Università degli Studi di Napoli *Federico II*

DOTTORATO DI RICERCA IN BIOLOGIA

PhD IN BIOLOGY

Cycle - XXIX

Novel methods for fertility preservation in cancer patients: Role of improved ovarian tissue cryopreservation and culture systems.

Candidate: Sam Sudhakaran

Tutor: Prof. Riccardo Talevi

Co tutor: Prof. Roberto Gualtieri

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Introduction:

Introduction

1. Cancer incidence worldwide and improvements in treatment

Cancer, among all other medical conditions still remains one among the main medical threats worldwide. WHO determined cancer to be the second biggest cause of death worldwide after cardiovascular diseases, claiming around 8.8 million lives in 2015 alone. (WHO GHO data 2015). Moreover, WHO also estimated 14.1 million new cancer cases diagnosed in 2012. These stimates show that if the present rates continue there would be a staggering 23.6 million cancer patients diagnosed each year by 2030 (WHO 2014). Studies suggest that, 1 in 51 women would have had an invasive cancer diagnosed by 39 years of age (Alketruse et al., 2007). Recent reports supported by the national cancer institute, have shown a 0.8% increase in cancer affected children during the past decade (Edwards et al., 2013).

On the upside, recent advances in chemotherapy, radiotherapy, and bone marrow transplantation can cure as high as 90% of women and children affected by cancer and other disorders requiring such treatment (Donnez et al., 2013). The five-year survival rates for cancer patients has improved to 68%, compared to a mere 50%, 30 years ago, owing to advancements in anti-cancer therapy. (National Cancer Institute, 2012). During the past 3 decades, survival rates of childhood cancers in particular have improved significantly due to both improved antineoplastic agents and radiotherapy. In Europe alone the 5 year childhood cancer survival rates have greatly improved to ~70–82% owing to multi-agent chemotherapy agents being used for treatment (Edgar et al., 2009; Gatta et al., 2014, The Childhood Cancer Foundation, 2015). In other words, this would translate to ~300,000 and 500,000 childhood and adolescent cancer survivors in Europe alone (Winther et al., 2015).

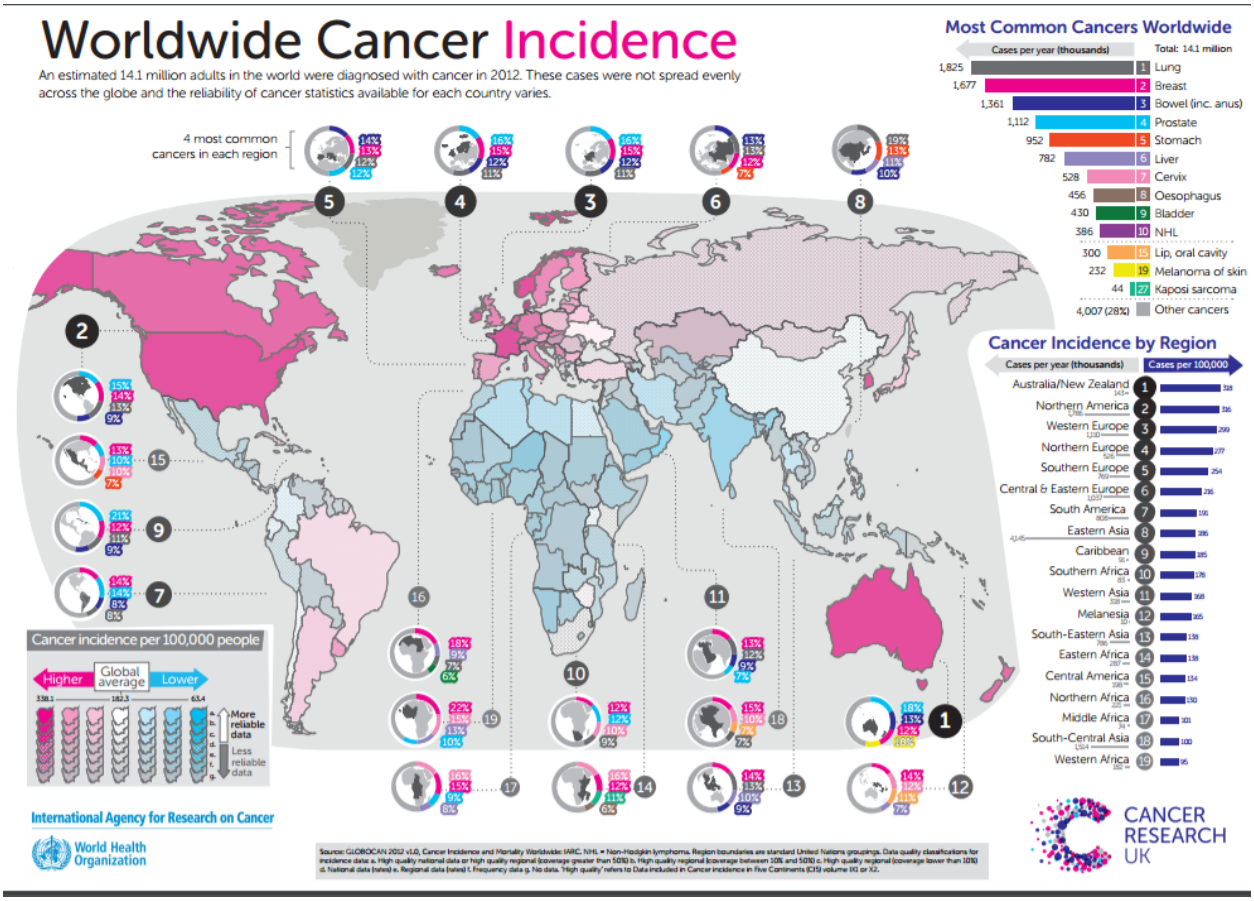


Chart. 1 Cancer Incidence worldwide. Cancer research UK

2. *Anti- cancer treatment and fertility*

Inspite of promising results obtained in anti-cancer treatment, ionizing radiation and aggressive chemotherapy, can result in some degree of premature ovarian failure in almost all patients requiring such therapy. The most common type of cancer in adult women today is breast cancer, affecting 1 in 9 women, with an estimated 25% of these women premenopausal at diagnosis (Stearns et al., 2006). The main problems associated with chemotherapy in female survivors include early menopause and increased incidence of infertility in women who do not loose complete ovarian activity post

chemotherapy (Letourneau et al., 2012a). Premature menopause can result in reduced quality of life (Letourneau et al., 2012b), and also has associated risks including osteoporosis (Bruning et al., 1990), cardiovascular disease (Jeanes et al., 2007) and psychosocial problems, such as depression (Carter et al., 2005).

Whole body irradiation coupled with intensive chemotherapy associated with bone marrow transplantation poses one of the greatest threats to the patient's fertility future (Donnez et al., 2013). Moreover, several studies have shown that a radiation dose as low as 5- 20 Gy, is sufficient to cause gonadal function impairment and particularly the human oocyte has been found to be extremely sensitive to radiation doses such that < 2Gy intensity could be enough to destroy 50% of primordial follicles (Wallace et al 2005).

In terms of chemotherapeutic agents the ovarian reserve is particularly sensitive to alkylating agents which are classified as high risk for later gonadal dysfunction (Schmidt et al 2011, Anderson et al 2013). On the other hand, treatment using platinum agents and plant alkaloids are considered safer (Byrne et al 1992). Cyclophosphamide, an alkylating chemotherapeutic agent has been proven to be one among the most devastating to oocytes and granulosa cells (Wallace et al 2003, Donnez et al 2011). The ovarian reserve is completely abolished after treatment regimes including alkylating agents such as busulphan wherein Teinturier et al reported a 0% recovery after its administration preceding bone marrow transplantation (Teinturier et al 1998).

2.1 Targets of chemotherapeutic damage

Chemotherapeutic agents possess various targets of damage within the ovarian tissue like oocytes, primordial or growing pool of follicles, somatic supporting cells (granulosa cells and stromal cells). While chemotherapy treatment may induce POF by directly destroying the primordial follicles, or indirectly via the loss of activated, growing follicles causing mass activation of the follicle pool (Meirow et al., 2010). The commitment of activated follicles to either ovulation or atresia means that their loss will directly contribute to determination of the reproductive lifespan. Growing follicles,

however, produce factors, such as AMH (Durlinger et al., 1999), which regulate the rate of follicle activation: thus acute loss of the growing follicle population is thought to result in increased recruitment of primordial follicles into the growing pool. This mechanism is recently termed as the mechanism of follicle burn out post chemotherapy (Kalich-Philosoph et al., 2013).

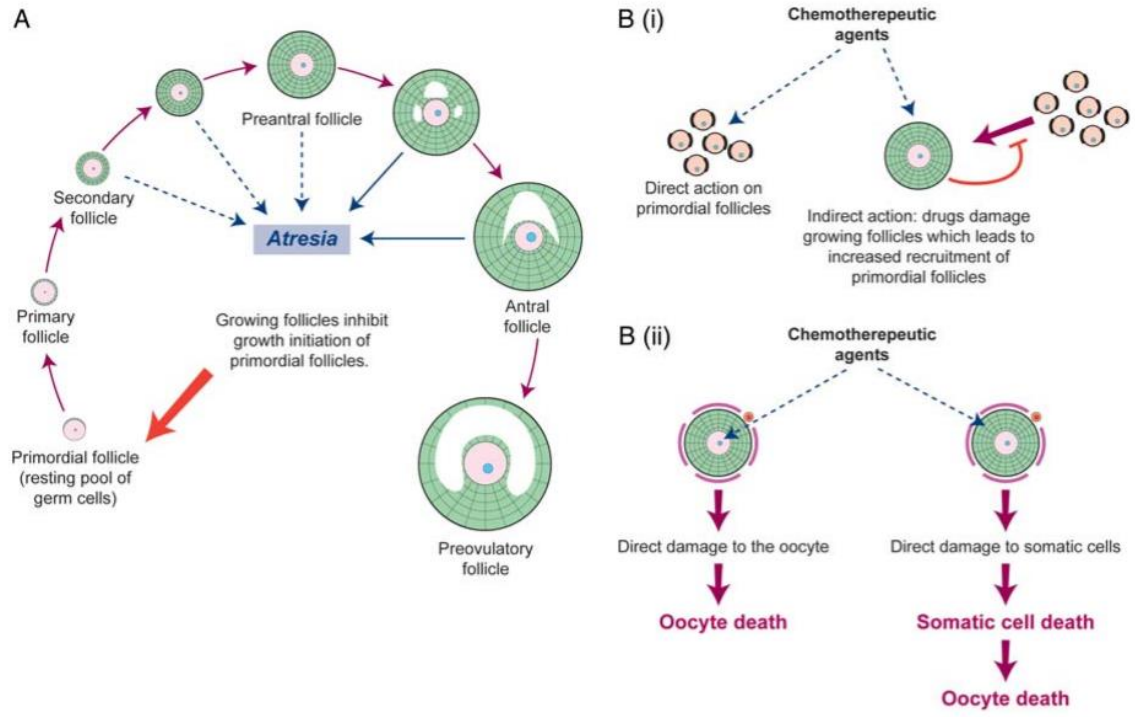


Chart .2 Chemotherapy diminishing the reserve follicle pool by direct or indirect induction of damage (Morgan et al 2012)

In fact, different classes of chemotherapeutic agents show varying mechanisms of damage to the follicle pool. In short anthracyclins like doxorubicin and alkylating agents like cyclophosphamide seems to pose a greater threat to rapidly dividing cells like granulosa cells (Down's et al 1999, Morgan et al 2012). Platinum compounds like cisplatin are known to form DNA adducts that if not cleared efficiently, results in the activation of the apoptotic cascade in oocytes (Siddik et al 2003). Topoisomerase inhibitors like Irinotecan and etoposide may affect follicular health by upregulating

apoptosis by upregulating the expression of Fas ligand (FasL) in granulosa cells of larger follicles (Utsonomiya et al., 2008). The Fas/FasL pathway has been implicated in granulosa cell apoptosis during the process of follicle atresia and has also been linked to p53-mediated apoptosis (Kim et al., 1999). Hence topoisomerase inhibitors are also believed to target granulosa cells to induce follicle loss.

Chemotherapy regimen	Author	Model species	Affected cell type	Affected follicle class
Doxorubicin	Perez *et al.* (1997)	Mouse	Oocyte	
	Jurisicova *et al.* (2006)	Mouse	Oocyte	
	Ben-Aharon *et al.* (2010)	Mouse	Granulosa cells	Secondary
	Bar-Joseph *et al.* (2010)	Mouse	Oocytes	
	Soleimani *et al.* (2011)	Human, Mouse	Oocyte, granulosa cells, stroma, blood vessels	Primordial, pre-antral
Cisplatin	Gonfloni *et al.* (2009)	Mouse	Oocyte	Primordial, primary
Cisplatin/paclitaxel	Yucebilgin *et al.* (2004)	Rat		Primordial
Irinotecan	Utsunomiya *et al.* (2008)	Mouse	Granulosa cells	Pre-antral, antral, pre-ovulatory
Cyclophosphamide	Zhao *et al.* (2010)	Rat	Granulosa cells	
	Desmeules and Devine (2006)	Mouse	Oocyte and granulosa cells	Primordial, primary
	Petrillo *et al.* (2011)	Mouse, rat	Mainly Oocyte	Primordial, primary
	Oktem and Oktay (2007a)	Human	Oocyte	Primordial
	Raz *et al.* (2002)	Human	Granulosa cells	
Cyclophosphamide, busulfan/carmustine, V, MTX, I-Fos, JET/ hydroxyurea, imatinib/HAM, AI, AIE/cyclophosphamide, MTX, I-Fos, etoposide, V/MTX, JET/ABVE, CVPP/ABVE/carmustine, V, MTX, E-PIE, JET/adriamycin, etopside, I-Fos, ACTD, cyclophosphamide, V/ VCAIE/BEA, CVPP/ABVE, BECOPP/ABVE, CVPP/ cyclophosphamide/VMTX/AC/VC/CAF/C, ADR, 5FU	Abir *et al.* (2008)	Human	Granulosa cells, oocyte	Pre-antral
ADE-GMTZ/CHOP/etoposide+ MTX/ABVD/CHOP+RT/ Idarubicin cytarabine/AC/BEACOPP	Oktem and Oktay (2007b)	Human		Primordial
Pr, V, DNR, asparaginase, 6MP, MTX, COAP/PVD, cyclophosphamide, asparaginase, 6MP, MTX/PVD, aspariginase, 6MP,MTX/Pr, V, 6MP, MTX/PVD, COAP, 6MP, MTX, cyclophophamide, /Pr, V, daunorubicin, asparaginase, 6TG, cytosine arabinoside, doxorubicin, 6MP, MTX/Pr, V, daunorubicin, cytosine arabinoside, 6TG, COAP	Marcello *et al.* (1990)	Human	Stroma, capillary	
ABVD/MECC/BEACOPP/VACOP-B/MINE-EASHAPP/MTX, V, P/ GMALL/melphalan, V, nitrogen mustard, gemcytabine, cisplatin, carmustine, etoposide, cytarabine/cytarabine, daunorubicin	Meirow *et al.* (2007)	Human	Blood vessel	

Chart . 3 Targets of follicle damage by chemotherapeutic drugs (Meirow et al 2012)

3. Fertility preservation and anti-cancer treatment

Fertility preservation refers to the prevention of damage or rescue of ovarian follicle population from the gonadotoxic effects of chemo and radiotherapy. Numerous strategies of fertility preservation have been proposed till date (Donnez et al., 2013, Levine et al., 2015)

3.1 Traditional strategies of fertility preservation

GnRH agoinsts

Attaining ovarian dormancy and a reversal to the pre-pubertal ovarian function was one among the earliest strategies for fertility preservation tried more than 3 decades ago. Blumenfeld and von Wolff speculated that by reducing levels of FSH, GnRH agonists could positively influence the vicious cycle of chemotherapy-induced depletion of the ovarian reserve, increase levels of FSH and accelerate recruitment of further follicles. However later analysis did not find enough proof to support the validity of this technique (Blumenfeld and von Wolff et al 2008). GnRH analogue adjuvant therapy was seen to be unsuccessful in reducing the risk of premature ovarian failure in lymphoma patients under norethisterone regimen in a large multi-centre study conducted in 2013 (Demeestere et al., 2013). Furthermore complete ovarian suppression using this technique usually requires weeks. Hence, the use of GnRH agonists for fertility preservation remains controversial and, according to the ASCO 2013 recommendations, GnRH agonists should not be relied on to preserve fertility (Loren et al 2013).

Ovarian transposition

Ovarian transposition before pelvic radiation has also been in practice suggested for all women of reproductive age requiring such treatment (Demesstere et al 2013) Ovarian transposition is a strategy offered to patients with advanced cervical cancer, but remains underused with only 28% of patients <40 years of age undergoing ovarian transposition before radiotherapy (Morice et al., 2000). The procedure is rather simple

and can be performed by laparoscopy with care to make sure that the blood supply to the ovary is not compromised. The ovaries are usually fixed to the anterolateral abdominal wall, 3–5 cm above the umbilicus. Lateral transposition is preferable to medial transposition. However, ovarian transposition become ineffective in cases of combined chemo and radiotherapy and this technique holds the possibility of seeding the tumor to other areas in case of potential ovarian involvement (Morice et., 2001).

Oocyte cryopreservation

Oocyte cryopreservation could be proposed as an option in cases where the anti-cancer treatment can be delayed. However, this in turn would risk worsening the cancer and the life of the patient. Moreover, around 20 vitrified oocytes are required to achieve a live birth, as the live birth rate per vitrified oocyte (in egg donation programmes) is 5.7% reported by the best teams in the world (Cobo et al., 2010, Rienzi et al., 2012, Vajta et al., 2010). Controlled ovarian stimulation becomes inevitable obtain such high number of oocytes and the supraphysiologic E2 levels resulting from ovarian stimulation may promote the growth of estrogen-sensitive tumors, such as endometrial and estrogen receptor–positive breast cancers (Reddy et al., 2012) Furthermore, cancer patients potentially have an elevated risk of thromboembolic events because of a hypercoagulable state induced by their malignancy and high E2 levels (Aurousseau et al., 1995) Malignancies with bone marrow infiltration or liver involvement may create a tendency toward bleeding during oocyte retrieval owing to thrombocytopenia, platelet dysfunction, or defective coagulation factor synthesis. Some of the cancer-related medical conditions, including respiratory dysfunctions due to tracheal compression, mediastinal mass, or large pleural effusion, and vascular disturbances, as in superior vena cava syndrome, may preclude safe administration of conscious sedation during oocyte retrieval making it a risky prospect for cancer patients. Ovarian stimulation is also not applicable to pre-pubertal patients excluding them from the scope of this technique

Embryo cryopreservation

Embryo cryopreservation is another well-established procedure that can be used to preserve fertility in women of reproductive age with an available partner (or women using donor sperm). Until 2012, embryo cryopreservation was the only fertility preservation option endorsed by the ASRM. The technique has proven to be effective in patients undergoing IVF treatment for various clinical reasons, including storage of supernumerary embryos, risk of ovarian hyperstimulation syndrome (OHSS), impaired endometrial development and impractical embryo transfer (Cakmak et al., 2013, Devroey et al., 2011). Embryo cryopreservation has reliable success rates, and reports even suggest that frozen embryo transfer is more successful than fresh embryo transfer, probably because of improved embryo–endometrium synchrony (Roque et al., 2013). In spite of promising application of embryo cryopreservation in conventional IVF programmes, this strategy holds all the risk factors similar to oocyte cryopreservation. It is further complicated by the fact that the patient should have a partner to derive embryos and is not useful in case of pre-pubertal patients.

4. Ovarian tissue cryopreservation

Ovarian tissue cryopreservation is the only fertility preservation option available for prepubertal girls and for women who cannot delay the start of chemotherapy. The possibility of restoring both endocrine and reproductive function of the gonads represents the main advantage of auto-transplantation of frozen/thawed OT However, since the follicular reserve of the ovary is age-dependent, the age of the patient should be considered before adopting this strategy. Worldwide consensus agrees on the upper limit of patients to be 35 years (Silber et al., 2005, 2012).

The size of the cortical tissue excised for preservation is usually dependent upon the estimated risk of ovarian failure relative to the planned treatment and existing ovarian volume. Total oophorectomy is advised in patients undergoing pelvic irradiation or total body irradiation and in those receiving high doses of alkylating agents. This procedure should also be performed in very young girls (that is, prepubertal girls) because of the small size of their ovaries (Jadoul et al., 2010) One entire ovary is usually maintained in place to enable subsequent orthotopic re-implantation (Donnez et al., 2011). Till date 86 live births have been described after ovarian tissue cryopreservation worldwide (Jensen et al., 2016) validating the future potential of this technique. An analysis of 60 case series of orthotopic reimplantation from three different countries including 60 patients revealed that restoration of ovarian activity was observed in 93% of the patients (Donnez et al., 2013). However, this procedure is still considered experimental, and further research is warranted to avoid cryodamage (ASRM 2013).

4.1 Cryopreservation protocols: Slow freezing vs vitrification

The efficiency of any cryopreservation strategy majorly depends on the freezing protocol used. Although vitrification has gained a wider acceptance than slow freezing in the cryopreservation of oocytes and embryos in human assisted reproduction, only recently has it been applied for clinical OT cryopreservation, relying on a limited number of biological comparative studies (Keros et al., 2009, Xiao et al., 2010, Amorim et al., 2012, Fabbri et al., 2014, Klocke et al., 2015), and two live births have been reported to date using the vitrification protocol (Kawamura et al., 2013, Suzuki et al., 2015).

