



Brussels, Belgium - December 10-12, 2009

## World Congress on Fertility Preservation

President of the Congress:  
Professor Jacques Donnez



**ISFP**





## GENERAL INFORMATION

### VENUE

The Congress will take place at the:  
**SHERATON BRUSSELS HOTEL**  
Place Rogier, 3 - Brussels 1210 - Belgium  
Phone:+39-2-224-31-11  
[www.starwoodhotels.com](http://www.starwoodhotels.com)

### LANGUAGE

The official language of this Congress will be English.

### TRAVEL INFORMATION

Brussels, officially the Brussels Capital-Region, is the de facto capital city of the European Union (EU) and the largest urban area in Belgium. Brussels has grown from a 10<sup>th</sup> century fortress town founded by Charlemagne's grandson into a metropolis of more than one million inhabitants. Since the end of World War II, Brussels has been an important centre for international politics. It hosts the main institutions of the European Union, and the headquarters of the North Atlantic Treaty Organization (NATO). Thus, Brussels is the polyglot home of many international organisations, diplomats and civil servants.

Serono Symposia International Foundation  
and International Society for Fertility Preservation

## WORLD CONGRESS ON FERTILITY PRESERVATION

Brussels, Belgium - December 10-12, 2009

### AIM OF THE CONGRESS

The increasing successes of oncologic treatments make implementation of procedures aimed at preserving fertility even more crucial. These procedures must not be limited to preserving and restoring fertility in patients undergoing therapies, but also be applied to any cases where the reproductive function is threatened. The aim of this congress is to offer an updated review of fertility preservation techniques that are currently available as well as those under evaluation. This Congress will involve members of the International Society for Fertility Preservation (ISFP) and world experts in the objective of developing a network among health care professionals interested in this challenging field.

### LEARNING OBJECTIVES

After attending this Congress, the participants will have an updated knowledge on:

- How to approach fertility preservation in adult and children.
- Impact of cancer treatment on gonads.
- Cryopreservation techniques.
- Other most recent laboratory procedures used in fertility preservation.
- Advantages and disadvantages of fertility preservation strategies using gonadic cells, tissues and whole organs.

### TARGET AUDIENCE

The Congress is addressed to clinicians and scientists involved in the fertility field, fertility preservation practice, oncologic patient management and endocrinologists interested in this topic.

### ACCREDITATION

Serono Symposia International Foundation ([www.seronosymposia.org](http://www.seronosymposia.org)) submitted this program **“World Congress on Fertility Preservation”** (Brussels, December 10-12, 2009) for accreditation by the European Accreditation Council for Continuing Medical Education (EACCME), the Royal College of Physicians and the Italian Ministry of Health.



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**SCIENTIFIC  
PROGRAM**



## THURSDAY - DECEMBER 10, 2009

- 08.00 Registration
- 08.15 Welcome on behalf of Serono Symposia International Foundation (SSIF)  
**Robert Fischer**, Germany
- 08.30 Welcome on behalf of International Society for Fertility Preservation (ISFP)  
**Jacques Donnez**, Belgium

### CANCER THERAPY AND FERTILITY PRESERVATION

*Chairmen: Jacques Donnez, Belgium - Roger G. Gosden, USA*

- 08.45 **L1:** **Opening Lecture:** Fertility preservation in children.  
Effect of radiotherapy on the uterus  
**W. Hamish Wallace**, UK
- 09.25 **L2:** Effect of cytotoxic therapies on the ovary  
**Dror Meiorow**, Israel
- 09.50 **L3:** Effect of cytotoxic therapies on the testis  
**Herman Tournaye**, Belgium
- 10.15 **L4:** Evaluation of ovarian function before and after chemotherapy - an approach to determine the cytotoxicity of different chemotherapy regimens?  
**Mikkel Rosendahl**, Denmark
- 10.40 *Coffee Break*

*Chairmen: S. Samuel Kim, USA - W. Hamish Wallace, UK*

- 11.10 **L5:** GnRH agonists, is there evidence for a protective effect on the ovary?  
**Yvon Englert**, Belgium
- 11.35 **L6:** Effect of chemotherapy and fertility preservation in women with breast cancer  
**Jehoshua Dor**, Israel
- 12.00 **L7:** The real risk of conservative surgery in women with borderline and malignant ovarian tumors  
**Philippe Morice**, France
- 12.25 *Lunch and Poster Visit Session*



## CRYOPRESERVATION AND TRANSPLANTATION OF OVARIAN TISSUE FROM OVARIAN TISSUE TO ISOLATED FOLLICLES AND WHOLE OVARY PRESERVATION

*Chairmen:* **Dror Meirow**, Israel - **Isabelle Demeestere**, Belgium - **Pasquale Patrizio**, USA

- 14.00 **L8:** Heterotopic autotransplantation of cryobanked human ovarian tissue: eight year clinical experience in cancer patients  
**S. Samuel Kim**, USA
- 14.25 **L9:** Orthotopic transplantation of ovarian cortex: review of the world experience  
**Jacques Donnez**, Belgium
- 14.50 **L10:** Transport of ovarian tissue prior to cryopreservation - Experience from Denmark  
**Claus Yding Andersen**, Denmark

*Selected oral communications on cryopreservation and transplantation*

- 15.05 **OC1:** Oncologists' practice and attitudes regarding fertility preservation in female cancer patients: a pilot study in the Netherlands  
**L.A. Louwé**, The Netherlands
- 15.15 **OC2:** Autotransplantation of cryopreserved ovarian tissue in a cohort of 12 Danish women  
**K. Tryde Schmidt**, Denmark
- 15.25 **OC3:** Ovarian cortex cryopreservation in girls under 16 years of age  
**P. Jadoul**, Belgium
- 15.35 **OC4:** Cryopreservation of ovarian tissue for fertility preservation in girls  
**K. Tryde Schmidt**, Denmark
- 15.45 **OC5:** How to provide a good vascularisation on ovarian tissue grafting and pregnancies  
**P. Piver**, France
- 15.55 *Coffee break*

*Chairmen:* **Claus Yding Andersen**, Denmark - **Marie-Madeleine Dolmans**, Belgium

- 16.30 **L11:** Risk of transplanting ovarian tissue in breast cancer  
**Antonio Pellicer**, Spain
- 16.55 **L12:** Risk of transplanting ovarian tissue in leukemia  
**Dror Meirow**, Israel

*Selected oral communications on cryopreservation and transplantation*

- 17.20 **OC6:** Systematic follow-up of young patients undergoing chemotherapy in order to assess the dynamics of follicular depletion: what should we learn? The experience of the Lille University Hospital  
**C. Decanter**, France
- 17.30 **OC7:** Microarray approach to investigate gene expression in human ovarian tissue after xenografting  
**L. Romeu**, Belgium
- 17.40 **OC8:** In vitro maturation and vitrification of immature oocytes combined with ovarian tissue cryopreservation: a new strategy of fertility preservation  
**G. Fasano**, Belgium
- 17.50 **OC9:** Localization of c-kit/kit ligand and anti-Müllerian hormone in ovarian follicles following 28 weeks of human ovarian tissue xenotransplantation  
**D. Anu**, Belgium
- 18.00 **OC10:** Successful propagation of human spermatogonial stem cells in vitro  
**H. Sadri-Ardekani**, the Netherlands
- 18.10 **OC11:** Morphological and ultra-structural features of cryopreserved ovine ovarian tissue: deleterious effect of PROH applying different thawing protocols  
**I.C. Oskam**, Norway
- 18.20 **OC12:** Minimal residual disease in cryopreserved ovarian cortex from patients with leukaemia  
**M. Rosendahl**, Denmark
- 18.30 **OC13:** From the analysis on 5571 autopsy findings of females under the age of 40 in Japan  
**K. Kyono**, Japan
- 18.40 End of the day

FRIDAY - DECEMBER 11, 2009

## WHOLE OVARY TRANSPLANTATION OR ARTIFICIAL OVARY: FROM ANIMAL STUDIES TO HUMAN

*Chairmen:* **Stephan Gordts**, Belgium - **Marie-Madeleine Dolmans**, Belgium - **Chii-Ruey Tzeng**, Taiwan

