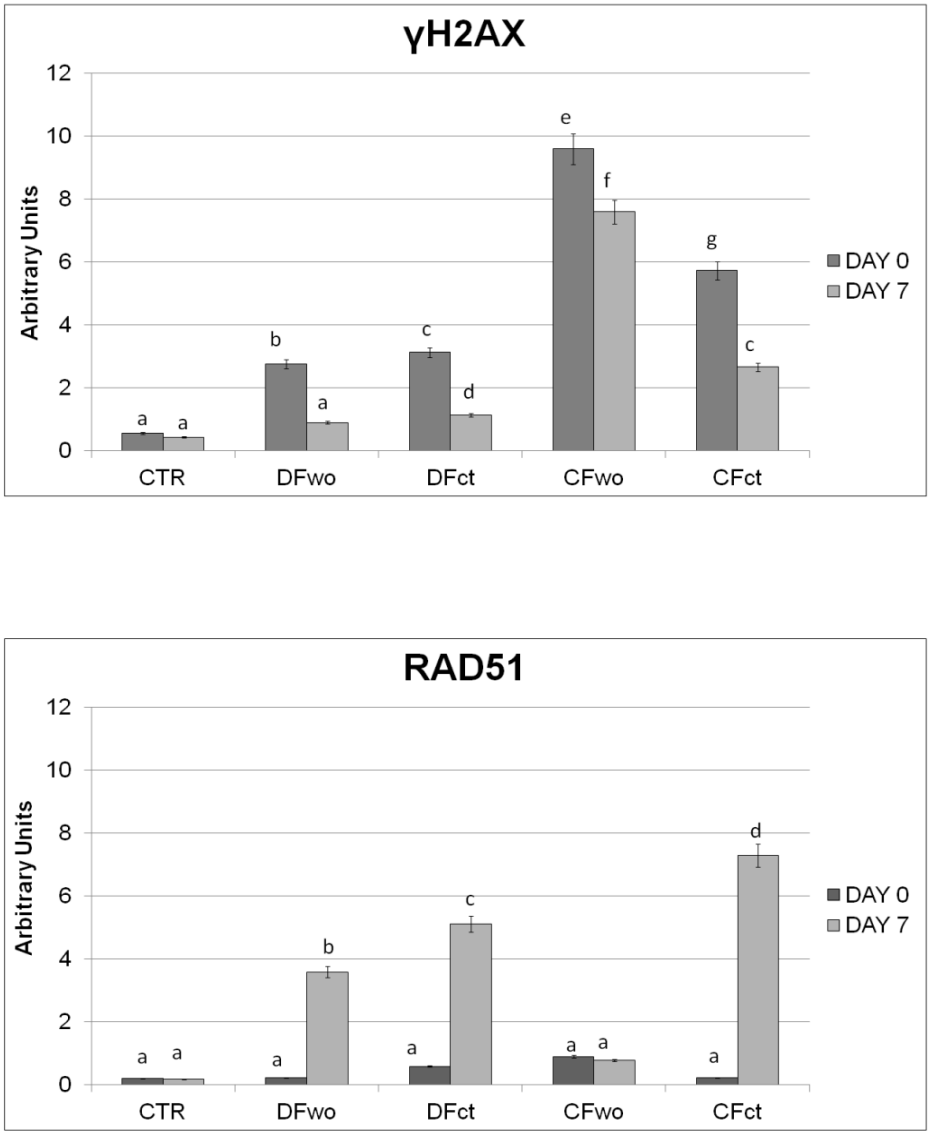

Figure 52. Quantitative assessment of γ-H2AX and RAD51 by image analysis. Values with different indices (a-g) are significantly different ($p<0.05$).

PART III: E*X VIVO* CULTURE OF WHOLE OVARY

Ovarian morphology

Follicle morphology was investigated after 2 and 4 days of *ex vivo* culture. The percentage of morphologically normal follicles in fresh and frozen ovaries cultured for 2 days (89% ± 2.9% and 87% ±2.4% respectively, p= 0.106) was not significantly different than that in un-cultured ovaries (Day 0) (95% ± 2.9% and 93%± 2,6% respectively, p=0.093). However, after 4 days of culture, a reduction (p=0.048) of intact follicles was observed in both fresh and frozen ovaries (79% ± 2.7% and 75% ± 2.1% respectively, p=0.095) (Figure 53).

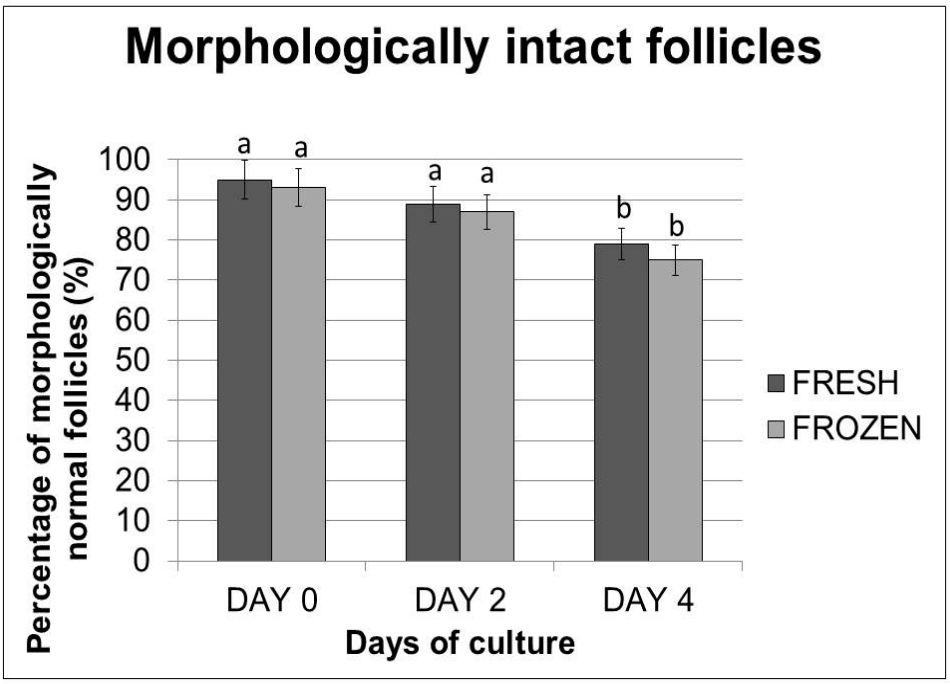

Figure 53. Overall distribution of morphologically normal follicles in fresh ovaries (FRESH) and in frozen/thawed ovaries (FROZEN) at different days of *ex vivo* culture. Values with different indices (a-b) are significantly different (P<0.05).

Cell proliferation and TUNEL assay

Cell proliferation was evaluated by counting Ki67-positive cells. No differences on cell proliferation rate were found between fresh and frozen ovaries for all days analyzed. After 2 days of culture, the percentage of cells expressing Ki67 was comparable (p=0.087) to un-cultured ovaries (Day 0) in both fresh and cryopreserved ovaries. Only after 4 days of culture, a reduction (p=0.048) of cell proliferation was observed in both fresh and frozen ovaries (21.3%±0.87% and 20.5%±0.83%, respectively, p=0.010) (Figure 54).

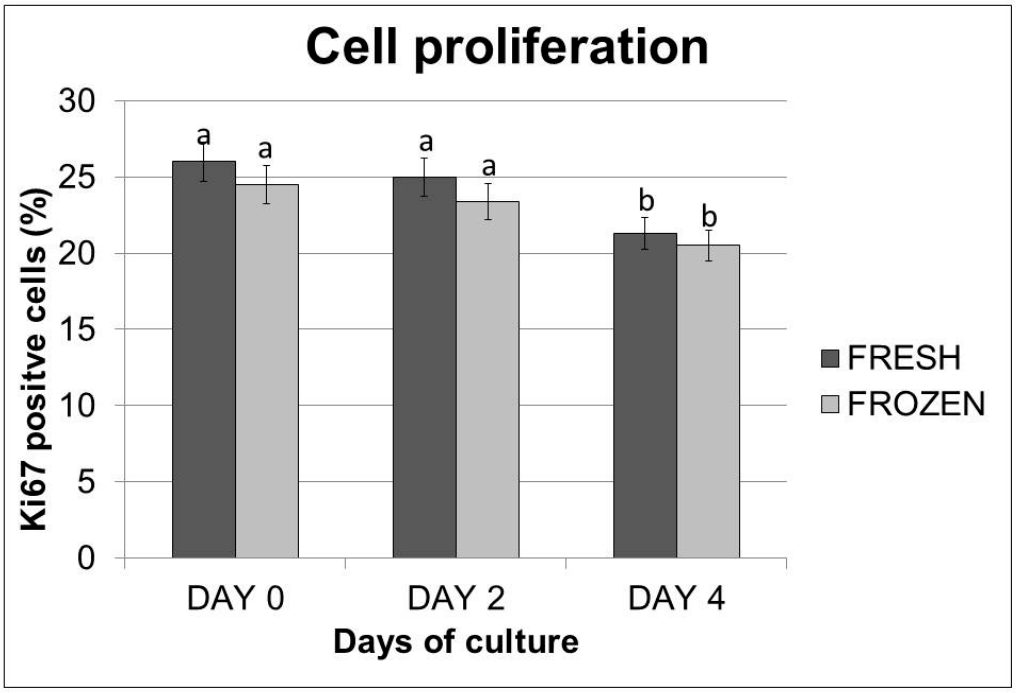

Figure 54. Percentage of immunofluorescence positive cells for Ki67 in fresh ovaries (FRESH) and in frozen/thawed ovaries (FROZEN) at different days of *ex vivo* culture. Values with different indices (a-b) are significantly different (P<0.05). n=1980 ± 231 nuclei counted for each section.

Similar results were observed for the apoptotic index. No significantly different (p=0.079) for apoptotic level were observed between day 0 and day 2 both in fresh and frozen ovaries. However, after 4 days of culture, the number of apoptotic cells per mm^2 increased (p=0.043) in both groups (Figure 55).

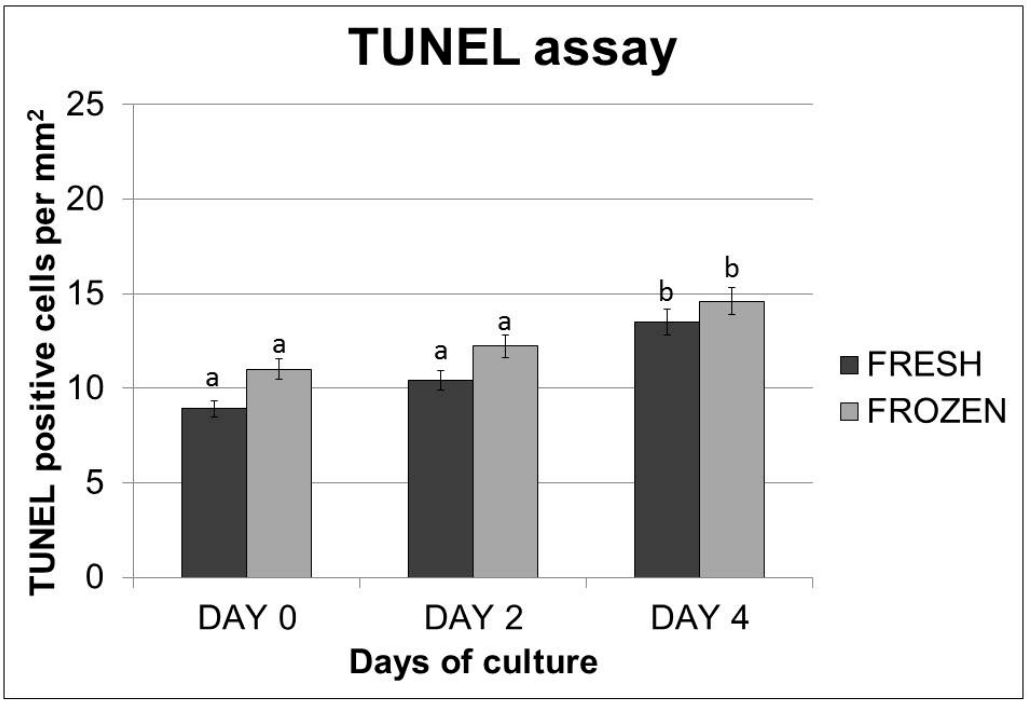

Figure 55. Number of TUNEL-positive cells per mm² in fresh ovaries (FRESH) and in frozen/thawed ovaries (FROZEN) at different days of *ex vivo* culture. Values with different indices (a-b) are significantly different (P<0.05). n=1980 ± 231 nuclei counted for each section.

5. DISCUSSION

Cryopreservation of ovarian tissue is the only available option for preserving female fertility in pre-pubertal girls or in a series of circumstances when gonadotoxic treatments cannot be delayed [186, 187]. This can be achieved through the excision and banking of whole organs or of ovarian cortical fragments.

In theory cryopreservation of whole ovaries with an intact vascular pedicle should prevent post-transplantation ischemic damage by enabling the anastomosis of the severed vessels. However, cryopreservation of whole organ is much more complicated than ovarian cortical fragments due to the heat transfer problems and the non-homogenous rate of cooling between the core and the periphery of the organ. Therefore, it is important to identify the technique that can provide the best cryopreservation of large specimens. Particularly in order to provide real advantages it is necessary that the three dimensional structure of the organ and its vasculature network remains largely intact.

This has proved problematic when an entire ovary is frozen with conventional slow freezing procedures that reduce the sample temperature through multidirectional heat transfer by convection which does not allow a uniform cooling between the core and the periphery of the specimen [98]. As a consequence ice growth takes

place with uncontrolled velocity thereby causing structural damages especially in the area with higher water content like vessels [102].

On the contrary directional freezing is based on the generation of a thermal gradient by a large mass of conductive material. The sample moves through it at a predetermined velocity resulting in a precise and uniform liquid-ice interface and in an efficient removal of the latent heat [102]. Therefore temperature gradient between the inner portion of the organ and its surface is reduced to a minimum, together with the formation of large disrupting ice crystals.

The results obtained in this thesis by directly comparing directional and conventional slow freezing for preserving whole ovaries support this notion.

The first benefit observed in our samples was a better preservation of vessel integrity that spanned all size categories and both inner and outer ovarian regions. Our results indicated that the perfusion of Indian Ink flowed with no noticeable difference between whole ovaries frozen with directional freezing and fresh controls (Figure 33).

Preservation of intact blood vessels was not the only advantage of directional freezing. The preservation of a stromal cell density indistinguishable from the fresh control (Figure 34) is particularly relevant since damages to the ovarian stroma are known to be caused by the freezing-thawing process when whole ovaries were analyzed [166, 188] and are characterized by stromal edema and

lower cell density. These alterations reflect a structural damage that compromises follicular survival and development [189]. Therefore the extended damage of the ovarian stroma that we observed after conventional freezing is consistent with similar alterations observed in previous experiments on sheep ovaries [166, 188, 189].

Alteration of the stromal structure was associated with the lower developmental competence exhibited by the primordial follicles of the whole ovaries frozen with conventional freezing at the end of the 7 days culture period (Figure 36). In fact, whole ovaries frozen with conventional freezing displayed a lower percentage of normal follicles as compared with directional freezing and fresh groups (Figure 37). These result are consistent with previous studies that reported a significant decrease in the percentages of morphologically normal follicles in sheep and cow whole ovaries frozen with conventional freezing [166, 190].

Furthermore, we observed a lower rate of cell proliferation in whole ovaries frozen with conventional freezing both at the beginning and at the end of the culture period (Figure 38). This is certainly related to the higher rate of DNA damage observed at the same time because the presence of double-strand breaks into DNA triggers a complex set of responses which include cell cycle arrest. We have examined the expression of the histone variant γH2AX, the phosphorylated form of H2AX which is formed within minutes of DNA damage [191]. The presence of γH2AX at the foci which form

at DNA break sites attracts BRCA2 which, in turn, binds to RAD51 and activates DNA repair [192].

Our results showed that DNA damage in directional freezing of whole ovaries was higher than in fresh controls. However upon culture progression, DNA repair activity was evident in directional freezing of whole ovaries leading to a substantial reduction of DNA damage, whereas DNA repair activity was virtually absent in whole ovaries frozen with conventional freezing, leaving the rate of DNA damage substantially unchanged (Figure 42). Evaluation of DNA damage and repair mechanisms allows a more sensitive assessment of cellular damage as compared to measuring apoptosis which is an irreversible process [193]. In this case the high levels of Rad51, a marker of DNA repair, observed in directional freezing of whole ovaries after 7 days of culture allows us to see that the DNA damage observed on day 1 of culture was reversible. The ability of directional freezing of whole ovaries to repair DNA damage is particularly relevant for its long term function after thawing since DNA double strand break repair efficiency has been identified as an important determinant of oocyte aging in women [194].

However, whereas no live births or pregnancies have been achieved following the cryopreservation of whole human ovaries, 24 live babies have been obtained after transplantation of frozen-thawed ovarian fragments [186]. This suggests that the use of ovarian fragments is the method of choice even if, at present, the average

functional life span of these samples ranges from 2 to 5 years [186, 195].

Therefore it was of interest to determine whether directional freezing could also improve the viability of ovarian fragments and how these would compare with whole ovaries frozen with the same method. For this reason, in this thesis ovarian cortical fragments were cryopreserved with both freezing methods and the data obtained were compared with whole ovaries frozen with the same method.

Our results, showed that the use of directional freezing significantly improved the integrity of all follicular structure from primordial to secondary stages and although the effect was evident in both whole organs and ovarian fragments, it was more efficient in whole organs (Table 10).

Functional analysis of *in vitro* growth of primordial follicles confirmed the same pattern. Directional freezing was beneficial for both entire ovaries and ovarian fragments but directional freezing of whole ovaries was the only group whose viability was the same as that of fresh tissue (Figure 43). This result is somehow unexpected since cryopreservation efficiency is inversely related to the size of the sample.