Slow cooling has been suggested to negatively affect the ovarian stroma (Camboni et al., 2008, Nottola et al., 2008, Schubert et al., 2008, Keros et al., 2009, Xiao et al., 2010, Amorim et al., 2011, Talevi et al., 2013), granulosa cells (GCs) (Eyden et al., 2004), and the development of the thecal layer during in vitro culture (Amorim et al., 2011). Compared with conventional slow cooling, vitrification avoids the development of ice

crystals, promoting the formation of an amorphous glassy solid state, through the use of higher cooling rates and cryoprotectant (CPA) concentrations (Pegg et al., 2007). However, the efficacy of vitrification is still controversial. (Klocke et al., 2015, Kagawa et al., 2009), and this is probably due to several variables, such as tissue size, choice and concentrations of CPAs, and nature of devices used to vitrify the tissue in open or closed systems that in turn affect the cooling rates (Herraiz et al., 2014).

4.2 Slush nitrogen vitrification

Cooling rates are one among the most critical factors in attaining optimal glassy state vitrification. The whole process of vitrification depends on crossing the temperatures at which ice formation occurs as quick as possible and reaching the glass transition temperature <-130ºC. A fundamental factor that can result in poor heat transfer and limits the cooling rate is represented by the Leidenfrost effect (Song et al., 2010). Indeed, as soon as an object is plunged into liquid nitrogen (LN), it enters into the so-called film boiling regime caused by the large temperature difference between the object and LN (Santos et al., 2012). Heat flow from the object to LN, causes the latter to boil in the immediate proximity of the object, generating a pocket of nitrogen vapor that acts as an ‘‘insulator’’ and further delays heat transfer.

Slush nitrogen (SN) has been recently proposed as a new strategy that increases the cooling rate, avoids the Leidenfrost effect or the boiling of liquid nitrogen around the sample, and allows the use of lower concentrations or time of exposure to toxic CPAs. The internal temperature of nitrogen falls to -207ºC from -196 ºC during its phase transition from liquid to solid under pressure. However, the main advantage in cooling rates come from avoiding the formation of the insulating vapor (Leidenfrost effect). When conventional liquid nitrogen can attain cooling rates of ~ 25,000ºC, SN vitrification offers a possibility to raise cooling rates to the order of 100,000ºC (Santos et al., 2012). Fresh and SN-vitrified mouse oocytes were reported to have the same ability to develop to, the blastocyst stage and produce healthy offspring, and SN has been shown to improve the clinical outcome of human oocyte vitrification (Santos et al.,

2012, Yoon et al., 2007, Lee et al., 2010, Criado et al., 2011). Although, the efficacy of SN vitrification on OT cryopreservation has not been evaluated to date. Hence, our aim was to apply the advantages of higher cooling rates attainable using SN vitrification in ovarian tissue cryopreservation and analyze the effects

4.3 Concerns in ovarian tissue cryopreservation and transplantation

Although, ovarian tissue cryopreservation and transplantation holds tremendous potential for both pre-pubertal and adolescent cancer affected women to preserve their future fertility, the most notable concern is the probability of re-introducing malignant cells back into the cured individual with very high chances of propagating the cancer again (Chung et al., 2013).

Ovarian metastases have been reported for most malignancies including breast cancer, lung cancer, renal tumors, neoblastomas, Ewing's sarcoma, Hodgkins's lymphoma, Non-Hodgkin's lymphoma, biliary duct cancer and other gastrointestinal cancers (Horvath et al., 1977, Insabato et al., 2003, Khan et al., 1986, Liu et al., 1989, Young et al., 1993, Hashimoto et al., 2003). Studies evaluating the incidence of ovarian metastasis in different cancers show that ovarian involvement is highest in gastric cancer (55.8%), colon cancer (26.6%), breast cancer (24.2%), pulmonary carcinoma (23.4%), lymphoma (13.3%) uterine cancer (13.1%) and leukaemia (8.4%) (Kyono et al 2010).

High risk	Moderate risk	Low risk
Leukemia	Breast cancer Stage IV Infiltrating lobular subtype	Breast cancer Stage I–II Infiltrating ductal subtype
Neuroblastoma	Colon cancer	Squamous cell carcinoma of the cervix
Burkitt lymphoma	Adenocarcinoma of the cervix	Hodgkin's lymphoma
	Non-Hodgkin lymphoma	Osteogenic carcinoma
	Ewing sarcoma	Nongenital rhabdomyosarcoma
		Wilms tumor

Chart. 4 Classification of cancers based on the risk of ovarian metastasis (Dolmans et al 2013)

Routine histological examination of the ovarian tissue fragments have proven to be inefficient in predicting the prevalence of malignant cells in the transplanted tissue (Dolmans et al., 2010). In this context, transplantation of stored ovarian tissue is particularly warranted in leukaemia, being a systemic disease with very high chances of metastasizing to the ovaries. In a study involving 18 leukaemia patients, routine histology and immunohistochemical analysis showed no presence of malignant cells in the biopsied ovarian tissue. However, highly sensitive reverse transcriptase PCR (RT-PCR) revealed the presence of molecular leukemic markers in the tissues of 9 in 16 of these patients, previously thought to be safe from ovarian metastasis (Dolmans et al., 2010). Although, there is no conclusive report demonstrating the re-introduction of malignant cells through ovarian tissue transplanted after storage, studies in animal models have shown growth of intraperitoneal masses after transplantation of ovarian tissue from leukemic (Dolmans et al., 2010). Hence, transplantation of stored ovarian tissue in general can only be offered with extreme caution for re-storation of fertility in cancer treated women. Considering these issues recent research in fertility preservation is focused on alternative approaches to circumvent this problem. Cryopreservation, culture and methods to re-implant isolated ovarian follicles rather than whole tissue, is one among the most focused topics in this context.

5. Concept of ovarian follicle in vitro culture

Depending on age, the ovarian cortex houses thousands of dormant primordial follicles that can be isolated (Kristensen et al., 2011). These immature follicles represent the largest population of ovarian follicles and are more resistant to cryopreservation than advanced stage follicles (Gougeon et al 1986). Furthermore, early stage follicles have shown to maintain normal morphology and ultrastructure following freezing, making them excellent candidates for long term preservation. As follicles are enclosed in a basement membrane that prevents direct contact between follicular cells and capillaries, white blood cells and nerve processes (Rodgers et al., 2003), their isolation will ensure that no malignant cells are returned back to the patient. The membrana granulosa of each ovarian follicle is encapsulated by a follicular basal lamina separating it from the surrounding stromal elements in primordial follicles or from the theca interna in antral follicles. In healthy follicles it excludes capillaries, white blood cells and nerve processes from the granulosa compartment until ovulation, at which time the basal lamina is degraded as the follicles develop into corpora lutea. The basal lamina also has size limit to the selective passage However, movement of cells from the thecal side across the basal lamina is restricted whilst it is aligned with healthy granulosa cells (Rodgers et al., 2003)

Hence, the most important advantage of isolated follicle culture and transplant arises from the fact that the malignancy cannot cross the basal lamina of the follicle and the oocyte is protected from cancer cell invasion (Abir et al., 2006). Moreover, improvements in follicular development *in vitro* can also help these patients to obtain a larger number of oocytes for *in vitro* fertilization techniques, hence overcoming the concerns of re-transplantation all together. Henceforth, improvements in the isolation, culture and re-introduction of ovarian follicles can greatly improve current fertility preservation strategies and can provide a risk-free method for the restoration of fertility in women affected by chemo/radiotherapy induced premature ovarian failure.

5.1 Two step ovarian follicle culture

Several strategies for in vitro follicular growth have been trialed over the years, mainly different permutations of isolated (Hornick et al., 2012) or in situ follicular culture O'Brien et al., 2003). Optimizing in vitro follicular growth right from the primordial stage has tremendous scope in view of fertility preservation, since this class of follicles constitute the major share of ovarian reserve in women of all age groups and they better cope with exogenous stresses like cytotoxic therapy and cryopreservation (Donnez et al., 2011). Isolated primordial follicle culture has been more successful in the murine model but commendable results are yet to be accomplished in higher mammals and human (Hornick et al., 2013, Monica et al., 2014). A relatively tough ovarian stroma in higher animals and human make enzymatic isolation avoiding follicle damage very complicated or infeasible (Wandji et al., 1996). Furthermore, follicle isolation strips away stromal and thecal cells that are crucial in supporting pre-antral follicle growth (Hovatta et al., 1999) and ovarian strip in situ culture of follicles has tremendous advantage over isolated follicle culture systems in this regard, such that the follicles are maintained within their natural habitat. The ovarian tissue itself is a regulating force, with an array of bio-mechanical signals (West-Farell et al., 2009) which are highly complex and dynamic to be accurately mimicked by any artificial matrix in vitro.

The two-step culture strategy is where the primordial follicles are activated within the cortical tissue till the secondary stage using the natural signals available for initial growth and isolating these follicles to individually culture them to mature competent oocytes for in vitro fertilization completely avoiding the need for transplantation and associated risk of cancer re-introduction (McLaughlin et al., 2010). Pioneering works in this stream has been done by Eppig et al as early as 1996, obtaining live births from cultured primordial follicles in the murine model (Eppig et al., 1996) and this technique was further developed by Telfer et al, applying this culture strategy in higher mammals

like bovine and human (McLaughlin et al., 2010, Telfer et al 2008). However, inspite of these early reports, complete folliculogenesis and live births using this strategy have only been attained in lower animals. Although, a recent report by Xiao et al, of obtaining mature human MII oocytes from pre-antral follicles looks promising (Xiao et al., 2015), this group initiated growth from advanced secondary/early antral follicles still leaving behind the true potential of complete in vitro folliculogenesis in vitro.

5.2 Scope for improvement in current ovarian tissue culture

A major limitation in realizing complete folliculogenesis in vitro arises from the fact that several groups have been unsuccessful in optimizing in situ follicle culture to obtain sufficient number of secondary follicles to mature further. In spite of assessing an array of growth and molecular factors to optimize media for cortical strip culture over the years (Smitz et al., 2010), the proportion of secondary follicles yielded rarely surpass 10% of the total follicle population obtained post culture (McLaughlin et al., 2010, Monica et al 2014). These shortcomings could be owed to the fact that, these culture systems have mainly emphasized on the nutritional and endocrine requirements of the tissue (Adriaens et al., 2004) neglecting basic requisites (Debra et al., 2014) like oxygen.

In addition to attaining folliculogenesis in vitro, several groups have also demonstrated positive effects of ovarian tissue culture after cryopreservation, reviving the tissue from stress and damage encountered during cryopreservation (Lara et al., 2015). In addition, an optimal culture system could greatly contribute to optimizing and assessing cryopreservation protocols (Isachenko et al., 2006). Hence, improvements in ovarian organ culture systems could revolutionize the way cryopreserved ovarian strips are processed in future where the culture system aids in tissue repair and assessing the quality and aiding the selection of strips to be used (Lara et al., 2015, Isachenko et al., 2006) in place of blind, unsuccessful transplantations.

6. Oxygen requirement in ovarian tissue culture

Occurrence of tissue necrosis due to inefficient transport of oxygen, is a major hurdle in establishing optimal ovarian organ cultures that hinders oocyte/follicle development (Morimoto et al., 2007). Follicle culture *in vitro* has been regularly conducted at atmospheric oxygen tension (20% v/v /140 mm Hg), (Xu et al., 2010). Theoretically, follicles should be maintained at around 5% oxygen tension owing to the low partial pressure of oxygen in the peritoneal cavity i.e. 40mmHg (Tsai et al., 1998). Low oxygen culture has been beneficial for the culture of rat pre antral follicles improving oocyte, viability maturation, parthenogenetic activation and fertilization rates *in vitro* (Heise et al., 2009). Caprine pre-antral follicles exhibited higher percentage of antrum formation at 5% oxygen as compared to 20% (Silva et al., 2010). Higher levels of reactive oxygen species are frequently associated with high partial pressure of oxygen and this oxidative stress induces cytotoxicity (Evans et al., 2004). Subsequently, culturing under low oxygen tension has seen to reduce cumulus cell apoptosis in canine oocyte cumulus complexes in culture (Silva et al., 2009). Lately, a higher number of healthy oocytes were also derived under low oxygen conditions, during *in vitro* culture of follicles (Xu et al., 2011). Perifollicular vascularity which in turn co-relates with follicular fluid oxygen content has been associated with oocyte developmental competence (Van Blerkom et al., 1997, 1998). On the other hand, high oxygen tensions are known to induce increased reactive oxygen species (ROS) levels and lose of oocyte granulosa cell junctions, which could in turn damage the oocyte (Cetica et al., 2001, Heise et al, 2009). In this context, a quantitative analysis of oxygen requirement in the growing follicle is generally lacking (Clark et al., 2006)

Oxygen diffusion in aqueous medium is known to be very slow with a diffusion coefficient of about 0.00003cm^2/s at 37 ºC (Himmelbau et al., 1964). A strong consensus seems to exist in literature, about this inverse relation between oxygen perfusion and media height in upright culture systems, often forcing ovarian tissue strips to be cultured in suspended tissue culture inserts with just microliters of media

covering the strips or very small culture drops (Jin et al., 2010) rather than in immersion. These systems could stress the cultured strips for lack of sufficient nutrients and a higher concentration of toxic metabolites in the tissue vicinity with an added risk of the tissue drying out. The fact that oxygen concentrations at the vicinity of the follicle must be maintained at a level at least equaling the rate of oxygen consumption of the follicle (Wycherley et al., 2004) makes it challenging to meet this balance.

Hence, the second aim of our study was to investigate the effects of oxygen delivery, one among the basic but critical/dynamic necessities of organ culture systems (Boland et al., 1994) on follicular morphology, progression, and viability during ovarian cortical strip culture. We made use of oxygen permeable dishes to efficiently modulate local oxygen tensions at the tissue vicinity and to study the effect of media volumes on the performance of the tissue strips cultured, without changing the ambient oxygen conditions.

Aims and objectives:

- **Improving the efficiency of ovarian tissue cryopreservation using slush nitrogen vitrification.**

- **Improving ovarian tissue in situ culture and analyzing the influence of optimal oxygen diffusion in ovarian strip in situ culture.**

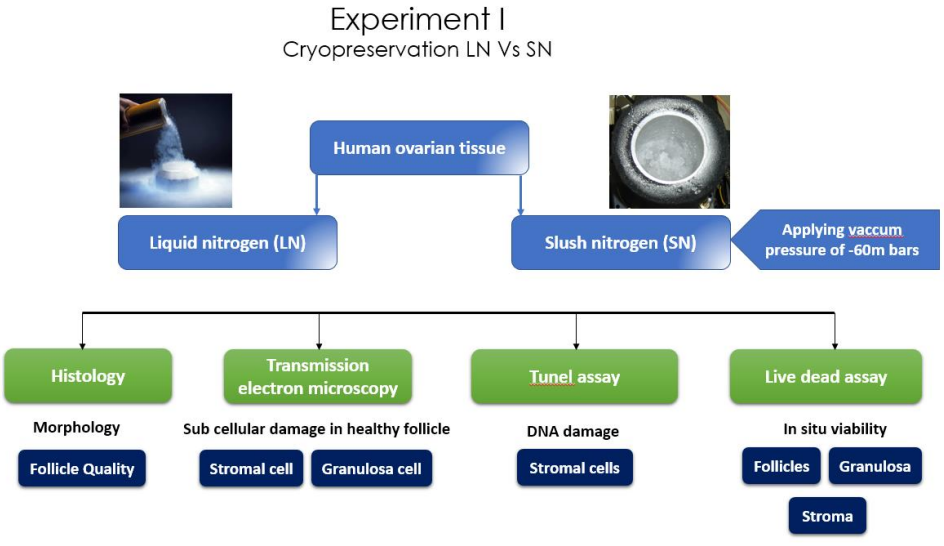
- **Merging optimal cryopreservation and culture protocols and assess the functional potential of SN vitrified strips during long term culture.**

Experimental design

The main study was divided into three different experiments.

Experiment I: Improving ovarian tissue cryopreservation using slush nitrogen vitrification

Experiment I was aimed at analyzing the efficiency of slush nitrogen (SN) vitrification of human ovarian strips in comparison with conventional liquid nitrogen (LN) vitrification. To this end human ovarian strips were cryopreserved using either of the two cryopreservation protocols. Post-warming the tissues were cultured for a brief period of 24 hours to stabilize the strips and to allow time for any in vitro recovery of stress related damage as mentioned below. The strips were processed for histological assessment of the follicle quality, tunel assay for DNA fragmentation assessment of stromal cells and granulosa cells, transmission electron microscopy (TEM) to assess ultrastructural damage and Live dead assay to analyze the in situ viability of the follicles. The results of this experimental section helped us to underline how slush nitrogen vitrification could be superior to conventional liquid nitrogen vitrification in better preserving the follicle quality, DNA integrity of ovarian somatic cells and the viability of follicles post warming hence overall improving the efficacy of these cryopreserved tissue strips



Experiment II: Improving ovarian tissue in situ culture by analyzing the influence of optimal oxygen diffusion during ovarian strip in situ culture

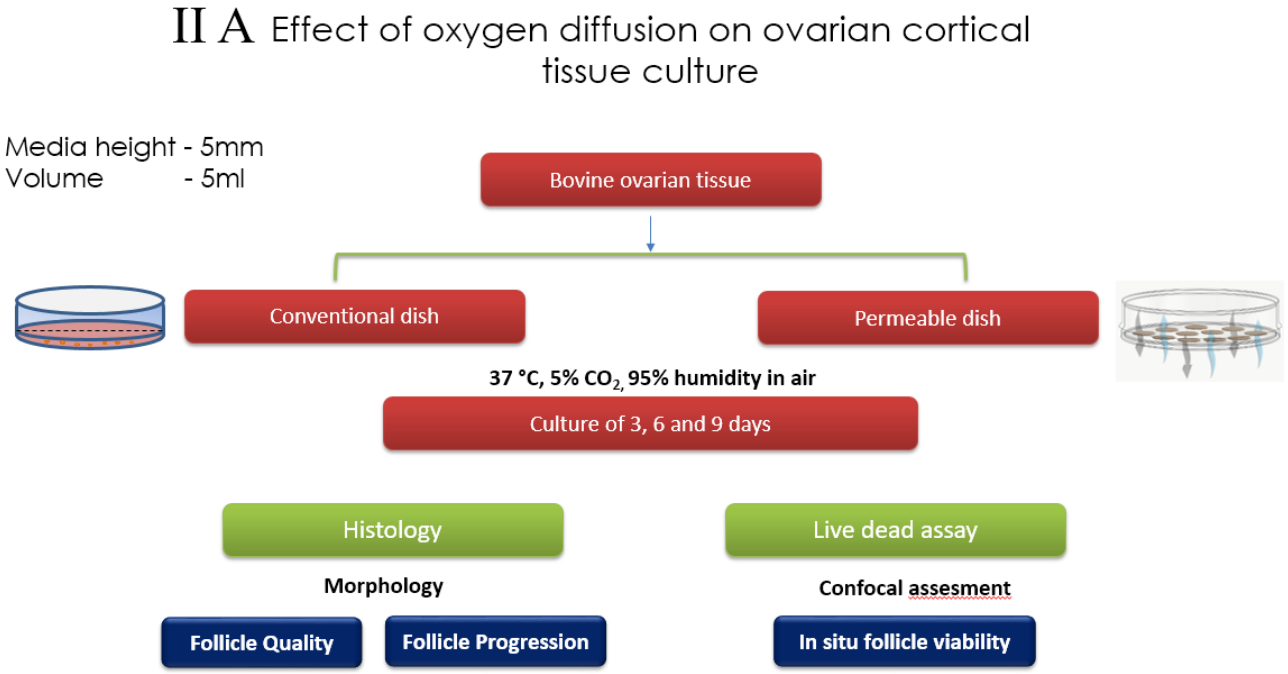
Initial efforts were also made to isolate primordial follicles from the cortical strips to try to encapsulate isolated primordial follicles into a 3-dimensional matrix like alginate. However, this strategy had to be abandoned since the isolated primordial follicles lost their viability under harsh enzymatic treatment and extruded the oocyte soon after isolation rendering them non-viable.

Hence all culture experiments were made on bovine and human ovarian cortical strips. Since human ovarian strips being precious and its availability being limited, all preliminary experiments aimed at identifying the best culture conditions for human strip culture were performed in the bovine model. All experiments were addressed to understand whether modulation of oxygen diffusion in culture media, through the use of oxygen permeable dish (PD) versus conventional dish (CD) with different media column height, had an effect on follicle quality, activation, i.e. the passage of primordial follicles to the primary stage, progression to the secondary stage, and viability.

Experiment II in total was further performed as 3 subsections.