- 08.00 **L13:** Lessons from experimental ovarian transplantation  
**Bruno Salle**, France
- 08.25 **L14:** The preservation of the whole ovary in humans: where we are  
**Pasquale Patrizio**, USA
- 08.50 **L15:** Transplantation of fresh ovarian tissue: cortical graft versus whole ovary?  
**Sherman J. Silber**, USA
- 09.15 *Coffee break*
- Selected oral communications on ovarian cortex and whole ovary transplantation*
- 09.45 **OC14:** Glucose/lactate metabolism as a general marker for ovarian tissue survival after cryopreservation of an intact ovary  
**H.R. Westphal**, The Netherlands
- 09.55 **OC15:** Which are the ideal donor and recipient vessels for a whole ovarian transplantation?  
**S. Ploteau**, Belgium
- 10.05 **OC16:** A decade of experience with fertility preservation at Karolinska University Hospital  
**K.A. Rodriguez-Wallberg**, Sweden
- 10.15 **OC17:** Heterotopic autotransplantation of ovarian cortex in cynomolgus monkeys  
**N. Suzuki**, Japan
- 10.25 **OC18:** In vitro growth of isolated follicles in three dimensional alginate-collagen matrix  
**R. Talevi**, Italy
- 10.35 **OC19:** Mixed origin of neovessels and host-graft vascular link-up in human ovarian xenografts  
**A.S. Van Eyck**, Belgium
- 10.45 **OC20:** Effects of ionizing radiation on ovulation rate and oocyte morphology in mouse  
**M. Sapmaz Metin**, Turkey
- 10.55 *Coffee break*



## TESTICULAR TISSUE PRESERVATION

*Chairmen:* **Sherman J. Silber**, USA - **Christine Wyns**, Belgium

11.30 **L16:** Testicular tissue preservation: a review  
**Christine Wyns**, Belgium

*Selected oral communications on male fertility preservation*

11.55 **OC21:** Reconstitution of spermatogenesis from testis failure after transplantation of germinal cells for male fertility preservation - a transgenic mouse model  
**C.H. Chen**, Taiwan

12.05 **OC22:** Effective cryopreservation of prepubertal mouse testicular tissue by vitrification  
**M. Curaba**, Belgium

12.15 **OC23:** Amifostine-doxorubicin association effects on prepubertal rat testes: long term damage on sperm DNA integrity and early embryo developmental delay  
**V. Vendramini**, Brasil

12.25 *Lunch and Poster Visit Session*

## ISOLATED FOLLICLES TRANSPLANTATION OR IN VITRO MATURATION

*Chairmen: Andrea Borini, Italy - Antonio Pellicer, Spain*

- 14.00 **L17:** Folliculogenesis in humans: in vivo and in vitro  
**Johan Smitz, Belgium**
- 14.25 **L18:** In vitro maturation: factors affecting oocyte quality  
**David F. Albertini, USA**
- 14:50 **L19:** Isolation and grafting of human isolated follicles: why and how? A place for an artificial ovary?  
**Marie-Madeleine Dolmans, Belgium**
- 15.15 **L20:** Bioengineering the ovary  
**Christiani Andrade Amorim, Belgium**
- 15.40 **L21:** Oncofertility: the preservation of fertility options for young people with cancer  
**Teresa K. Woodruff, USA**
- 16.05 *Coffee break*

*Chairmen: Pedro N. Barri, Spain - Johan Smitz, Belgium - Teresa K. Woodruff, USA*

- 16.30 **L22:** Closing the gap between in vitro growth (IVG) and IVM using fresh and cryopreserved tissue  
**Evelyn E. Telfer, UK**
- 16.55 **L23:** Fresh human ovarian tissue: from primordial to antral follicle in culture  
**Outi Hovatta, Sweden**
- 17.20 **L24:** Impact of cryopreservation and grafting on folliculogenesis  
**Debra Gook, Australia**
- 17.45 **L25:** In-vitro maturation and oocyte vitrification for the preservation of fertility in cancer patients  
**Seang Lin Tan, Canada**

*Chairmen: S. Samuel Kim, USA - Jacques Donnez, Belgium*

- 18.15 **Key note lecture:** Potential for stem cell renewal of function in sterile ovaries and testes  
**Roger G. Gosden, USA**
- 19.00 End of the day

## SATURDAY - DECEMBER 12, 2009

*Chairmen:* **Christiani Andrade Amorim**, Belgium - **W. Hamish Wallace**, UK

*Selected oral communications on cryopreservation and transplantation*

- 08.00 **OC24:** Human meiotic spindle alterations following slow-cool cryopreservation  
**J.J. Bromfield**, USA
- 08.10 **OC25:** Ultra-rapid vitrification supported follicle morphologies of cynomolgus monkeys after freezing compared to conventional vitrification and slow freezing  
**S. Hashimoto**, Japan
- 08.20 **OC26:** Efficiency of oocyte cryopreservation  
**E. Borghi**, Italy
- 08.30 **OC27:** Clinical grade vitrification of human ovarian tissue  
**M. Sheikhi**, Sweden
- 08.40 **OC28:** Vitrified human ovaries harbor less primordial follicles and produce less antimüllerian hormone (AMH) than slow frozen ovaries  
**O. Oktem**, Turkey

## VITRIFICATION VERSUS SLOW FREEZING

*Chairman:* **Roger G. Gosden**, USA

*Co-chairmen and discussants:*

**Anna Veiga**, Spain - **Pedro N. Barri**, Spain - **Outi Hovatta**, Sweden

### Oocytes

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- 09.00 **L26:** Slow freezing versus vitrification - What is more efficient?  
**Andrea Borini**, Italy
- 09.20 **L27:** Pros and cons  
**Anna Veiga**, Spain
- 09.30 *Discussion*
- 09.45 *Coffee Break*

## Embryos

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- 10.15 **L28:** Slow freezing versus vitrification - What is more efficient?  
**Etienne Van Den Abbeel**, Belgium
- 10.35 **L29:** Pros and cons  
**Pedro N. Barri**, Spain
- 10.45 *Discussion*

## Ovarian tissue

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- 11.00 **L30:** Slow freezing versus vitrification - What is more efficient?  
**Bruno Salle**, France
- 11.20 **L31:** Pros and cons  
**Outi Hovatta**, Sweden
- 11.30 *Discussion*

## ETHICS: FINAL LECTURE

*Chairmen:* **S. Samuel Kim**, USA - **Dror Meirow**, Israel - **Pasquale Patrizio**, USA

- 11.45 Introduction: **Jacques Donnez**, Belgium
- L32:** The next debate: Future, is there a place for ovarian tissue and oocyte cryopreservation for social reasons?  
**Guido Pennings**, Belgium
- 12.30 *Prize for oral presentation and poster - Closing Ceremony*  
**Jacques Donnez**, Belgium and ISFP Board Directors

## DISCLOSURE OF FACULTY RELATIONSHIPS

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<b>David F. Albertini</b>	Declared receipt of honoraria or consultation fees from Editor in Chief/J. Assisted Reproductive Genetics / Springer.
<b>Christiani Andrade Amorim</b>	Declared no potential conflict of interest.
<b>Claus Yding Andersen</b>	Declared no potential conflict of interest.
<b>Pedro N. Barri</b>	Declared no potential conflict of interest.
<b>Andrea Borini</b>	Declared no potential conflict of interest.
<b>Isabelle Demeestere</b>	Declared no potential conflict of interest.
<b>Marie-Madeleine Dolmans</b>	Declared no potential conflict of interest.
<b>Jacques Donnez</b>	Declared no potential conflict of interest.
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<b>Stephan Gordts</b>	Declared no potential conflict of interest.
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<b>Mikkel Rosendahl</b>	Declared no potential conflict of interest.
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<b>Johan Smitz</b>	Declared no potential conflict of interest.
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<b>Herman Tournaye</b>	Declared no potential conflict of interest.



<b>Chii-Ruey Tzeng</b>	Declared no potential conflict of interest.
<b>Etienne Van den Abbeel</b>	Declared no potential conflict of interest.
<b>W. Hamish Wallace</b>	Declared no potential conflict of interest.
<b>Teresa K. Woodruff</b>	Declared no potential conflict of interest.
<b>Christine Wyns</b>	Declared no potential conflict of interest.

The following faculty have provided no information regarding significant relationship with commercial supporters and/or discussion of investigational or non-EMEA/FDA approved (off-label) uses of drugs as of November 20, 2009.

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**Dror Meirow**  
**Philippe Morice**  
**Bruno Salle**  
**Evelyn E. Telfer**  
**Anna Veiga**

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**ABSTRACTS  
(L1 - L32)**

# L1

## FERTILITY PRESERVATION IN CHILDREN. EFFECT OF RADIOTHERAPY ON THE UTERUS

**W. Hamish Wallace**

Department of Haematology/Oncology, Royal Hospital for Sick Children, Edinburgh, UK

Childhood cancer is rare, with an incidence of around 110 cases per million children per year. Although survival from these malignancies was very poor in the 1960's, major advances, both in treatment, thanks to the use of multi-agent chemotherapy, and in supportive care, achieved markedly improved rates of cure over recent decades. Current data suggest that around 80% of children with cancer will stay alive five years from diagnosis. As a result, the number of long-term survivors is increasing, and fertility preservation has become a major issue for the young patient about to start treatment for cancer.