In fact, large biological samples suffer from a long isothermal period caused by the massive release of latent heat during the process of ice formation [162, 196]. The phenomenon is caused by the energy

generated by water molecules when they rejoin to form an ice crystal. Such energy is released in the form of heat that causes a temperature rise of the surrounding structures [116]. The heat is normally transferred to the ice crystals that have just been formed because these are made of conductive material. The consequence is a transient thawing followed by refreezing in a sequence that is repeated several times through the thickness of the sample causing severe cell damage [197, 198]. Damage is usually reduced by keeping the ratio of surface to volume as high as possible so that the excessive heat is removed by adjusting the cooling rate to achieve uniform freezing. The thinner the sample the faster the heat, released from its inner parts, is removed minimizing the damage. However the need to have samples as thin as possible is in contrast with the requirement of having a large follicle population upon thawing and it is impossible when using low surface to volume ratio samples like whole ovaries. The large mass of conductive material in the MTG apparatus serves the purpose of quickly removing the latent heat from large samples thereby preventing the thawing and freezing sequence [109].

These considerations largely explain the better results of directional freezing compared to conventional slow freezing described in our experiments, but cannot account for the better performance obtained with whole ovaries than with smaller ovarian fragments. We hypothesize that the different ways that the cryoprotectant was

administered may have a role. The presence of the ovarian pedicle in entire ovaries enabled an extensive uniform perfusion through the vasculature [199].

Consistent with the favorable effect of directional freezing on primordial follicle viability *in vitro* we observed a higher cell proliferation rate compared to samples that underwent conventional freezing (Figure 44).

In an attempt to understand the mechanisms that may have caused this effect we studied known responses to temperature stress, which is inherent to any form of cryopreservation. One of the best characterized responses to thermal stress is the expression of HSPs [200]. HSPs bind to other proteins, termed clients, which are damaged by thermal or other stress conditions and mediate their transport to target organelles for repair or degradation [201].

Our results show that the HSP machinery was significantly stimulated over baseline levels only in the case of conventional freezing of whole ovaries (Figure 46). This is consistent with particularly extensive protein damage in this group since the client-chaperone binding and release cycles are repeated several times until the client has reached its final active conformation, or has entered the proteolytic system [202].

The presence of extensive cellular damage in whole ovaries frozen with conventional freezing was confirmed by the significant increase in apoptotic cells after 7 days of culture (Figure 45), consistent with

the notion that the accumulation of damaged proteins, if unresolved, can lead to apoptosis [203]. However protein damage is not the only cause of apoptosis as it can also be activated by a failure of DNA repair mechanisms [204]. In fact the high levels of DNA damage associated with a lack of DNA repair activity observed in conventional freezing of whole ovaries is consistent with the high apoptotic rate observed in the same group.

In contrast to HSPs, γ-H2AX and RAD51 are not visible in the nuclei of intact cells therefore it was possible to detect their increased expression in our experimental samples. Results were consistent with the pattern of follicular viability since DNA damage was significantly lower in directional freezing samples followed by more intense DNA repair activity suggesting that the DNA damage observed on day 1 of culture was reversible (Figure 52).

Our results, indicated that directional freezing improves the viability of cryopreserved ovarian tissue both in whole organs and cortical fragments, but we observed a better preservation of follicles when the samples were frozen as whole ovaries.

However it must be remembered that a high risk of retransmission of malignancy exists if autotransplantation of ovarian tissue were performed in women who have suffered from systemic hematological malignancies [187], [205]. In this respect whole ovary cryopreservation cannot offer any improvement in case of autotransplantation, but it could provide some appealing alternative

for the development of methods aimed at grafting isolated follicles in an artificial ovary [206] or other, yet to be described, methods.

Vanacker et al., developed the first biodegradable artificial ovary with the aim to offer an environment where isolated follicles and ovarian cells can survive and grow [206]. However, to achieve this, the artificial ovary needs to hold isolated follicles in their original three-dimensional form, since it is essential to maintain intercellular interactions between granulosa cells and oocytes, which regulate many aspects of oocyte growth and development. Furthermore, as human ovarian follicles are exceptional in that they can grow to around 600× their size during folliculogenesis , the artificial ovary, unlike other artificial organs, should also be biodegradable. Furthermore, for these reasons, more studies are required to assess the value of incorporating isolated pre-antral follicles and evaluate their survival and interaction with ovarian cells.

In the experiment described in this thesis, we developed a perfusion system for *ex vivo* culture of whole ovaries. This represents an experimental situation which is close to an *in vivo* situation, with preserved ovarian tissue architecture and intravascular flow.

Fresh and frozen whole ovaries were cultured for 2 or 4 days and our results showed that none of the parameters considered, were affected by cryopreservation.

Morphological analysis showed unaltered histological appearance and absence edema after 2 or 4 days of culture (Figure 53).

The high rate of Ki67 positive cells (Figure 54), associated with a small percentage of TUNEL positive cells (Figure 55), detected until day 2, demonstrated that our culture system can support ovarian tissue survival and proliferation. More experiments are needed in order to extend the culture period since a gradual decrease in expression of Ki-67 in concomitance with apoptotic index increment were detected from day 2 to 4.

Previous studies were carried out in human postmenopausal [207] and in rat [208] ovary for a short culture period (8-10 hours). The experiment described in this thesis, constituted the first attempt to maintain alive a large animal ovary outside the body.

Based on these successful preliminary results, we believe this culture system can also be used for future studies aiming to stimulation of primordial follicles growth through *ex-vivo* perfusion for future *in vivo* or *in vitro* use.

6. CONCLUSION

Data obtained indicated that directional freezing improves the viability of cryopreserved ovarian tissue not only when used with whole organs but also with ovarian fragments. This provides a way to further improve the efficiency of this procedure.

The direct comparison of cortical fragments and whole organs, unexpectedly revealed that the latter show better preservation of early follicles in many aspects almost identical to those recorded in fresh control samples. This encourages a reconsideration of the possible use of whole ovary cryopreservation as a viable alternative to cortical fragments.

Data obtained from the *ex vivo* culture, demonstrated that it is possible to culture fresh and frozen-thawed whole ovaries for up to 4 days, suggesting that in the future this technique could provide appealing alternative to the re-transplantation of cryopreserved ovarian tissue, with the purpose to eliminate the risk of transmission of cancer cells in women who have systemic hematologic malignancies.

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APPENDIX A

Direct comparative analysis of conventional and directional freezing for the cryopreservation of whole ovaries

Sara Maffei, M.Sc.,[a] Maike Hanenberg, M.Sc.,[b] Georgia Pennarossa, Ph.D.,[c] José Roberto V. Silva, D.V.M., Ph.D.,[d] Tiziana A. L. Brevini, D.Pharm., Ph.D.,[a] Amir Arav, D.V.M., Ph.D.,[e] and Fulvio Gandolfi, D.V.M.[a]

[a] Laboratory of Biomedical Embryology, Università degli Studi di Milano, Milan, Italy; [b] Wageningen University and Research Center, Wageningen, the Netherlands; [c] Department of Veterinary Medicine, Università degli Studi di Sassari, Sassari, Italy; [d] Biotechnology Nucleus of Federal University of Ceara, Ceara, Brazil; and [e] Core Dynamics, Ness Ziona, Israel

Objective: To compare conventional slow equilibrium cooling and directional freezing for cryopreservation of whole ovaries.
Design: Experimental animal study.
Setting: Academic research environment.
Animal(s): Adult ewes.
Intervention(s): Eighty-one ovaries were randomly assigned to fresh control, conventional freezing (CF), and directional freezing (DF) group. Ovaries of CF and DF groups were perfused via the ovarian artery with Leibovitz L-15 medium, 10% fetal bovine serum, and 1.5 M dimethyl sulfoxide for 5 minutes. Each ovary was inserted into a glass test tube containing 10 mL of the same solution and cooled to −100°C or −70°C, respectively. Ovaries were stored in liquid nitrogen for a minimum of 2 weeks.
Main Outcome Measure(s): Structural integrity of cortical and medulla regions, vascular integrity, follicle in vitro development, cell proliferation, and DNA damage and repair.
Result(s): All examined parameters indicate that the structure of DF ovaries remains largely intact and comparable to fresh controls, whereas significant damages were observed in CF ovaries.
Conclusion(s): Directional freezing allows good preservation of whole ovaries, with most of the parameters taken into consideration almost identical to those recorded in fresh control samples. This encourages a reconsideration of the possible use of whole-ovary cryopreservation as a viable alternative to cortical fragments. (Fertil Steril® 2013;100:1122–31. ©2013 by American Society for Reproductive Medicine.)
Key Words: Whole ovary, cryopreservation, directional freezing, sheep

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Current cancer treatments improve the long-term survival rates of young women with malignancies. However, many of these therapies have lasting effects on fertility because they cause severe injuries to the ovarian reserve, which may lead to consequent premature ovarian failure, with a negative impact on life quality of young cancer survivors (1, 2).

Cryopreservation of ovarian tissue has been suggested as an alternative to oocyte and embryo freezing for preserving fertility of these patients, and two options are available: cryopreservation of ovarian cortical fragments and cryopreservation of the whole ovary. The first technique is currently the most widely used, owing to the higher efficiency of the cryopreservation methods and to the less-invasive nature of the retransplantation procedures (3–8). On the other hand, the relatively small size of grafted cortical fragments and their avascular nature may limit their functional longevity upon transplantation (9). However, recent follow-up studies of ovarian fragment transplantation suggest that this may not be the case because, in some cases,

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Reprint requests: Fulvio Gandolfi, D.V.M., Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy (E-mail: fulvio.gandolfi@unimi.it).

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both fresh and cryopreserved tissue may remain functional for up to 7 years (10, 11).

Whole ovary cryopreservation with vascular reanastomosis, in theory, represents an appealing method to reduce ischemic time and prolong graft longevity (12). In fact, successful cryopreservation and transplantation of whole ovaries have been reported in different experimental animal species (mice, rat, rabbit, dog, and sheep) (13–17). However, the method suffers from a number of drawbacks. In particular, orthotopic transplantation has proved technically very challenging and cannot prevent ischemic damage (18, 19). In addition, the possibility exists to reintroduce malignant cells, even if this risk is also present when ovarian fragments are transplanted (20).

Nevertheless, it is important to improve the efficiency of cryopreserving whole organs, because in large mammals and humans this has been more problematic than in small animals, owing to heat transfer in such large organs (21) caused by the large thermal gradient existing between the surface and the interior of the system (22). At present there are two main methods for cryopreserving whole ovary: conventional freezing (CF) and directional freezing (DF). Conventional freezing operates on the principle of heat transfer by convection (23, 24). Directional freezing is an alternative approach to preserve the large organs, based on the physical concept of DF, which enables precise adjustment of the temperature gradients to achieve an accurate cooling rate through the entire tissue (17, 25, 26). This method has solved the problems associated with heat release by maintaining a uniform cooling rate through the entire organ. Although whole ovary cryopreservation with DF followed by successful retransplantation has been performed once in sheep (17), data regarding this new method are limited. In particular a direct comparison with CF has never been performed. The aim of this study was to compare the efficiency of CF with DF of whole ovaries. On the basis of our results (27) and those in the literature (17, 28), the sheep ovary was chosen as a model because of its similarity to the human ovary.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich.

Ovaries Collection

Sheep female reproductive tracts were collected at the local slaughterhouse and transported to the laboratory in cold (0–4°C) 0.9% saline solution. A total of 81 ovaries were randomly assigned to a fresh control group (CTR, n = 27), CF group (n = 27), or DF group (n = 27). Upon arrival to the laboratory, ovaries of the CF and DF groups were perfused via the ovarian artery with Ringer's solution and 10 UI/L heparin for 10 minutes, followed by perfusion with cryoprotectant solution containing Leibovitz L-15 medium (Life Technologies), 10% fetal bovine serum (vol/vol; Life Technologies), and 1.5 M dimethyl sulfoxide for 5 minutes (29). Each ovary was inserted into a 16-mm-diameter glass test tube (Manara) containing 10 mL of the same solution.

Conventional Freezing

Conventional freezing was performed in a Kryo 560M apparatus (Planer) with the following program: [1] from 4° to −40°C using a cooling rate of 0.5°C/min (seeding was induced at −7°C), [2] from −40° to −100°C at 5°C/min, and [3] immersion in liquid nitrogen (29).

Directional Freezing

Directional freezing was performed with a Multi-Thermal-Gradient (IMT). Briefly, the technology operates on the physical concept of DF and is based on a series of four heat-conductive blocks arranged in a line. Different temperatures are set along the blocks, thereby imposing a temperature gradient. Freezing tubes were pushed along the thermal gradient (+4° to −70°C), and the speed was set at 0.01 mm/s, resulting in a cooling rate of 0.3°C/min, after which samples were plunged into liquid nitrogen (17, 25).

Ovarian Thawing

Ovaries frozen with CF and DF were thawed by plunging the test tubes into a 68°C water bath for 20 seconds and then into a 37°C water bath for 2 minutes. The cryoprotectant solution was rinsed out of the ovaries by perfusion via the ovarian artery with 10 mL *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–Tyrode's albumin lactate pyruvate medium (HEPES-TALP medium), supplemented with 0.5 mol/L sucrose and 10 IU/mL heparin at 38°C, followed by another 10 mL rinse with no sucrose (17, 25).

Morphologic Analysis

Nine ovaries from each group were allocated to morphologic and immunochemical analysis. To improve fixation, ovaries were cut in half, embedded in paraffin, and stained with hematoxylin and eosin. Samples were observed under an Eclipse E600 microscope (Nikon), and pictures were acquired with Nis Elements Software (version 4.0). Follicles were classified as primordial (1 layer of flattened granulosa cells around the oocyte), intermediate (1 layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Follicles were further classified as normal, when a spherical oocyte with a non-pyknotic nucleus was surrounded by granulosa cells organized in discrete layers; or degenerated, when a misshapen oocyte with or without vacuolation with a pyknotic nucleus was surrounded by disorganized granulosa cells detached from the basement membrane (30). Stromal cell density was quantified by counting nuclei with ImageJ software (National Institutes of Health) as described below.

Immunohistochemistry

Ovarian cortical fragments were fixed with 10% formaldehyde, embedded in paraffin, and sectioned before immunostaining. Slides were then boiled for 5 minutes in antigen-unmasking solutions (Vector Laboratories) and then blocked with a

solution of 1% bovine serum albumin. Samples were incubated over night at 4°C with the following primary antibodies: rabbit polyclonal antibody specific for Ki67 marker of cell proliferation (1:50 dilution; Abcam) cross-reacts with the sheep antigen (31, 32); mouse monoclonal anti-γH2AX, a DNA damage marker, which specifically reacts with sheep (1:200; Abcam); and rabbit monoclonal anti-RAD51, a DNA repair marker (1:200; Santa Cruz Biotechnology). We validated its specificity for the ovine antigen with a Western blot analysis of ovarian proteins, which revealed a 37-kDa signal, the expected molecular weight (data not shown). Sections were washed three times with phosphate-buffered saline and incubated with suitable secondary antibodies (Alexa Fluor; Life Technologies) for 45 minutes. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Samples were observed under an Eclipse E600 microscope (Nikon), pictures were acquired with Nis Elements Software (version 4.0), and the images were analyzed using ImageJ software as described below.

Blood Vessel Integrity

Vessel integrity was assessed using Indian ink. Fresh ovaries were perfused with 10 mL Ringer's solution supplemented with 10 IU/mL heparin, and thawed ovaries were perfused as described above. Thereafter, six ovaries from each group were perfused via the ovarian artery with a solution of 25% Indian ink in 0.9% NaCl supplemented with 167 IU heparin/mL for 5 minutes (33), with a flow rate of 1.3 mL/min. A perfusion was considered successful if Indian ink was macroscopically seen to perfuse the whole ovary. After perfusion ovaries were fixed in 10% formaldehyde and embedded in paraffin for hematoxylin and eosin staining. Perfused vessels were classified as intact or damaged, according to the presence or absence of Indian ink in the lumen, respectively. Vessels were divided into three categories according to their size: small (Ø $\leq$20 μm), medium (Ø 21–74 μm), and large (Ø $\geq$75 μm). Vessel number was determined in six randomly selected fields per section of both medulla and cortical tissue of each ovary. For each experimental group a minimum of 200 vessels were counted.

Image Analysis

Pictures were taken with constant exposure parameters, to ensure reproducible analysis with the image analysis software ImageJ (http://rsbweb.nih.gov/ij/index.html) after conversion in 8-bit grayscale. A minimum cut-off for intensity and particle size was established. Nuclei on border edges were excluded.

Follicle Culture

Culture procedure was as previously described (34). Briefly, six fresh and frozen/thawed ovaries for each group were cut in half, and large antral follicles, corpora lutea, and medulla were removed. The isolated cortex of each ovary was divided into several fragments of approximately 2 × 2 × 1 mm. Cryoprotectant was removed from CF and DF fragments through stepwise washings of 10 minutes each in Alpha Minimum Essential Medium (α-MEM) supplemented with sucrose (0.5 M, 0.25 M, 0.125 M, and 0 M). Three fragments, for each ovary, were immediately fixed in 10% formaldehyde solution for histologic studies (time 0, control). Other fragments were placed into 24-well culture dishes (Sarstedt) and individually cultured in 1 mL of medium for 7 days at 39°C under an atmosphere of 5% CO_2 in air. The culture medium was composed of α-MEM (Life Technologies) supplemented with ITS (6.25 μg/mL insulin, 6.25 μg/mL transferrin, and 6.25 ng/mL selenium) (Life Technologies), 2 mM glutamine (Life Technologies), 0.23 mM pyruvate, 2 mM hypoxantine, 1.25 mg/mL bovine serum albumin, 100 μg/mL penicillin G, 100 μg/mL streptomycin, 200 mIU/mL sheep FSH, and 50 ng/mL human recombinant basic fibroblast growth factor (R&D Systems) (34). Medium was replaced every 2 days.