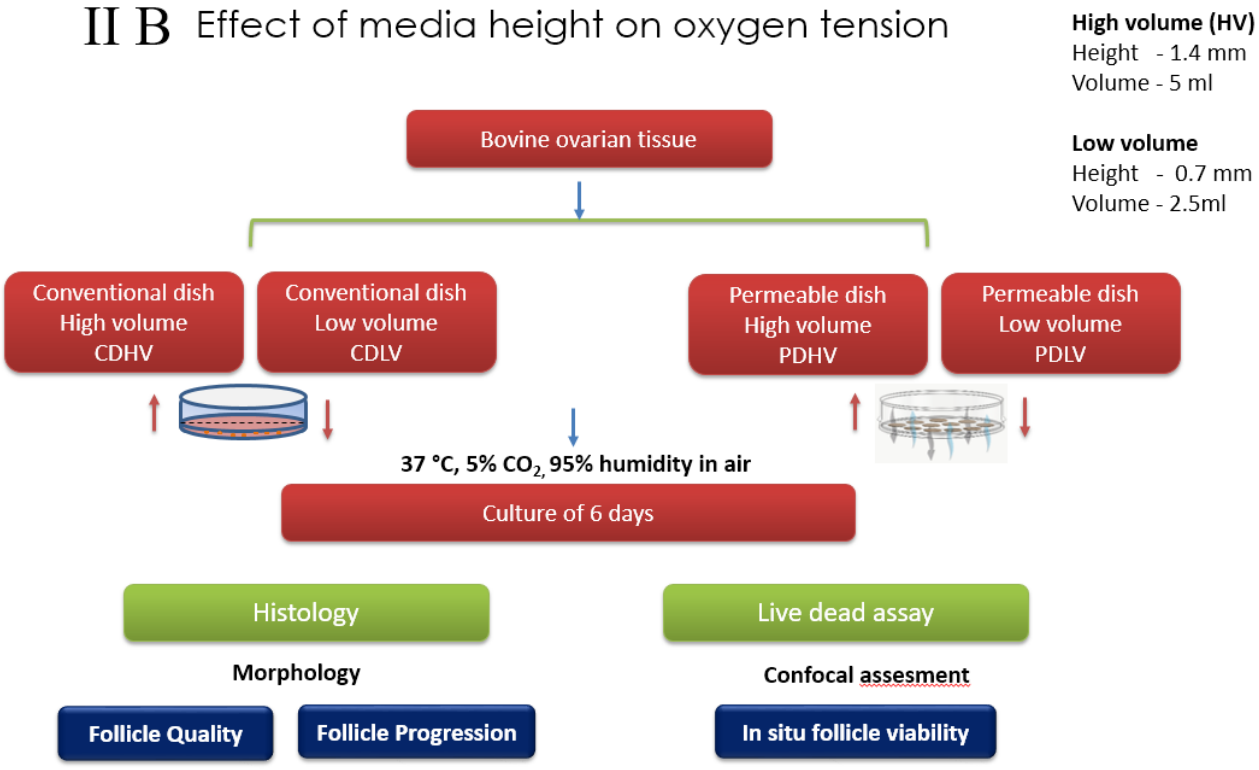
Experiment IIA.

During experiment IIA (n=3) bovine cortical strips were cultured as described below in 5ml (~1.4 mm media height) for 3, 6 and 9 days in conventional dish high volume (CDHV) versus permeable dish high volume (PDHV).



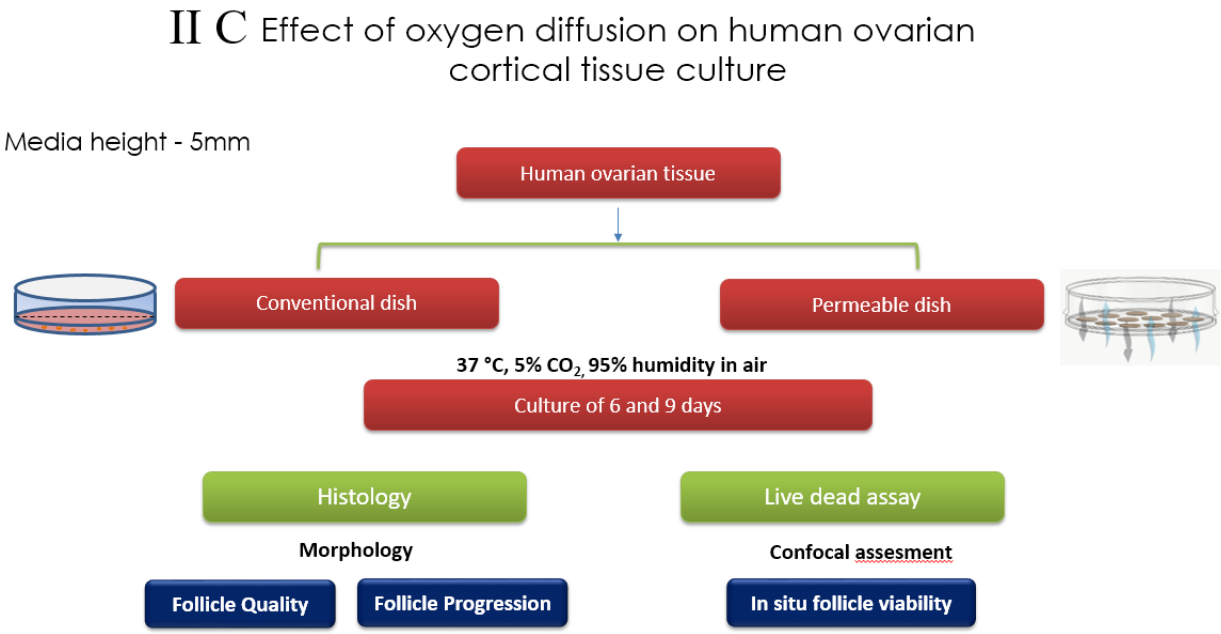
Experiment IIB.

As results of experiment IIA demonstrated a significantly higher follicle quality and viability in PD versus CD starting from day 6, experiment IIB (n=3) was addressed to understand whether increase of oxygen levels in the tissue vicinity through the use of a lower volume (LV) of medium (2.5ml, ~0.7 mm media height) in PD (PDLV) and CD (CDLV) had an effect on follicle quality, activation, viability and progression culturing the strips for 6 days. To this end, bovine cortical strips from the same ovary were cultured in PDHV, PDLV, CDHV and CDLV for 6 days and then assessed for follicle quality, activation, progression to the secondary stage, and viability.



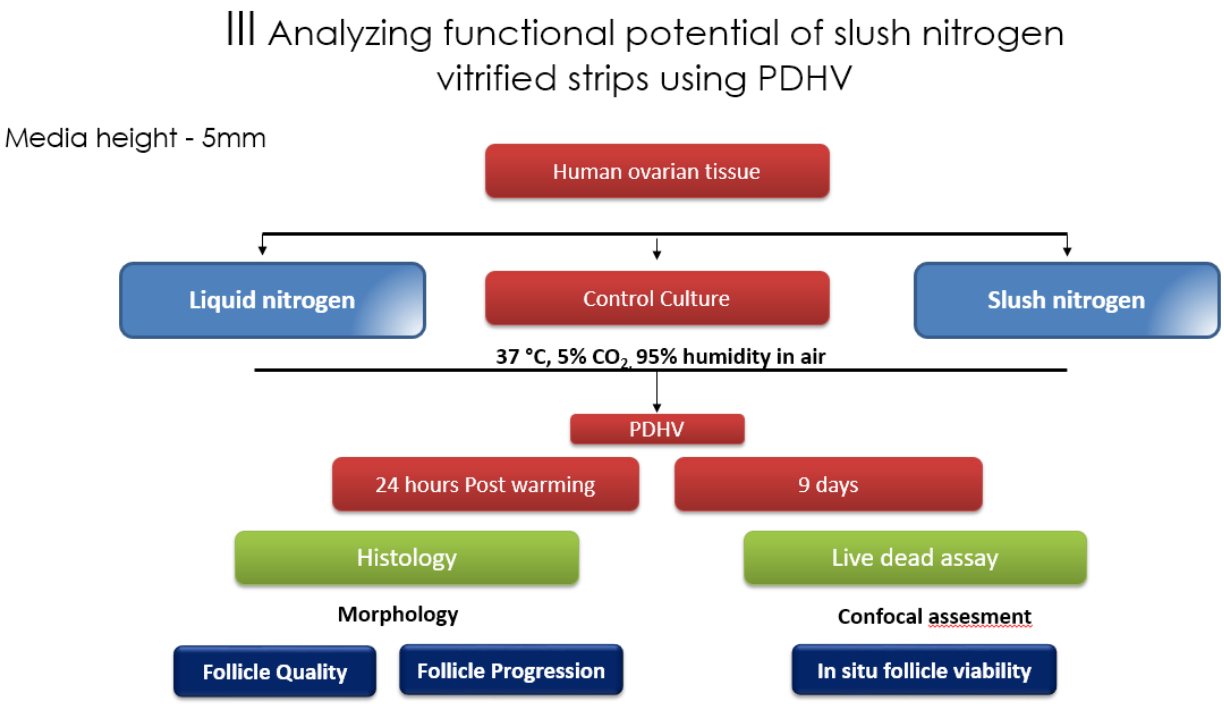
Experiment IIC.

The aim of experiment IIC (n=6) was to adopt the best culture condition found in the preliminary bovine experiments (PDHV), to understand whether modulation of oxygen levels in the tissue vicinity during culture also affected follicle quality, viability, activation, progression and viability of human cortical strips. As a control, human strips from the same ovaries were parallelly cultured in CDHV.



Experiment III: Merging optimal cryopreservation and culture protocols

Experiment III was designed to couple the results obtained in the first two sections and to assess the functional potential of SN vitrified strips using long term culture in PDHV. Functional potential of vitrified strips is an important facet that could determine the long term efficiency of the strips in resuming endocrine and reproductive functions of the transplanted ovaries or follicles. To this end human ovarian cortical strips cryopreserved using (LN) or (SN) were cultured post warming were cultured using (PD) under high media volume found to be the best culture condition from experiment II. To attain base line data fresh tissue from the respective ovaries were also cultured for the same time duration. Data was monitored as Day 0 (fresh control), Control D9 (fresh culture), SN2 time 0 and LN2 time 0 (time 0 post thaw), SN2 day9 and LN2 day9 (culture post warming).



Materials and methods

Collection and Processing of OT

The use of human tissue in this study was approved by the Ethics Committee of the Azienda Ospedaliera San Giuseppe Moscati (Avellino, Italy; reference number CE 6-2009, approval granted June 5, 2009). Tissues from a total of 9 patients were utilized during Experiment I (aged 14–35 years) and a total of 6 patients each during Experiment 2 and 3 (aged 18-34) after informed consent during laparoscopic surgery for benign gynecologic conditions. The tissue was transported to the laboratory within 2 hours in minimum essential medium (Sigma Aldrich), supplemented with 5% human serum albumin (Octapharma) at 4_C. Bovine ovarian tissue was obtained from a local abbatoir and transported to the laboratory in Leibovitz's L-15 medium supplemented with penicillin-streptomycin (Pen-Strep) (1%) and Amphotericin-B (1µg/ml) at 4ºC within two hours of slaughter. Bovine and human ovarian biopsies were transferred to handling media (Leibovitz's L-15 media supplemented with 2mM glutamine, 3mg/ml BSA, 1% Pen-Strep and 1µg/ml amphotericin B. The cortical OT was manually dissected from medullar tissue and divided using a scalpel into cortical strips avoiding areas with visible antral follicles to ensure a predominantly uniform primordial follicle population within the strips

Isolation of primordial follicles

Ovarian cortical strips with their medulla removed were dissected into(1mm x 1mm x 0.5mm). Ovarian fragments obtained were transferred to 50 ml conical tubes containing 10 ml of Leibovitz's medium L15 (Sigma) supplemented with 1% fetal calf serum (Sigma) (FBS), 1 mg/ml collagenase type IA (Sigma), 0.25mg/ml Dnase I (Roche) and incubated in a water bath at 37°C for 30 min with gentle agitation. The ovarian digest was periodically (every 15 min) shaken with a pipette to mechanically disrupt the digested tissue. Digestion was completed by the addition of an equal volume of Leibovitz's L-15 medium supplemented with 10% FBS. The solution was transferred to Petri dishes and investigated for follicles under a stereomicroscope (Leica, Van Hopplynus Instruments, Brussels, Belgium). The follicles were picked up using a

135-µm-diameter flexipet (COOK) and washed three times in L15 medium supplemented with 10% FBS in order to avoid introduction of stromal cells into the alginate matrix. Follicles were then transferred in culture medium, consisting of α- MEM supplemented with 20% FBS, 0.47mM pyruvic acid (Sigma), 1% insulin-transferrin-selenium (GIBCO) and 1% penicillin-streptomicin (Sigma), for 30' at 38°C. Follicle diameter was measured using NIS elements advanced software (Nikon). Follicles were assessed for viability using Live dead assay.

Cryopreservation of human ovarian tissue

Ovarian tissue slices for vitrification were manually dissected into approximately 5x2 mm and 1 mm thick strips. Fresh Cryopreservation of human ovarian tissue was performed by both liquid nitrogen (LN) and slush nitrogen (SN) vitrification protocols. The two protocols differed only in the use of either liquid or slush nitrogen to attain ultra-low temperatures. For each patient, fresh tissue was directly fixed or vitrified and cultured after warming as detailed below

Vitrification and warming were performed with minimum essential medium (Sigma Aldrich) supplemented with 20 mg/mL human serum albumin as basal medium (BM). Exposure to VS and WS was performed at room temperature (RT) and at 37_C, respectively (Amorim et al., 2012). The vitrification solution (VS) consisted of BM supplemented with 10% dimethyl sulfoxide (Sigma Aldrich), 26% ethylene glycol (Sigma Aldrich), 2.5% polyvinylpyrrolidone (Sigma Aldrich), and 1 M sucrose (Sigma Aldrich). Cortical strips were equilibrated in 25% VS (5 minutes), 50% VS (5 minutes), and transferred into 100% VS (1 minute). The strips were then blotted on aseptic gauze to remove the remaining VS, dropped into LN or SN, and then stored in Nunc Cryotubes (Sigma Aldrich). The SN was obtained, exposing a polystyrene container holding 750 mL of LN to a negative pressure of 65–70 mBar for 15 minutes in a vacuum chamber (Vacutherm, Thermo Scientific Heraeus). Strips were dropped into SN within 5 minutes after returning to normal atmospheric pressure.

Warming of the cryopreserved tissue

For warming, strips were transferred in warming solutions (WS) consisting of BM supplemented with different concentrations of sucrose. Briefly, the strips were immersed in WS1 (1 M) for 15 seconds, then in WS2 (0.5 M), WS3 (0.25 M), and BM for 5 minutes each. Warmed strips were cultured for 24 hours in BM supplemented with insulin, transferrin, and selenium at 37ºC, 5% CO2 in air and then fixed for histology, TEM, or assessment of viability as detailed below during experiment I. Strips for experiment III post thaw long term culture were further dissected and cultured as described below.

Ovarian tissue culture

Bovine ovaries and fresh/frozen human ovarian strips meant for long term culture experiments were further sliced into (1mmx1mmx0.5mm) cortical strips using an automated tissue chopper (Mcilwain, Mickle Laboratory Engineering Company, Ltd, Surrey, UK). The strips were washed by 2 passages in fresh handling media and 10 strips were randomly distributed into each culture dish. For each experiment the cortical strips (10/dish) derived from the same ovary were cultured in α-MEM media supplemented with 3mM glutamine, 0.1% BSA, 1% Pen-Strep, 1% ITS (10 µg/ml Insulin, 5.5 µg/ml transferrin and 6.7 ng/ml selenium), 1µg/ml amphotericin-B and 50µg/ml ascorbic acid at 37 °C, 5% CO_2 and 95% humidity in air. Half of the media volume was changed every 48h. Ovarian tissue strips were cultured either in a conventional (CD) or permeable (PD) culture dish and cultured as described in the experimental outline. Fresh/cultured control strips from each ovary were processed for histology and viability assessment as described below.

Histology

Tissue strips for histological assessment were fixed in Bouin's solution, dehydrated by sequential passages in increasing concentrations of alcohol, embedded in paraffin wax

and 5µm serial sections were cut and stained with hematoxylin & eosin. Follicles were graded and staged on serial sections from each tissue strip by a blinded expert observer. Each follicle was carefully graded and staged only when the germinal vesicle was visible preventing chances for re-counting. Follicle quality was graded as previously reported (Talevi et al., 2013) into; grade 1: Spherical oocyte with granulosa cells homogenously distributed; homogenous oocyte cytoplasm and slightly granular nucleus with a dense, spherical condensed chromatin at the centre; grade 2: oocyte still spherical but granulosa cells missing, pulled away from the follicle and grade 3: misshapen oocyte with or without vacuolation, granulosa cells/oocytes with pyknotic nuclei. Follicles were staged based on Gougeon's criteria (Gougeon et al 1986) as follows: primordial follicle, a single layer of flattened granulosa cells surrounding the ooocyte; primary follicle, oocyte surrounded by one complete layer of cuboidal granulosa cells; secondary follicle: two or more layers of cuboidal granulosa cells surrounding the oocyte.

Transmission Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde (SIC) in 0.1 M sodium cacodylate (Sigma Aldrich) at pH 7.3 for 1.5 hours at RT, washed 3 _ 10 minutes in the same buffer, postfixed in 1% osmium tetroxide (SIC) in 0.1 M sodium cacodylate at pH 7.3 on ice, washed 3 _ 10 minutes in the same buffer, treated with 0.1% tannic acid in buffer for 10 minutes, and dehydrated in increasing concentrations of ethanol (Sigma Aldrich) on ice. All samples were treated 2 _ 5 minutes with propylene oxide (Fluka), infiltrated with propylene

oxide/agar 100 (1:1; Agar Scientific) overnight, and individually embedded in fresh resin. Thick (0.5–1 mm) and thin sections (60–80 nm) were cut with a diamond knife (Diatome) at a Reichert-Jung Ultracut E ultramicrotome and collected on glass slides or 200-mesh thin bar copper grids (SIC). Thick sections were stained with 0.1% toluidine blue in sodium borax, examined by light microscopy, and photographed using a DScooled camera head (DS-5Mc) connected to a DS camera control unit (DS-L1; Nikon). Follicles with atretic or unsurvived oocytes were excluded from TEM analysis. Thin sections\were

stained with saturated uranyl acetate in methanol and Reynold's lead citrate and observed and photographed with an EM 208 S electron microscope (Philips) at 80 kV. The percentage of damaged mitochondria (i.e., swollen and/or with a reduced electron density of the matrix) was determined on images of fresh and cryopreserved tissue, acquired at a magnification of 11,500x. The percentage of intact GCs was determined on images of primordial/primary follicles acquired at a magnification of 3,500_. The percentage of intact

SCs was determined scoring 400–600 cells per treatment on images acquired at a magnification of 3,500x.

TUNEL Assay

After histologic analysis of follicle quality, two slides with 8– 10 sections for each sample were labeled to assess DNA fragmentation by the In Situ Cell Death Detection Kit FITC conjugated (Roche) according to the manufacturer's protocol. After rehydration, sections were incubated in blocking solution, consisting of Tris HCl 0.1 M supplemented with 20% fetal calf serum and 3% bovine serum albumin, for 30 minutes at RT. After washing three times in PBS, for 5 minutes each, sections were incubated with TUNEL reaction mixture for 1 hour at 37ºC, in a humidified chamber. After counterstaining with Hoechst 33342 (Sigma Aldrich) for 7 minutes at RT, sections were washed as above, mounted, and observed on a Nikon TE 2000 fluorescence microscope. Images were acquired using a Nikon DS-cooled camera head (DS-5Mc) connected to a Nikon DS camera control unit (DS-L1), using the same exposure conditions. Negative controls were carried out by omitting terminal deoxynucleotidyl transferase from the reaction mixture, and positive controls were performed before treating the sections with 1 mg/mL DNAse for 10 minutes at RT. Follicles with more than 50% of their GCs positive for TUNEL or with TUNEL-positive oocytes were considered apoptotic. Percentage of TUNEL-positive follicles and SCs was determined on images acquired with a 40x objective.

Live dead fixed far viability assay:

Briefly, tissue strips designated for viability assessment were incubated in Dulbecco's PBS (dPBS) with 1µg/ml Live Dead Fixable Far Red Assay Kit "Molecular probes (Invitrogen) labelling dead cells and nuclear stain (Hoeschst 33342) for 3 hours at 4ºC under gentle agitation (Talevi et al., 2016). The pieces were then fixed with 4% Paraformaldehyde in PBS for 2 hours at room temperature, washed in fresh PBS and incubated in PBS with 10µg/ml Hoechst 33342 at 4ºC overnight. The live dead probe is resistant to formaldehyde fixation, reacts with free amines both in the cell interior and on the cell surface and is excluded by cells with intact membranes. The slices were then optically cleared using a clearing protocol (Meng-Tsen Ke et al., 2013). Briefly, samples were serially incubated in 5 mL of 20%, 40%, and 60% (wt/vol) fructose, each for 3 hours with gentle shaking at room temperature (RT). Samples were then incubated in 80% (wt/vol) fructose for 12 hours, 100% (wt/vol) fructose for 12 hours, and finally in 115% (wt/vol) fructose for 24 hours with gentle shaking at RT. All fructose solutions were supplemented with 0.5%. α-thioglycerol. The tissues were mounted in 115% fructose on a glass slide with 3 spacer coverslips (0.17mm) placed on either side of the strip and covered with another coverslip such that the strips were not compressed during observation.

Analysis was carried out with a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems) using the 405-nm diode laser for visualizing the nuclear label (Hoechst 33342) and the 633-nm helium neon laser for the live–dead probe. Each tissue field was traversed using the z-position control to visualize the interior of the tissue slices. Follicle viability and imaging was performed using a 63x glycerol immersion objective with a working distance of 300µm.

Respirometry of ovarian strips

For analysis of oxygen consumption rate of ovarian strips in culture, bovine cortical strips (1mmx1mmx0.5mm) were polarographically analysed analysed using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.), maintained in a chamber at 37ºC. The strips were suspended in 3 ml Leibovitz's L-15 media supplemented with 0.1 per cent (w/v) bovine serum albumin for analysis. Strips were stirred until oxygen equilibrium of atmospheric concentration was reached within the respirometer chamber. At this point the system is hermetically closed and the oxygen consumption rate is derived as a function of the drop in initial oxygen concentrations each minute. Drops in oxygen saturation was measured for atleast 4 consecutive minutes, to calculate the average consumption of oxygen by the strips per minute. Calculations were made based on the fact that the initial concentration of oxygen saturation in the media would be 1.9444E-04 mol/L when in equilibrium with 21% atmospheric oxygen.