Currently the only established option to preserve fertility for males is cryopreservation of spermatozoa before commencing treatment. Patients for whom this procedure is suitable must be peri- or post-pubertal and sexually mature. In addition they must be able to give consent for the storage of the specimen. There is currently no established option to preserve fertility for the pre-pubertal young boy.

Female fertility preservation provides significantly different challenges to that for the male. Embryo freezing is now an accepted and well-established procedure in many centres, but is not available for children who do not have a partner. Cryopreservation of mature oocytes has become increasingly successful. However, for the young patient, cryopreservation of ovarian cortical tissue is extremely promising with around 30 cases of auto-transplanted frozen-thawed ovarian tissue reported leading to the birth of seven live infants so far. Ovarian tissue cryopreservation has the potential advantages of preserving a large number of oocytes within primordial follicles, it does not require hormonal stimulation when time is short, and is appropriate for the pre-pubertal girl. Disadvantages include the need for an invasive procedure, and the uncertain risk of ovarian contamination in haematological and other malignancies.

Radiotherapy causes both ovarian and uterine damage. There are no reports of uterine damage after chemotherapy. Uterine damage manifest by impaired growth and blood flow is a likely consequence of radiation to a field that includes the pelvis. Exposure of the pelvis to radiation is associated with: an increased risk of miscarriage, mid-trimester pregnancy loss, preterm birth and low birth weight. The optimal dose and delivery route of oestrogen replacement required to facilitate uterine growth in adolescent women treated with total body irradiation, needs to be established.

In this lecture I will discuss our experience with 36 women, highlighting issues of patient selection especially in the young, and uncertainties over the effects of cancer treatments on subsequent fertility. Of these 36 women, 11 have died, but 5 have had spontaneous pregnancies, with to date none having requested re-implantation of their stored ovarian tissue. Ovarian cryopreservation appears to be a potentially valuable method for fertility preservation, but the indications and approaches best used remain unclear.

In addition to the many scientific and technical issues to be overcome before clinical application of these techniques, a number of ethical and legal issues must also be addressed to ensure a safe and realistic prospect for future fertility in these patients.



## L3

## EFFECT OF CYTOTOXIC THERAPIES ON THE TESTIS

**Herman Tournaye**

Centre for Reproductive Medicine, Universitair Ziekenhuis Brussels, Brussels, Belgium

The testis has both an endocrine and exocrine function. The endocrine output depends on an adequate Leydig cell function. For the exocrine function, i.e; spermatogenesis, both a functional Sertoli cell and spermatogonial stem cell pool are necessary. Spermatogonial stem cells are undifferentiated cells, that give rise to the spermatogenic cells and, finally, the spermatozoa. Even though there is a continual loss of differentiated cells, the spermatogenic cell lineage maintains its cell number thanks to the adult spermatogonial stem cells, which produce both new stem cells (self-renewal) and differentiating cells.

Both chemo- and radiotherapy can cause long-term or even permanent gonadal failure.

Leydig cells are rather resistant to chemo- and radiotherapy. While elevated LH indicates Leydig cell dysfunction, most men will show normal testosterone levels and will retain a normal bone density. While routine testosterone supplementation after gonadotoxic treatment is not indicated, men should have endocrine monitoring after cancer treatment and in selected cases testosterone substitution may be required. Leydig cells seem more vulnerable to chemo- and radiotherapy in pre-pubertal life.

Also Sertoli cells show a good resistance to chemo- and radiotherapy, except in the postnatal and pubertal period when their numbers are increasing through mitosis. Increases in FSH are due to indirect effects through loss of the germ cell pool.

The extent of the gonadotoxicity on this germ cell is strongly related to the nature of the specific agents that are used and their dose or to the intensity of the radiation and the place of the body where it was administered. Transient reductions of sperm count can occur even after mild forms of chemotherapy or low doses of gonadal radiation, due to the destruction of the sensitive differentiating spermatogonia. Stronger chemotherapeutic regimes or higher doses of gonadal irradiation, however, lead to prolonged reduction in sperm count or complete azoospermia. Whether sperm production will eventually recover depends on the survival of the spermatogonial stem cells and the integrity of their ability to differentiate. High doses of radiation to the testis (>2.5 Gy) cause DNA damage and cell death. The most damaging chemotherapeutic agents in the adult man are the alkylating agents. Many therapeutic agents will only lead to a temporary reduction in sperm counts or have no effect at all on sperm production. These include other antimetabolites, topoisomerase inhibitors, corticosteroids, interferons ...

Even though the gonadotoxic effect of most anti-cancer treatments is known, it is difficult to predict the final effect on the fertility potential of the patient. There remain important interindividual differences in response to the treatment and even if a regimen with low gonadotoxicity is started, it is possible that eventually a more gonadotoxic treatment has to be administered because of earlier treatment failure

Even if the anti-cancer treatment does not lead to infertility, single gene and chromosomal mutations might have been induced in the surviving germ cells and will be passed on to the offspring where they may lead to genetic diseases.

Since spermatogenesis starts only at puberty, the only spermatogenic cells that are present in the seminiferous tubules before the onset of puberty are the spermatogonia. Although it has been reported that the prepubertal testis, assumed to be in a quiescent stage, is less sensitive to the gonadotoxic effects of chemo- and radiotherapy, recent findings have shown that even in prepubertal life, the seminiferous tubules are mitotically active. Therefore even in prepubertal boys, fertility preservation is an important issue. Fertility preservation in prepubertal boys lies within the preservation of their spermatogonial stem cells. Three options for fertility preservation through the application of spermatogonial stem cells are currently under research: the spermatogonial stem cell transplantation, the grafting of testicular tissue pieces and the in vitro proliferation and/or maturation of spermatogonia. Each technique has of course its (dis)advantages, but each one of them might eventually find its way to the clinic.

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## EVALUATION OF OVARIAN FUNCTION BEFORE AND AFTER CHEMOTHERAPY - AN APPROACH TO DETERMINE THE CYTOTOXICITY OF DIFFERENT CHEMOTHERAPY REGIMENS?

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### Introduction

The three main factors to consider when evaluating a woman's risk of chemotherapy induced amenorrhoea and infertility are: the age of the woman, the type and dose of chemotherapy. Nevertheless, inter-individual variations may result in a different outcome in two women of equal age receiving the same type and dose of chemotherapy.

This presentation focuses on two such possible inter-individual variations:

1. Do the pre-treatment levels of ovarian markers – and do their alterations during treatment – predict the outcome with regards to ovarian function?
2. Is the risk of chemotherapy induced amenorrhoea in breast cancer patients related to the degree of chemotherapy induced bone marrow suppression and hence possibly related to inter-individual variations in the bioavailability of the cytotoxic substances?

### Materials and methods

To answer **Question 1**, 17 women with cancer had ovarian markers (i.e. AMH, FSH, antral follicles) recorded before, during and after combination chemotherapy.

To answer **Question 2**, 1016 premenopausal women received 7 series of CEF chemotherapy for breast cancer. Those patients with sub-optimal bone marrow suppression after the first series of chemotherapy were randomised to either increased or unchanged doses of FEC. Those with initial optimal bone marrow suppression continued with unaltered dosage. Bone marrow suppression and menstrual regularity was monitored.

### Results

In study 1, we discovered a significant and immediate chemotherapy-induced drop in AMH and Inhibin B. These were, however, not predictive of the outcome. Irrespective of age, the pre-treatment AMH level was predictive of the AMH level one year after the end of chemotherapy.

In study 2, for younger patients, a more pronounced bone marrow suppression was – irrespective of the dose – significantly related to a higher risk of amenorrhoea.

### Conclusions

The three most important factors of development of chemotherapy induced amenorrhoea remain the age of the patient and the type and dose of chemotherapy. However, individual counselling may be guided by the pre-treatment AMH level and for younger patients it seems that a higher bioavailability of the chemotherapy increases the patient's risk of amenorrhoea.

## L5

**GnRH AGONISTS, IS THEIR EVIDENCE FOR A PROTECTIVE EFFECT ON THE OVARY?****Y. Englert<sup>1</sup>, P. Brice P.<sup>2</sup>, F. Peccatori<sup>3</sup>, B. Dominique<sup>4</sup>, S. Tsepelidis<sup>1</sup> and I. Demeestere<sup>2</sup>;**

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Since the first publication of Ataya *et al.* (1985) describing a protective effect of GnRH against chemotherapy-induced gonadal damage in rats, various papers have confirmed the protective effect on ovarian function (and sometime on fertility) in animal models, including non human primates, against ovariotoxic chemotherapy, such as alkylant agents. Human literature is much weaker; especially because nearly all publications describe comparative studies between groups of patients and historical retrospective controls, in case control settings. Nevertheless, more than 300 patients received GnRH agonists in these studies and performed better than controls regarding to ovarian function. Much fewer prospective randomized studies were published and will be examined in details, including an ongoing European multicentric study with more than 100 included patients coordinated by the authors and conducted in 15 centers. In this study patients with hematologic cancer, that provide the informed consent, are randomized either to GnRH agonist or to a control group. An intermediary analysis at 18 months follow up will be presented.