Semiquantitative Polymerase Chain Reaction

Total RNA was extracted from the cortical region of six ovaries for each group using TRIzol Reagent (Life Technologies) and reverse transcribed into complementary DNA in a total volume of 20 μL reaction mixture containing 1 μL oligo(dT)$_{12-18}$ (500 ng/μL). Reverse transcription was performed with 200 U/μL Superscript II reverse transcriptase (Life Technologies) for 1 hour at 42°C. Complementary DNA amplifications were carried out in an automated thermal cycler (iCycler; Bio-Rad), using the appropriate conditions for Ki67-specific primers (forward 5′-atggggagtgagaaggaggt-3′; reverse 5′-tccaagttccccttgcatac-3′; Tm 59°C). Sheep DNA was used as positive control. The optimal cycle number at which the transcript was amplified exponentially was established running a linear cycle series, and the number of PCR cycles was kept within this range. An aliquot of each complementary DNA sample was amplified by PCR with β-actin gene-specific primers (forward primer: 5′-ccaaggccaaccgtgagaag-3′; reverse primer 5′-ccatctcctgcttcgaagtcc-3′; Tm 57°C). The reverse transcription–polymerase chain reaction products were subjected to electrophoresis on a 2% agarose gel, and the intensity of each band was assessed by densitometric analysis performed with Quantity One software (Bio-Rad). The relative amount of Ki67transcript was calculated by dividing the intensity of the Ki67 band by the intensity of the β-actin band.

Statistical Analysis

All experiments were performed in at least six biologically independent replicates. Data are reported as mean ± SEM. Mann–Whitney *U* test (SPSS 19.1; IBM) was used to analyze vessel numbers, and one-way analysis of variance followed by Turkey's post hoc tests (SPSS 19.1; IBM) were used for all the remaining experiments. Differences were considered statistically significant at $P<.05$.

RESULTS

Morphologic Assessment Immediately after Thawing

The percentage of morphologically normal follicles in each group is shown in Figure 1A. The rate of intact

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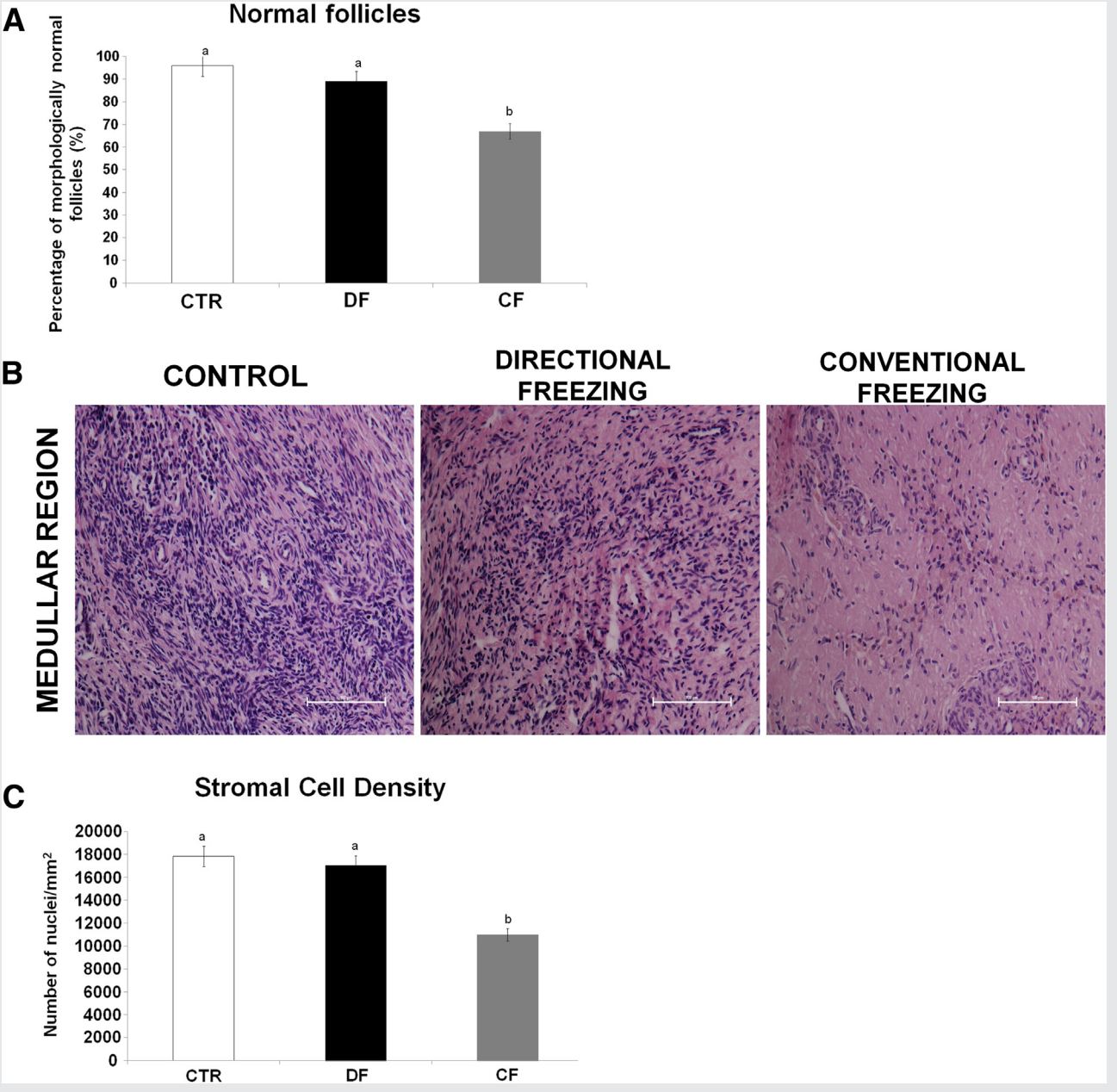
FIGURE 1



Morphologic analysis of ovarian cortical and medullar regions in fresh control and frozen–thawed ovaries. (A) Overall distribution of morphologically normal follicles in CTR and in DF or CF groups after thawing. (B, C) Representative pictures of the medullar region of the different experimental groups (B) and the quantitative results of image analysis (C). Values with different indices (a–b) are significantly different ($P<.05$).

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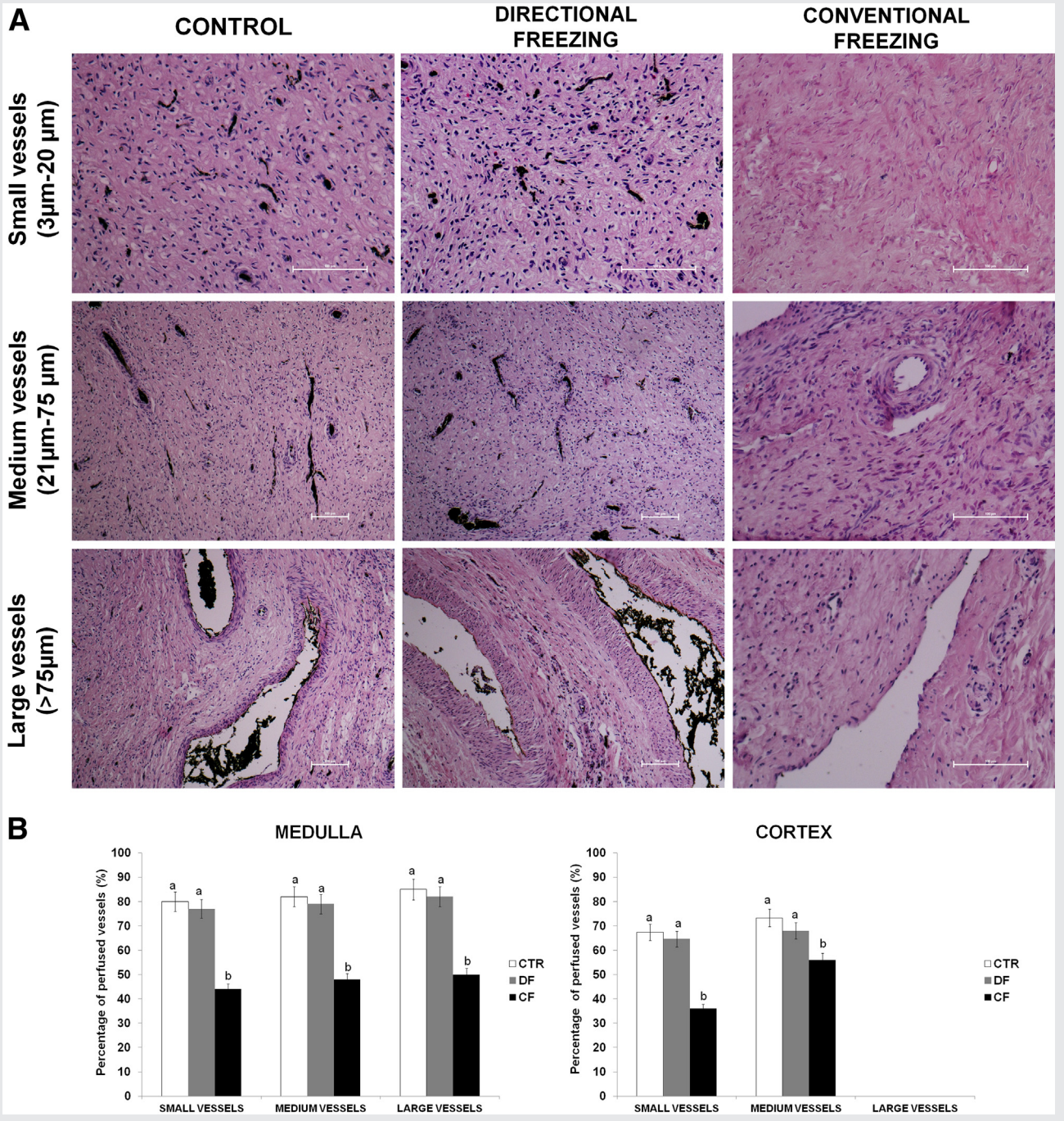
follicles in the DF group (89% ± 1.7%) was significantly higher ($P<.05$) than that in the CF group (61% ± 3.4%), and it was not different ($P=.75$) from that in fresh control tissue (96% ± 1.5%) (Fig. 1A). Quantitative assessment of stromal cell density indicated that DF ovaries had values (17,041 nuclei per mm^2) similar to those of fresh organs (17,833 nuclei per mm^2) but significantly higher than in CF ones (10,875 nuclei per mm^2) (Fig. 1B and C).

Indian ink was observed only in blood vessels and not in the surrounding tissue, indicating an optimal perfusion (Fig. 2A). The DF and CTR ovaries displayed the same rate of intact vessels in ovaries, both in the medulla (stromal vessels) and in the cortex (perifollicular vessels) (Fig. 2B). Conversely, CF ovaries displayed a significantly lower number of intact vessels ($P=.009$). No significant differences between ovarian medulla and cortex vessels were observed, either in control or in cryopreserved samples (Fig. 2B).

FIGURE 2



(A, B) Representative pictures of blood vessels in the different experimental groups classified in three diameter categories (A) and their distribution in the medulla and cortex regions (B). Values with different indices (a–b) are significantly different ($P<.05$). Scale bars = 100 μm.

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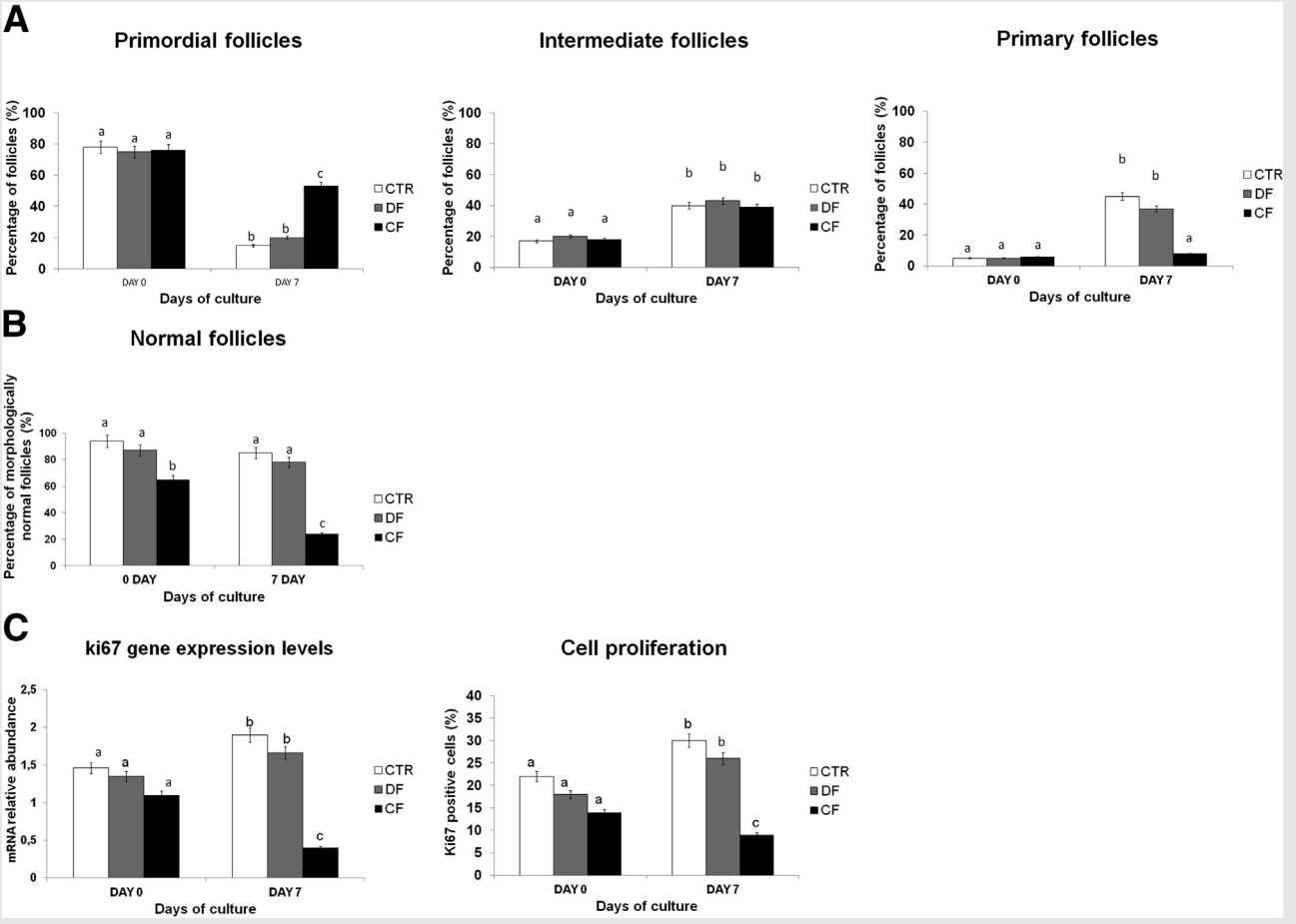
Functional Assessment

After 7 days of culture the rate of primordial follicles that resumed their development reaching the primary stage was higher in DF than in CF ovaries. The developmental competence of DF ovarian fragments was the same as in fresh control tissue, whereas CF fragments showed a significant decrease of the overall follicle number ($P=.038$; Fig. 3A).

The percentage of intact follicles in ovarian tissue before and after in vitro culture is show in Figure 3B. Consistent with their developmental capacity, the rate of morphologically normal primordial, intermediate, and primary follicles in the DF group was not different from that in the CTR group upon thawing. In contrast, the CF group displayed a higher rate ($P<.05$) of degenerated follicles and oocytes with

FIGURE 3



Functional assessment of cortical fragments cultured in vitro for 7 days. (A) Distribution of primordial, intermediate, and primary follicles at the beginning (day 0) and at the end (day 7) of the culture period. (B) Overall distribution of morphologically normal follicles during culture in the three experimental groups. (C) Quantitative evaluation of cell proliferation assessed both as Ki67 messenger RNA levels (*left*) and as percentage of immunopositive cells for the encoded protein (*right*). Values with different indices (a–c) are significantly different ($P<.05$).

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pyknotic nuclei. Seven days later a significant reduction in the percentage of histologic normal follicles was found in the CF group (24% ± 5.7%) compared with the DF (77% ± 2.8%) and CTR groups (85% ± 2.43%).

After 7 days of culture abundance of Ki67 transcripts significantly increased in DF and CTR tissue ($P<.05$); by contrast, a reduction in Ki67 messenger RNA level was detected in CF tissue ($P<.05$) (Fig. 3C, left). Ki67 immunofluorescence results were in agreement with the observed transcript levels (Fig. 3C, right). The percentage of proliferation was comparable among the different groups at day 0. After 7 days of culture an average of only 9% ± 0.85% of cells expressed the Ki67 antigen in CF tissue, compared with 30% ± 0.73% and 26% ± 0.64% for fresh and DF tissue sections, respectively (Fig. 3C, right).

Coherent with this pattern are the data observed investigating the occurrence of DNA double-strand breaks. These were obtained through the analysis of the expression of γ-H2AX, a marker of DNA damage, and of RAD51, a marker of DNA repair, and are shown in Figure 4. As expected, virtually no γ-H2AX and RAD51 signals were detected in fresh tissue. Clear signals of DNA damage, in the form of γ-H2AX foci, were observed in DF tissue at the beginning of culture, together with a weak staining for DNA repair. The DNA damage, however, significantly decreased after 7 days concomitantly with a sharp increase of DNA repair, as indicated by a clear RAD51 immune signal (Fig. 4A and B). By contrast, a very strong immune staining for γ-H2AX was observed in CF ovaries immediately after thawing, indicating widespread DNA damage, but this was not accompanied by RAD51 labeling either at the beginning or at the end of culture, suggesting a lack of DNA repairing ability (Fig. 4A and B).

DISCUSSION

This article describes the first direct comparison between directional and conventional slow freezing for preserving whole ovaries. We have used sheep ovaries because their size and texture is comparable to human ovaries, although their volume, on average, is smaller (25, 35–42).