Statistical analysis:

For each experiment data is presented as cumulative percentages. Overall, analysis was performed by Fisher's exact test or by the estimate model of analysis of variance followed by the Tukey's honestly significant difference test for pairwise comparisons when overall significance was detected.

Results

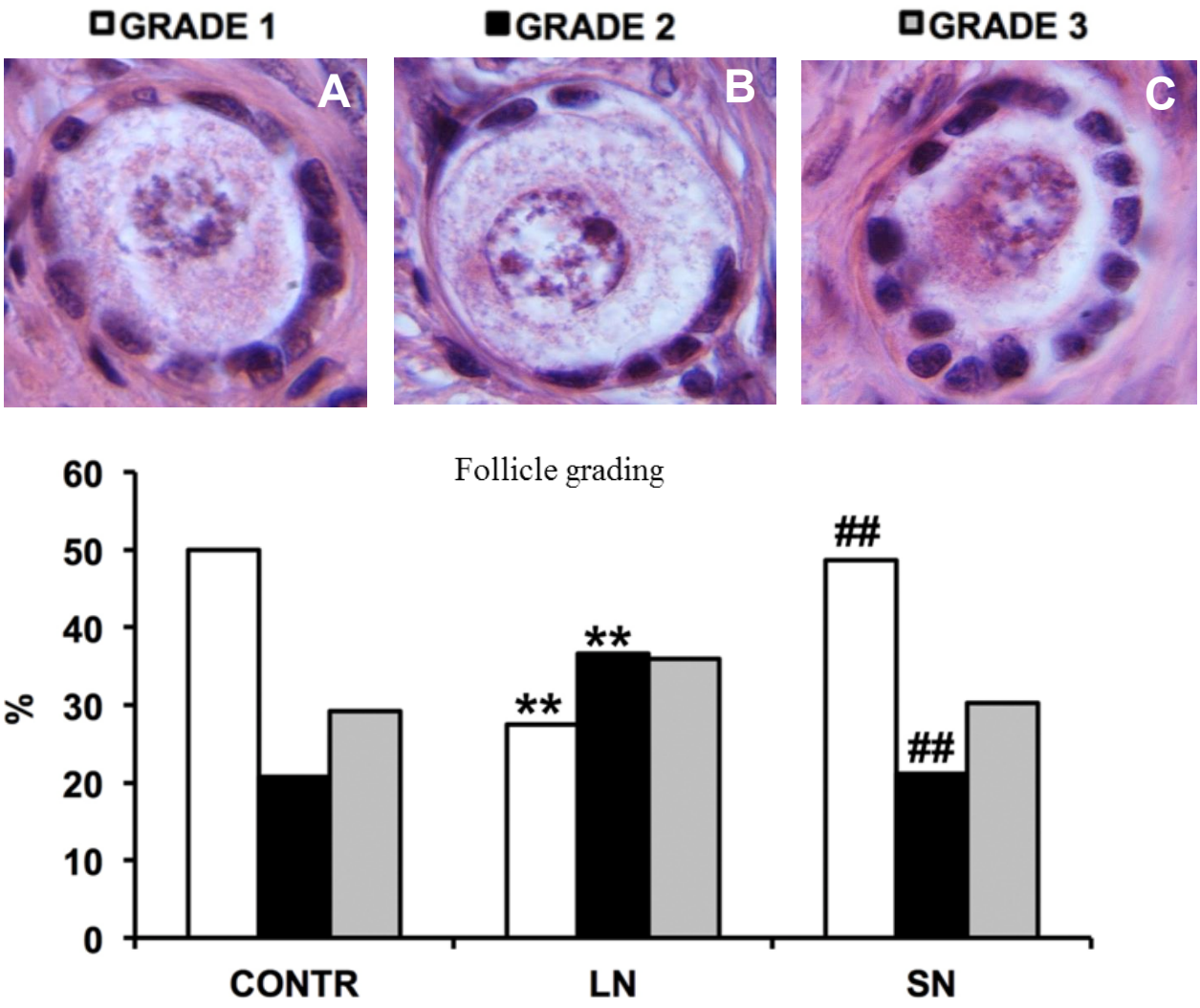
Experiment I:

Histology:

To evaluate the effect of the SN vitrification on the preservation of follicle morphology, the distribution of follicular grades in fresh samples (n = 240) was compared with that in vitrified–thawed samples (LN 476; SN 327). In fresh tissues, the percentage of grade-1 follicles (Fig. 1) was 50%. A highly significant decrease of grade 1 follicles (P<.01) and a concomitant significant increase of grade 2 follicles (Fig. 1) (P<.01) occurred after LN vitrification, compared with fresh samples.

In contrast, the percentages of grade 1 and 2 follicles in SN were not significantly different from those observed in fresh controls. The percentage of grade 3 follicles (Fig. 1) was not significantly different among samples. These findings indicate that SN vitrification adopted herein better preserves the quality of follicles. Follicular densities were not significantly different in fresh and vitrified strips (fresh tissue, 2.45±0.7; LN, 1.86±1.96; SN, 2.21 ± 1.57/mm2; P=.89) (Table. 1). Classification of the different follicular stages showed that primordial follicles represented the largest proportion of the follicle pool both in fresh and vitrified samples. Analysis of follicle stages at 24 hours of culture after warming showed a significant transition of primordial toward primary follicles in SN samples. No differences were detected in SCs morphology at the histologic level among samples.

Fig.1



**** p<0.001 vs CONTR**

p<0.001 vs LN

Table. 1 Distribution of follicular stages within strips

Sample	Primordial	Primary	Secondary
Fresh	84.3	13.4	2.2
Liquid nitrogen	80.5	16.4	3.1
Slush nitrogen	73.1[a]	21.8[a]	5.1

[a] *P*<.05 vs. fresh control.

Transmission Electron Microscopy:

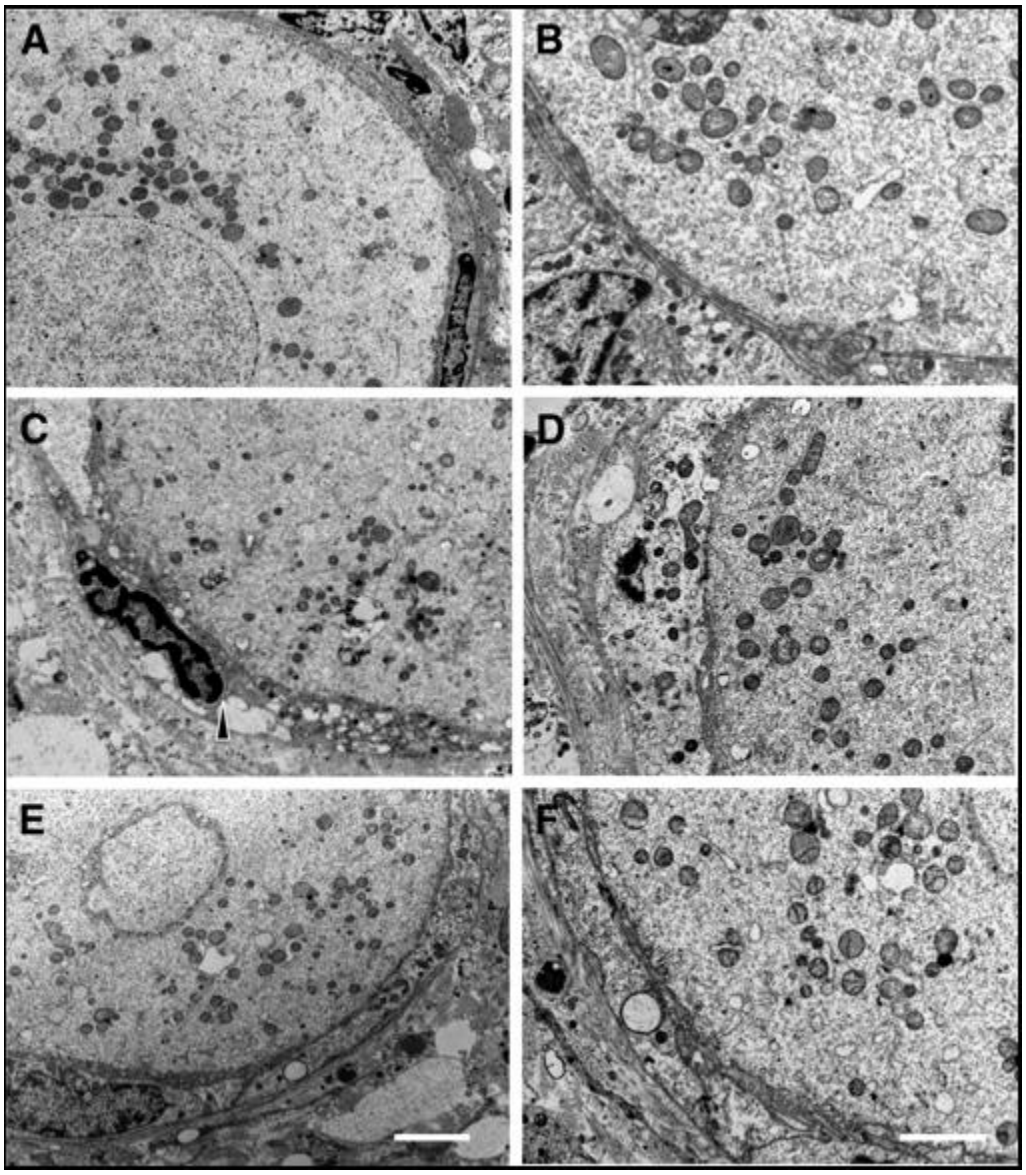
Cortical strips of fresh and vitrified human OT were evaluated under TEM to analyze the influence of the different protocols on oocyte and granulosa cell (GC)/stromal cell (SC) ultrastructural damage. Fresh primordial/primary follicles (n=14), were surrounded by a well organized layer of GCs showing projections interdigitated with the oocyte microvilli. The oocyte contained a large vesicular nucleus with dispersed chromatin and one or more dense nucleoli. Mitochondria, endoplasmic reticulum vesicles, and annulate lamellae had a perinuclear distribution. Mitochondria showed the typical morphologic features described in human oocytes (Motta et al., 2000, Gualtieri et al., 2011) (Figs. 2A, 2B and 3A). In LN samples, the primordial/primary follicles (n=13) showed a discontinuous layer of GCs, not regularly surrounding the oocyte, and interdigitations appeared regressed (Figs. 2C, 2D and 3C). No signs of cryodamage were detected in the oocyte cytoplasm. In SN samples, the primordial/primary follicles (n=15) were well preserved and had an uninterrupted layer of GCs interdigitated with the oocyte plasma membrane. Oocytes presented normal-appearing nuclei, and the endoplasmic reticulum vesicles and annulate lamellae had the typical distribution and morphologic features seen in fresh tissue (Figs. 2E, 2F and 3E).

In cryopreserved human oocytes and OT, mitochondria are shown to represent a specific target of cryodamage (Gualiteri et al., 2009, 2011). Herein, a morphometric analysis of oocyte mitochondria in fresh and vitrified cortical strips was performed. Mitochondria occur as spherical/oval elements, approximately 0.5 μm in diameter, and typically contain a few short cristae rarely crossing a medium electron-dense matrix. In contrast, damaged mitochondria appeared swollen, with few cristae and a low-density matrix. Data analysis showed no differences in the proportion of intact mitochondria in oocytes of fresh and vitrified follicles (percentage intact mitochondria: control, 93.1; LN, 93.6; SN, 96.9).

Cryopreservation may affect the morphology and viability of GCs and SCs (Keros et al., 2009). Therefore, the number of intact and damaged GCs and SCs at an ultrastructural level was morphometrically analyzed in fresh and vitrified samples. Intact GCs showed flat nuclei containing euchromatin in the inner part and small peripheral patches of heterochromatin. The cytoplasm contained abundant mitochondria with cristae, and rough endoplasmic reticulum vesicles. The GCs had smooth surfaces closely apposed to the basal membrane and to neighboring cells, whereas the surface facing the oocyte had projections interdigidated with the oocyte plasma membrane (Figs. 2A, 2B, 2E, 2F and 3A, 3E). Damaged GCs had heterochromatic nuclei, a vacuolized cytoplasm, and few or disrupted interdigitations with the oocyte plasma membrane (Figs. 2C, 2D and 3C). The percentage of intact GCs in fresh samples (cells counted 112, 92%) was dramatically decreased after vitrification with LN (cells counted 120, 45%; $p<.001$). and only slightly though significantly reduced in SN samples (cells counted 131, 73%; $p<.01$) Fig. 4A.

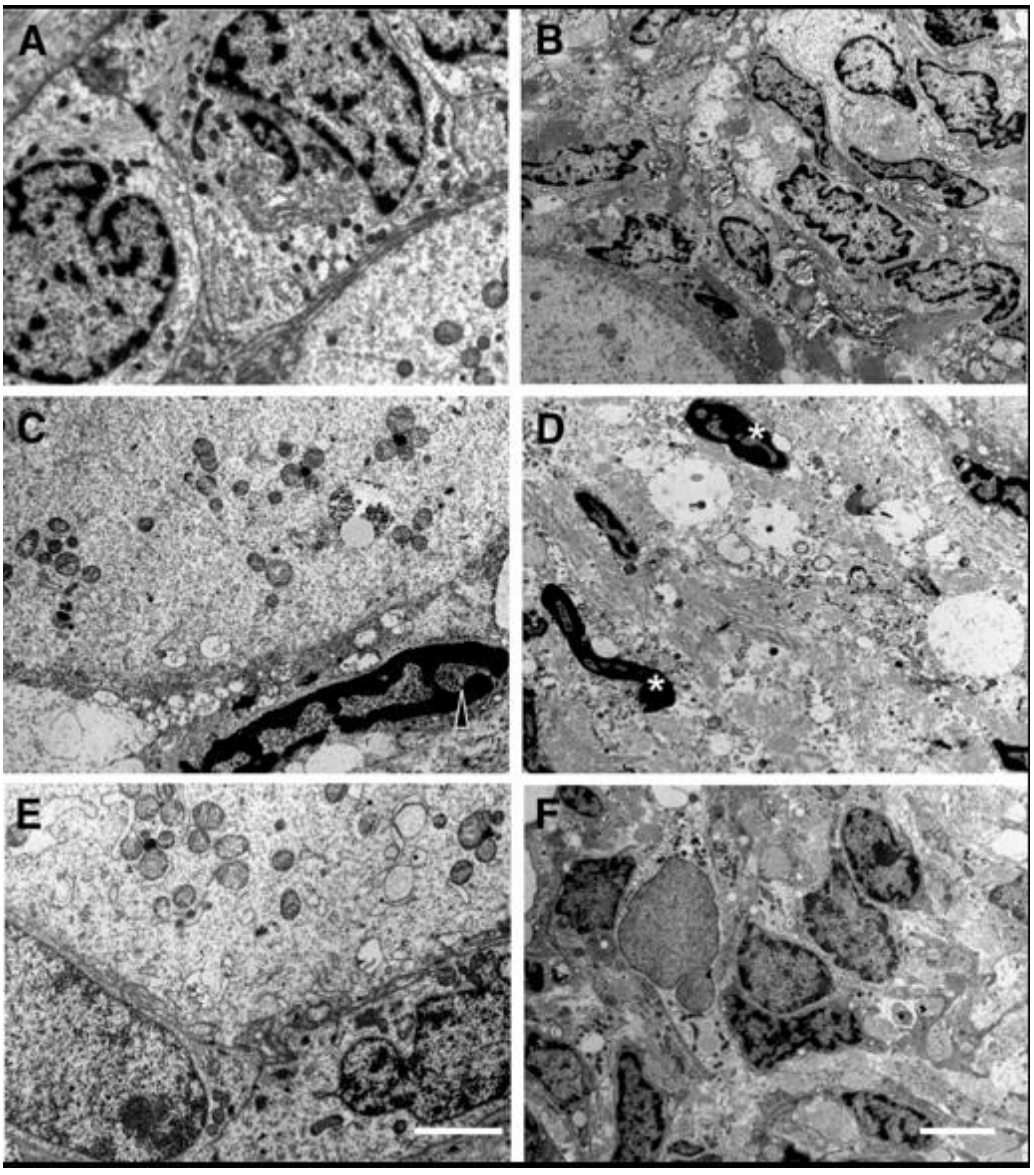
Intact SCs (Fig. 3B and 3F) had elongated and/or convoluted nuclei with central euchromatin and peripheral patches of heterochromatin. The cytoplasm showed abundant mitochondria with a highly electrondense matrix, and rough endoplasmic reticulum vesicles. Damaged SCs were retrieved with different frequencies both in fresh and vitrified samples and were characterized by the complete loss of the cytoplasm and highly heterochromatic nuclei directly facing collagen bundles (Fig. 3D). Morphometric analysis demonstrated a highly significant decrease of intact SCs (cells counted in fresh samples 204, 59.8%) after vitrification both in LN ($p<0.001$) and in SN ($p<0.05$). However, SCs were clearly better preserved in samples vitrified in SN (cells counted 226, 48.7%) than in LN (cells counted 200, 24%; $p<.001$) Fig. 4B.

Fig. 2 TEM images (follicles)



Transmission electron micrographs of representative follicles in fresh (A, B) and vitrified ovarian tissue with liquid (C, D) or slush (E, F) nitrogen. *Arrowhead,* damaged GCs. In A, C, and E, bar = 4 μm; in B, D, and F, bar = 2 μm.

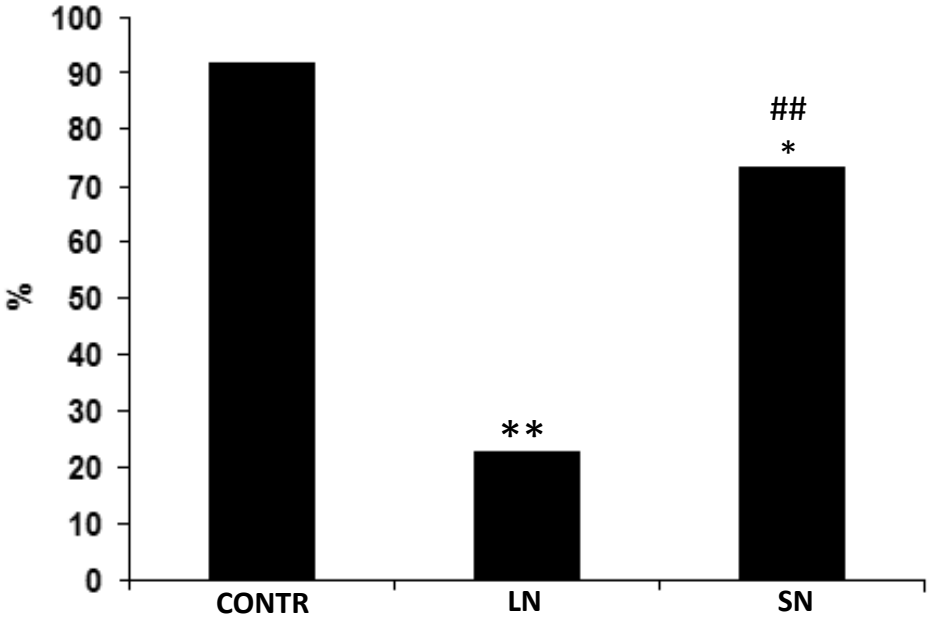
Fig. 3 TEM images (granulosa cells)



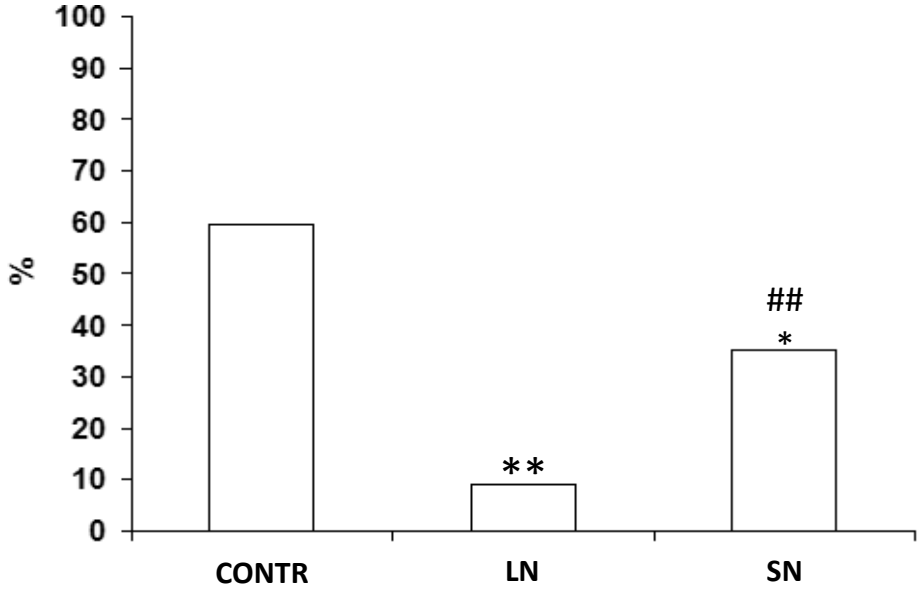
Transmission electron micrographs of GCs (A, C, E) and SCs (B, D, F) in fresh (A, B) and vitrified ovarian tissue with liquid (C, D) or slush (E, F) nitrogen. *Arrowhead,* damaged GC. *Asterisks* (*) indicate damaged SCs. In A, C, and E, bar = 2 µm; in B, D, and F, bar = 4 µm.