All these studies, randomized as well as case controls, are focused up to now on ovarian function and it will be stressed that menstruating is a necessary but not sufficient criterion for fecundity, the ultimate goal of protection of the ovarian function in young women and children. GnRH agonist strategy to protect ovarian function is still today a matter of debate, not only because of lack of definitive clinical evidence, but also because of the FSH independence of primordial follicles recruitment, target to be protected against toxic effect of anti mitotic drugs. It has been however hypothesized no less than 5 possible explanations to understand why GnRH analogs may decrease toxic effect of alkylant drugs on the ovarian primordial follicles. The recent surprising publications on the description of undifferentiated germ line stem cells in the adult ovary as well as the unfavorable outcome reported for the use of antagonists in animal model will be put in perspective with these hypotheses to revisit them, one by one. Safety of the use of GnRH analogs during chemotherapy will be also examined.





## L7

**THE REAL RISK OF CONSERVATIVE SURGERY IN WOMEN WITH BORDERLINE AND MALIGNANT OVARIAN TUMORS****Philippe Morice**

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Conservative treatment of borderline ovarian tumors (BOT) increases recurrence rate (compared to radical treatment). This risk is estimated, in literature, between 0 and 25%. It is also elevated after cystectomy (between 12 to 58%). But data in literature seem to confirm that, even though the risk of relapse is majored after conservative treatment in BOT, survival of patients is not altered with conservative management. So, conservative surgery could be safely performed in young patients treated for early stage BOT and carefully followed-up. Such conservative treatment should be considered in patients with serous BOT and non-invasive peritoneal implants. Pregnancy rates varied in literature between 30% and 80%. In Vitro Fertilization (IVF) procedures could be considered in patients treated for early stage borderline disease and having a persistent infertility after the surgical treatment.

Conservative treatment has a large place in the management of a majority of non epithelial cancer (even in the case of advanced stage disease). But the indication and modalities of such treatment depends on the tumor histological subtype.

Conservative surgery for patients with epithelial ovarian cancer could be considered in patients with stage IA grade 1 or 2 disease but should not be proposed in stage IA grade 3 disease. Recent data seems to suggest that such treatment could be safely proposed in selected cases of patients with stage IC grade 1 disease.

This treatment should not performed in patients with FIGO stage > I. Patients are selected on careful histological analysis of the tumor specimen (in order to precise the histological subtypes and tumor grade), absence of extra-ovarian spread determined after (re)staging surgery and absence of inherited syndrome predisposition to ovarian cancer. In Vitro Fertilization (IVF) procedures are contraindicated in patients treated for epithelial cancer and having a persistent infertility after the surgical treatment.

## HETEROTOPIC AUTOTRANSPLANTATION OF CRYOBANKED HUMAN OVARIAN TISSUE: EIGHT YEAR CLINICAL EXPERIENCE IN CANCER PATIENTS

### S. Samuel Kim

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Ovarian tissue cryobanking followed by autotransplantation has been proven to be a valuable strategy to restore ovarian function and fertility for cancer patients. There are still many unknowns including its efficacy, since our experience with human ovarian transplantation is limited. Frozen-thawed ovarian tissue can be transplanted either to an orthotopic site or heterotopic site. To date, orthotopic autotransplantation of frozen-thawed ovarian tissue resulted in seven live births worldwide. On the other hand, no baby has been born after heterotopic autotransplantation. It is however worthy to continue investigating this technology even with skepticism, as several studies showed the return of ovarian function and potential fertility after heterotopic transplantation. Besides, heterotopic transplantation is easy, convenient, less invasive, and can be cost effective if repeated transplantation is required.

We transplanted frozen-thawed ovarian tissue heterotopically in cancer patients during the past 8 years to assess the long term ovarian function and restoration of fertility. Four study patients with the age range between 28 and 35 (three with cervical cancer, one with breast cancer) were identified and consented for heterotopic transplantation. All ovarian tissue had been cryopreserved using a slow freezing method before cancer treatment. Heterotopic ovarian transplantation (to the space between rectus muscle and fascia in the abdomen) was performed between 2002 and 2005. The restoration and maintenance of ovarian function was confirmed by a serial blood test (FSH, LH, estradiol, progesterone, testosterone) and ultrasound monitoring. To investigate the restoration of fertility, three patients were stimulated with gonadotropin and oocyte retrievals were attempted, once restoration of normal ovarian function was established. The retrieved oocytes were matured *in vitro* and fertilized with partner's sperm.

The hormonal profile of all four patients was consistent with the postmenopausal level before transplantation. The return of the ovarian function was evidenced by the elevation of serum estradiol levels and by the decrease of FSH levels below 20 mIU/ml between 12-20 weeks after transplantation in all patients. However, restored ovarian function lasted only 3-5 months, and all three patients (except one with relapsed disease) agreed to undergo second transplantation. The return of ovarian function after second transplantation was faster in all three patients (between 1-3 months).

In contrast to first transplantation, we observed the establishment of long term ovarian function (lasting for 9-50 months) after second transplantation. All three patients maintained the FSH levels below 15 mIU/ml during this period. We were able to retrieve 7 oocytes (two GV, four MI, one MII) from ovarian grafts in 2 patients between August 2003 and November 2006. Three of four MI oocytes were developed to full maturity *in vitro*. All four MII oocytes were fertilized and cultured *in vitro* for 2 or 3 days before cryopreservation. Currently, 4 embryos (at 6-cell, 3-cell, 2-cell, PN stage) are stored in liquid nitrogen.

Although our results are encouraging, it is a real challenge to make it a clinically practicable technology since the environment of heterotopic sites may not be as optimal for follicle development.

## L9

**ORTHOTOPIC TRANSPLANTATION OF OVARIAN CORTEX: REVIEW OF THE WORLD EXPERIENCE****Jacques Donnez**

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It is our duty to evaluate the effects of specific cancer therapies on fertility and discuss fertility preservation options with young women requiring such treatment prior to its initiation.

The different cryopreservation options available for fertility preservation in cancer patients are: embryo cryopreservation, oocyte cryopreservation and ovarian tissue cryopreservation.

The only established method of fertility preservation is embryo cryopreservation, but this requires the patient to be of pubertal age, have a partner, and be able to undergo a cycle of ovarian stimulation.

Cryopreservation of ovarian tissue is the only option available for prepubertal girls, and for woman who cannot delay the start of chemotherapy.

Thirty cases of orthotopic reimplantation of cryopreserved ovarian tissue have so far been reported and seven live births have been achieved, yielding a pregnancy rate of almost 25%. In our department, six women have undergone orthotopic reimplantation of cryopreserved tissue either once or twice. Restoration of ovarian function, proved by follicular development and estradiol secretion, occurred in all cases. A time interval of 3.5 to 5 months was observed between reimplantation and the first signs of ovarian graft activity in all cases.

Two ongoing pregnancies were naturally obtained from this series of 6 patients. Graft activity was found to persist for 2.5 to 4 years. In non-pregnant patients, IVF was performed, but the quality of oocytes and embryos was not optimal (see Dolmans *et al.*, Hum Reprod 2009). Prognostic factors (age, previous chemotherapy) are therefore discussed.

With all the advances in ovarian tissue, such as cryobanking and reproductive technology, fertility preservation is now a real possibility for patients whose gonadal function is threatened by radiotherapy or chemotherapy. For this reason, it should be a medicolegal obligation for gynecologists, oncologists and pediatricians to systematically propose cryopreservation before initiating cancer therapy that could impair future fertility

## TRANSPORT OF OVARIAN TISSUE PRIOR TO CRYOPRESERVATION - EXPERIENCE FROM DENMARK

### Claus Yding Andersen

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Survival rates of patients suffering from a malignant disease have steadily increased during this decade. This is largely, a result of the use of more aggressive and effective chemo- and radiotherapies. However, as a side effect, these therapies also exert a toxic effect on the pool of ovarian follicles. If the ovaries become depleted of follicles the woman will become sterile, lose menstrual cycles and many women experience profound effects on the physical and psychological status. For young girls it may further imply that pubertal development fails.

A number of methods are now under development in order to preserve fertility in patients receiving potential gonadotoxic treatment. Cryopreservation of ovarian tissue is a new method that can be employed on a short notice and is now gaining ground as a valid alternative to embryo and oocyte cryopreservation.