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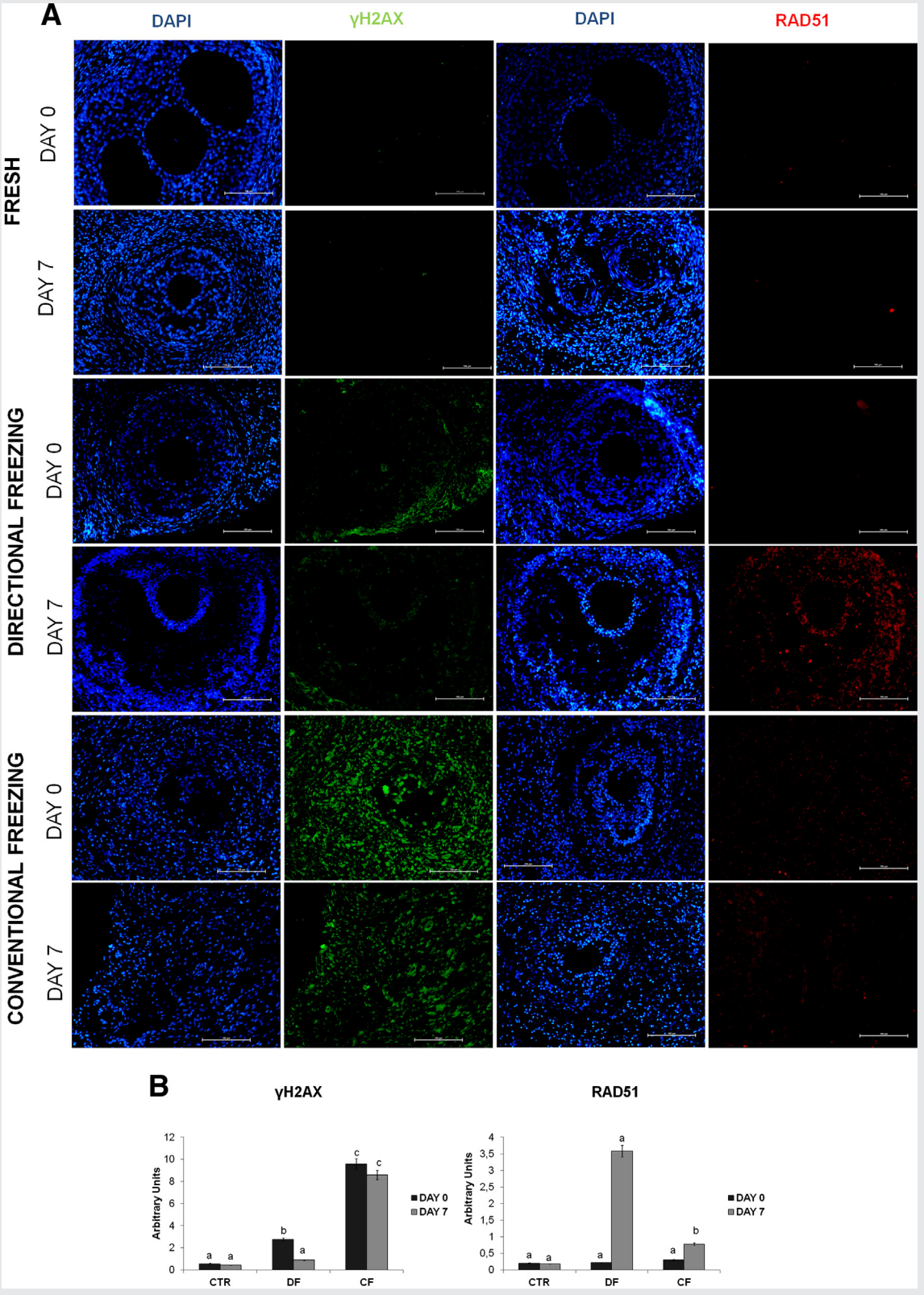
FIGURE 4



Representative pictures of cells positive for γH2AX (marker of double-strand DNA breaks; *green*) and RAD51 (marker of repair ability; *red*). (A) Immunostaining was carried out at day 0 and day 7 of in vitro culture. (B) Quantitative assessment by image analysis. Values with different indices (a–c) are significantly different ($P<.05$). Scale bars = 100 μm.

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In theory, cryopreservation of whole ovaries with an intact vascular pedicle should prevent post-transplantation ischemic damage by enabling the anastomosis of the severed vessels. However, previous attempts showed that a large loss of follicles, up to 98%, takes place even after vascular anastomosis (15). The fact that extensive damage also occurs with fresh ovaries suggests that ischemia induced by thromboembolism in the ovarian vascular system plays a major role and is due to the technically challenging nature of this complex surgery. This not only causes extensive tissue necrosis but may cause increased morbidity and mortality. In addition, the prolonged surgical procedures due to the technical complexity of the vascular surgery may cause the so-called warm ischemia, which determines more or less extensive follicular losses. Although advances in transplantation techniques will decrease current limitations of the surgical procedures, it is important to identify the technique that can provide the best cryopreservation of large specimens.

In particular, to provide real advantages it is necessary that the 3-dimensional structure of the organ and its vasculature network remain largely intact. This has proved problematic when an entire ovary is frozen with conventional slow freezing procedures that are efficiently used for smaller entities like oocytes or embryos. Conventional freezing devices reduce the sample temperature through multidirectional heat transfer by convection, which does not allow a uniform cooling between the core and the periphery of the specimen (22). As a consequence ice growth takes place with uncontrolled velocity, thereby causing structural damage, especially in the area with higher water content, like vessels (26).

Our data support this notion as specifically indicated by the high rate of damaged blood vessels observed after CF, both in the medullary and in the cortical region.

Conversely, DF is based on the generation of a thermal gradient by a large mass of conductive material. The sample moves through it at a predetermined velocity, resulting in a precise and uniform liquid–ice interface and in an efficient removal of the latent heat (43). Therefore temperature gradient between the inner portion of the organ and its surface is reduced to a minimum, together with the formation of large disrupting ice crystals. The first benefit observed in our samples was a better preservation of vessel integrity that spanned all size categories and both inner and outer ovarian regions. Our results indicated that the perfusion of Indian ink flowed with no noticeable difference between DF and fresh controls. This aspect is particularly important in view of a possible in vivo retransplantation because it would ensure a smooth resumption of the circulation. Indeed a fully functional vascular supply was demonstrated by magnetic resonance imaging in one sheep ovary 24 months after its transplantation after cryopreservation with DF (25). However, the success rate remains low (from 30% to 50%) (15, 17, 44), leaving open the options for a fair risk evaluation between a long-term functional whole ovary and a failed transplantation, which leaves no alternative options.

However, preservation of intact blood vessels was not the only advantage of DF. The preservation of a stromal cell density indistinguishable from that in the fresh control is particularly relevant because damages to the ovarian stroma are known to be caused by the freezing–thawing process when whole ovaries or cortical fragments were analyzed (45, 46) and are characterized by stromal edema and lower cell density. These alterations reflect a structural damage that compromises follicular survival and development (47). Therefore the extended damage of the ovarian stroma observed after conventional slow cooling is consistent with similar alterations observed in previous experiments on sheep ovaries (45–47). Alteration of the stromal structure was associated with the lower developmental competence exhibited by the primordial follicles of the CF group at the end of the 7 day culture period. Because follicles were cultured within small fragments of ovarian cortex a structural damage of the stroma cannot be considered a likely cause of this observation. Nor is the higher rate of damaged blood vessels in the cortical region a probable direct cause because in this experiment the cryoprotectant was removed by rinsing the isolated fragments with culture medium rather than through vascular perfusion. Two observations indicate direct cellular damage as a more plausible reason. In fact, ovaries frozen with CF displayed a lower percentage of normal follicles as compared with DF and CTR groups. These result are consistent with previous studies that reported a significant decrease in the percentages of morphologically normal follicles in sheep and cow whole ovaries frozen with CF (24, 46). We observed a lower rate of cell proliferation in CF ovarian fragments both at the beginning and at the end of the culture period. This is certainly related to the higher rate of DNA damage observed at the same time because the presence of double-strand breaks into DNA triggers a complex set of responses that includes cell cycle arrest.

Indeed, DNA damage in DF samples was higher than in fresh controls, even if no differences were observed in the structural integrity between the two groups. However, upon culture progression, DNA repair activity was evident in the DF sample, leading to a substantial reduction of DNA damage, whereas DNA repair activity was virtually absent in the CF samples, leaving the rate of DNA damage substantially unchanged. Evaluation of DNA damage and repair mechanisms allows a more sensitive assessment of cellular damage as compared with measuring apoptosis, which is an irreversible process (48). In this case the high levels of Rad51 observed in DF samples after 7 days of culture allow us to see that the DNA damage observed on day 1 of culture was reversible. In fact, the formation of a Rad51–single-stranded DNA nucleofilament is an essential step that leads to DNA repair (49). The ability of DF ovarian tissue to repair DNA damage is particularly relevant for its long-term function after thawing because DNA double-strand break repair efficiency has been identified as an important determinant of oocyte aging in women (50).

In conclusion, this study indicates that DF allows good preservation of whole ovaries, with most of the parameters taken into consideration almost identical to those recorded in fresh control samples. This encourages a reconsideration of the possible use of whole ovary cryopreservation as a viable alternative to cortical fragments.

However, it must be remembered that a high risk of retransmission of malignancy exists if autotransplantation

of ovarian tissue were performed in women who have had systemic hematologic malignancies (18, 20). In this respect whole ovary cryopreservation cannot offer any improvement in case of autotransplantation, but it could provide some appealing alternative for the development of methods aimed at grafting isolated follicles in an artificial ovary or for the stimulation of primordial follicle growth through ex vivo perfusion for future in vivo or in vitro use.

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APPENDIX B

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human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Beneficial effect of directional freezing on *in vitro* viability of cryopreserved sheep whole ovaries and ovarian cortical slices

S. Maffei[1], G. Pennarossa[2], T.A.L. Brevini[1], A. Arav[3], and F. Gandolfi[1,*]

[1]Laboratory of Biomedical Embryology, Department of Health, Animal Science and Food Safety, Università Degli Studi di Milano, 20133 Milano, Italy [2]Department of Veterinary Medicine, Università Degli Studi di Sassari, 07100 Sassari, Italy [3]Core Dynamics, 70400 Ness Ziona, Israel

*Correspondence address. E-mail: fulvio.gandolfi@unimi.it

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STUDY QUESTION: Does directional freezing improve the structural and functional integrity of ovarian fragments compared with conventional slow freezing and to whole ovary cryopreservation?

SUMMARY ANSWER: Compared with slow freezing, the use of directional freezing significantly improves all structural and functional parameters of ovarian fragments assessed *in vitro* and, overall, whole ovaries were better preserved than ovarian fragments.

WHAT IS KNOWN ALREADY: Directional freezing has been developed to provide an alternative way to cryopreserve large biological samples and it is known to improve the structural and functional integrity of whole ovaries. Conventional slow freezing of ovarian fragments is the procedure more widely used in clinical settings but it causes substantial structural damage that limits the functional period after transfer back into the patient.

STUDY DESIGN, SIZE, DURATION: We performed a 2 × 2 factorial design experiment on a total of 40 sheep ovaries, divided into four groups ($n = 10$ ovaries per group): (i) directional freezing of whole ovary (DFwo); (ii) directional freezing of ovarian fragments (DFof); (iii) conventional freezing of whole ovary (CFwo); (iv) conventional freezing of ovarian fragments (CFof). An additional eight ovaries were used as fresh controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Ewe ovaries were randomly assigned to one of the experimental groups and frozen accordingly. Upon thawing, ovarian tissue was examined morphologically and cultured *in vitro* for 7 days. Samples were analyzed for cell proliferation and apoptosis, for DNA damage and repair activity, and for the presence of a panel of heat shock proteins (HSPs) by immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: Most studied parameters were significantly improved ($P < 0.05$) in all samples cryopreserved with directional compared with slow freezing. The proportion of primordial follicles, which developed to the primary stage in whole ovaries (53 ± 1.7%) and in ovarian fragments (44 ± 1.8%) cryopreserved with directional freezing, was greater than with slow frozen whole ovaries (6 ± 0.5%, $P = 0.001$) or fragments (32 ± 1.5%, $P = 0.004$). After 7 days of culture, cell proliferation in DFwo (28 ± 0.73%) was the highest of all groups ($P < 0.05$) followed by DFof (23 ± 0.81%), CFof (20 ± 0.79%) and CFwo (9 ± 0.85%). Directional freezing also resulted in a better preservation of the cell capacity to repair DNA damage compared with slow freezing both in whole ovaries and ovarian fragments. Apoptosis and HSP protein levels were significantly increased only in the CFwo group. Direct comparison demonstrated that, overall, DFwo had better parameters than DFof and was no different from the fresh controls.

LIMITATIONS, REASONS FOR CAUTION: The study is limited to an *in vitro* evaluation and uses sheep ovaries, which are smaller than human ovaries and therefore may withstand the procedures better.

WIDER IMPLICATIONS OF THE FINDINGS: Improved integrity of ovarian morphology may translate to improved outcomes after transplantation. Alternatively, the particularly good preservation of whole ovaries suggests they could provide a source of ovarian follicles for *in vitro* culture in those cases when the presence of malignant cells poses a substantial risk for the patient.

STUDY FUNDING/COMPETING INTEREST(S): Supported by: Associazione Italiana per la Ricerca sul Cancro (AIRC) IG 10376, Carraresi Foundation and by Legge 7 Regione Autonoma Sardegna (R.A.S). There are no conflicts of interest.

Key words: cryopreservation / directional freezing / whole ovaries / ovarian fragments / follicles

Introduction

A continuous improvement of cancer treatments is steadily increasing the chances of survival in patients of all ages but this positive outcome, at the same time, increases the challenges related to the patients' quality of life. In young women this is often related to the maintenance of fertility (Reinmuth *et al.*, 2008) since the probability of normal full-term pregnancy is reduced to 30–50% in cancer survivors compared with the corresponding healthy general population (Chung *et al.*, 2013).

In particular, bone marrow transplantation (BMT), a common therapeutic procedure for several hematologic procedures, requires aggressive chemotherapy and or radiotherapy, both of which are primary causes of gonadal dysfunction and failure (Jadoul and Donnez, 2012). The use of alkylating agents associated with abdominal ionizing radiation in the range of 5–20 Gy will render infertile almost 100% of the patients prepared for BMT (Anderson and Wallace, 2013; Donnez *et al.*, 2013; Wallace *et al.*, 2005). The damaging effect will increase exponentially from the age of 25 years and a model can predict with reasonable accuracy the age of menopause based on the radiation doses and the age at the time of treatment (Wallace *et al.*, 2005). The model clearly shows that, irrespective of the parameters applied, the risk of premature ovarian failure is high.

All this brings an increasing demand for suitable treatments for fertility preservation in female cancer patients which are strongly influenced by the type of cancer, the type of treatment and age at the time of treatment. Ovarian stimulation followed by oocyte or embryo banking is a possible choice if it can be performed before initiation of cancer therapies, if the tumor is not hormone sensitive, if the patient is sexually mature and if there is a high risk that metastatic cells may have colonized the ovary (Chung *et al.*, 2013).

Ovarian tissue cryopreservation represents another alternative even if it is still considered to be experimental by the American Society for Reproductive Medicine. Indeed this approach may represent the only option when dealing with pre-pubertal girls or with patients who cannot delay the cancer treatment (Donnez and Dolmans, 2010, 2011; Grynberg *et al.*, 2012; Silber, 2012; Donnez *et al.*, 2013). It is possible to cryopreserve cortical strips or the entire organ; however, to date, ovarian cryopreservation and subsequent retransplantation in humans has been limited to avascular cortical pieces (Demeestere *et al.*, 2009; Anderson and Wallace, 2011; Grynberg *et al.*, 2012; Donnez *et al.*, 2013).

Current preference for the clinical use of avascular pieces of ovarian cortex is based on several factors. The smaller size of tissue pieces in comparison with the whole organ facilitates the adequate penetration of the cryoprotectant crossing the stroma to reach granulosa cells and oocytes (Hovatta, 2005). It also facilitates heat transfer during cooling and warming which typically takes place by convection using slow-programmable freezers (Grynberg *et al.*, 2012; Donnez *et al.*, 2013), even if vitrification is beginning to be considered as a viable alternative (Bordes *et al.*, 2005; Keros *et al.*, 2009). However, direct comparisons between slow freezing and vitrification in human have produced conflicting results (Gandolfi *et al.*, 2006; Isachenko *et al.*, 2009, 2010). Transplantation of ovarian fragments either on the exposed medulla of the contralateral ovary (Donnez *et al.*, 2006) or in a peritoneal pocket (Donnez *et al.*, 2012) is technically easier than the vascular anastomosis required for the transplantation of an entire ovary (Kim, 2010). Finally, the cryopreservation of several fragments of the same ovary allows the possibility to repeat the autotrasplantation procedure should the transplanted pieces cease to function. At the same time the use of ovarian fragments suffers from some limitations, the main one being the loss of over 50% of the primordial follicles due to tissue ischemia that takes place after transplantation (Van Eyck *et al.*, 2009; Van Eyck *et al.*, 2010). This, together with the damages caused by cryopreservation, leads to a functional lifespan that, depending on the follicle density at the time of the procedure, can vary, on average, from 2 to 5 years (Bromer and Patrizio, 2009; Donnez *et al.*, 2013). In addition, since this procedure is largely devoted to cancer patients, there is the real danger that the ovarian tissue contains malignant cells posing a severe risk to the patient (Meirow *et al.*, 2008; Dolmans *et al.*, 2010; Greve *et al.*, 2012).

Transplantation of fresh whole ovary has been attempted with success in human (Leporrier *et al.*, 1987; Hilders *et al.*, 2004; Silber *et al.*, 2008) and in several animal species (Bromer and Patrizio, 2009) demonstrating that anastomosis of the ovarian pedicle is difficult but feasible. However, cryopreservation of the entire organ has proved more difficult, largely due to the physical constraints that limit an appropriate heat transfer between the core and the periphery of the organ (Arav and Natan, 2009). In addition, the large volume of the whole organ poses some limitation to the perfusion and diffusion of cryoprotectants (Falcone and Bedaiwy, 2005; Torre *et al.*, 2013). Both are essential for preventing intravascular ice formation which would irreversibly compromise a rapid and efficient resumption of the blood supply (Pegg, 2010). An efficient protocol for the cryopreservation and re-anastomosis of the entire ovary could potentially reduce ischemic damage. However, data obtained from experiments mostly performed in sheep show some positive results (Grazul-Bilska *et al.*, 2008) and pregnancies (Salle *et al.*, 2002) but also highlight stromal and vascular damage following either slow freezing (Onions *et al.*, 2009; Wallin *et al.*, 2009) or vitrification (Salle *et al.*, 2003; Courbiere *et al.*, 2006).

This has prompted the development of an alternative way to cryopreserve large samples, such as whole ovaries, based on directional cooling (Arav and Natan, 2009).