Fig. 4

A. Intact follicular cells



B. Intact stromal cells



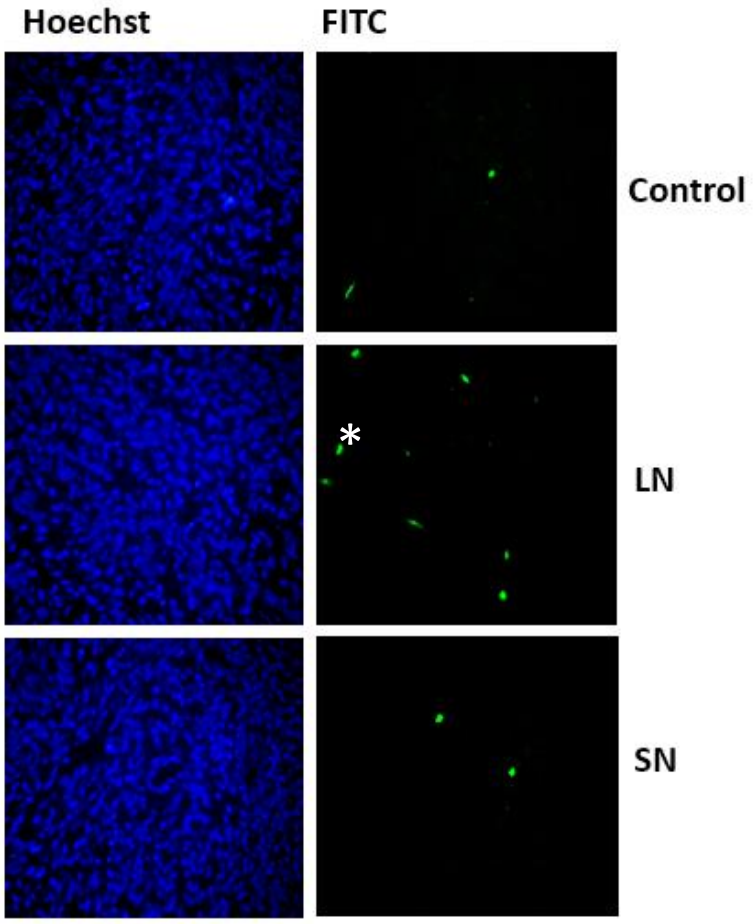
**** p<0.001, * p<0.01 Vs fresh control**

p<0.001, Vs LN

TUNEL Assay

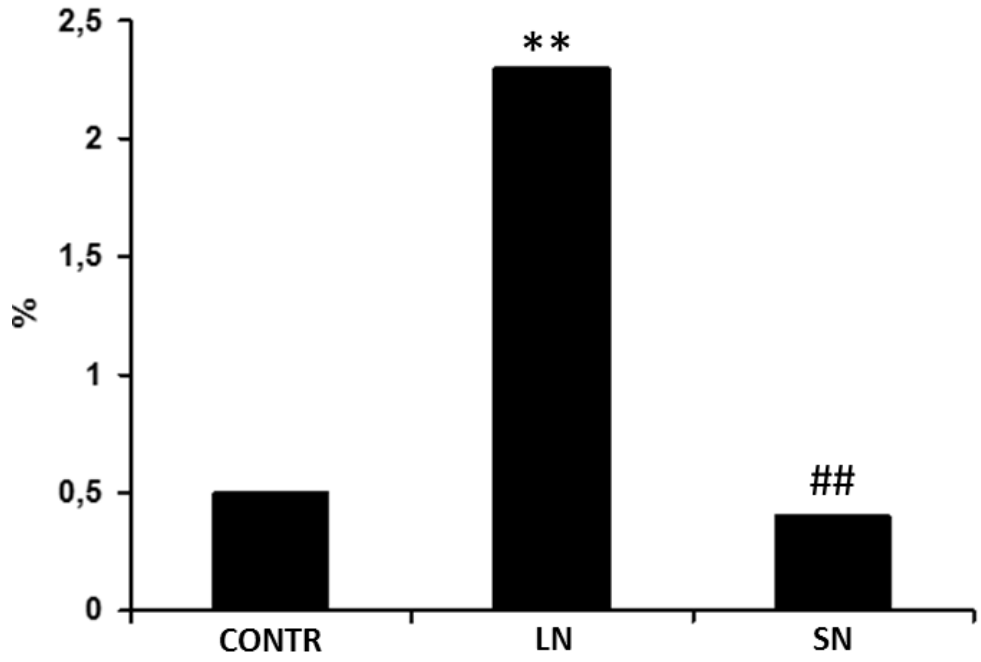
In situ analysis of DNA fragmentation through the TUNEL assay was performed on a limited number of primordial/primary follicles, and no significant differences were found among samples (TUNEL-positive/total follicles: fresh, 0/21; LN, 2/16; SN, 1/20; p>.05). The percentage of TUNELpositive SCs (Fig. 5) was evaluated on 5,663, 4,971, and 4,459 cells in fresh, LN, and SN samples, respectively. No significant differences in the percentages of DNA fragmented SCs were detected in SN (0.4%) vs. fresh tissue (0.5%), whereas a highly significant increase of DNA fragmented cells occurred after vitrification in LN (2.3% vs 0.5%; p<.01 Fig. 6).

Fig. 5 TUNEL assay images



Representative fluorescent micrographs of Tunel assay. * DNA fragemented stromal cell.

Fig. 6 Tunel positive stromal cells



**** p<0.001, Vs fresh control**

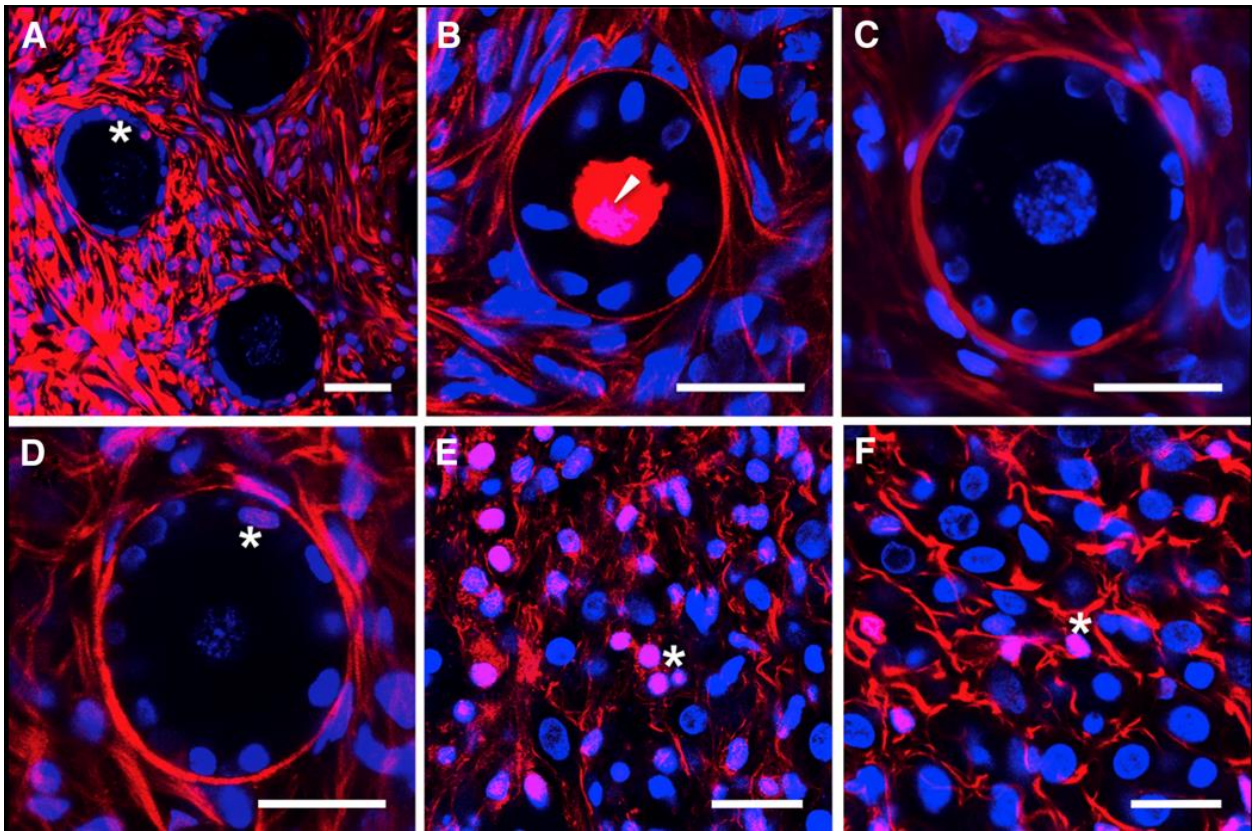
p<0.001, Vs LN

Viability assessment

As mentioned above, a fundamental endpoint that should be evaluated during development of ovarian cryopreservation methods is the viability of the thawed tissue. Live and dead oocytes were clearly visualized throughout the tissue depth at the confocal microscope. Analysis of blue–red merged images showed (Fig. 7A) that the live–dead probe stained the collagen bundles, the follicle basal lamina, and dead oocytes (Fig. 7B), GCs (Fig. 7A and 7D), and SCs in red (Fig. 7E and 7F), whereas in live follicles (Fig. 7A, 7C and 7D) it was unable to penetrate healthy GCs and oocytes that were visualized by the nuclear blue stain. In merged images, dead cells were recognized by a red cytoplasm and a pink nucleus (overlap of the blue and red signal). Data collected on 337 primordial/primary follicles (fresh, 120; LN, 105; SN, 112), demonstrated that SN vitrification did not affect oocyte viability (SN, 87%; fresh, 90%;

p=.53) Fig. 8A, whereas it was significantly decreased after LN vitrification (63%) compared with both fresh and SN samples p<0.001. Data demonstrated that both vitrification protocols significantly affect GC and SC viability, although to a different extent. In fact, the percentage of viable GCs in fresh samples was markedly higher compared with LN (P<.001) and only slightly increased compared with SN samples (P<.001) (fresh, 575 of 618, 93%; LN, 263 of 495, 53%; SN, 450 of 556, 81%) Fig. 8B, and the same was observed for SC viability (fresh, 334 of 531, 63%; LN, 169 of 563, 30%; SN, 261 of 502, 52%; p<.001 for all comparisons Fig. 8C).

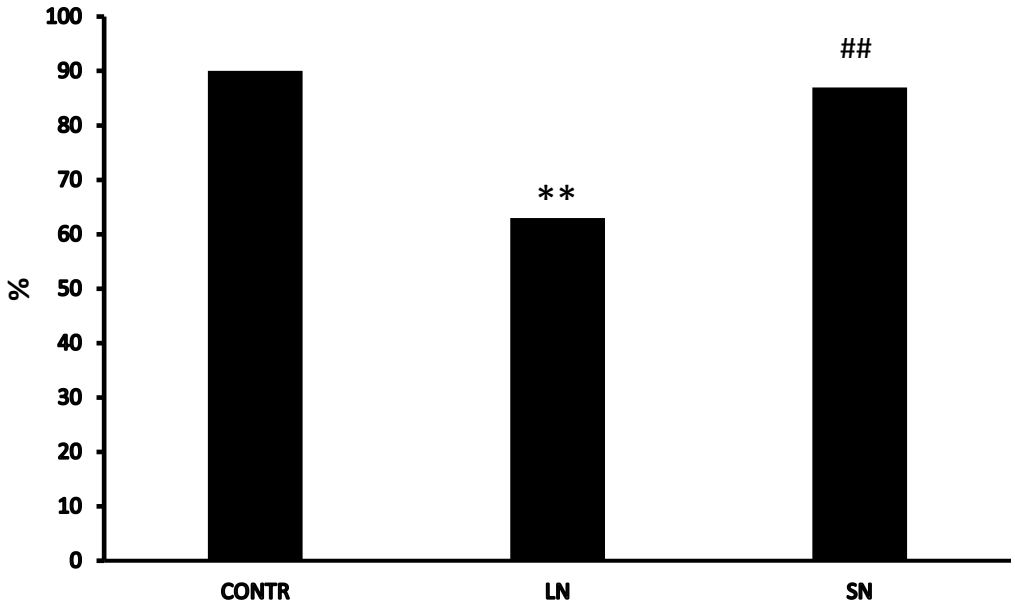
Fig.7 Live dead assay images



Representative confocal micrographs of ovarian tissue viability (*red*: live–dead far-red probe; *blue*: Hoechst 33342–stained nuclei). (**A**) Low magnification of three follicles with viable oocytes (*asterisk* [*] indicates dead GC). (**B**) High magnification of a follicle with a dead oocyte (*arrowhead* indicates the germinal vesicle). (**C**, **D**) High magnifications of follicles with live oocytes (*asterisk* indicates dead GC).

(**E**, **F**) High magnification of LN (**E**) and SN (**F**) vitrified ovarian stroma with dead SCs in *pink* (overlap of the nuclear stain and live–dead far-red probe). Bar = 25 μm.

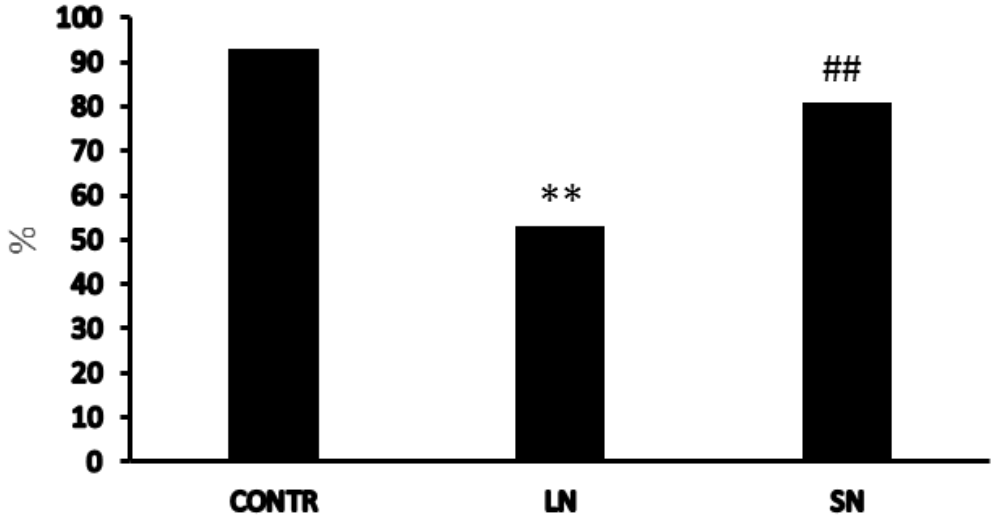
Fig. 8A Oocyte viability



**** p<0.001, Vs fresh control**

p<0.001, Vs LN

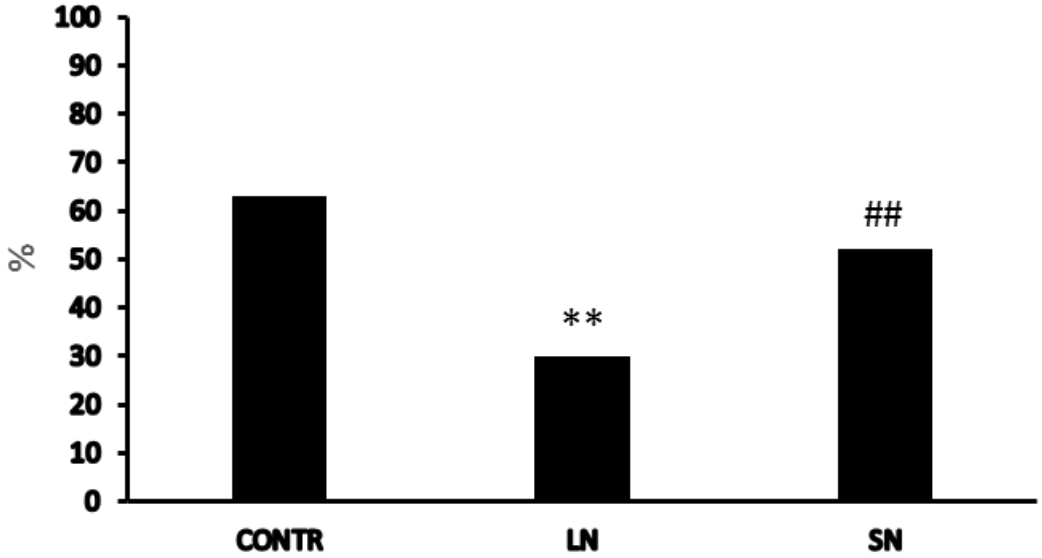
Fig. 8B Granulosa cell viability



**** p<0.001, Vs fresh control**

p<0.001, Vs LN

Fig. 8C Stromal cell viability



**** p<0.001, Vs fresh control**

p<0.001, Vs LN

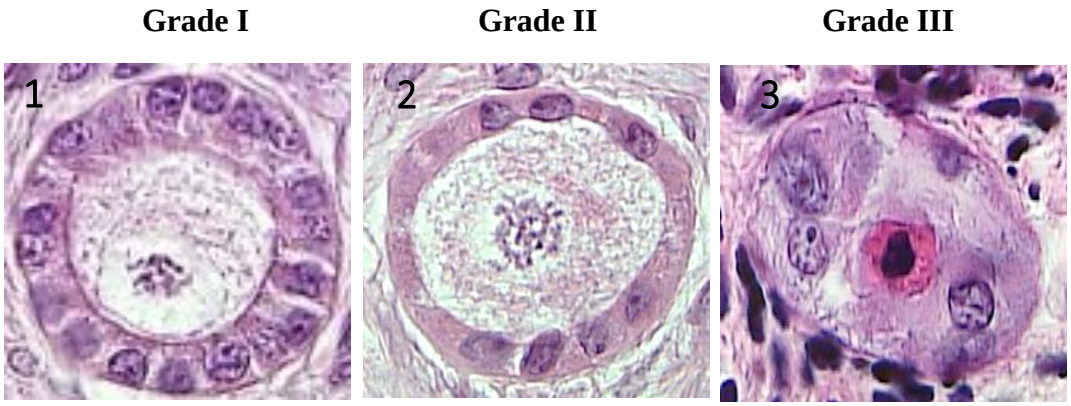
Experiment IIA

Histology:

A total of 1837 bovine follicles from Day 0 control and 3, 6, 9 days cultures were histologically assessed (Fig.1A). Although, superior in PDHV, follicle grading (Fig. 9A) on day 3 was not seen to be significantly different from CDHV. However, strips cultured in PDHV harboured a significantly higher proportion of grade I follicles both on day 6 and 9 of culture, $p<0.0001$ (PDHV vs CDHV, Day 6: 35% vs 10%; Day 9: 40% vs 7%) (Fig.9B). Furthermore, by day 9 of culture the proportion of atretic grade III follicles in CDHV, significantly increased compared to day 0 tissues ($p<0.01$), whereas in PDHV it was seen to be consistently lower throughout the culture period (Fig.9B). This could be a simultaneous effect of re-absorption of atretic follicles during culture, coupled with no further addition of atretic follicles in PDHV.

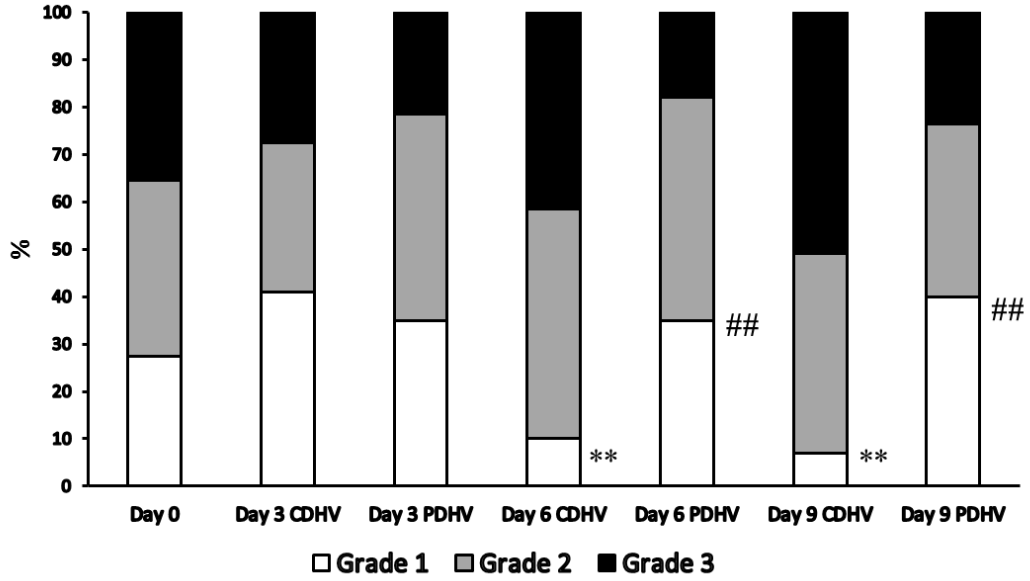
In terms of follicular staging (Fig.10A), both groups at day 3 showed a marked and significant decrease of primordial follicles and a corresponding significant increase of primary follicles compared to day 0 tissue, ($p<0.0001$; Fig. 10B). Interestingly, follicular activation to the primary stage on day 3 was significantly better in PDHV compared to CDHV (98 vs 82.8%, $p<0.001$), suggesting a role of increased oxygen diffusion in follicular activation. Both groups showed a significantly higher proportion of secondary follicles by Day 9 compared to day 0 (Day 0, 3%; Day 9, CD 8%; PD 8.5%; $p<0.05$) (Fig. 10B).