Cryopreservation of ovarian tissue involves removal of one ovary or parts of one ovary prior to treatment. When the women have been cured and are considered fit, the thawed ovarian tissue, with a viable pool of follicles, can be transplanted to women who entered menopause. The pool of surviving primordial follicles will be reactivated and the patients will regain fertility and will experience cyclic variation in sex hormone levels.

Laboratory of Reproductive Biology at University Hospital of Copenhagen is the only center in Denmark offering cryopreservation of ovarian tissue as a treatment in close collaboration with three fertility clinics round the country. The ovarian tissue is extracted at the local hospital and transported on ice to our laboratory, where cryopreservation and storage is performed. This transport model has now been used for more than 250 cases and totally in Denmark almost 400 girls and women have had ovarian tissue cryopreserved.

In Denmark a total of fifteen women (10 having their tissue transported prior to cryopreservation) have experienced transplantation of frozen/thawed ovarian tissue. Three women recently received transplantation and the outcome is currently unknown but the remaining 12 women all regained ovarian function. Over a period of 20 - 25 weeks, FSH gradually return to pre-menopausal levels and menstrual cycles are regained. The longevity of the tissue depends on the age of the woman at tissue retrieval and the amount of tissue transplanted. Most women experience return of ovarian function for some years with just a fraction of tissue from one ovary being replaced.

Four of the women have been pregnant; in most cases as a result of assisted reproduction. Two women have delivered three healthy babies as a result of transplanted frozen/thawed ovarian tissue. In the latter two cases the tissue was transported 4-5 hours prior to cryopreservation. The presentation will review our experiences and recent results.

## L11

**RISK OF TRANSPLANTING OVARIAN TISSUE IN BREAST CANCER****Antonio Pellicer, Maria Sánchez and Juana Crespo**

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Ovarian tissue preservation and transplantation are intended for women undergoing aggressive regimens of chemical and/or radiological therapy, bone marrow transplantation or stem cell transplantation. Main indications for the procedure are neoplastic diseases and autoimmune disorders, breast cancer being the most frequently addressed among them. We established a program of fertility preservation (FP) in 2005 and today >300 women, with a mean age of 28.25yrs (11-39), have been treated and ovarian cortical tissue cryopreserved. Approximately 55% are breast cancer patients, 25% Hodgkin disease, and 20% other non malignant or malignant diseases. The procedure is based on the removal of the right ovarian cortex by laparoscopy and cryopreservation, before cancer treatment begins. Pieces of ~2 x 3 cm are frozen, while additional tissue from the right, as well the left ovary is sent to Pathology to search for the presence of malignant cells in the ovaries. Once the patient is free of disease, the right ovarian cortex is thawed and implanted onto left ovarian medulla (after extraction at the same surgical time of left ovarian cortex).

The main concern of this procedure is the possibility of reintroducing metastatic cells within the implant, an issue that has not been addressed systematically. Thus, a study was designed to analyse the presence of ovarian metastases in breast cancer patients undergoing ovarian tissue cryopreservation. Morphological and immunohistochemical studies following the concept of the sentinel lymph node (SLN) were performed on 100 cortical ovarian biopsies and on six frozen-thawed entire cortex from patients with the diagnosis of infiltrating ductal breast carcinoma undergoing ovarian cortex extraction and cryopreservation. The antibody panel included Cytokeratin CAM 5.2, Gross Cystic Disease Fluid Protein-15 (GCDFP15), Wilms tumour antigen-1 (WT1) and Mammaglobin 1 (MGB1). Employing only morphologic criteria, suspicious neoplastic cells were detected in the biopsies of 5 cases, but in none of the 6 entire cortex analysed. These 5 cases were reclassified as hyperplastic surface epithelium-inclusion cysts (CAM 5.2+, WT1+) or apoptotic granulosa cells (CAM 5.2-, GCDFP15+, WT1-).

To date, a total of 8 reimplantations have been performed, two of them very recently. The remaining patients have a normal endocrine function. One of them is a 36 yrs old patient diagnosed of atypical medullar breast cancer, negative for estrogen, progesterone and HER2 receptors. She underwent ovarian tissue cryopreservation before chemo and radiotherapy. Menses occurred sixty-three days after transplantation, but because she was infertile already before treatment and because she was 39 yrs old after treatment, ART was performed using vitrification because the life span of the transplanted tissue was uncertain. A total of 16 mature oocytes were obtained out of four stimulations. All vitrified oocytes survived after warming and 77.7% fertilized; two day-3 embryos were replaced and two healthy boys were born at 34 weeks. In conclusion, using the methodology of the SLN our data suggest the absence of tumour cells in biopsies obtained from patients undergoing ovarian cortex cryopreservation to preserve their fertility potential, although future methods of cancer screening may change our perception of this procedure. Ovarian tissue cryopreservation and grafting preserves fertility and oocyte vitrification can be simultaneously employed to increase the success of ART in poor prognosis patients and to avoid the consequences of a short lifespan of the transplanted tissue.





## THE PRESERVATION OF THE WHOLE OVARY IN HUMANS: WHERE WE ARE

P. Patrizio<sup>1</sup> and A. Arav<sup>2</sup>;

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Cryopreservation and re-transplantation of ovarian cortical strips have been successful, to some extent, by documenting pregnancies and births. However, the lifespan of the transplanted avascular grafts is limited by the ischemia and the consequent loss of primordial follicles that occurs during the re-establishment of a blood supply. Thus, to overcome this problem for patients that desire long term resumption of endocrine function, we began to perform (in vitro) studies on the cryopreservation of the whole ovary with their intact vascular pedicle.

This paper will review the recent advances in whole ovary cryopreservation mostly on human specimens, with a focus on surgical technique for removal, choice of cryoprotectants, freezing protocols, and histological and immunochemistry results post-organ thawing (see summary table). Two main issues in organ cryopreservation and re-transplantation had to be solved before progressing into whole ovary cryopreservation: the first, the creation of an efficient and reproducible cryopreservation protocol; the second, the demonstration that it is feasible to re-establish the vascular anastomosis of the organ to re-transplant. The first issue has been solved by utilizing the same Multi-Thermal-Gradient device and slow cooling-rapid thawing protocol described earlier (Arav *et al.* 2005) for whole sheep ovaries. With this method, ovaries from premenopausal women have been successfully cryopreserved for times ranging from 48 to 96 hours following either laparotomy, laparoscopic, or robotic assisted laparoscopic oophorectomy. The ovarian artery has a very narrow diameter (between 0.4- 0.5 mm) therefore it is important to leave a long pedicle (at least 4 cm long) for a successful organ perfusion with cryoprotectant (ethylene glycol). Compared to the fresh contra lateral ovary used as control, the frozen/thawed ovary was histologically indistinguishable. Immunohistochemistry and Western Blot assays did show some increase in anti-caspase 3 and p53 phospho-serine expression, suggesting some increased level of apoptosis in the frozen/thawed specimens. The functional meaning of these changes can only be assessed with re-transplant experiments.

Concerning the second issue, feasibility of whole organ re-transplant, a recent paper (Silber *et al.* 2008) has shown that with a whole fresh ovary the re-transplant is technically doable. However, there are no data yet of human cases of whole ovary re-transplantation after cryopreservation/thawing.

**Table:** Summary of whole ovary cryopreservation experiments in humans.

	N=	Surgical Method	Freezing Method	Cryoprotectant	Outcomes Measured
Martinez-Madrid <i>et al.</i> , 2004	3	Laparoscopy	Slow Cooling	DMSO	Follicle, stromal cell, vascular viability, Histological morphology
Bedaiwy <i>et al.</i> , 2006	2	Laparoscopy	Slow Cooling (ovaries bisected)	DMSO	Follicle viability, Apoptosis
Martinez-Madrid <i>et al.</i> , 2007	3	Laparoscopy	Slow Cooling	DMSO	Apoptosis, Ultrastructural assessment
Jadoul <i>et al.</i> , 2007	9	Laparoscopy	Slow Cooling	DMSO	Technical feasibility of oophorectomy and freezing
Patrizio <i>et al.</i> , 2008	12	Laparoscopy, laparotomy	Slow Cooling	Ethylene Glycol	Apoptosis, Histology, IHC and WB



# L15

## TRANSPLANTATION OF FRESH OVARIAN TISSUE: CORTICAL GRAFT VERSUS WHOLE OVARY?

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A series of monozygotic (MZ) twin pairs discordant for premature ovarian failure presented an unusual opportunity to study ovarian transplantation. Ten MZ twin pairs requested ovarian transplantation and nine have undergone transplantation with cryopreservation of spare tissue. Eight had a fresh cortical tissue transplant, one of whom received a second frozen-thawed transplant after the first ceased functioning at three years. One had a fresh microvascular transplant. All recipients reinitiated ovulatory menstrual cycles and normal Day 3 serum FSH levels by 77-142 days. Seven patients have already conceived naturally (three twice). Currently, seven healthy babies have been delivered out of ten pregnancies. The oldest transplant ceased functioning by three years, but then she conceived again after a frozen-thawed secondary transplant. There was no apparent difference in return of ovarian function between the nine fresh ovarian grafts and the one frozen graft. Ovarian transplantation appears to restore ovulatory function robustly. Ten pregnancies, leading to seven healthy babies, including one after cryopreservation, bode well for application to fertility preservation.