The technology for directional cooling is based on a series of heat conductive blocks at set temperatures that are arranged in a line along which the sample proceeds at a pre-set velocity. The blocks have different temperatures so that a gradient temperature can be imposed along the track. The samples to be frozen are placed inside glass test tubes and moved along the temperature gradient formed by the blocks. The freezing rate is determined by the combination of temperature gradient and speed of the sample along the track (Arav and Natan, 2012). If the velocity of the sample is slower than the speed at which the heat is removed from the center of the sample towards its periphery, heat transfer is quickly removed in the direction opposite to that of the sample movement, thanks to the large mass of the conductive material which forms the blocks. All this results in a uniform cooling rate throughout the sample (Gavish *et al.*, 2008). This method has been used before for the cryopreservation of sheep whole ovaries with good success and maintenance of ovarian function for 6 years (Arav *et al.*, 2005, 2010). However, the number of ovaries cryopreserved with this method is limited and it has never been applied to ovarian fragments.

To move forward the cryopreservation of both avascular ovarian fragments and of whole ovaries must improve (Donnez *et al.*, 2013). Therefore, the aim of the present study was to perform a direct comparison between a convection-based programmable slow-freezer and the multi-thermal-gradient apparatus for the cryopreservation of ovarian

cortical strips and of whole ovaries. Based on our own results (Gandolfi *et al.*, 2006) and those in the literature (Shaw and Trounson, 2002; Arav and Natan, 2009) all experiments were performed on sheep ovaries owing to its similarity with the human ovary.

Materials and Methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (Italy).

Sheep female reproductive tracts were collected at the local slaughterhouse and transported to the laboratory in cold (0–4°C) 0.9% saline solution.

Eight ovaries were randomly assigned to fresh control group (CTR) and 10 to each experimental group. The experimental groups included the following: (1) directional freezing of whole ovary (DFwo); (2) directional freezing of cortical tissue (DFof); (3) conventional freezing of whole ovary (CFwo) and (4) conventional freezing of cortical tissue (CFof).

Sample preparation

Upon arrival to the laboratory, ovaries were randomly divided in three groups, i.e. fresh controls, ovaries to be frozen as whole organs or as ovarian fragments. Samples to be frozen as whole organs were perfused via the ovarian artery with Ringer's solution and 10 IU/l heparin for 10 min, followed by perfusion with cryoprotectant solution containing Leibovitz L-15 medium (Life Technologies, Italy), 10% fetal bovine serum (v/v; Life Technologies, Italy) and 1.5 M dimethylsulfoxide for 10 min at a flow rate of 1.3 ml/min (Grazul-Bilska *et al.*, 2008) at room temperature.

Ovarian fragments (10 × 5 × 1 mm) were sliced from the cortical region and immersed in the same cryoprotectant solution. Each ovary and all fragments (8–10) from a single ovary were inserted into a 16 mm diameter and 80 mm long glass test tube (Manara, Israel) containing 10 ml of the same solution. The tube wall is 1 mm thick and made of quartz. Tubes were closed with specifically designed silicon plugs. All samples were equilibrated at +4°C before the freezing process.

Conventional slow freezing

Conventional slow freezing was performed in a Kryo 560M apparatus (Planer, UK) with the following program: (i) from 4° to −40°C using a cooling rate of 0.5°C/min (seeding was induced at −7°C), (ii) from −40° to −100°C at 5°C/min and (iii) immersion in liquid nitrogen (Grazul-Bilska *et al.*, 2008).

Directional freezing

Directional freezing was performed with a Multi-Thermal-Gradient (MTG, IMT Ltd., Ness Ziona, Israel). The three thermal blocks were set at 4°, −10° and −70°C, respectively, thereby imposing a temperature gradient around the tubes. Freezing tubes were pushed lengthwise along the thermal gradient and the speed was set at 0.01 mm/s resulting in a cooling rate of 0.3°C/min down to −70°C, after which samples were plunged into liquid nitrogen (Arav *et al.*, 2005, 2010).

Thawing

Samples were thawed by plunging the test tubes into a 68°C water bath for 20 s and then into a 37°C water bath for 2 min. The contents of the tube were quickly emptied into a petri dish containing Leibovitz L-15 medium at 37°C. Whole ovaries were immediately perfused through the ovarian artery with Leibovitz L-15 medium at 37°C supplemented with decreasing sucrose concentrations (0.25, 0.125 and 0 M) at 3 ml/min for a total of 30 min (10 min for each step; Jeremias *et al.*, 2002; Bedaiwy *et al.*, 2003).

Ovarian fragments were washed three times for 10 min each in Leibovitz L-15 supplemented with the same sucrose concentrations (0.25, 0.125 and 0 M). The material was then randomly allocated for further analysis. Each experiment was performed on a minimum of three independent biological replicates and all samples were blinded for analysis.

Follicle culture

The *in vitro* culture of the thawed and fresh control samples was performed as previously described (Matos *et al.*, 2007). Several ovarian fragments of ~2 × 2 × 1 mm were derived from whole ovaries and isolated cortical pieces. Three randomly selected fragments from each ovary were immediately fixed in 10% formaldehyde solution for histological studies (time 0, control). Other randomly selected fragments were placed into 24-well culture dishes (Sarstedt, Italy) and individually cultured in 1 ml of medium for 7 days at 39°C under an atmosphere of 5% CO_2 in air. The culture medium was composed of α-Minimum Essential Medium (Life Technologies, Italy) supplemented with ITS (6.25 μg/ml insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenium; Life Technologies), 2 mM glutamine (Life Technologies, Italy), 0.23 mM pyruvate, 2 mM hypoxantine, 1.25 mg/ml bovine serum albumin (BSA), 100 μg/ml penicillin G, 100 μg/ml streptomycin, 200 mIU/ml sheep FSH and 50 ng/ml human recombinant basic fibroblast growth factor (R&D Systems, Italy) (Matos *et al.*, 2007). The medium was replaced every 2 days.

Morphological analysis

Samples collected before or after cryopreservation and at Days 0 and 7 of follicle culture were fixed with 10% formaldehyde, embedded in paraffin and the sections either stained with hematoxylin and eosin or used for the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay or immunohistochemistry, as described below. Slides were observed under an Eclipse E600 microscope (Nikon, Japan) and pictures were acquired with Nis Elements Software (Version 4.0). For each measure, a minimum of four sections, 200 μm^2 apart, from each sample were examined. Follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte), intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte) or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Follicles were further classified as normal when a spherical oocyte with a non-pyknotic nucleus was surrounded by granulosa cells organized in discrete layers, or degenerated when a mis-shapen oocyte, with or without vacuolation, with a pyknotic nucleus was surrounded by disorganized granulosa cells detached from the basement membrane (Silva *et al.*, 2004).

Immunohistochemistry

Sections were boiled for 5 min in antigen unmasking solutions (Vector Laboratories, Italy) and then blocked with a solution of 1% BSA. Non-specific sites were blocked with a solution of phosphate-buffered saline (PBS) containing 5% BSA and 10% non-immune serum (Life Technologies). Samples were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal antibody specific for the Ki67 marker of cell proliferation (1:50 dilution; Abcam, UK); mouse monoclonal anti-γH2AX, a DNA-damage marker (1:200; Abcam) and rabbit monoclonal anti-RAD51, a DNA-repair marker (1:200; Santa Cruz) (Maffei *et al.*, 2013). Sections were washed three times with PBS and incubated with suitable secondary antibodies (Alexafluor; Life Technologies) for 45 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Blinded samples were observed under an Eclipse E600 microscope (Nikon, Japan), pictures were acquired with Nis Elements Software (Version 4.0) and the images were analyzed using ImageJ software as described below.

TUNEL assay

To detect DNA fragmentation, sections were placed on silanized slides (Bio-Optica, Italy) and apoptotic cells were detected by *In situ* Cell Death Detection Kit, TMR red (Roche, Italy) according to the manufacturer's instructions. Positive controls were treated with DNAse I recombinant (50 U/mL; Roche) in 50 mM Tris–Hcl, pH 7.5, 1 mg/ml BSA for 10 min at +25°C before DNA end labeling. For negative controls, TDT was omitted from the reaction mixture. TUNEL-positive cells were calculated as cells per area of ovarian tissue using the image analysis software ImageJ, as detailed below.

Cell proliferation and TUNEL were evaluated per mm^2 therefore every cell type present in the examined area was considered.

Image analysis

Pictures were taken with constant exposure parameters in order to be analyzed with the image analysis software ImageJ (http://rsbweb.nih.gov/ij/index.html). Threshold adjustments were applied to generate a black and white image which was analyzed to quantify fluorescent signal. In each case, marker expression was normalized by DAPI fluorescence.

Western blots

Ovarian fragments (~2 mm^3) derived from five experimental groups at Days 0 and 7 of culture were homogenized individually, lysed and constitutive proteins were extracted using ReadyPrep™ Protein Extraction Kit (Bio-Rad, Italy). Protein concentration was assessed with DC Protein Assay (Bio-Rad). Aliquots of 50 μg proteins were prepared and resuspended in (1:1) 2× Laemmli sample buffer (4% (w/v) sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% (w/v) glycerol, 0.004% bromophenol blue and 0.125 M Tris–HCl (pH 6.8). Samples were loaded and electrophoresed on 8 or 10% SDS-polyacrylamide gels depending on the molecular weight of the target molecule. Proteins were then transferred onto 0.45-μm pore size nitrocellulose filters (Life Technologies) according to Towbin *et al.* (1979) using 0.5 A/cm^2. Equal sample loading and transfer efficiency were confirmed by staining of the membrane with Ponceau Red. The membrane was probed with the primary antibodies listed in Table I, as described by Pennarossa *et al.* (2012) and previously validated in sheep. Monoclonal anti-β-actin was used as a loading control (data not shown). Protein bands were visualized by Western Breeze® chemiluminescent kit (Life Technologies, Italy).

Statistical analysis

All experiments were performed on a minimum of three independent biological replicates. Data are reported as mean ± SEM and analyzed using one-way analysis of variance followed by Turkey's *post hoc* tests (Statistical Package for the Social Sciences 20, IBM). Differences were considered statistically significant if $P < 0.05$.

Table I List of antibodies used for western blot analysis of proteins extracted from sheep ovaries.

Antibody	Host species	Company	Catalog number	WB working dilution
HSF1	Goat	Santa Cruz Biotech	sc-8061	1:1000
HSP40	Rabbit	Abcam	ab69402	1:10 000
HSP70	Mouse	Abcam	ab5439	1:5000
HSP90	Mouse	Abcam	ab13492	1:1000
β-Actin	Mouse	Sigma	a5441	1:1000

HSF1, heat shock transcription factors 1; HSP40, heat shock protein 40; HSP70, heat shock protein 70; HSP90, heat shock protein 90; WB, western blotting.

Results

All groups were compared with each other and with a fresh control, shortly after thawing and after 7 days of *in vitro* culture.

In vitro follicle development

The percentage of follicle growth in each group, after 7 days of culture, is shown in Fig. 1. The proportion of primordial follicles which developed to the primary stage both in whole ovaries (53 ± 1.7%) and in ovarian fragments (44 ± 1.8%) cryopreserved with directional freezing was significantly greater than with conventionally frozen whole ovaries (6 ± 0.5%, $P = 0.001$) or fragments (32 ± 1.5%, $P = 0.004$).

The percentage of developing follicles in whole ovaries was higher than in ovarian fragments when frozen with directional freezing ($P = 0.005$) but lower when frozen with conventional freezing ($P = 0.001$). Finally, whole ovaries frozen with directional freezing had a rate of growing follicles not significantly different from fresh ovaries (55 ± 1.9%, $P = 0.106$).

Morphological assessment of cryopreservation damage

The percentage of morphologically normal follicles in each group immediately after thawing and cryoprotectant removal is shown in Table II. The percentage of intact follicles was inversely related to the stage of development, in all groups including fresh controls. There were more intact follicles of all developmental stages following directional freezing when compared with conventional freezing. Ovarian fragments showed a higher rate of normal morphology compared with whole ovaries when frozen with conventional freezing but the opposite was observed when whole ovaries and ovarian fragments were preserved with directional freezing. The percentage of morphologically normal follicles in the DFwo group (89.93 ± 4.10%) was not significantly different from the fresh control (92.54 ± 4.36%).

Cell proliferation and apoptosis

Differences in developmental competence of primordial follicles were consistent with differences in cell proliferation rate as examined by the expression of Ki67. The percentage of proliferating cells was comparable among the different groups at Day 0 (Fig. 2A). After 7 days of culture, Ki67-positive cells in fresh tissue (30 ± 0.78%) and in group DFwo (28 ± 0.73%) were the highest of all groups ($P < 0.05$). The rate of cells expressing Ki67 in group DFof (23 ± 0.81%) was significantly higher than those in group CFof (20 ± 0.79%, $P = 0.03$) and an average of only 9 ± 0.85% of Ki67-positive cells was found in group CFwo (Fig. 2A).

TUNEL-positive cells were detected both at Days 0 and 7 of culture in all experimental groups but apoptotic levels were not related to cell proliferation. Immediately after thawing and the removal of the cryoprotectant, the number of apoptotic cells per mm^2 was low and comparable among all groups. The TUNEL assay showed that the number of positive cells after 7 days in culture was comparable for all experimental groups

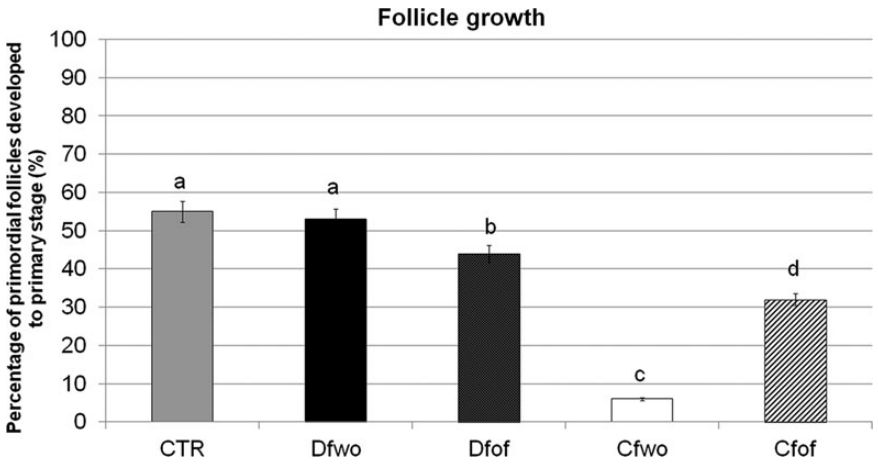

Figure 1 Functional assessment of sheep cortical fragments cultured *in vitro* for 7 days. Percentage (mean ± s.e.m.) of primordial follicles developed in the primary stage after 7 days of culture. Values with different indices (**a**–**d**) are significantly different ($P < 0.05$); one-way analysis of variance followed by Turkey's *post hoc* tests. CTR, control ($n = 280$); DFwo, directional freezing whole ovary ($n = 280$); DFof, directional freezing ovarian fragments ($n = 260$); CFwo, conventional freezing whole ovary ($n = 230$); CFof, conventional freezing ovarian fragments ($n = 250$).

Table II Morphologically normal follicles.

Morphologically normal follicles (%)					
	Follicle type				
	Primordial	Intermediate	Primary	Secondary	All follicles
CTR	97.5 ± 4.5[a] ($n = 600$)*	91.2 ± 4.34[a] ($n = 350$)*	88.40 ± 4.12[a] ($n = 300$)*	71.5 ± 4.44[a] ($n = 60$)*	92.54 ± 4.36[a] ($n = 1310$)*
DFwo	94.8 ± 4.15[a] ($n = 600$)*	89.28 ± 4.29[a] ($n = 350$)*	85.23 ± 3.95[a] ($n = 300$)*	68.62 ± 3.34[a] ($n = 60$)*	89.93 ± 4.10[a] ($n = 1310$)*
DFof	88.7 ± 4.12[b] ($n = 300$)*	80.17 ± 4.30[b] ($n = 150$)*	72.91 ± 3.68[b] ($n = 100$)*	53.25 ± 3.21[b] ($n = 20$)*	82.24 ± 4.82[b] ($n = 570$)*
CFwo	78.4 ± 3.97[c] ($n = 600$)*	58.10 ± 3.32[c] ($n = 350$)*	43.5 ± 3.21[c] ($n = 300$)*	23.4 ± 1.92[c] ($n = 60$)*	62.46 ± 3.52[c] ($n = 1310$)*
CFof	84.40 ± 4.03[d] ($n = 300$)*	69.72 ± 3.27[d] ($n = 150$)*	64.20 ± 3.34[d] ($n = 100$)*	42.70 ± 2.76[d] ($n = 20$)*	75.53 ± 3.52[d] ($n = 570$)*

Percentage (mean ± s.e.m) of morphologically normal primordial, intermediate, primary and secondary follicles in fresh tissue (CTR) and in experimental groups immediately after thawing. DFwo, directional freezing of whole ovary; DFof, directional freezing of ovarian fragments; CFwo, conventional slow freezing of whole ovary; CFof, conventional slow freezing of ovarian fragments.
*Number of evaluated follicles.
[a,b,c,d]Different superscripts in the same column indicate a significant difference amongst experimental groups for the same follicle type as determined by one-way analysis of variance followed by Turkey's *post hoc* tests ($P < 0.05$).

except CFwo, where a significantly higher percentage of TUNEL-positive cells was visible after 7 days (29.34 ± 1.13, $P = 0.002$) (Fig. 2B).

Expression level of heat shock-related molecules

The effect on heat stress response after cryopreservation was examined by quantitative analysis of HSP40, HSP70, HSP90 and HSF1 proteins at the end of the warming and dilution/cryoprotectant removal steps and after 7 days of culture. Changes were only detected in cortical tissue from the conventionally cryopreserved whole ovaries after 7 days of culture, where the protein levels were significantly higher than on Day 0 (Fig. 3), indicating that conventional freezing applied to the whole ovary induced the activation of proteins involved in stress-response pathways. There were no differences within or between the other groups (Fig. 3).