Fig. 9A Follicle morphological grades



Histological grading of bovine follicles from hematoxylin eossin stained sections 1: Grade I follicle showing healthy follicular characteristics. 2: Grade II follicles with missing granulosa cells. 3: Grade III atretic follicle showing a pyknotic oocyte nucleus and misshapen oocyte. Bar=10µm

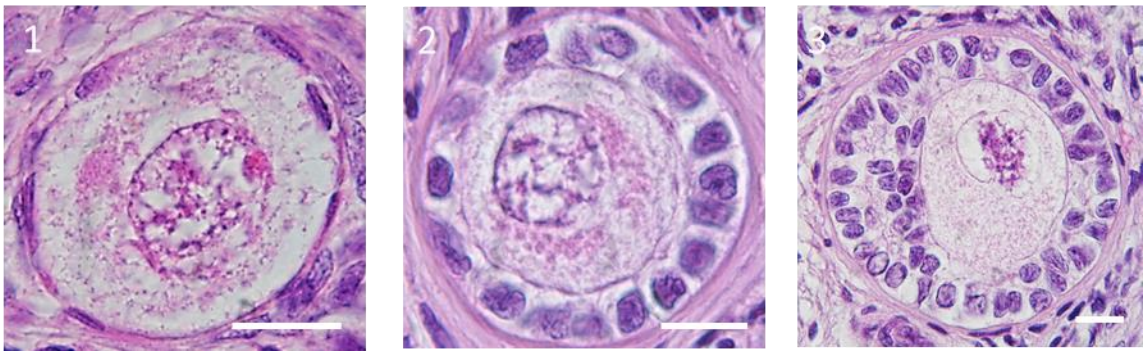
Fig.9B Follicle grading



**** p<0.001, Vs Day 0**

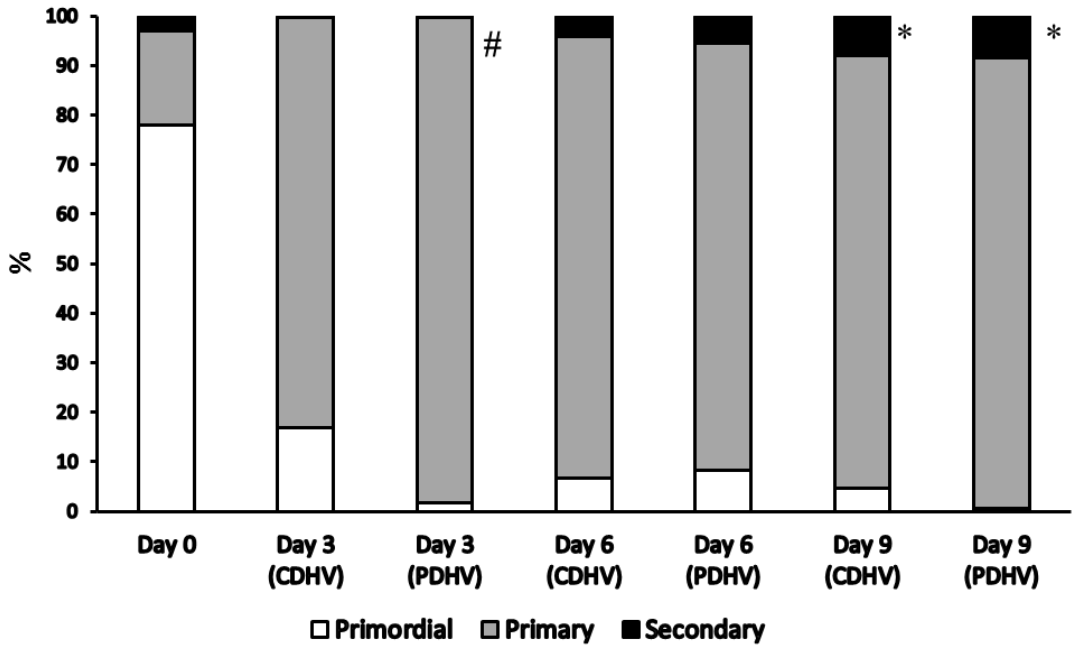
p<0.001, Vs CDHV

Fig. 10A Follicle progressional stages



Representative images from hematoxylin eosin stained sections, depicting bovine follicular develeopmental stages.1: Primordial, 2: Primary, 3: Secondary. Bar=10µm

Fig. 10B Follicle staging



*** p<0.01, Vs Day 0**

 # p<0.01, Vs CDHV

Follicle Viability:

Data for follicle viability Fig. 11A was obtained from a total of 1969 follicles from control and cultured strips (Fig. 11B). Fresh tissue had a follicle viability of 92.5%. A significant reduction of viability occurred during culture under both conditions. However, the viability throughout the culture was about 80% in PDHV and only around 50% in CDHV (PDHV vs CDHV, $p<0.0001$; Fig.11B).

Fig. 11A Live dead assay images

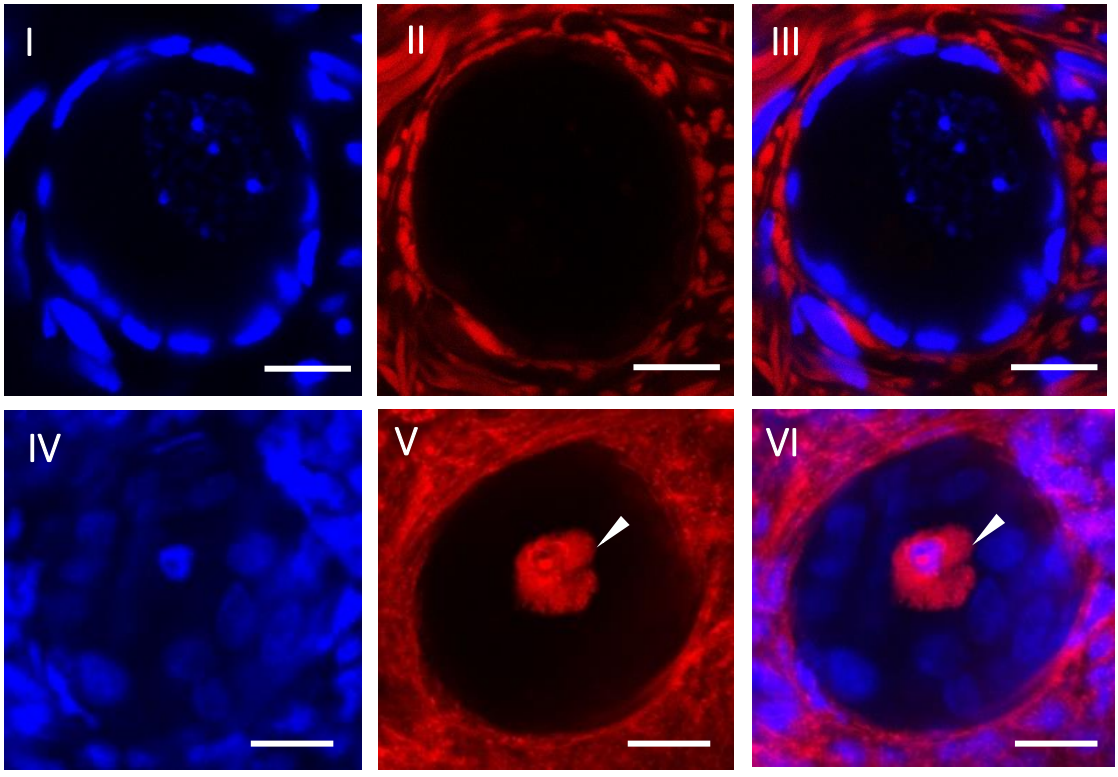
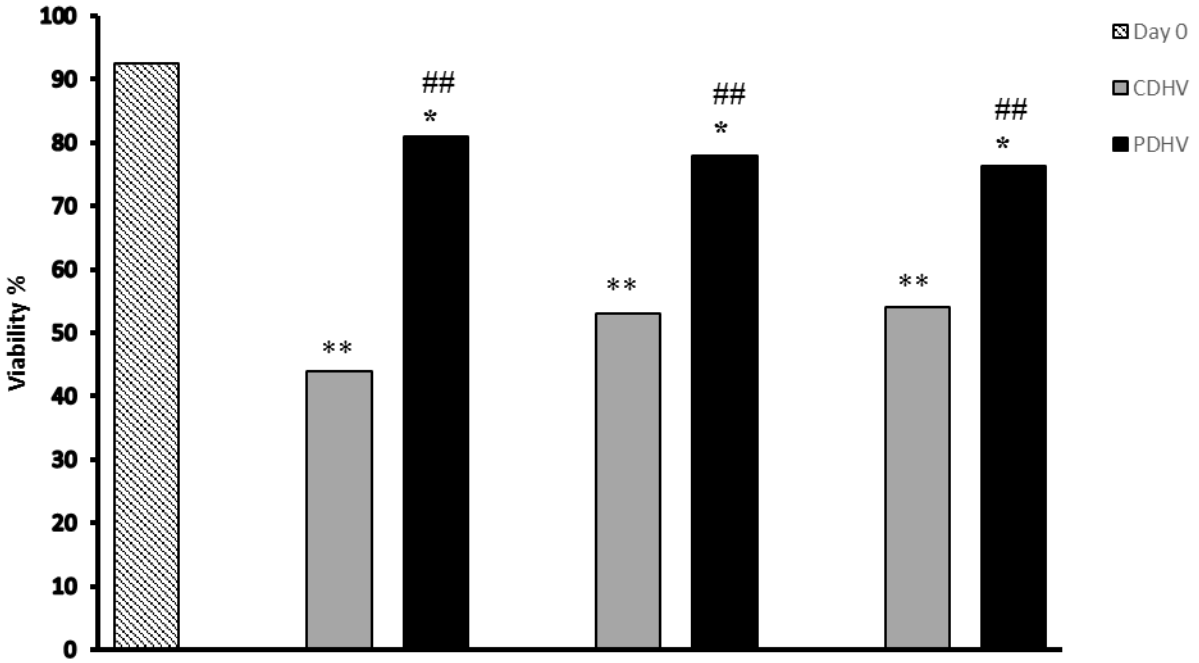


Fig. 11B Follicle viability



**** p<0.001, * p<0.01, Vs fresh control**

p<0.001, Vs LN

Experiment IIB

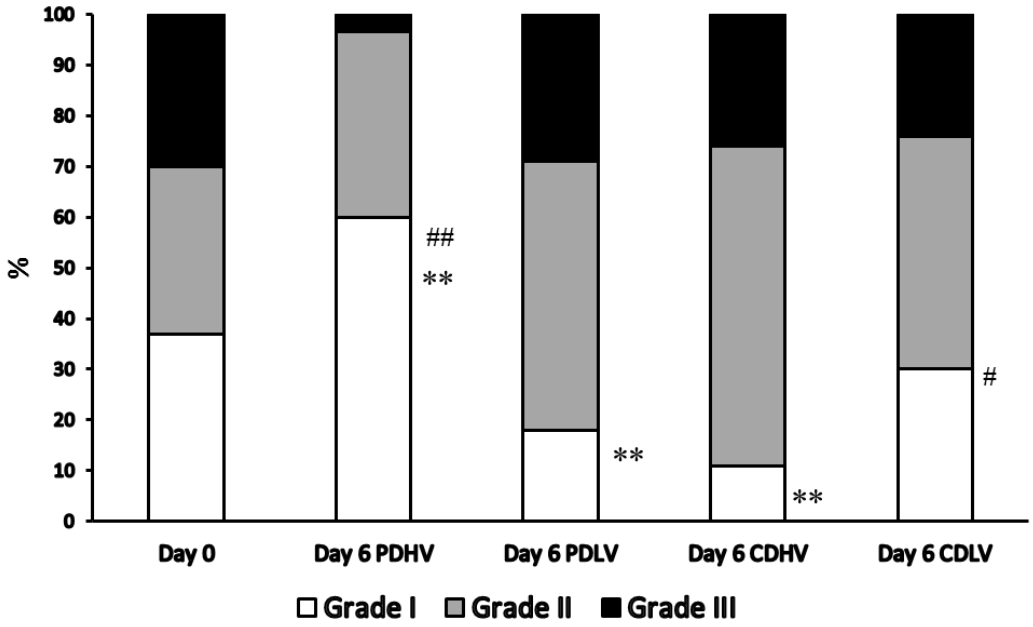
Histology:

A total of 818 bovine follicles were morphologically assessed to study the effect of media height on oxygen perfusion and culture performance in both culture dishes. On day 6 of culture PDHV performed significantly better than all other groups, harbouring significantly higher proportion of grade I follicles, $p<0.0001$ (Day 0 - 37%, Day 6 PDHV – 60%, PDLV – 18%, CDHV – 11%, CDLV – 30%, Fig 12).

Interestingly CDLV outperformed CDHV in terms of grade I follicles, $p<0.001$ possibly showing a positive effect of better oxygen perfusion in the conventional dish due to the reduced media height over longer culture. CDHV and PDLV with lowest and highest oxygen tensions remained the worst culture groups at day 6

By day 6 of culture, majority of the primordial follicles were activated to the primary stage with no significant differences between the groups (Fig.13). Primary follicles were found to be significantly higher in all culture conditions compared to Day 0, $p<0.001$, showing that all culture conditions could support initial follicle activation.

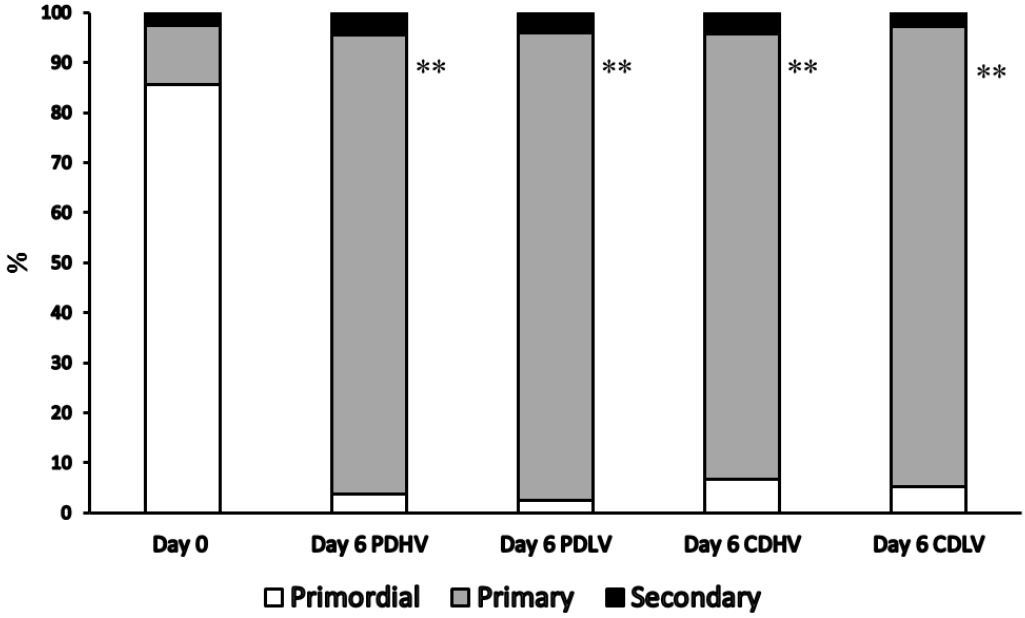
Fig. 12 Follicle grading



**** p<0.001, Vs day 0 control**

p<0.001, # p<0.01 Vs test groups

Fig. 13 Follicle staging

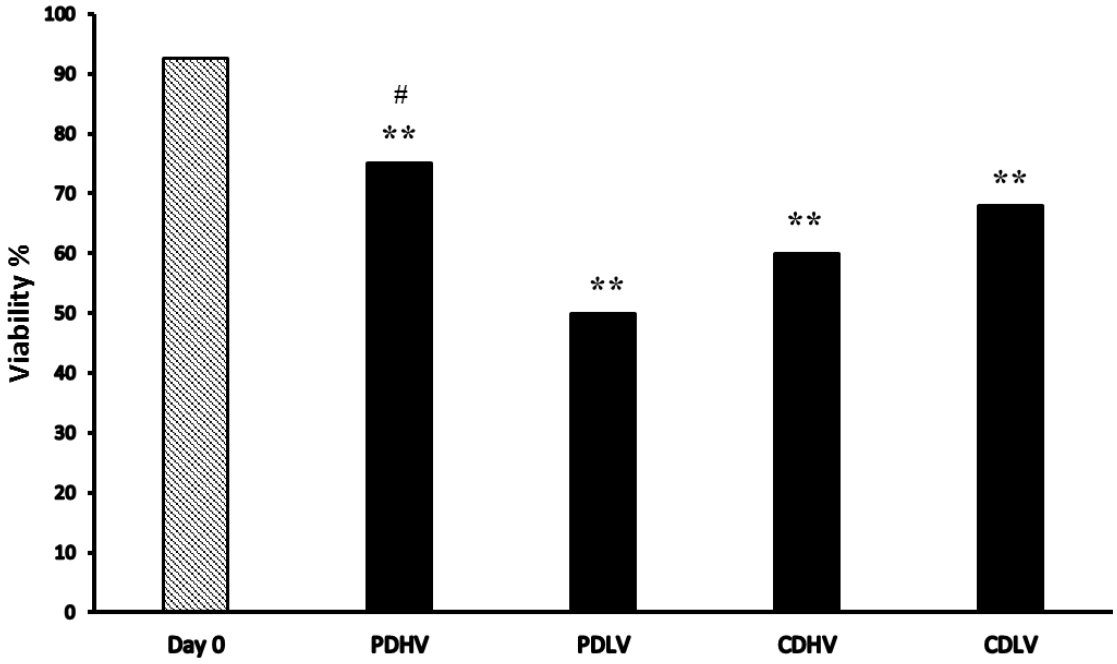


**** Primary follicles, Day 0 Vs other groups, p<0.001**

Follicle Viability:

Data for follicle viability was obtained from a total of 1969 follicles from control and cultured strips (Fig.3). Fresh tissue had a follicle viability of 92.5%. A significant reduction of viability occurred during culture under both conditions. However, the viability throughout the culture was about 80% in PDHV and only around 50% in CDHV (PDHV vs CDHV, $p<0.0001$; Fig.13).

Fig. 13 Follicle viability



**** p<0.001 Vs Day 0,**

 # PDHV Vs PDLV, CDHV, p<0.01

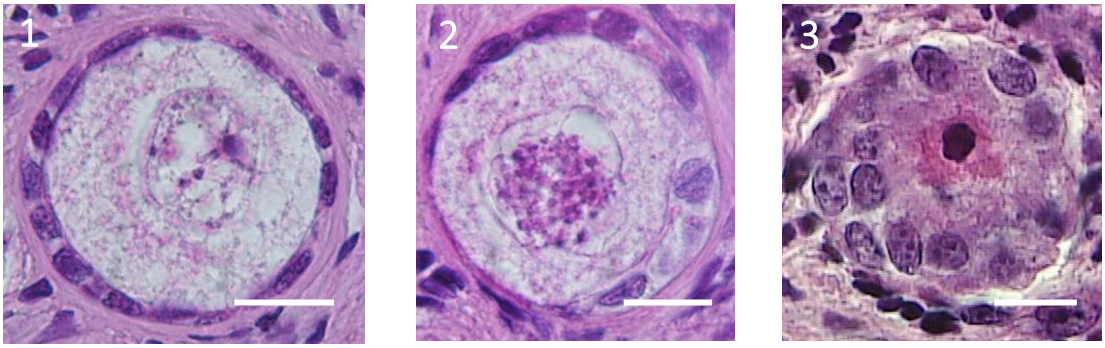
Experiment IIC

Histology:

A total of 1383 human follicles from control and cultured human strips were assessed morphologically (Fig. 14). A significant drop in follicle quality was observed both in PD and CD cultures compared to day 0 tissues, (Day 0 - 46.65%, Day 6 CDHV-12.25%, Day 6 PDHV-38.2%, Day 9 CDHV-19%, Day 9 PDHV- 44.8% $p<0.0001$ (Fig. 15). However, PDHV was still the best culture condition and the proportion of grade I follicles were seen to be superior to CDHV both at day 6 and day 9, $p<0.001$ (Fig. 15).

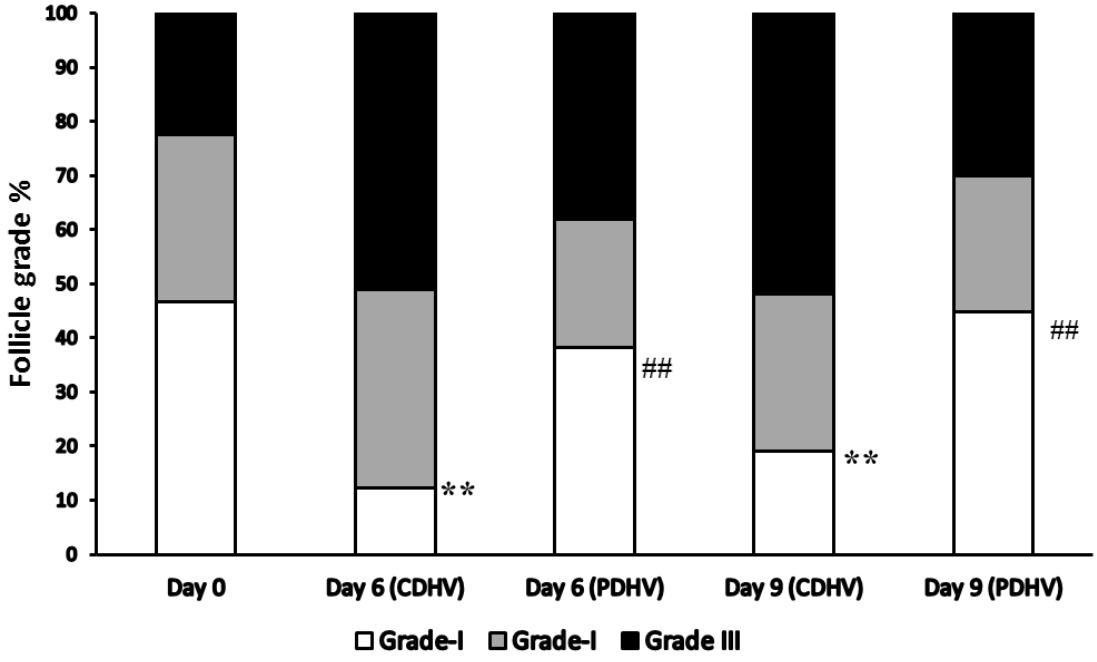
Follicle staging (Fig.16) demonstrated that, as seen in the bovine experiments majority of primordial follicles were recruited to the primary stage by day 6 with no significant differences among the culture groups. Interestingly, as opposed to the bovine experiments the proportion of secondary follicles in PDHV rose significantly higher than day 0 and CDHV groups, $p<0.001$, (Fig. 17), both at day 6 and 9 of culture.