## TESTICULAR TISSUE PRESERVATION: A REVIEW

**Christine Wyns**

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Fertility in adult life may be severely impaired by gonadotoxic therapies. For young boys who do not yet produce spermatozoa, cryopreservation of immature testicular tissue is an option to preserve their fertility, albeit still experimental. Existing approaches to immature testicular tissue cryopreservation and fertility restoration in the literature are extensively reviewed and current knowledge on *in vivo* spermatogonial stem cell (SSC) protection is updated. Different cryopreservation protocols and fertility restoration options from frozen tissue, i.e. cell suspension transplantation, cell aggregate grafting, tissue grafting and *in vitro* maturation, are presented. Results obtained in humans are discussed in the light of lessons learned from animal studies.

Advances in reproductive technology have made fertility preservation a real possibility in young patients whose gonadal function is threatened by gonadotoxic therapies. The putative indications for such techniques, as well as their limitations according to disease, are outlined.

## L17

## FOLLICULOGENESIS IN HUMANS: IN VIVO AND IN VITRO

Johan Smitz

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Although still experimental, the practice of oocyte and ovarian tissue cryopreservation has been spreading fastly, since most female cancer patients of reproductive age do not have the option of utilizing established assisted reproductive technologies as IVF and embryo cryopreservation to safeguard their fertility. Indeed, embryo preservation is not an option for prepubertal girls or single women.

Although knowledge of mammalian ovary regulation has exponentially increased through the availability of molecular techniques in the last two decades, it is still impossible today to obtain - with a high efficiency - oocytes that could be developed into a normal embryo. Initiatives, such as the Web-based Ovarian Kaleidoscope Database, offer unique opportunities with up-to-date information on the function and regulation of ovarian genes. Having a more precise knowledge on the major mechanisms driving the earliest stages of follicle growth has incited few laboratories to attempt cultures of small pieces of ovarian cortex. While we are obliged by 'numbers' to focus on cultures of primordial follicles, it is still precisely unknown how their first step of growth is regulated. Making use of defined matrices and purified growth factors, systematic attempts have been undertaken, which can bring - still with very low efficiency - primordial follicles up to early secondary follicles. Encouraging results using alginate matrix and defined media components have demonstrated that starting - off with one and a half - to two layered early preantral follicles, early antral follicles could be obtained in sheep (Muruvi *et al.*, 2009), rhesus monkey (Xu *et al.*, 2009) and human (Xu *et al.*, 2009). For each species and each follicle growth stage, another culture system will probably have its advantages. A next step in the challenge will be further optimization of the culture conditions (as was also needed for the first live born mice by the Eppig Laboratory) in order to increase the number of oocytes capable of undergoing GVBD. At this point, we might apply the techniques used for In Vitro Maturation (IVM), which are - although still less effective as IVF - capable to provide healthy babies.

There is evidence from research in the bovine model that, in large mammal's oocytes, quality depends in events that occur before GVBD. By the time that chromosomes start to be condensed, the oocyte has generally accumulated the appropriate information for fertilisation and early embryonic development (Sirard *et al.*, 2001; Dieleman *et al.*, 2002).

The hypothesis is: in-vitro culture system could be improved to support better the final maturation stages of the oocyte. It is possible that oocytes are probably already predetermined at their retrieval. It is currently unexplored whether therapeutic interventions in this earlier part of folliculogenesis might be helpful in providing more optimal starting material for culture.

The selection of cumulus oocyte complexes (COC) for culture is a crucial process that determines outcome. New tools are QPCR analysis of cumulus cells to provide a more precise way to evaluate the progression in oocyte maturity. The availability of large numbers of oocytes from animal models are useful material, as a first approach, to select genes related to development that could be subsequently tested on precious human oocytes. However species-specific differences will have to be taken into account. Cumulus cells could perhaps reflect oocyte health and bear the markers for developmental competence. Such markers could be helpful to select the adequate culture system. Refinement of the culture technique for COC becomes accessible when there is a better control over meiosis.

The improvements in ovarian tissue-, follicle- and oocyte-cumulus culture go hand in hand with the increased knowledge generated from the molecular analyses of gametogenesis and early embryo development.

Prolonged culture systems need to be proactively tested to uncover eventual epigenetic changes due to in-vitro manipulations.

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## IN VITRO MATURATION: FACTORS AFFECTING OOCYTE QUALITY

**David F. Albertini**

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Fertility preservation strategies place an increasing demand on proving the safety and efficacy of human ARTs including those that bear directly on the use of oocyte in vitro maturation protocols. While in some mammalian species the use of IVM often results in the production of developmentally competent ova capable of sustaining term gestation, for most species this is not the case. Two factors have emerged as primary determinants of oocyte quality when considering the deployment of IVM: **(1)** the history of the follicular milieu for a given oocyte, and **(2)** the meiotic status of oocytes at the time they are placed in culture. This presentation will develop the hypothesis that the quality of an oocyte will be a byproduct of intrinsic and extrinsic influences that exert their effects just prior to or during the resumption of meiosis. It follows then, that conditions required to optimize IVM for clinical use must emphasize the particular demands for oocytes whose legacies draw upon patient variations in age, metabolic status, or disease conditions that could in any way disturb the normal course of oogenesis in that individual.

Forces that are intrinsic to the oocyte that specify their quality status generally fall into the category of maturation competencies. Amongst these, we suggest abandoning the traditional view that cytoplasmic and nuclear maturation include distinct events that must occur in coordination with each other to satisfy acquisition of oocyte developmental competence. Rather, we view the oocyte as a semi-autonomous entity (see below) that uses complex signaling networks to discriminate and synergize "maturation" of chromatin, the cytocortex, and metabolic components that are collectively designed to address the needs of the zygote and not that of the oocyte per se. For the oocyte to assume autonomy in preparing for embryogenesis implies that the driving forces for the completion of the meiotic cell cycle must take their origin from somatic elements of the follicle especially those represented by the cumulus oophorus. Thus, extrinsic control over oocyte quality is indirectly satisfied by virtue of three properties of the granulosa cell syncytium: **(1)** translating the stimulus from LH into the metabolic activation of the cumulus-oocyte complex, **(2)** effecting a signaling pathway that both inactivates cell cycle arrest and encourages the largely post-translational activation of the meiotic cell cycle machinery, and **(3)** induce substantial changes in the molecular composition and structure of the oocyte cortex as a result of physical interactions with the oolemma and the ends of transzonal projections. Only with a deeper understanding of the balance between oocyte intrinsic and extrinsic forces will it be possible to tailor human IVM for the various pre-conditions that fertility preservation strategies demand.

# L19

## ISOLATION AND GRAFTING OF HUMAN ISOLATED FOLLICLES: WHY AND HOW? A PLACE FOR AN ARTIFICIAL OVARY?

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Reversing treatment-related premature ovarian failure using autotransplantation of frozen-thawed ovarian tissue harvested before chemoradiotherapy is becoming an increasingly realistic prospect for clinical application, since 7 live births have already been reported with this technique. Our objective is to offer young patients at risk of premature ovarian failure after treatment, safe fertility preservation options.

One major concern raised by the transplantation of ovarian cortical fragments in cancer patients is the potential risk that the cryopreserved ovarian tissue might harbor malignant cells that could induce a recurrence of the disease after reimplantation.

Hematological malignancies and breast cancer determine the most frequent indications for ovarian tissue cryopreservation. Both carry the risk of ovarian metastasis.

We therefore decided to conduct a study to evaluate the presence of breast cancer cells and leukemic cells in human cryopreserved ovarian tissue from patients with advanced breast cancer disease and chronic myeloid leukemia or acute lymphoblastic leukemia.

In each case, analysis of routine histology, PCR for disease-specific markers and xenografting were used to test the frozen-thawed ovarian tissue. Results show that malignant cells may be present in ovarian tissue from leukemic patients, but also from breast cancer patients.