DNA damage and repair

The expression of γ-H2AX, a marker of DNA damage, and of RAD51, a marker of DNA repair are shown in Figs 4 and 5. Virtually no γ-H2AX or RAD51 signals were detected in fresh tissue. Clear signals of DNA damage, in the form of γ-H2AX foci, were observed in groups DFwo, DFof and CFof at the beginning (Day 0) of culture, together with a weak staining for DNA repair. After 7 days DNA damage was significantly decreased while DNA repair activity increased, as indicated by a clear RAD51 signal. Very strong staining for γ-H2AX was observed in group CFwo at Day 0 indicating widespread DNA damage but RAD51 labeling was low at that time, suggesting a lack of DNA repair.

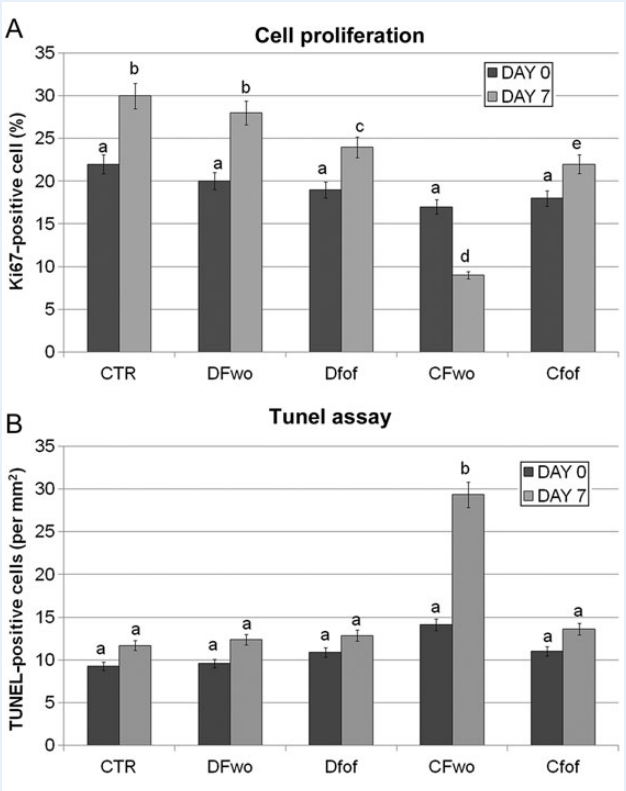

Figure 2 Quantitative evaluation of Ki67 and TUNEL at the beginning and the end of *in vitro* culture. Percentage (mean ± s.e.m) of immunopositive cells for Ki67 (**A**). Number of TUNEL-positive cells per mm^2 (**B**). Values with different indices (a–e) are significantly different ($P < 0.05$); one-way analysis of variance followed by Turkey's *post hoc* tests; $n = 2630 \pm 340$ nuclei counted for each section. CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments.

Discussion

Cryopreservation of ovarian tissue is now a well-established method for preserving female fertility in a series of circumstances when gonadotoxic treatments cannot be delayed or in patients before puberty (Grynberg *et al.*, 2012; Donnez *et al.*, 2013). This can be achieved through the excision and banking of whole organs or of fragments of the cortical region. However, whereas no live births or pregnancies have been achieved following the cryopreservation of whole human ovaries, 24 live babies have been obtained after transplantation of frozen-thawed ovarian fragments (Donnez *et al.*, 2013). This clearly suggests that the use of ovarian fragments is the method of choice even if, at present, the average functional life span of these samples ranges from 2 to 5 years (Bromer and Patrizio, 2009; Donnez *et al.*, 2013). In theory, the cryopreservation of whole ovaries followed by the vascular anastomosis of the ovarian pedicle of the thawed organ should provide a larger follicular reserve and a longer life span of the transplant. However, currently available data do not support this hypothesis. This is largely due to the damage caused by the freezing procedures applied to a sample as large as a human ovary. We have recently performed a direct comparison of sheep ovarian tissue viability using whole ovaries cryopreserved with a conventional programmable slow freezer or a directional freezing apparatus (Maffei *et al.*, 2013). Our results indicated that ovarian structure and function were significantly better preserved by the directional freezer. Therefore, it was of interest to determine whether directional freezing could also improve the viability of ovarian fragments and how these would compare with whole ovaries frozen with the same method.

In the present study, the morphological analysis of ovarian tissue immediately after thawing showed that the use of directional freezing significantly improved the integrity of all follicular structure from primordial to secondary stages. Since it is well known that the major cause of cellular damage is intracellular ice formation (Mazur, 1977; Pegg, 2010), this observation suggests that the controlled direction of the ice front, from one end to the other of the samples, was effective in maintaining a precise cooling rate thereby preventing the formation of intracellular ice. Although the effect was evident in both whole organs and ovarian fragments, it was more efficient in whole organs since the rate of morphologically intact follicles was not significantly different from that recorded in the fresh samples. Functional analysis of *in vitro* growth of primordial follicles confirmed the same pattern. Directional freezing was beneficial for both entire ovaries and ovarian fragments but DFwo was the only group whose viability was the same as that of fresh tissue. This result is somehow unexpected since cryopreservation efficiency is inversely related to the size of the sample. Large biological samples, in fact, suffer from a long isothermal period caused by the massive release of latent heat during the process of ice formation (Balasubramanian and Coger, 2005; Pegg, 2010). The phenomenon is caused by the energy generated by water molecules when they rejoin to form an ice crystal. Such energy is released in the form of heat that causes a temperature rise of the surrounding structures (Petersen *et al.*, 2006). The heat is normally transferred to the ice crystals that have just been formed because these are made of conductive material. The consequence is a transient thawing followed by refreezing in a sequence that is repeated several times through the thickness of the sample causing severe cell damage (Barratt *et al.*, 1998; Koshimoto and Mazur, 2002). Damage is usually reduced by keeping the ratio of surface to volume as high as possible so that the excessive heat is removed by adjusting the cooling rate to achieve uniform freezing. The thinner the sample the faster the heat, released from its inner parts, is removed minimizing the damage. However the need to have samples as thin as possible is in contrast with the requirement of having a large follicle population upon thawing and it is impossible when using low surface to volume ratio samples like whole ovaries. The large mass of conductive material in the MTG apparatus serves the purpose of quickly removing the latent heat from large samples thereby preventing the thawing and freezing sequence (Gavish *et al.*, 2008).

These considerations largely explain the better results of directional freezing compared with conventional slow freezing described in our experiments but cannot account for the better performance obtained with whole ovaries than with smaller ovarian fragments. We hypothesize that the different ways that the cryoprotectant was administered may have a role. The presence of the ovarian pedicle in entire ovaries enabled an extensive uniform perfusion through the vasculature (Maffei *et al.*, 2013). A study to compare outcomes of conventional to directional freezing after vascular perfusion of cryoprotectants is needed. In this study the ovarian fragments were directly immersed into the cryoprotectant thus relying on diffusion. Our experiments were performed in this way in order to mimic the different way

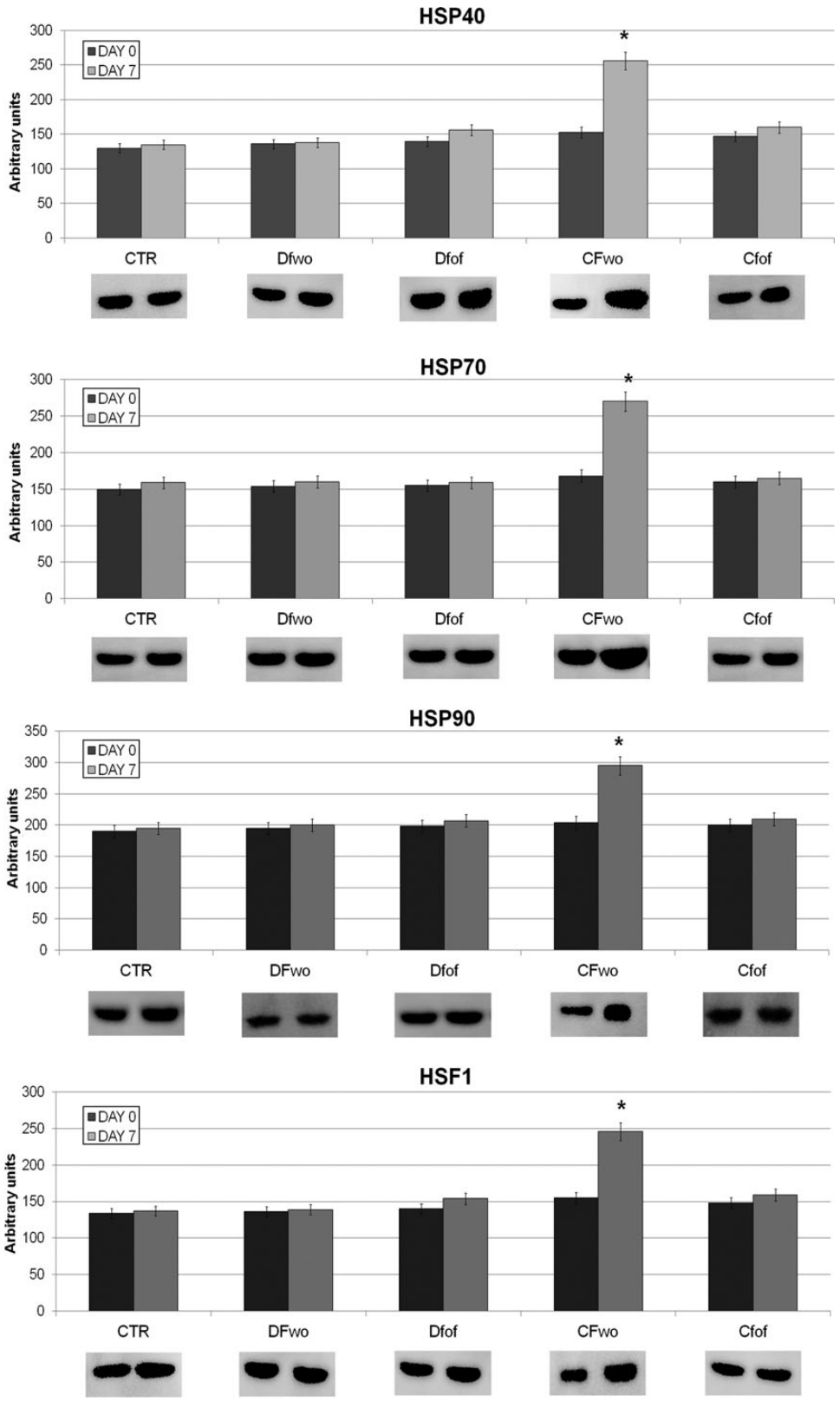

Figure 3 Heat stress response after cryopreservation. Western blots and densitometric analysis of heat shock proteins in sheep ovarian proteins extract from all experimental groups at Days 0 and 7 of culture. Values are expressed as mean ± s.e.m. of three replicates measured in arbitrary units. * $P < 0.05$ determined with one-way analysis of variance followed by Turkey's *post hoc* tests; $n = 10$ fragments (2 × 2 × 1 mm) for each replicate. HSP40, heat shock protein 40; HSP70, heat shock protein 70; HSP90, heat shock protein 90; HSF1, heat shock transcription factor 1. CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments.

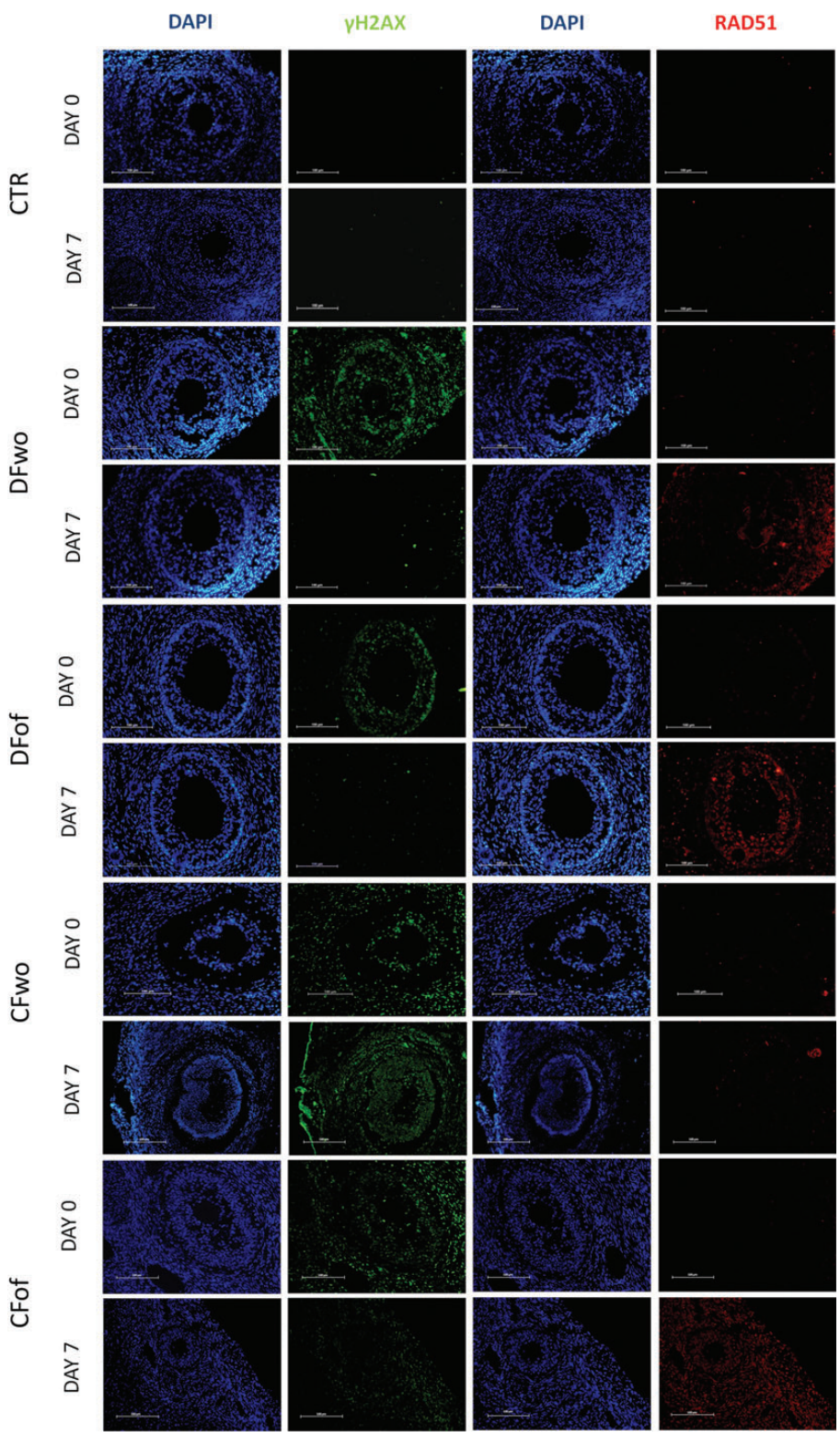

Figure 4 Representative pictures of cells positive for gamma H2A histone, member X (γH2AX, marker of double-strand DNA breaks, green) and RAD51 recombinase (RAD51, marker of repair ability, red). Immunostaining was carried out at Days 0 and 7 of *in vitro* culture. CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments. Scale bar = 100 μm.

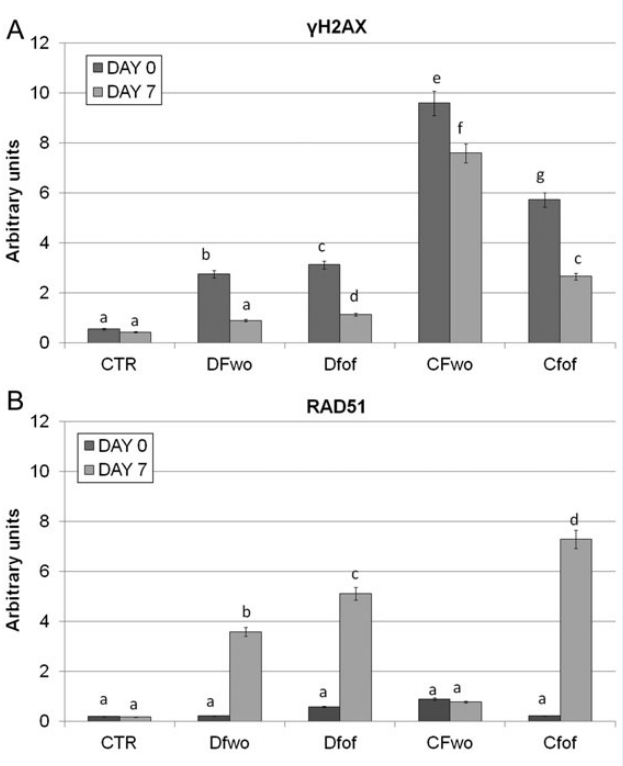

Figure 5 Quantitative assessment of γH2AX and RAD51 by image analysis. Values are expressed as mean ± s.e.m. of three replicates measured in arbitrary units. Different superscripts (**a**–**g**) indicate significant differences ($P < 0.05$) as determined with one-way analysis of variance followed by Turkey's *post hoc* tests. γH2AX, gamma H2A histone, member X; CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments.

samples would be treated in a clinical setting. Based upon our findings it will be interesting to study the effect of cryoprotectant administration in more detail.

Consistent with the favorable effect of directional freezing on primordial follicle viability *in vitro* we observed a higher cell proliferation rate compared with samples that underwent conventional freezing. In an attempt to understand the mechanisms that may have caused this effect we studied known responses to temperature stress, which is inherent to any form of cryopreservation.

One of the best characterized responses to thermal stress is the expression of HSPs (Rupik *et al.*, 2011). HSPs bind to other proteins, termed clients, which are damaged by thermal or other stress conditions and mediate their transport to target organelles for repair or degradation (Vabulas *et al.*, 2010). They have also physiological roles and are constitutively expressed in the absence of stress during many processes including gametogenesis and embryogenesis (Hartl and Hayer-Hartl, 2009).