Fig.14 Follicle morphological grades



Histological grading of human follicles from hematology eosin sections. 1: Grade 1 follicle showing healthy follicular characteristics. 2: Grade 2 follicle with missing granulosa cells. 3: Grade 3 atretic follicle showing a pyknotic oocyte nucleus and misshapen oocyte. Bar=10µm

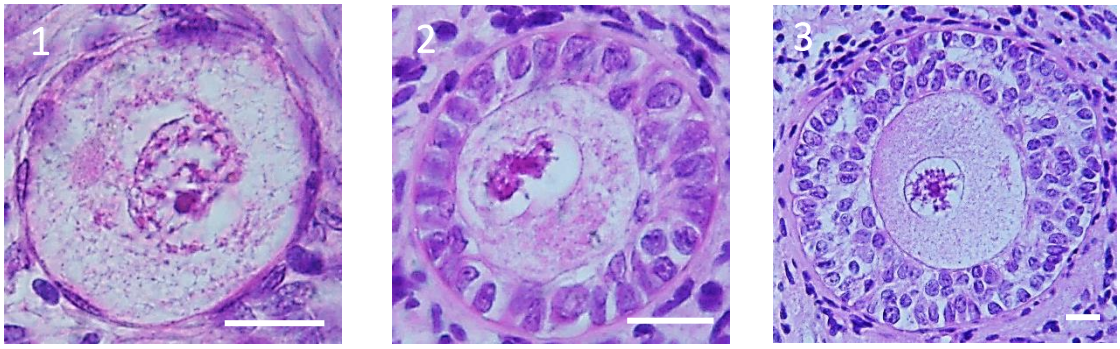
Fig. 15 Follicle grading



**** p<0.0001, Vs Day 0**

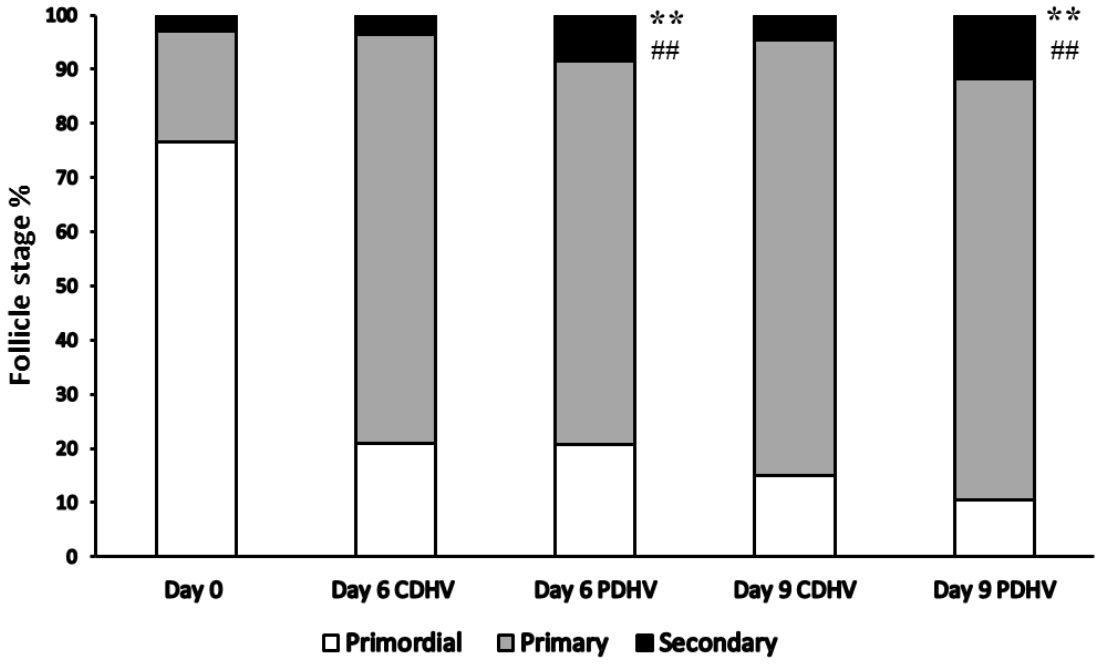
p<0.001, Vs CDHV

Fig. 16 Follicle progressional stages



Representative images from hematoxylin eosin stained sections, depicting human follicular developmental stages 1: Primordial, 2: Primary, 3: Secondary. Bar=10µm

Fig.17 Follicle staging



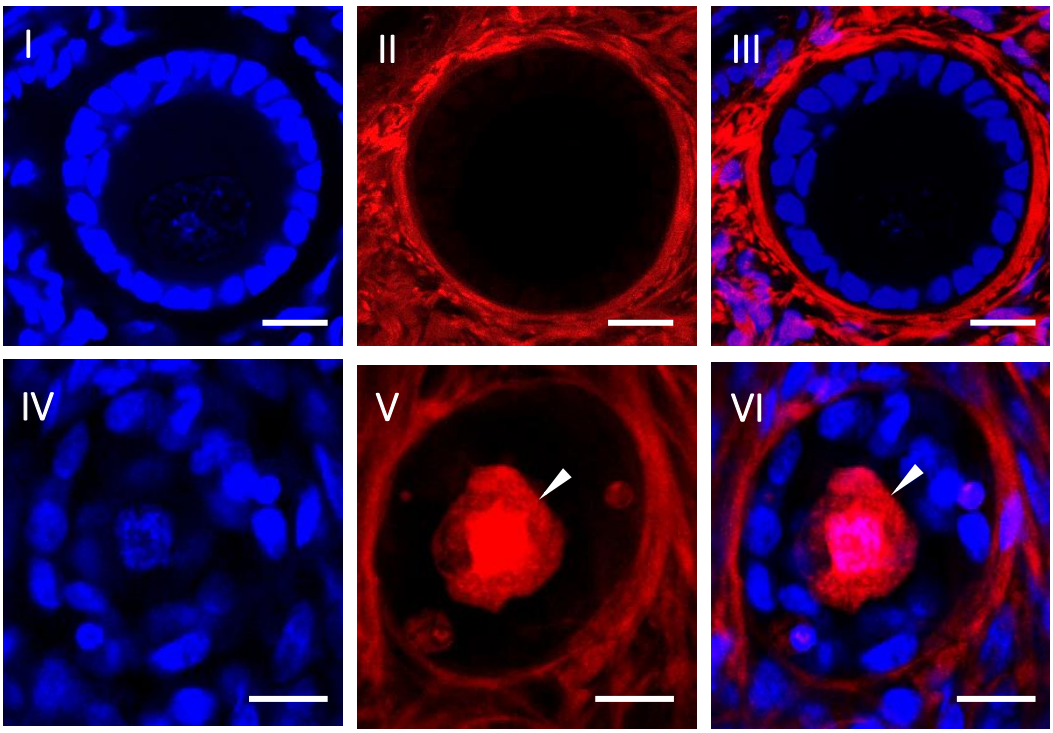
**** p<0.001, Vs Day 0**

p<0.001, Vs CDHV

Follicle Viability

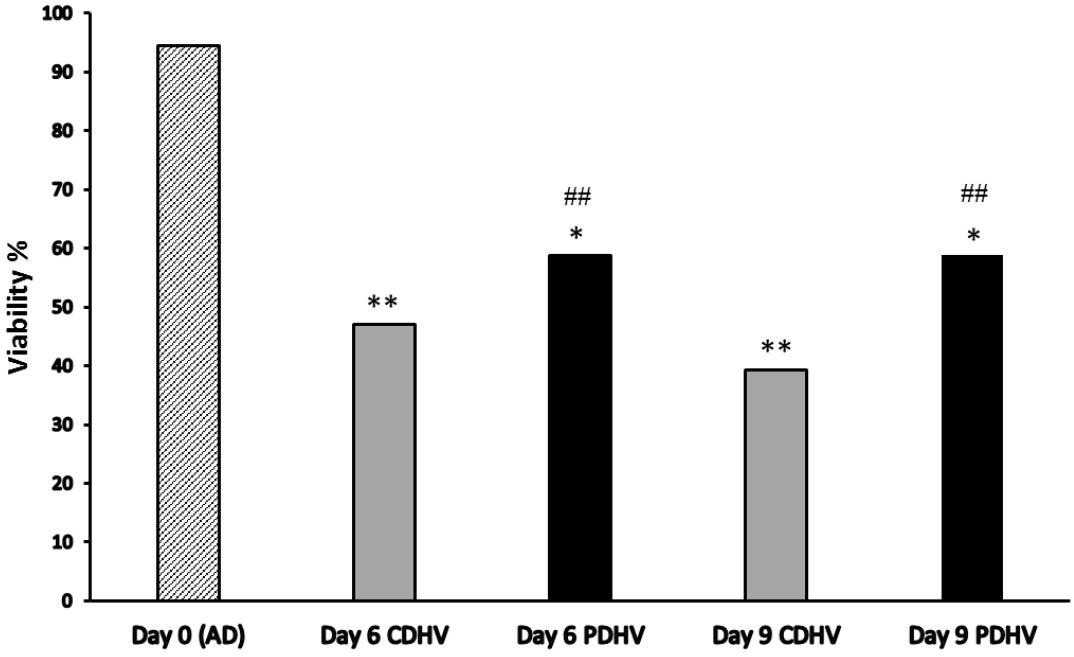
Follicle viability (Fig.18), was in agreement with the histology findings such that both culture conditions showed a fall in viability compared to Day 0 tissues (Fig.19). However, among the cultured groups PDHV, remained the better culture condition with a significantly higher follicle viability compared to CDHV both at Day 6 and Day 9, $p<0.001$ (Fig.19)

Fig. 18 Live dead assay images



Live dead confocal analysis of human follicles. I, IV: Nucleus, II, V: Far red, III, VI: Merge. Upper row represents a viable follicles with no far red nuclear stain and the lower panel represents a non-viable follicle with (arrowhead) intense staining in the far red spectrum. Bar=10µm

Fig. 19 Follicle viability



**** p<0.001, Vs Day 0**

p<0.001, Vs CDHV

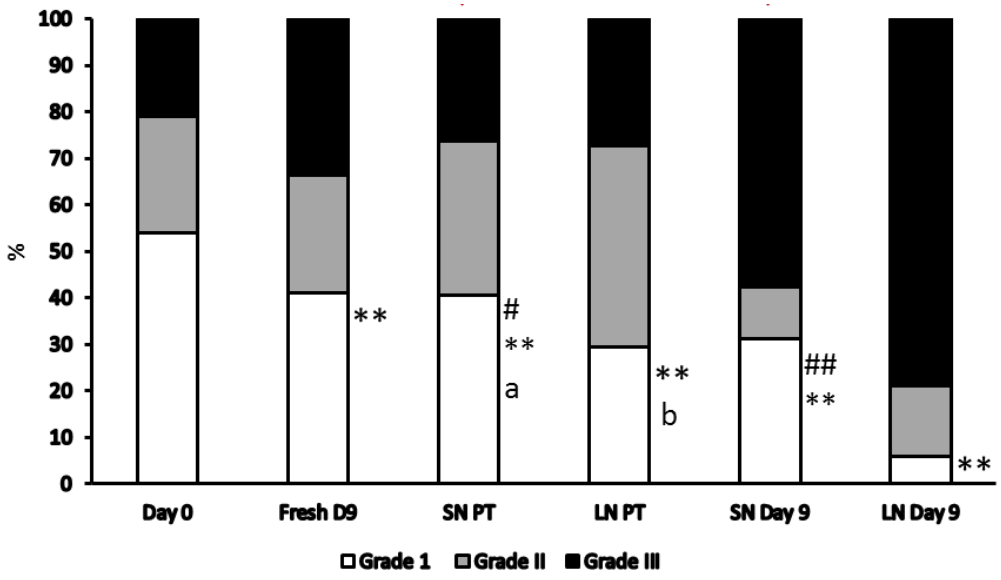
Experiment III

Histology:

A total of 2650 follicles were analyzed for follicle grading and staging. Fresh tissue at Day 0 harbored 54% grade I follicles (Fig. 20). All fresh and cryopreserved samples showed a significant decrease in the proportion of grade I follicles compared to the day 0 controls $p<0.001$. As demonstrated during experiment I SN vitrified strips were significantly superior to LN strips in terms of follicle quality 24 hours post warming (Grade I follicles SN PT – 40.5% Vs LN PT – 29.3%, $p<0.05$, Fig. 20). Interestingly, a similar trend was observed even after culturing these strips and by 9 days of culture the differences between the two groups were seen to be extremely different (Grade I follicles: SN Day 9 – 31.3%, LN Day 9 - 6%, $p<0.001$, Fig. 20). Long term culture had caused a fall in follicle viability in both groups compared to post 24 hour culture. However, this effect was seen to be drastic in LN vitrified strips (Fig. 20 a, b).

Interestingly follicle progression to the secondary stage in SN vitrified strips was also seen to be superior both compared to fresh tissue post 9 day culture and LN vitrified strips at day 9 (Secondary follicles: SN Day 9 - 36.6 Vs Fresh D9 - 26.8 %, $p<0.05$; Vs LN Day 9 – 25.1%, $p<0.05$, Fig. 21).

Fig. 20 Follicle grading



** p<0.001, Vs Day 0

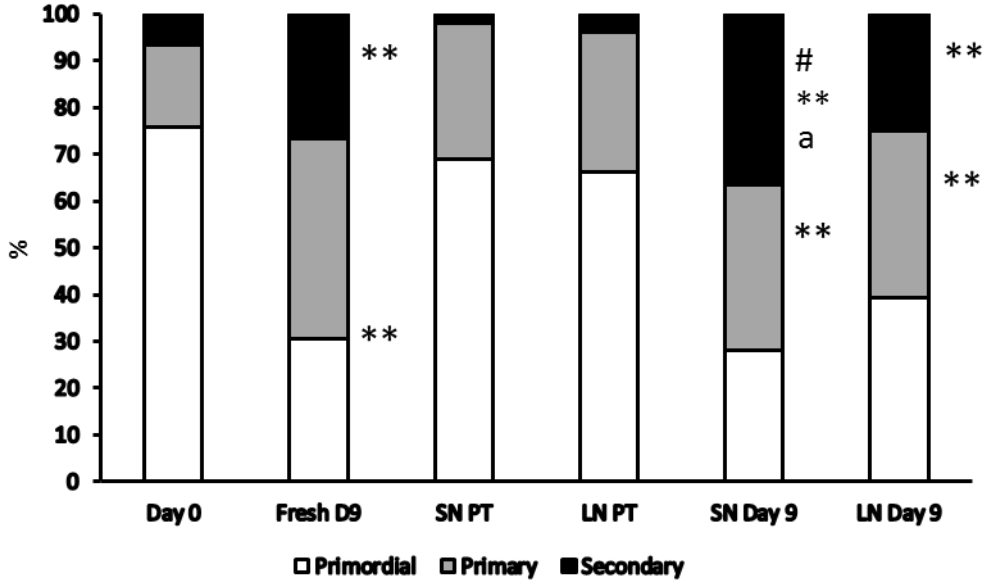
p<0.0001, Vs LN

# p<0.05, Vs LN

a p<0.05, Vs SN Day 9

b p<0.001, Vs LN Day 9

Fig. 21 Follicle staging



** p<0.001, Vs Day 0

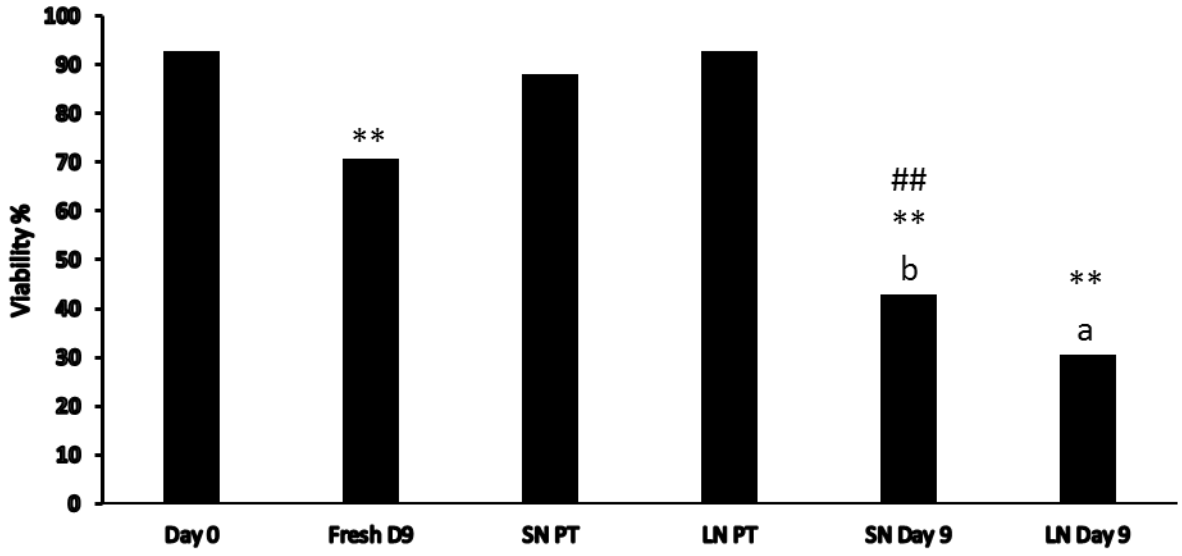
# p<0.05, Vs LN

a p<0.05, Vs Control D9

Viability:

All groups cultured for 9 days showed a fall in viability compared to the day 0 controls (Day 0 – 92.7% Vs Fresh D9 – 70.8%, SN Day 9 – 43%, LN Day 9 – 30.5%, $p<0.001$, Fig. 22) and this fall in viability was particularly evident for cryopreserved strips (Fig. 22 a, b). Although 24 hours post warming, viability of both SN and LN vitrified strips were not different from fresh strips at day 0, both groups showed a significant fall in viability following 9 days of culture. However, SN vitrified strips demonstrated a significantly higher follicle viability compared to LN tissues after long term culture. (SN Day 9 – 43% Vs LN Day 9 – 30.5%, $p<0.001$).

Fig. 22 Follicle viability



**** p<0.001, Vs Day 0**

a p<0.001 Vs Fresh D9

p<0.001, Vs LN

b p<0.001 vs Fresh D9

Respirometry:

Respirometry of ovarian strips demonstrated a mean oxygen consumption rate of 1.2745E-11 mol/s for each individual strip. Oxygen consumption rate predicted by the mathematical simulation model was seen to be close to the measured value at 0.878E-11 mol/s.

Discussion and Conclusions

Optimizing ovarian tissue cryopreservation using SN:

Although a substantial number of births has been achieved after OT re-transplantation, ovarian cryopreservation is still considered an experimental procedure and needs to be refined (ASRM 2013, 2014) to reduce cryodamage and the consequent depletion of follicles and SCs that may impair a full recovery of ovarian function. Because the evaluation of its clinical efficacy is considerably delayed by the time between OT cryopreservation and re-implantation, which may span several years (Revelli et al., 2013, Oktay et al., 2016), basic biological studies are needed to define more appropriate procedures aimed at minimizing the injuries to oocytes, GCs, and SCs.

Herein, SN was used to perform the ultrarapid vitrification of the human ovary to understand whether the increase in cooling rates minimizes the injuries to ovarian follicles and stroma observed in parallel samples subjected to LN vitrification. To this end we chose the vitrification protocol 2 developed by Amorim et al. (Amorim et al., 2012) because it was demonstrated to preserve the quality, viability, and ability to resume folliculogenesis of preantral follicles after xenografting. Our main findings, demonstrates that SN vitrification improves (1) the recovery of grade 1 follicles; (2) the ultrastructural preservation of GCs and SCs; (3) the DNA integrity of SCs; (4) the viability of oocytes, GCs, SCs and (5) To better preserve the functional potential of the vitrified strips and in turn the follicles to mature to the secondary stage. To our knowledge this is the first study in which SN was used to vitrify human or animal OT.

Although there is a general agreement on the negative effects of both slow freezing and vitrification on the quality of follicles and SCs in thawed tissues (Ischenko et al., 2009, Xiao et al., 2013), some slow-freezing and vitrification procedures were reported to not damage the follicle quality (Keros et al., 2009, Klocke et al., 2015). Herein, follicle quality in fresh and vitrified sample was evaluated classifying follicles into three grades on the basis of histologic analysis (Talevi et al., 2013). Compared with fresh tissue, findings showed a high and significant decrease of grade 1 follicles and a concomitant increase of grade 2 follicles in LN- and not in SN-vitrified samples. This finding clearly

depends on the improvement of heat transfer during SN vs. LN vitrification, as already shown in human and mouse oocytes (Yoon et al., 2007, Lee et al., 2010, Criado et al., 2011, Minasi et al., 2012). Follicular development inside the ovary is a highly complex process involving fundamental cross-talk among the oocytes, GCs, and SCs (Gougeon et al., 2010, Palma et al., 2012). Therefore, the integrity of GCs and SCs in thawed OT is fundamental for the recovery of ovarian function after re-implantation.