Nevertheless, when appropriate, these patients should still benefit from fertility preservation techniques. Ovarian tissue cryopreservation is often the only option we can offer to leukemic patients, due to the lack of time for ovarian stimulation or because of their prepubertal age. For these patients, follicle culture with in vitro maturation could be a solution. Another option could be grafting of isolated follicles, enzymatically purified from the cryopreserved ovarian tissue. This technique opens the door to the development of an artificial ovary, made from isolated follicles embedded in a scaffold. Research in this exciting field has to continue, in order to develop different possibilities for fertility preservation that will allow us to propose the most appropriate therapeutic option to patients, according to disease.

## BIOENGINEERING THE OVARY

### Christiani Andrade Amorim

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Ovarian tissue cryopreservation and transplantation has been implemented worldwide as a routine procedure to safeguard fertility in cancer patients. However, grafted ovarian fragments may have a very short lifespan due to the irregular distribution of primordial follicles<sup>1</sup> and their significant loss after transplantation<sup>2</sup>. Furthermore, there are still concerns about its application in patients with certain types of disease because of the risk of cancer cell transmission<sup>3</sup>. An alternative would be grafting of isolated preantral follicles. This would allow the assessment of the follicular population before grafting, and the entire process of follicular development would occur in the graft recipient. Promising results obtained after isolation and xenografting of human preantral follicles embedded in plasma clot<sup>4,5</sup>, showed that this approach is feasible and encouraged us to pursue this path, focusing on the development of a scaffold that would mimic the ovary. Applying the principles of tissue engineering, we aim to design a scaffold to embed isolated follicles and act as a template to guide tissue growth up to its final form, allowing attachment, proliferation and migration of cells, angiogenesis and transport of molecules, oxygen and nutrients, and metabolic waste removal. In addition, it would have all the positive features of a plasma clot, such as fast degradation, biocompatibility and ability to maintain the original three-dimensional structure of follicles. Finally, as an off-the-shelf living tissue product, it would be easy to handle and graft. However, developing a scaffold that aims to recreate such a complex organ requires coordinated efforts of different professionals, including clinicians, engineers, biologists, chemists, surgeons and physicists, coupled with creative ideas on the relationship between follicular survival and development, material degradation and host response. Although this challenging and exciting approach is still in its infancy, we believe that, in the near future, it will become a new field in assisted reproductive technology.

**Keywords:** Tissue engineering, transplantation, preantral follicles, scaffold.

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## L21

**ONCOFERTILITY: THE PRESERVATION OF FERTILITY OPTIONS FOR YOUNG PEOPLE WITH CANCER****Teresa K. Woodruff**

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Cancer is now a disease with a variety of treatment options, which are leading to longer and more productive lives by survivors. Globally, there are more than 10 million people diagnosed with cancer: 10% of these newly diagnosed men and women are under the age of 45 years old. Infertility can be a consequence of many of the more aggressive chemo- and radiation therapies that prolong and save lives. The ability to easily preserve sperm prior to cancer treatment provides hope at the time of diagnosis and families later in life for male survivors. A notable example is Tour de France winner Lance Armstrong who has three children conceived using sperm frozen days before he underwent the massive chemo- and radiation therapy that saved his life. Unlike sperm, the female germ cell, the oocyte or egg must be retrieved surgically. Moreover, the vast majority of collected oocytes will be immature and cannot be used immediately by a woman who is ready to start a family. The *overall hypothesis of the program* is that effective fertility-extending options can be provided to young women undergoing life-preserving cancer treatment.

The purpose of our work is to bring physicians, medical ethicists, social scientists and basic scientists together to develop new strategies for fertility preservation for female cancer survivors under the new discipline of *oncofertility*. And even as the lexicon is being established, complex bioethical issues face both providers and parents. At the basic science level, complex issues of ovarian function and preservation must be addressed including the problem of follicle growth and development *in vitro*. Our investigative group has pioneered the development of a 3-dimensional system that supports follicle development, largely, we believe, because the links between the egg and its surrounding cells are maintained. Using a tissue-engineered approach, we have developed an *in vitro* follicle growth system that supports the maturation of the enclosed oocyte, which can be fertilized and results in live, healthy and reproductively competent mice. The goal of our program and the broader Oncofertility Consortium is to explore and expand the reproductive options available to young people facing a fertility-threatening but life-preserving cancer treatment.

The Oncofertility Consortium logo is a **trademarked advocacy ribbon** that reflects the growing concern for the reproductive future of cancer patients. The intertwining spring green and hearty purple represents **blossoming hope** and **uncompromised dedication** to improving fertility preservation options for cancer patients. The lower tip of the ribbon emerges shows an emergence of eggs or embryos, as well as sperm, **welcoming the translation** of current research to the improvement of fertility options for all cancer patients. The ribbon has a slightly 'bowed' shape, providing a subliminal imagery of a **fertile state**.



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<http://oncofertility.northwestern.edu/>

## CLOSING THE GAP BETWEEN IN VITRO GROWTH (IVG) AND IVM USING FRESH AND CRYOPRESERVED TISSUE

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The ability to develop human oocytes from the earliest follicular stages through to maturation and fertilisation in vitro would revolutionise fertility preservation practice. This has been achieved in mouse where in vitro grown (IVG) oocytes from primordial follicles have resulted in the production of live offspring. However, developing IVG systems to support complete development of human oocytes has been more difficult because of differences in scale of timing and size. Successes in growing human oocytes in vitro are being made in a step wise manner and the challenge now is to bridge the gaps between IVG steps and IVM.

Our lab has been working on a multi step culture system to support growth and development of bovine and human oocytes from primordial through to fully grown using fresh and cryopreserved ovarian cortical tissue. Our recent work has shown that human and bovine primordial follicles can be activated in vitro within ovarian cortical pieces and grow to multilaminar preantral (secondary) stages within 6 days (Step 1). These preantral follicles can be isolated and have the potential to grow to the antral stage (Step 2) within a total culture period of 10 days. Further 18 days in vitro results in fully grown oocytes in the bovine model. This time scale makes the complete in vitro development of oocytes from human tissue a practical and viable prospect. In this lecture, the approaches being taken to obtain complete in vitro development of human oocytes will be presented and assessment of subsequent growth rate and health of IVG oocytes discussed.



# L23

## FRESH HUMAN OVARIAN TISSUE: FROM PRIMORDIAL TO ANTRAL FOLLICLE IN CULTURE

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In human, culture of primordial follicles to antral stage in vitro has been more difficult than in mouse, where it was successful already 15 years ago. Isolated human primordial follicles proved difficult grow. In 1977 (Hum Reprod), we managed in getting them to initiate their growth and grow further within slices of human ovarian cortical tissue to secondary, and occasionally to early antral stages. The growth was better if we cultured them partially three-dimensionally in Matrigel-coated inserts. Collagen and laminin proved later to give similar support. To optimize the culture media, we supplemented it with several hormones and growth factors. Of these, follicle stimulating hormone (FSH), Growth Development Factor 9 (GDF 9), Insulin and insulin-like growth factors were clearly supportive. Antimüllerian hormone inhibited the initiation of growth. Secondary follicles are regularly seen in these cultures 1-2 weeks after the beginning of the culture, and after that the follicles could be cultured for at least six weeks. There has been progressing follicular atresia during this long period. However, a few follicles have always been healthy looking, multilayer secondary follicles or early antral follicles. Both the oocytes and the granulosa cell layers have been growing. We have hypothesized that the fast activation and relatively fast growth of the follicles was caused by removal of inhibition when the follicles were removed from the ovary. Within slices they are still surrounded by stromal and other follicles, which probably still inhibit their growth. Telfer et al. (Hum Reprod 2008) isolated the growing follicles from the surrounding stroma six days after initiating the cultures, and this increased their growth rate significantly. Adding a relative high concentration of Activin at the medium significantly increased the growth and development, in both fresh and frozen-thawed tissue. We can probably still influence the development by inhibiting the various inhibiting signals. Doing this we probably can go to the last step: maturation of the oocytes in vitro.

## IMPACT OF CRYOPRESERVATION AND GRAFTING ON FOLLICULOGENESIS

### Debra Gook

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Recent success following autografting of human cryopreserved ovarian tissue has resulted in 7 births. Typically, sporadic ovarian function is temporarily restored within 4-6 weeks after grafting but, generally, resumption of cycling is not observed until 6-8 months following grafting and may only be short lived. Unequivocal evidence of folliculogenesis in cryopreserved ovarian tissue has been demonstrated by the collection of mature oocytes from grafted tissue at heterotopic sites. However, follicular growth and oocyte quality appear to be impaired. The results have provided proof of this principle but improvements in methodology are necessary to advance this area. A prerequisite for improving methodology in a rational manner is an understanding of the relative impact of cryopreservation and the grafting process on the integrity of the grafted ovarian tissue. Xenografting of human ovarian tissue into immunodeficient mice offers a model by which this can be assessed.