We examined the expression of HSP70 because it is the most abundant and the most highly conserved (Vabulas *et al.*, 2010). This and other members of the family act through repeated client-chaperone binding and release cycles sustained by ATP hydrolysis and nucleotide exchange reactions. Heat shock responses in vertebrates are regulated at the transcriptional level by the heat shock transcription factor (HSF) family composed of several members, with HSF1 regulating heat shock gene expression in mammals (Fujimoto and Nakai, 2010). When a mammalian cell is exposed to thermal stress, HSF1 molecules present in the cytoplasm as inactive monomers combine to form biologically active trimers. These bind the heat shock response elements (HSEs), which are present upstream of each heat shock gene and activate their transcription (Fujimoto and Nakai, 2010). Our results show that the HSP machinery was significantly stimulated over baseline levels only in the case of conventional freezing of whole ovaries. This is consistent with particularly extensive protein damage in this group since the client-chaperone binding and release cycles are repeated several times until the client has reached its final active conformation, or has entered the proteolytic system (Kampinga and Craig, 2010). The presence of extensive cellular damage in CFwo samples was confirmed by the significant increase in apoptotic cells after 7 days of culture, consistent with the notion that the accumulation of damaged or misfolded proteins, if unresolved, can lead to caspase-8 oligomerization, activation and apoptosis (Pan *et al.*, 2011). However, protein damage is not the only cause of apoptosis as it can also be activated by a failure of DNA repair mechanisms (Batista *et al.*, 2009). In fact the high levels of DNA damage associated with a lack of DNA repair activity observed in CFwo samples is consistent with the high apototic rate observed in the same group. This implies that the amount of DNA damage taking place in cells of CFwo ovaries is sufficient to overwhelm their capability for DNA repair and lead to their elimination through apoptosis.

Indeed the analysis of the dynamics of DNA repair mechanisms in our different experimental groups enabled the identification of different levels of cellular damage and was more sensitive than measuring apoptosis, which is an irreversible process (Kale *et al.*, 2012). We have examined the expression of the histone variant γH2AX, the phosphorylated form of H2AX which is formed within minutes of DNA damage (Yuan *et al.*, 2010). The presence of γ-H2AX at the foci which form at DNA break sites attracts BRCA2 which, in turn, binds to RAD51 and activates DNA repair (Huen and Chen, 2010). In contrast to HSPs, γ-H2AX and RAD51 are not visible in the nuclei of intact cells; therefore, it was possible to detect their increased expression in our experimental samples. Results were consistent with the pattern of follicular viability since DNA damage was significantly lower in directional freezing samples followed by more intense DNA repair activity suggesting that the DNA damage observed on Day 1 of culture was reversible. In fact, the formation of an Rad51-ssDNA nucleofilament is an essential step that leads to DNA repair (Seeber *et al.*, 2013). The ability of directional freezing to preserve the ability of ovarian tissue to repair DNA damage is particularly relevant for its long-term function after thawing since DNA double-strand break repair efficiency has been identified as an important determinant of oocyte aging in women (Titus *et al.*, 2013).

In conclusion, this study indicated that directional freezing improves the viability of cryopreserved ovarian tissue not only when used with whole organs but also with ovarian fragments. This information provides a way to further improve the efficiency of the cryopreservation procedure. The direct comparison of ovarian fragments and whole organs unexpectedly revealed that the latter show better preservation of early follicles, in many aspects almost identical to those recorded in fresh control samples. However, the persistent technical difficulties linked to the surgical procedures required for retransplantation is likely to still

limit the use of whole ovaries in clinical settings. On the other hand, the increasing concerns related to the danger of transferring malignant cells with transplanted frozen–thawed ovarian tissue may lead to a re-evaluation of the technique in terms of using whole ovaries as a source of high-quality isolated follicles to be grown in artificial ovaries (Vanacker *et al.*, 2012) or other, yet to be described, methods.

Authors' roles

S.M. and G.P. performed the research; T.B., A.A. and F.G. designed the research and T.B and F.G wrote the paper.

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Conflict of interest

There are no conflicts of interest to declare.

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APPENDIX C

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1. **Maffei S.**, Pennarossa G.,. Brevini T.A.L, Gandolfi F. Effect of High Hydrostatic Pressure on ovarian tissue fragments cryopreservation. 4th COST Action, GEMINI General Conference Maternal Interactions with Gametes and Embryos. 30th September – 2nd October 2011, Gijon, Spain.
2. **S. Maffei**, J. R. V. Silva, M. Hanenberg, G. Pennarossa, T. A. L. Brevini, A. Arav, and F. Gandolfi. Whole-ovary cryopreservation: A direct comparison of conventional and directional freezing. 39th International Embryo Transfer Society (IETS). 19-22 January 2013, Hannover, Gemany.
3. T. A. L. Brevini, **S. Maffei**, G. Pennarossa, A. Arav, and F. Gandolfi. Multi-thermal gradient freezing allows the cryopreservation of sheep whole ovaries with the same efficiency of ovarian fragments. 39th International Embryo Transfer Society (IETS). 19-22 January 2013, Hannover, Gemany.
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Scientific award:

"Peter W. Farin Student Travel Award" of the 39th Annual Conference of the International Embryo Transfer Society, January 19-22, 2013 in Hannover, Germany.

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APPENDIX D

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1. Pennarossa, **S. Maffei**, M.M. Rahman, A. Vanelli, T.A.L Brevini, F.Gandolfi. Expression and localization of the chaperone protein MRJ in pig gametes and gonads. Systems Biology in Maternal Communication With Gametes and Embryos. GEMINI RESEARCH TRAINING SCHOOL. May 2010, Opatija, Croatia.
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Review

Parthenogenesis in non-rodent species: developmental competence and differentiation plasticity

T.A.L. Brevini*, G. Pennarossa, A. Vanelli, S. Maffei, F. Gandolfi

Unistem, Centre for Stem Cell Research, Laboratory of Biomedical Embryology, Università degli Studi di Milano, Italy

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Abstract

An oocyte can activate its developmental process without the intervention of the male counterpart. This form of reproduction, known as parthenogenesis, occurs spontaneously in a variety of lower organisms, but not in mammals. However, it must be noted that mammalian oocytes can be activated *in vitro*, mimicking the intracellular calcium wave induced by the spermatozoon at fertilization, which triggers cleavage divisions and embryonic development. The resultant parthenotes are not capable of developing to term and arrest their growth at different stages, depending on the species. It is believed that this arrest is due to genomic imprinting, which causes the repression of genes normally expressed by the paternal allele. Human parthenogenetic embryos have recently been proposed as an alternative, less controversial source of embryonic stem cell lines, based on their inherent inability to form a new individual. However many aspects related to the biology of parthenogenetic embryos and parthenogenetically derived cell lines still need to be elucidated. Limited information is available in particular on the consequences of the lack of centrioles and on the parthenote's ability to assemble a new embryonic centrosome in the absence of the sperm centriole. Indeed, in lower species, successful parthenogenesis largely depends upon the oocyte's ability to regenerate complete and functional centrosomes in the absence of the material supplied by a male gamete, while the control of this event appears to be less stringent in mammalian cells. In an attempt to better elucidate some of these aspects, parthenogenetic cell lines, recently derived in our laboratory, have been characterized for their pluripotency. *In vitro* and *in vivo* differentiation plasticity have been assessed, demonstrating the ability of these cells to differentiate into cell types derived from the three germ layers. These results confirmed common features between uni- and bi-parental embryonic stem cells. However data obtained with parthenogenetic cells indicate the presence of an intrinsic deregulation of the mechanisms controlling proliferation vs. differentiation and suggest their uni-parental origin as a possible cause.

Keywords: Parthenogenesis; Mammalian; Pluripotency; Differentiation; Centriole

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* Corresponding author. Tel: 0039(8) 02 503179880; fax: 0039(8) 02 50317989.
E-mail address: tiziana.brevini@unimi.it (T.A.L. Brevini).

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Isolation, Characterization and Differentiation Potential of Cardiac Progenitor Cells in Adult Pigs

A. Vanelli · G. Pennarossa · S. Maffei · G. B. Galvez · G. Cossu · M. Rahaman · F. Gandolfi · T. A. L. Brevini

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Introduction

Cardiovascular disease (CVD) remains as the first cause of death worldwide [1, 2]. It is known that a rising heart failure incidence is associated with unhealthy life styles and increasing life expectance. Current therapies are typically symptomatic and, even though they provide some survival benefit, they cannot reverse the loss of contractile cardiac tissue due to ischemic injury. For this reason, high expectances are associated to the recent developments in stem cell biology and regenerative medicine that promise to replace damaged or lost cardiac muscle with healthy tissue and thus to improve the quality of life and survival in patients with various cardiomyopathies [3].

The recent identification of different classes of cardiac progenitor cells suggests that the heart, classically considered a terminally differentiated, post-mitotic organ, may rather contain a stem cell compartment, responsible for both tissue turn-over and regeneration, which follows acute or chronic damage to the cardiac tissue [4–6]. Several groups have already reported the isolation of different types of cardiac stem-like cells based on distinct cell surface markers, such as c-kit, Isl-1 and Sca-1 [7–11] or the ability to generate genuine cardiomyocytes and integrate into heart tissue as induced pluripotent stem cells [12], or also in relation to their capacity to influence the neovascularization of the ischemic tissue, as satellite cells [13]. These cells are able to restore cardiac function after ischemic injury, although with variable efficiency [7, 14]. Another type of cells associated with the heart was mesoangioblast, a class of vessel-associated stem cells that can differentiate into various mesoderm cell types. They were originally described in the mouse embryonic dorsal aorta [15] and later similar cells have been identified and characterized from postnatal small vessels of human skeletal muscle [16] and mouse and human heart [4, 17].

However, the experiments carried out until now were predominantly performed on mice and human. This restricts significantly the possibility to apply the results obtained in preclinical studies that cannot be performed using the human as a model and, at the same time, are limited by the evident differences between mouse and humans, such as their size, mice heart rate and their general anatomy [18, 19]. In particular, human and mouse hearts diverge in the coronary architecture, the variations of which are much bigger in humans compared to mice. As a consequence, whereas the size and location of the ischemic area are fairly constant in the mouse, a much larger variation exists in the human [20]. Differences can also be appreciated at the cellular level, as indicated by the higher capillary density and the larger cross sectional area of the myocytes in human, in comparison to the mouse [21, 22]. Consequently, extrapolation of murine systems, particularly after induction of cardiovascular stress, must be meticulously monitored, when applied clinically, because of the obvious differences

A. Vanelli, G. Pennarossa and S. Maffei equally contributed to the present work

A. Vanelli · G. Pennarossa · S. Maffei · M. Rahaman · F. Gandolfi · T. A. L. Brevini (✉)
Unistem, Laboratory of Biomedical Embryology,
Università degli Studi di Milano,
via Celoria 10,
Milan, Italy
e-mail: tiziana.brevini@unimi.it

G. B. Galvez
Centro Nacional de Investigaciones Cardiovasculares (CNIC),
Madrid, Spain

G. Cossu
Stem Cell Research Institute, San Raffaele Hospital,
Milan, Italy

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Centrosome Amplification and Chromosomal Instability in Human and Animal Parthenogenetic Cell Lines

Tiziana A. L. Brevini · Georgia Pennarossa · Sara Maffei · Gianluca Tettamanti · Arianna Vanelli · Sara Isaac · Amir Eden · Sergio Ledda · Magda de Eguileor · Fulvio Gandolfi

Abstract Parthenotes have been proposed as a source of embryonic stem cells but they lack the centriole which is inherited through the sperm in all mammalian species, except for rodents. We investigated the centrosome of parthenotes and parthenogenetic embryonic stem cells using parthenogenetic and biparental pig pre-implantation embryos, human and pig parthenogenetic and biparental embryonic stem cells, sheep fibroblasts derived from post implantation parthenogenetic and biparental embryos developed in vivo. We also determined the level of aneuploidy in parthenogenetic cells. Oocytes of all species were activated using ionomycin and 6-dimethylaminopurine (6-DMAP). Over 60 % of parthenogenetic blastomeres were affected by an excessive number of centrioles. Centrosome amplification, was observed by microscopical and ultrastructural analysis also in parthenogenetic cell lines of all three species. Over expression of PLK2 and down regulation of CCNF, respectively involved in the stimulation and inhibition of centrosome duplication, were present in all species. We also detected down regulation of spindle assembly checkpoint components such as BUB1, CENPE and MAD2. Centrosome amplification was accompanied by multipolar mitotic spindles and all cell lines were affected by a high rate of aneuploidy. These observations indicate a link between centrosome amplification and the high incidence of aneuploidy and suggest that parthenogenetic stem cells may be a useful model to investigate how aneuploidy can be compatible with cell proliferation and differentiation.

Keywords Centrosome · Chromosome instability · Human · Pig · Sheep · Parthenogenesis · Cell lines · Embryos · Foetus

T. A. L. Brevini (✉) · G. Pennarossa · S. Maffei · A. Vanelli · F. Gandolfi
Laboratory of Biomedical Embryology, Centre for Stem Cell Research (UniStem), Università degli Studi di Milano, Via Celoria 10, 20133 Milan, Italy
e-mail: tiziana.brevini@unimi.it

G. Tettamanti · M. de Eguileor
Department of Biotechnology and Life Sciences, Università dell' Insubria, Varese, Italy

S. Isaac · A. Eden
Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, Israel

S. Ledda
Department of Veterinary Pathology and Clinic, Università degli Studi di Sassari, Sassari, Italy

Introduction

Mature oocytes can be activated in vitro leading to the generation of parthenotes that will form blastocysts morphologically indistinguishable from those derived from fertilized eggs.

Parthenotes have been used as a source of embryonic stem cells (ESC) in different species including mouse [1], rabbit [2, 3], pig [4] non-human primates [5] and human [6–11].

Parthenogenetic stem cells (ParthESC) show the traditional features associated with their biparental counterpart, including expression of pluripotency markers, telomerase activity, embryoid body formation, in vitro differentiation and, in most cases, teratoma formation.

It has been suggested that the use of ParthESC for therapeutic applications may have some advantages. The main one would be the reduced variability of the major histocompatibility alleles, due to their monoparental origin [12], which, in turn, would greatly facilitate the matching between cell lines and patients [13]. Moreover, parthenogenetic cell lines would provide an exact match to the oocyte donor's genome (both nuclear and mitochondrial). The non embryonic nature of the

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Why is it so Difficult to Derive Pluripotent Stem Cells in Domestic Ungulates?

F Gandolfi, G Pennarossa, S Maffei and TAL Brevini

Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, Milan, Italy

Content

Pluripotent stem cells are the focus of an extremely active field of investigation that is bringing new light on our understanding of the mechanisms that control pluripotency and differentiation. Rodent and primates are the only species where true, or *bona fide*, pluripotent stem cells have been derived. The attempts to derive pluripotent stem cells from domestic ungulates have been going on for more than 20 years with little progress. Cell lines from these species present a series of limitations that have precluded their use for both basic and clinically oriented studies. However, in the last 3 years, some substantial progress have been made making the currently available ungulate pluripotent stem cells closest than ever before to their human and mouse counterpart. This result has been achieved through both conceptual and technical progress that will be illustrated and discussed in this review.

Introduction

Organs counteract the physiological wear and tear thanks to a small population of cells known as organ-specific or adult stem cells. They are classified on the basis of their potency that can span from unipotency when only a single cell type can be generated to multipotency when a stem cell can originate to all or many cells of a single germ layer. When a stem cell can originate, all cells from all three germ layers are defined as pluripotent. Pluripotency is limited to the epiblast, a transient tissue that exists only for brief stage of embryonic development; therefore, stable pluripotent cells are not a physiological component of the body but exist only *in vitro* (Smith 2001).

The translation of the knowledge on stem cell biology into clinical applications is taking place at an increasingly fast pace. The ease of such translation depends on a delicate balance that includes the degree of plasticity of the stem cell of interest, its physiological rate of proliferation and its accessibility. As a result, clinical applications of stem cells of say hematopoietic (Sheppard et al. 2012) or epithelial (De Luca et al. 2006; Pellegrini et al. 2009) origin are well advanced and in many case have already entered the level of accepted procedures, whereas the use of stem cells such as those of muscle (Tedesco et al. 2010) or neuronal origin (Lindvall and Kokaia 2010) are still far from a clinical application both because their proliferation potential is more limited and because they are not easy to access especially those located in the central nervous system. These limitations could be overcome by the use of pluripotent stem cells that are attractive because easy to propagate *in vitro* and therefore readily available.

An important step for the translation of basic research into clinical applications is the use of animal models that are intermediate between laboratory rodents and humans. Domestic ungulates like ruminants and pig have often been used for pre-clinical research and their use in regenerative medicine could be beneficial as well.

However, whereas the derivation of organ-specific stem cells has been successful (Spencer et al. 2011), pluripotent stem cells have so far been difficult to obtain in these species.

True Embryonic Stem Cells

It is widely acknowledged that true, so-called *bona fide,* embryonic stem cells (ESC) can only be derived from mouse (Evans and Kaufman 1981), human (Thomson et al. 1998) and non-human primate embryos (Thomson et al. 1995).

Cell lines derived from these species share some major properties such as unlimited replication *in vitro* (self-renewal), capacity to differentiate into any of the different tissues that make the body, expression of core pluripotency factors such as *OCT4, SOX2* and *NANOG*, formation of teratomas if injected into immunodeficient mice.

However, in the years following the derivation of primate ESC, it became progressively clear that they present substantial differences when compared to mouse ESC (mESC), even if they share the same definition of ESC.

Differences begin in the culture dish where the presence of LIF and BMP4 in the culture medium is required to maintain the undifferentiated state of proliferating mouse ESC through the activation of the JAK/STAT3 pathway (Ying et al. 2003); however, the stimulation of the same pathways in primate ESC leads to a rapid differentiation (Xu et al. 2005). On the contrary, primate ESC require the presence in the culture medium of activin A and/or basic fibroblast growth factor (bFGF or FGF2) and depend on the activation of the activin/Nodal pathway (Xu et al. 2008). Mouse ESC can be derived only from a small number of 'permissive' strains, whereas primate ESC, and human ESC in particular, show no limitations related to a specific genetic background. Mouse and primate ESC differ also in their morphology. Small, compact and domed colonies are typically formed by mESC as opposed to primate ESC, which grow in larger, flat colonies. mESC colonies are propagated after dissociation to single cells, but the same treatment would rapidly kill primate ESC whose colonies need to be detached from the feeder layer and fragmented mechanically. In general, mESC grow more vigorously and can easily adapt to clonal culture conditions enabling the derivation of lines from single cells. This

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Pluripotency Network in Porcine Embryos and Derived Cell Lines

TAL Brevini, G Pennarossa, S Maffei and F Gandolfi

Laboratory of Biomedical Embryology, Centre for Stem Cell Research, Università degli Studi di Milano, Milan, Italy

Contents

Huge amounts of work have been dedicated to the establishment of embryonic stem cell lines from farm animal species since the successful isolation of embryonic stem cells from the mouse and from the human. However, no conclusive results have been obtained so far, and validated lines have yet to be established in domestic animals. Many limiting factors have been suggested and need to be studied further to isolate truly pluripotent cell lines from livestock. In this review, we will discuss the difficulties in deriving and maintaining embryonic stem cell lines from farm animal embryos and how can this lack of success be explained. We will summarize results obtained in our laboratory regarding derivation of pluripotent cells in the pigs. Problems related to the identification of standard methods for derivation, maintenance and characterization of cell lines will also be examined. We will focus our attention on the need for appropriate stemness-related marker molecules that can be used to reliably investigate pluripotency in domestic species. Finally, we will review data presently available on functional key pluripotency-maintaining pathways in farm animals.