Transmission electron microscopy analysis was carried out for assessment of fine morphologic changes in follicles that survived vitrification/warming. Although previous studies showed that oocyte and OT cryopreservation could affect the ultrastructure of survived oocytes (Gualtieri et al., 2009, Gualtieri et al., 2011, Gook et al., 1999, Bonetti et al., 2011), in our study fresh and vitrified samples had a similar ultrastructure. However, LN vitrification affected GCs at a different extent, leading either to the regression of plasma membrane interdigitations with the oocytes or to cell death. Taken together, these findings suggest that the increase of freezing rates during SN vitrification can effectively avoid GCs cryodamage.

As stated above, SCs represent one of the main targets of cryoinjury during OT cryopreservation. In our set of patients, the number of intact SCs in fresh tissue was rather low, and this is in agreement with other studies (Keros et al., 2009).

Although vitrification had a clear detrimental effect on viability of SCs, the number of intact SCs after SN vitrification was two times higher compared with LN samples. Ultrastructural features of damaged SCs suggest that cell death was due to necrosis rather than apoptosis. To confirm this hypothesis, we evaluated the percentages of TUNELpositive SCs in fresh and vitrified OT samples. In agreement with recent studies obtained with different vitrification methods (Fabbri et al., 2014, Salehnia et al., 2012, Rahimi et al., 2009), our findings showed a very low number of DNA-fragmented SCs, substantiating the hypothesis that cell death occurred through necrosis both in fresh and vitrified samples. However, conventional vitrification in LN not only induces higher SC

necrosis but also an apoptosis rate approximately fivefold higher compared with both fresh and SN samples.

The possibility to assess the viability of oocytes, GCs, and SCs in ovarian cortical strips in situ is fundamental to evaluate the impact of cryopreservation on tissue health. The calcein AM assay, which was first used in situ, allowed the detection of follicle and not GC/SC viability (Cortvrindt et al., 2001). Others used calcein AM or trypan blue to count live follicles after enzymatic tissue digestion (Sanfilippo et al., 2011). A noninvasive neutral red staining method has been recently developed to quantify follicle density and viability on thin (80–100 mm) ovarian cortical slices in ovine and human (Chambers et al., 2010). However, none of these methods allow the visualization of both follicles and ovarian somatic cells in situ. Herein, the live–dead far-red viability assay allowed a clear-cut distinction of dead and live oocytes, GCs, and SCs throughout the tissue depth. Data on oocyte survival were consistent with histologic follicle grading, whereas data on GCs and SCs were in good agreement with that obtained through electron microscopy, demonstrating that SN vitrification better preserves OT viability.

Assessment of follicle stages in fresh and warmed samples indicates that primordial follicles are capable of reactivating after warming/culture and progress toward the primary stage. In light of recent concerns on the duration of graft survival after OT transplantation (Roness et al., 2013), the slight but significant increase of primary follicles in SN vs. LN samples could be a negative effect of SN vitrification because the ovarian reserve might be depleted more quickly at re-implantation before the tissue is successfully re-vascularized. On the other hand, because SN tissues have a healthier condition in many respects compared with LN tissue, it could be argued that the latter has a decreased potential to restore ovarian function at re-implantation.

Our culture experiments helped us identify an optimal culture condition for the culture of cortical strips based on the right oxygen diffusion to the strip vicinity. SN vitrified strips when cultured in PDHV ensured long term follicle quality, viability and a better follicle progression to the secondary stage. Quite interestingly, SN vitrified strips had a

commendable secondary follicle progression of 35.6% at Day 9 of culture, which has never been reported before in literature validating potential of these strips to support resumption of folliculogenesis.

Data reported herein on the vitrification of human ovarian cortical strips demonstrates that the increase of cooling rate using SN improves the recovery of healthy oocytes, GCs, SCs and functional potential of the tissues.

Optimizing ovarian tissue in situ culture:

Successful isolation and culture of primordial follicles to obtain viable oocytes and offspring have been reported in lower animal models like mice (Obrien and Epigg 2003 Higuchi et al 2015), although most studies in higher animals and human that have attained significant oocyte maturation *in vitro* often commence with late secondary or even early antral follicles (Gupta and Ravindra et al 2008, Xiao and Woodruff et al 2015). Human folliculogenesis in vivo, is a complex process stretched over a period of 8 months (Gougeon et al 1986). Although, follicle growth *in vitro* is exceptionally accelerated (Xu et al 2009), to realize complete *in vitro* folliculogenesis, an optimal culture system that supports long term tissue viability and follicle quality would be inevitable. Furthermore, the two-step culture strategy (O'Brien et al., 2003), being one among the most promising techniques to realize complete *in vitro* folliculogenesis, optimizing cortical strip culture has tremendous scope in improving the efficiency of in situ follicle activation and progression to secondary stages.

Studies that have investigated the optimal oxygen concentrations for culture of oocytes and follicles have till date not reached a consensus (Park et al., 2005, Eppig et al., 1995, Gwatkin et al., 1974). Our study suggests that this could mainly be due to the fact that oxygen concentration near the follicle/tissue cultured can never be directly expressed as the gas tension maintained inside the incubator. Several factors like the media column height, surface area of media-air interphase, size of the strips cultured (representing total

cell number), follicular consumption rate, rate of gas diffusion etc could be factors influencing the actual amount of oxygen available to the follicles. In ovarian strip cultures, this is further complicated by the fact that only the cells at the periphery are exposed to the concentration of gases diffused in the surrounding media (Gigli et al., 2006).

Our experiment was focused on analyzing the effects of modulating oxygen diffusion on ovarian strips in culture, by using oxygen permeable dishes and altering the media height. To this end, bovine strips were first cultured in conventional versus permeable dishes under different media heights, to analyze the effect of different oxygen diffusion rates. The oxygen permeable dish under a high volume (PDHV) was revealed to be the most optimal condition in terms of follicle quality and viability, and this was further validated using human cortical strips.

Human and bovine culture experiments equivocally showed that strips cultured in PDHV could maintain superior follicle quality until 9 days of culture compared to conventional dishes. PDHV performed consistently and maintained a significantly higher proportion of healthy and viable follicles compared to the conventional dish, during the entire period of culture. This suggests that these strips could overcome necrosis to a greater extend during extended culture possibly owing to the optimal oxygen environment provided by the permeable dish. Wycherley et al proposed that a higher oxygen consumption by the cultured follicles, could lower the local oxygen concentrations, in turn increasing the flux of oxygen from the air media interphase to the cultured follicles. However, if this rate of diffusion is slower than the follicular oxygen consumption, follicles would progressively experience a hypoxic environment. This condition is further aggravated for cells at the interior of the follicle (Wycherley et al., 2004). Quite evidently, a media column of ~1.4mm (high volume) seems to hinder the sufficient passage of oxygen to the tissue vicinity and this in turn affects the morphological quality and viability of follicles. However, the permeable dish could better replenish oxygen levels, substituting for the

slow oxygen diffusion through the media column better preserving follicle quality and viability.

The role of ensuring an optimal oxygen concentration at the tissue vicinity was further evidenced by experiment IIB were culture was simultaneously performed at a low (2.5ml ~ 0.7mm) and a high (5ml ~1.4mm) media height, to analyze if lowering the media height would improve oxygenation and in turn the culture condition. This media height was chosen to mimic an interphase like culture system frequently applied for the culture of strips on tissue inserts, with media just covering the tissue surface (Higuchi et al 2015). Our results suggested that follicles within strips, prefer an optimal range of oxygen concentration for long term sustenance such that, PDHV was still seen to be the superior culture condition, harboring a significantly higher proportion of grade I and lower grade III atretic follicles.

Interestingly, reducing the media height in the permeable dish (PDLV) had a negative effect on follicle health, supposedly due to a prevalent hyperoxic condition. Fainstat et al made a similar observation that an insufficient oxygen concentration led to central necrosis whereas an elevated concentration caused tissue damage (Fainstat et al., 1968) when he analyzed the effect of various oxygen tensions on cultured ovarian tissue viability and follicular development. Several reports have shown detrimental effects like reduction in growth of follicles (Xu et al., 2011) antrum formation (Silva et al., 2010) and lose of granulosa cell – oocyte cell junctions (Heise et al., 2009) as a result of follicle culture under atmospheric oxygen concentrations (20%). Hence, culture in CDHV could have created a hypoxic environment and in PDLV a low media height together with the permeable base could have created a hyperoxic condition at the tissue vicinity both consequently damaging the follicles within. Although, reducing media height in conventional dishes (CDLV) was seen to improve oxygen diffusion, it was still seen to be inferior to PDHV in maintaining optimal oxygen concentration.

PDHV being the best culture condition when validated in the human model (experiment IIC) performed better compared to conventional culture dishes. Furthermore, experiment III demonstrated that culture in PDHV was also superior for culture of vitrified strips in terms of follicle quality, viability and attaining commendable progression to the secondary stage.

Follicular oxygen requirement is a highly dynamic process such that primordial and primary follicles show a rapid consumption of oxygen suggesting the role of both tricarboxylic acid cycle and oxidative phosphorylation in primordial follicle activation (Ishikawai et al., 2014) However, as the granulosa layers thickens the follicles could be deprived of oxygen in culture, eventually leading to granulosa cell death and follicle atresia (Hishfield et al., 1991). Our data also suggests this positive correlation between follicle progression and oxygen concentration such that PDHV (experiment IIA) showed a significantly higher percentage of bovine primary follicles at day 3 of culture illustrating a higher rate of follicle recruitment. Interestingly, during experiment IIB although PDLV negatively affected both follicle viability and quality, follicles readily activated in this condition. This observation is in agreement with other studies that suggest that supranormal levels of oxygen can support granulosa cell proliferation and hence primordial to primary transit, but these follicles eventually undergo necrosis and could compromise oocyte viability (Qvist et al., 1990)). In short our experiments underline ovarian tissues demand a critical balance of oxygen to ensure long term follicle quality, survival and progression. Fresh/cryopreserved human strip culture validated this finding such that PDHV was superior in terms of follicle progression to the secondary stage both at day 6 and 9 of culture.

Several studies aimed at improving follicle progression in ovarian tissue cultures have reported that supplementation of factors like FBS, FSH or activin were not particularly effective in augmenting the growth of primary follicles to the secondary stages (Fortune et al., 1998, 1999, 2000).

Our preliminary findings suggest a very decisive role of oxygen in follicle activation and progression to the secondary stage. Culture of ovarian strips using the gas permeable dish under the optimal media height, enabled us to maintain a higher follicle viability over 9 days of culture. Hence, this culture system could prevent tissue necrosis to a greater extend, ensuring long term follicle/tissue survival.

Merging optimal cryopreservation and culture systems:

As discussed earlier, the final aim of our experiments were to propose an optimal system to improve both cryopreservation and culture of human ovarian tissues. This is particularly important considering that till date, thousands of ovarian tissue samples have been cryopreserved worldwide. An optimal ovarian tissue culture system can only be proposed after having considered the efficiency of the system for the culture of frozen strips also. Furthermore, long term functional potential of vitrified strips is an important facet to be analyzed for any cryopreserved material. Conventional methods of growth potential include xenotransplantation of the warmed strips into various animal models. However, this is a challenging process often with disappointing results since the strips are out of vision and could be affected by various other factors like graft acceptance, ischemic damage that could be caused due to delay in neovascularization and even sub-optimal surgical conditions and skills. *In vitro* culture hence offers a powerful tool to assess the growth potential of follicles harbored within the vitrified strips.

Hence, we merged SN vitrification along with PDHV culture, both as a tool to assess the efficiency of both using the other and as an optimal protocol combination that could eventually be adopted in a clinical fertility preservation setup. SN vitrification provides a superior cryopreservation protocol that preserves both the somatic and germ cell compartments of the ovarian tissue compared to conventional LN vitrification. Culture in PDHV could maintain a higher follicle quality, viability and

impressive secondary follicle progression during long term culture. Such systems could be used for long term human cortical cultures, necessary to increase the efficiency of two step culture systems by improving the quality and yield of secondary follicles for isolated culture, obtaining mature oocytes for *in vitro* fertilization. These cultures could also act as a quarantine system that both stabilizes and aids in tissue selection for further transplantation.

In short, such innovations in ovarian cortical cultures could radicalize fertility preservation in many ways, completely avoiding concerns over tissue transplantation and re-introduction of malignant cells during such procedures, which could push the boundaries of the current state of art in cancer fertility preservation.

The data disclosed herein and other work done during my PhD has been either published or is in course of publishing as follows.

ORIGINAL ARTICLE: FERTILITY PRESERVATION

Successful slush nitrogen vitrification of human ovarian tissue

Riccardo Talevi, Ph.D.,[a] Vincenza Barbato, Ph.D.,[a] Ilaria Fiorentino, Ph.D.,[a] Sabrina Braun, Ph.D.,[a] Cristofaro De Stefano, M.D.,[b] Raffaele Ferraro, M.D.,[c] Sam Sudhakaran, M.Sc.,[a] and Roberto Gualtieri, Ph.D.[a]

[a] Dipartimento di Biologia, Università di Napoli "Federico II," Complesso Universitario di Monte S Angelo, Napoli, Italy; [b] Unità di Fisiopatologia della Riproduzione e Sterilità di Coppia, Azienda Ospedaliera San Giuseppe Moscati, Avellino, Italy; and [c] Iatropolis, Genesis Day Surgery Sterilità della Coppia, Caserta, Italy

Objective: To study whether slush nitrogen vitrification improves the preservation of human ovarian tissue.
Design: Control vs. treatment study.
Setting: University research laboratory.
Patient(s): Ovarian biopsies collected from nine women (aged 14–35 years) during laparoscopic surgery for benign gynecologic conditions.
Intervention(s): None.
Main Outcome Measure(s): Ovarian cortical strips of 2 × 5 × 1 mm were vitrified with liquid or slush nitrogen. Fresh and vitrified cortical strips were analyzed for cryodamage and viability under light, confocal, and transmission electron microscopy.
Result(s): Compared with liquid nitrogen, vitrification with slush nitrogen preserves [1] follicle quality (grade 1 follicles: fresh control, 50%; liquid nitrogen, 27%; slush nitrogen, 48%); [2] granulosa cell ultrastructure (intact cells: fresh control, 92%; liquid nitrogen, 45%; slush nitrogen, 73%), stromal cell ultrastructure (intact cells: fresh control, 59.8%; liquid nitrogen, 24%; slush nitrogen, 48.7%), and DNA integrity (TUNEL-positive cells: fresh control, 0.5%; liquid nitrogen, 2.3%; slush nitrogen, 0.4%); and [3] oocyte, granulosa, and stromal cell viability (oocyte: fresh control, 90%; liquid nitrogen, 63%; slush nitrogen, 87%; granulosa cells: fresh control, 93%; liquid nitrogen, 53%; slush nitrogen, 81%; stromal cells: fresh control, 63%; liquid nitrogen, 30%; slush nitrogen, 52%).
Conclusion(s): The histology, ultrastructure, and viability of follicles and stromal cells are better preserved after vitrification with slush nitrogen compared with liquid nitrogen. (Fertil Steril® 2016;105:1523–31. ©2016 by American Society for Reproductive Medicine.)
Key Words: DNA fragmentation, follicle, histology, ovarian cryopreservation, ultrastructure

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Riccardo Talevi, Vincenza Barbato, Ilaria Fiorentino, Sabrina Braun, Cristofaro De Stefano, Raffaele Ferraro, Sam Sudhakaran, Roberto Gualtieri. Successful slush nitrogen vitrification of human ovarian tissue. Fertil Steril. 2016 ;105(6):1523-1531

Isolated ovarian follicle culture: a promising strategy for fertility preservation

Sam Sudhakaran
Roberto Gualtieri
Vincenza Barbato
Illaria Fiorentino
Sabrina Braun
Anna Merolla
Riccardo Talevi

Department of Biology
"Federico II" University, Napoli, Italy

Address for correspondence:
Sam Sudhakaran, PhD
Department of Biology
"Federico II" University, Napoli, Italy
E-mail: roschers007@gmail.com

Summary

Ovarian tissue cryopreservation represents one among the most preferred strategies for fertility preservation currently. However, concerns regarding the transmission of malignant cells during the transplantion of stored ovarian tissues, is a major restraint in recommending the procedure to patients diagnosed with all kinds of malignant disorders. On the contrary, use of isolated follicles for restoration of fertility in such patients could completely evade the possibility of cancer re-introduction after treatment. Follicles housed in the ovarian environment *in vivo* prevail under the mechanical and the chemical/nutritional support of the ovary. Although not complete, recent knowledge about the dynamics of follicular progression has led to improvements in the culture system adopted. This review aims at summarising the culture of isolated follicles *in vitro*, particularly emphasising the efforts made to mechanically and nutritionally support the follicle. Advances in follicular culture systems could prove useful to highly improve the efficiency of current fertility restoration strategies and evade the concerns associated with the same.

KEY WORDS: fertility preservation, follicle culture, three dimensional support, isolated follicles.

Introduction

Cancer prevalence among women is till date, a major medical concern. Studies suggest that, 1 out of 51 women would have had an invasive cancer diagnosed by 39 years of age (1). Recent reports supported by the national cancer institute, have shown a 0.8% increase in cancer affected children during the past decade (2). However, recent advances in chemotherapy, radiotherapy, and bone marrow transplantation can cure as high as 90% of women and children affected by cancer and other disorders requiring such treatment. On the other side, ionizing radiation and aggressive chemotherapy can result in some degree of premature ovarian failure in almost 100% of patients requiring such therapy (3). Whole body irradiation coupled with intensive chemotherapy associated with bone marrow transplantation, poses one of the greatest threats to treated patients. The ovarian reserve is completely abolished after treatment regimes including alkylating agents such as busulphan (4). Moreover, several studies have shown that a radiation dose as low as 5- 20 Gy, is sufficient to cause gonadal function impairment (5-7).

Concerns in routine ovarian tissue cryopreservation

Cryopreservation of the ovarian tissue is one of the mainstays in fertility preservation strategies adopted today among cancer affected women. The fact that ~25 live births have been reported till date using this procedure (8), could easily ar-

Mini review in Current trends in clinical embryology

Engineering analysis of culture experiments of ovarian cortical tissue suggests that bioreactor design affects follicle activation *in vitro*

Gerardo Catapano[1], Roberto Gualtieri[2], Giuseppe Falvo D'Urso Labate[1], Sam Sudhakaran[2], Riccardo Talevi[2]

Abstract

Ovarian tissue cryopreservation and re-implantation of thawed tissue is currently the only option to preserve the fertility of pre-pubertal girls or patients in need of immediate cancer therapy. To avoid cancer transmission in case of blood-borne or metastatic malignancies, it has been proposed to culture *in vitro* strips of cortical tissue until follicle progression to the secondary stage, followed by follicle isolation and culture in three-dimensional matrices until the antral stage, and *in vitro* oocyte maturation. Bioreactors currently used *in vitro* are not optimized, assuming that they have no effect on culture outcome, and do not permit culture of viable and functional thawed ovarian cortical tissue for a long enough time to efficiently promote growth until the antral stage.

Aim of this paper is to discuss on theoretical grounds how to optimize bioreactor design and to show that, consistent with theory, literature reports provide evidence that bioreactor design affects the outcome of *in vitro* ovarian tissue culture. In particular, theoretical analysis of oxygen transport to follicles in a strip suggests that perifollicular oxygen concentration may be controlled and monitored only by accounting for all occurring mass and flow transport phenomena and cells metabolism. Consistent with that predicted by theory, literature reports show that follicle viability and progression in culture dishes is significantly affected by medium height and stirring, and by the strip thickness and shape. These evidences call for a rational and multidisciplinary approach to bioreactor design for ovarian tissue culture *in vitro* to exploit its full fertility potential.

KEYWORDS: bioreactor; design; follicle growth; *in vitro* culture; ovarian tissue

Under review in artificial organs

Health and progression of human follicles during ovarian tissue in vitro culture are improved by oxygen availability

ABSTRACT

Objective: To investigate the role of oxygen as a limiting factor in the quality , viability and progression of follicles to the secondary stage during ovarian follicle culture in situ

Design: Control vs. treatment study.

Setting: University research laboratory.

Patient(s): Ovarian biopsies collected from six women (aged 9 –34 years) during laparoscopic surgery for benign gynecologic conditions and /or fertility preservation.

Animal(s): Bovine ovaries collected from slaughtered animals

Intervention(s): Bovine cortical strips of 1 x 1 x 0.5 mm were cultured in conventional (CD) and gas permeable (PD) dishes with media column height of 1.4 (HV) and 0.7 mm (LW) for 3, 6 and 9 days. Human cortical streps were cultured in CDHV and PDHV for 3, 6 and 9 days.

Main Outcome Measure(s): Follicular quality, activation, progression to the secondary stage and viability

Result(s): The quality, activation and viability of bovine follicles were better preserved culturing strips in PDHV. Results in the animal model were confirmed in the human and in addition culture in PDHV also promoted follicle progression to the secondary stage.

Conclusion(s): The oxygen levels obtained through in vitro culture of human follicles in PDHV promote a higher quality, viability ,activation and progression to the secondary stage that is fundamental for subsequent culture of isolated follicles to obtain competent oocytes.

Key Words: Fertility preservation, ovarian follicle, in vitro culture, histology,viability

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