Introduction

The derivation of embryonic stem cells (ESCs) in farm animal species could represent a great improvement in developmental biology as well as applied biomedical research. In particular, farm animal ESCs could provide a powerful tool for genetic engineering aimed at improved production traits and products, for disease resistance and for biopharming (Keefer et al. 2007). These cells could also represent an excellent experimental model in pre-clinical trials, where the feasibility and the clinical potential of stem cell therapies could be studied. The close morphological and physiological resemblance to humans could be greatly advantageous for creation of biomedical models of human diseases and cell transplantation therapies. Although huge amounts of work have been dedicated to the establishment of ESC lines from farm animals over the past 20 years, no conclusive results have been obtained so far. Indeed, although progress towards the derivation of ESCs from porcine, bovine, caprine, ovine and equine species has been made and despite the many peer-reviewed journal articles describing ungulate ES or ES-like cell lines over the past 20 years, validated lines have yet to be established in farm animal species.

Why is it so difficult to derive and maintain ESCs from farm animal embryos? How can this lack of success be explained? Several concomitant factors have been suggested that hamper the process and still need to be elucidated. In this review, we will discuss some of the issues to be addressed and the hurdles to be overcome, related to the identification of standard methods for the derivation, maintenance and characterization of cell lines, the use of appropriate markers and elucidation of functional key pluripotency-maintaining pathways (Fig. 1). In particular, we will focus on the information available for the pig, which is a desirable species to create pluripotent cell lines, because of its striking similarities in terms of anatomy, physiology, metabolism and organ development with primates, and may therefore represent an important pre-clinical model for cell therapy approaches (Yang et al. 2000; Aleem Khan et al. 2006; Brevini et al. 2007a; Keefer et al. 2007).

Timing of Derivation

The first differentiation event in the mouse occurs at the late morula stage, when cells forming the outer compartment adopt an epithelial fate and the first two lineages, trophectoderm and inner cell mass (ICM), can be appreciated. Further differentiation of the ICM leads to formation of the epiblast and the primitive endoderm. In the interval between days 3.5 and 4.5, the hypoblast begins its evolution towards the extra embryonic endoderm and the yolk sac, while the epiblast develops into the embryo proper. The timing of this process is different in other species, and in particular, the interval between fertilization and the formation of the three early embryonic lineages is more protracted in porcine and bovine embryos, where epiblast formation begins at hatching and is complete towards day 12 (Vejlsted et al. 2005, 2006). This implies that no defined epiblast is present in these species before hatching. However, once formed, it will persist until day 13, when the major part of the epiblast differentiates into neural ectoderm and forms the neural plate. This process is accompanied by a gradual down-regulation of OCT4 and the onset of beta-tubulin III expression, which is correlated with neural differentiation, but, at the same time, indicates that embryos at this stage are no longer suitable for ESC derivation. Altogether, these observations indicate that farm animal species have a longer interval during which the epiblast is accessible and suggest that ICMs obtained from farm animal pre-hatching blastocysts may not be exactly equivalent to mouse epiblasts, usually isolated for ESC derivation.

What is the optimal time for isolation and establishment of porcine ESC lines? The available data are controversial. Chen et al. (1999) showed that recently hatched blastocysts had a higher success rate for establishment of pig ES-like cell cultures when compared with late-hatched blastocysts. Very poor outgrowth formation ability was detected in 5- to 6-day embryos, whereas 10- to 11-day embryos led to establishment of ES-like cell lines (Hochereau-de Reviers and Perreau 1993). Pluripotent epiblast cell cultures were

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Characterization of the Constitutive Pig Ovary Heat Shock Chaperone Machinery and Its Response to Acute Thermal Stress or to Seasonal Variations[1]

Georgia Pennarossa,[3,4] Sara Maffei,[3,4] Mahbubur M. Rahman,[4] Giovanna Berruti,[5] Tiziana A.L. Brevini,[4] and Fulvio Gandolfi[2,4]

[4]*Laboratory of Biomedical Embryology, Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, Milan, Italy*
[5]*Department of Biology, Università degli Studi di Milano, Milan, Italy*

ABSTRACT

Reduced oocyte competence causes the lower fertility reported in domestic sows during the warm months of the year. Somatic cells express heat shock proteins (HSPs) to protect themselves from damage caused by thermal stress. HSPs are classified as molecular chaperones and control the correct folding of newly synthesized or damaged proteins. The present work performed a comprehensive survey of the different components of the heat shock chaperone machinery in the pig ovary, which included the HSP40, HSP70, HSP90, and HSP110 families, as well as heat shock factors (HSF) 1 and 2. Pig ovarian follicles constitutively expressed different members of these families; therefore, we examined their ability to respond to heat stress. In order to take into account the role of the complex follicular architecture, whole pig ovaries were exposed to 41.5°C for 1 h. This exposure significantly disrupted oocyte maturation and determined the upregulation of the *HSP70*, *HSP40*, *HSPH1*, *HSPA4*, *HSPA4L*, *HSF1*, and *HFS2* genes, whereas expression levels of *HSP90A* and *HSP90B*, as well as those of genes unrelated to heat stress were not altered. Unexpectedly HSP and HSF expression levels changed only in oocytes but not in cumulus cells. Cumulus-oocyte complexes isolated from ovaries collected in summer showed the same pattern as those collected in winter. We conclude that the HSP chaperone machinery is constitutively fully operational in the pig ovary. However, following thermal stimuli or seasonal variations, cumulus cell HS-related gene expression remains unchanged, and only oocytes activate a response, suggesting why this mechanism is insufficient to preserve their competence both in vitro and in vivo.

cumulus cells, HSF1, HSF2, HSP110, HSP40, HSP70, HSP90, oocyte, oocyte competence, ovary, seasonal infertility

INTRODUCTION

The domestic pig exhibits a period of depressed reproductive performance during late summer and early autumn months [1–4]. This seasonal infertility is characterized by a reduction in farrowing rate [4], an extended weaning-to-estrous interval [5], and a delay in the onset of puberty [6]. All these manifestations cause a considerable economic damage to the swine industry.

It has been difficult to determine a specific cause for this phenomenon because many different parameters are involved, including heat stress, photoperiod, humidity, genetic background, and management [7]. A disruptive effect of heat stress on the oocyte has been extensively described in the cow (e.g., see ref. [8]) and more recently in pig [2, 9]. However most cells developed mechanisms based on the expression of heat shock proteins (HSPs) to protect themselves from damage during cellular stress [10].

HSPs are classified as molecular chaperones because they bind to other proteins, named clients, which are damaged by the stress conditions and mediate their transport to target organelles for repair or degradation [11]. They are grouped in six families of structurally unrelated molecules [12] whose members also have physiological roles and are constitutively expressed in the absence of stress in many processes including gametogenesis and embryogenesis [13].

The HSP70 family (also known as HSPA) is found in all eukaryotic cells and is the most abundant and most highly conserved complex [11]. Members of this family act through repeated client-chaperone binding and release cycles sustained by ATP hydrolysis and nucleotide exchange reactions. However HSP70s never work alone, rather they require at least the presence of the two cofactors DnaJ protein (HSP40) and nucleotide exchange factor (NEF) [14]. The DnaJ protein facilitates client binding, stimulating ATPase activity, whereas client dissociation is prompted by the ADP dissociation stimulated by NEFs. Members of the HSP110 family, which includes HSPA4, HSPA4L, and HASH1, are known NEFs that remove ADP after ATP hydrolysis, enabling a new HSP70 interaction cycle with non-native protein substrate [15].

Another highly conserved and highly expressed HSP family is HSP90, which in mammals, includes the two cytosolic isoforms HSP90α and HSP90β [16]. Similar to HSP70, HSP90 proteins also exert their function in collaboration with a number of cochaperones that regulate HSP90 activity in several ways such as the recruitment of client proteins and the regulation of the ATPase cycle [17].

Heat shock response in vertebrates is regulated at the transcription level by the heat shock transcription factors (HSFs) family, composed of several members, with HSF1 regulating heat shock gene expression in mammals [18]. When a mammalian cell is exposed to heat stress, HSF1 molecules,

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[2]Correspondence: Fulvio Gandolfi, Laboratory of Biomedical Embryology, Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, Via Celoria 10, Milan, 20133, Italy. E-mail: fulvio.gandolfi@unimi.it
[3]These authors contributed equally to this work.

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Brief demethylation step allows the conversion of adult human skin fibroblasts into insulin-secreting cells

Georgia Pennarossa[a], Sara Maffei[a], Marino Campagnol[a], Letizia Tarantini[b,c], Fulvio Gandolfi[a], and Tiziana A. L. Brevini[a,1]

[a]Laboratory of Biomedical Embryology, UniStem, Center for Stem Cell Research, [b]Department of Clinical and Community Sciences, and [c]Istituto di Ricerca e Cura a Carattere Scientifico Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Università degli Studi di Milano, Italy

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The differentiated state of mature cells of adult organisms is achieved and maintained through the epigenetic regulation of gene expression, which consists of several mechanisms including DNA methylation. The advent of induced pluripotent stem cell technology enabled the conversion of adult cells into any other cell type passing through a stable pluripotency state. However, indefinite pluripotency is unphysiological, inherently labile, and makes cells prone to culture-induced alterations. The direct conversion of one cell type to another without an intermediate pluripotent stage is also possible but, at present, requires the viral transfection of appropriate transcription factors, limiting its therapeutic potential. The aim of this study was to investigate whether it is possible to achieve the direct conversion of an adult cell by exposing it to a demethylating agent immediately followed by differentiating culture conditions. Adult human skin fibroblasts were exposed for 18 h to the DNA methyltransferase inhibitor 5-azacytidine, followed by a three-step protocol for the induction of endocrine pancreatic differentiation that lasted 36 d. At the end of this treatment, 35 ± 8.9% fibroblasts became pancreatic converted cells that acquired an epithelial morphology, produced insulin, and then released the hormone in response to a physiological glucose challenge in vitro. Furthermore, pancreatic converted cells were able to protect recipient mice against streptozotocin-induced diabetes, restoring a physiological response to glucose tolerance tests. This work shows that it is possible to convert adult fibroblasts into insulin-secreting cells, avoiding both a stable pluripotent stage and any transgenic modification.

pancreatic beta cell | cell plasticity

Regenerative medicine requires new cells that can be delivered to patients for repairing and renovating degenerated or damaged tissues (1). When such cells are not readily available, two main strategies have been developed to obtain them: directed differentiation, by which pluripotent cells, exposed to specific cell culture conditions designed to mimic natural events, assume a specific cell fate, and transdifferentiation, also referred to as reprogramming, which enables a fully differentiated cell type to be converted into another without going through an undifferentiated pluripotent stage (2).

Induced pluripotent stem cell (iPSC) technology showed that the stability of a mature phenotype can be overcome when transforming a somatic cell of any patient in an unlimited source of autologous pluripotent cells. The elimination of the immune rejection risk provided by iPSCs immediately boosted the clinical potential of directed differentiation (3). However, the requirement of permanent integration of viral vectors into the host genome to generate iPSCs poses a severe limit to their current therapeutic use (1). This has stimulated the development of several protocols for a virus-free iPSC derivation, but at present, these approaches are generally more technically demanding and less efficient, and therefore have not gained a widespread adoption (2). Similar limitations apply to recent examples of successful transdifferentiation, as the direct conversion of one mature cell type into another has been obtained only with virus-based transfection protocols. As for iPSC derivation, virus-free transdifferentiation protocols would be highly desirable and are being actively pursued (4, 5).

The differentiated state of mature cells of adult organisms is acquired through a gradual loss of differentiative potency (6), leading to a progressive restriction in their options (7), and is physiologically very stable. Differentiation is achieved and maintained through the epigenetic regulation of gene expression, which consists of different mechanisms including DNA methylation, histone modification, nucleosome packaging and rearrangements, higher-order chromatin structures, and the interplay between chromatin and nuclear lamina (8). For this reason, the complete reversal of this process requires extensive reprogramming that makes it inefficient and prone to errors (9).

We reasoned that part of the problem may derive from considering the achievement of a stable pluripotent state as an indispensable step, even if this does not occur during embryonic development, where pluripotency is limited to a short window of time.

Among the different mechanisms involved in lineage specification and cellular reprogramming, DNA demethylation plays a major role both during early embryonic development and during somatic cell reprogramming (10). Therefore, we tested whether a short exposure to a demethylating agent is sufficient to allow the direct conversion of an adult mature cell into another differentiated cell type. To this purpose, we selected the cytidine analog 5-azacytidine (5-aza-CR), a well-characterized DNA methyltransferase inhibitor known to activate the expression of silent genes (11) and to alter the differentiation state of embryonic (12) and mesenchymal cell lines (13).

This approach would be useful for therapeutic applications only if it can be applied to easily accessible primary cells; therefore, we worked on adult dermal fibroblasts that can be simply propagated in vitro with a stable phenotype and that are little prone to genetic instability. 5-aza-CR-treated skin fibroblasts have been exposed to an endoderm differentiation protocol, and we observed the efficient formation of pancreatic beta cell-like cells. We monitored cell morphology and gene expression pattern (Table S1) during the process and confirmed that fibroblasts were transiting along a brief dedifferentiation state and were readdressed to the new cell lineage. Pancreatic converted cells (PCCs) were shown to express early and mature pancreatic lineage specific markers. A functional beta-cell phenotype was validated by their intracellular production and storage of insulin. Furthermore, exposure to glucose triggered a dynamic response, inducing active insulin release in cell supernatants. On transplantation into SCID, mice PCCs were able to protect them against streptozotocin (STZ)-induced hyperglycemia.

Results

Fibroblasts obtained from skin biopsies formed a monolayer (Fig. 1*A*), displaying the vigorous growth in culture typical of this cell population (doubling time, 18–24 h; Fig. S1*A*) and a standard elongated morphology. Uniform immunostaining with the fibroblast-specific marker vimentin (Fig. 1*A*) and total absence of pluripotency or endoderm/pancreatic markers expression allowed us to determine that we worked with homogeneous cell populations (Fig. 2 and Figs. S2–S4).

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[1]To whom correspondence should be addressed. E-mail: tiziana.brevini@unimi.it.

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Reprogramming of Pig Dermal Fibroblast into Insulin Secreting Cells by a Brief Exposure to 5-aza-cytidine

G. Pennarossa · S. Maffei · M. Campagnol · M. M. Rahman · T. A. L. Brevini · F. Gandolfi

Abstract Large animal models provide useful data for pre-clinical research including regenerative medicine. However whereas the derivation of tissue specific stem cells has been successful. pluripotent stem cells so far have been difficult to obtain in these species. A possible alternative could be direct reprogramming but this has only been described in mouse and human. We have recently described an alternative method for reprogramming human somatic cells based on a brief demethylation step immediately followed by an induction protocol. Aim of the present paper was to determine whether this method is applicable to pig in the attempt to achieve cell reprogramming in a large animal model for the first time. Pig dermal fibroblasts were exposed to DNA methyltransferase inhibitor 5-aza-cytidine (5-aza-CR) for 18 h. After a brief recovery period, fibroblast were subjected to a three-step protocol for the induction of endocrine pancreatic differentiation that was completed after 42 days. During the process pig fibroblast rapidly lost their typical elongated form and gradually became organized in a reticular pattern that evolved into distinct cell aggregates. After a brief expression of some pluripotency genes, cells expression pattern mimicked the transition from primitive endoderm to endocrine pancreas. Not only converted cells expressed insulin but were able to release it in response to a physiological glucose challenge in vitro. Finally they were able to protect recipient mice against streptozotocin-induced diabetes. This work shows, that the conversion of a somatic cell into another, even if belonging to a different germ layer, is possible also in pig.

Keywords Pre-clinical model · Epigenetic conversion · Diabetes

Introduction

The use of animal models that are intermediate between laboratory rodents and humans is an important step for translating basic research into clinical applications [1]. Domestic ungulates like ruminants and pig have often been used for several kind of pre-clinical research and their use could be beneficial in regenerative medicine as well. However whereas the derivation of organ specific stem cells has been successful [2, 3], pluripotent stem cells have so far been difficult to obtain in these species.

Despite the first attempts to obtain embryonic stem cells (ESC) in pig date back more than 20 years ago [4, 5] so far it has been very difficult if not outright impossible to derive bona fide embryonic stem cells [6–10]. In most cases what have been defined as ES-like cell lines, showed several major deficiencies ranging from short life in culture to lack of controlled pluripotency or of true chimerism [10].

At present it is not clear whether the lack of ungulate ESC is due to the lack of appropriate culture conditions or the epiblast from these species is inherently different from that of rodent and primates so that "suspending" it in vitro to derive stable cell lines may not be possible [11].

A recent attempt showed promising results culturing day 10–12,5 elongated pig blastocysts in medium supplemented with FGF2 instead of LIF [12]. The resulting cell lines showed a robust self-renewal and the ability to differentiate into precursor cells derived from all three germ layer as well as into trophectoderm and germ cell precursors. However, as for all previous attempts neither chimera nor fully differentiated cell types were obtained with these lines.

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G. Pennarossa · S. Maffei · M. Campagnol · M. M. Rahman · T. A. L. Brevini · F. Gandolfi (✉)
Laboratory of Biomedical Embryology - Department of Health, Animal Science and Food Safety and Center for Stem Cell Research, Università degli Studi di Milano, Via Celoria 10, Milan 20133, Italy
e-mail: fulvio.gandolfi@unimi.it

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