Previous studies using a xenografting system have established preservation of development potential of human primordial follicles within cryopreserved ovarian tissue by demonstrating growth to antral stages, ovulation and final oocyte maturation<sup>1</sup>. More recent studies have focused on the very early histological changes following grafting using the xenograft system. Tissue is examined for morphological changes by conventional histology and the health of the tissue is assessed using a marker of DNA damage (apoptosis), and normal folliculogenesis using antibodies to the zona pellucida (ZP) proteins<sup>2</sup>.

A dramatic loss of normal ovarian architecture together with an associated high proportion of apoptotic stromal tissue is apparent as early as day 2 following grafting. This response is irrespective of whether the tissue has been cryopreserved or not (fresh) and also independent of the grafting site. Follicle morphology is also poor with elevated levels of ZP proteins within the oocyte; an indication of atresia, and most pre-granulosa cells are swollen with some apoptotic nuclei. This is not cryopreservation associated damage but likely to be due to ischemic conditions prior to neovascularisation of the tissue. However, by day 4, when the environment would still be expected to be hypoxic<sup>3</sup>, both follicular and stromal morphology are more normal together with a reduced level of apoptosis. Improvement in tissue morphology appears to be independent of neovascularisation. Although the size of the graft is reduced with time post grafting the morphology appears more similar to normal ovarian cortex and follicles which have survived the initial damage are capable of growth to the antral stage.

With the aim of reducing the duration of ischemia, tissue has been coated with angiogenic factors (VEGF, PDGF) prior to grafting. Addition of angiogenic factors has induced migration of red blood cells and platelets to the surface of the tissue but the initial damage (Day 2) within the tissue is more extensive than in the absence of the factors. As a consequence, the numbers of follicles present are reduced.

Although there is some damage to stromal cells and follicles following cryopreservation, the vast majority of damage appears to be as a result of ischemia. This has been reported by others<sup>4-6</sup> and is clearly the obstacle which must be overcome to increase the longevity of folliculogenesis in autografted tissue.

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# L25

## IN-VITRO MATURATION AND OOCYTE VITRIFICATION FOR THE PRESERVATION OF FERTILITY IN CANCER PATIENTS

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Fertility declines with increasing age and is markedly accelerated following gonadotoxic therapy for cancer. Many women whose current circumstance precludes a pregnancy find themselves facing potential childlessness. ASCO and ASRM have endorsed IVF and embryo cryopreservation (EC) as the only method of female fertility preservation. However, this requires 2-5 weeks to complete, produces relatively high estradiol levels, which may be deleterious in certain malignancies, and requires a male partner. Cryopreservation of an entire ovary has not been successfully performed in humans. Ovarian tissue cryopreservation with orthotopic transplantation requires two operations, and in cancer patients carries a small but finite risk of re-introducing malignant cells. Oocyte cryopreservation represents the least invasive option for patients without a male partner. Recent advances in vitrification techniques have markedly improved the efficacy of oocyte cryopreservation. In a clinical trial at the McGill Reproductive Center (MRC) involving 38 infertile women, oocyte vitrification (OV) using the McGill cryoleaf resulted in a mean survival rate of 81% post-thawing, a 76% fertilization rate, a CPR/C of 45%, an LBR/C of 40%, and 22 healthy babies. In a review of 165 pregnancies and 200 infants conceived following OV, the birth weight and the incidence of congenital anomalies (2.5%) were comparable to those following spontaneous conception or IVF treatment. A novel fertility preservation strategy involves immature oocyte retrieval in an unstimulated menstrual cycle or from ovarian tissue biopsies, followed by in vitro maturation (IVM) and OV or EC. IVM has become an effective treatment option for many infertile women, resulting in the birth of well over 1000 healthy infants without any increase in fetal abnormality or miscarriage rates in comparable patients. In a pilot study at the MRC on IVM-OV, an LBR/C of 20 % per cycle was achieved, including the world's first four live births from vitrified IVM oocytes. Advantages of IVM-OV or EC include: 1) eliminating expensive drugs and monitoring; 2) completing treatment within 2 to 10 days; 3) avoiding hormone-sensitive tumors; and 4) retrieving oocytes at any phase of the menstrual cycle. To date, the MRC has provided fertility preservation to over 130 patients with breast, hematological, brain, soft tissue, colorectal and gynecological cancers.



# KEY NOTE LECTURE

## POTENTIAL FOR STEM CELL RENEWAL OF FUNCTION IN STERILE OVARIES AND TESTES

**Roger G. Gosden**

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Germline stem cells (GSCs) emerging from the epiblast of early embryos provide progenitors for gamete formation in postnatal male and female gonads after they have undergone migration to the gonadal anlagen, multiplication, epigenetic reprogramming and cytodifferentiation. While GSCs persist in testes throughout life as spermatogonia, they have been regarded as a transient population in the ovaries of all mammalian species studied to date, disappearing perinatally after formation of a limited number of oocytes when they are represented by the reserve of primordial follicles that must serve the full reproductive lifespan until it is exhausted at the menopause. New evidence has appeared that conflicts with this well-established theory, claiming that circulating precursor cells constantly 'seed' the ovaries with new germ cells derived from bone marrow. The question of follicular renewal in adult ovaries remains controversial, but it is hard to refute the possibility that small numbers of quiescent GSCs persist after birth which can be activated under certain conditions. Discovery of candidate GSCs has been claimed on the basis of protein biomarkers and oocyte-like cells derived in vitro from the surface epithelium or cortex of human and mouse ovaries. Stronger evidence was obtained recently using neonatal and adult mouse ovaries to harvest GSC candidates which were clonally expanded in culture and, after transplantation to sterile hosts, underwent folliculogenesis to form ovulable oocytes that could be fertilized to create viable offspring<sup>1</sup>. The possibility of restoring spermatogenesis in irradiated mouse testes has also been investigated by transplanting mouse embryonic stem cells (mES) or bone marrow, but neither cell type was able to replace lost spermatogonia. When cells resembling germ cells were generated from mES cells in vitro, those of male origin became haploid after meiosis and capable of fertilization. Although large, oocyte-like cells were formed spontaneously from female mES cells in culture, their competence for reproduction was more doubtful, perhaps because cytoplasmic maturation is so much more complex and critical in female compared to male gametes. Lastly, the current optimism for future clinical applications of induced pluripotent cells (iPS) for regenerative medicine now extends to reproduction since mice created from iPS cells derived from skin and by tetraploid complementation are both viable and fertile<sup>2</sup>. If human GSCs could be created de novo using a comparable technology they might revolutionize the treatment of female and male sterility and minimize the need for donor gametes.

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# L26

## OOCYTES: SLOW FREEZING VERSUS VITRIFICATION - WHAT IS MORE EFFICIENT?

**Andrea Borini**

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Studies conducted in the last few years suggest that oocyte cryopreservation can be applied in a systematic and reproducible manner, in some cases with success rates which appear to compete with those routinely achieved with embryo freezing. Vitrification techniques, recently introduced in the field of human IVF, have further enhanced the hope of developing oocyte storage as a viable assisted reproduction strategy. The overall ability of oocyte cryopreservation to generate a viable pregnancy depends on the inclusion in the calculation of all the events of attrition at pre- and post-storage stages. Only under such conditions, certain differences between alternative methods become apparent. For example, it is well known that a major improvement (from 35-40% to 70-75%) in the survival rate of oocytes, frozen and then thawed via slow cooling, may be obtained by increasing the sucrose concentration in the freezing solution from 0.1 to 0.3 mol/l (Fabbri *et al.*, 2001). This change also improves the rate of fertilization (Borini *et al.*, 2006; De Santis *et al.*, 2007; Levi Setti *et al.*, 2006). However, the different degree of attrition at the steps of survival after thawing and on fertilization in the 0.3 mol/l sucrose protocol is counterbalanced by a higher implantation rate of embryos generated by the protocol involving the lower sucrose concentration (Borini *et al.*, 2004; De Santis *et al.*, 2007). The overall outcome, considered as the proportion of implantations per thawed oocyte, ultimately makes the efficacy of the two methods very similar (2.4 versus 2.6%) and in any case insufficient for competing with embryo freezing whose efficiency per used oocyte is approximately 5-6%. Our group reported an implantation rate per thawed oocyte used of 5.9%, following the application of a protocol based on differential concentration of sucrose in the freezing (0.2 mol/l) and thawing (0.3 mol/l) solutions (Bianchi *et al.*, 2007). A similar implantation rate per thawed oocyte has been more recently reported by another Italian group (Parmegiani *et al.*, 2008). In recent years, the alternative cryopreservation approach of vitrification has raised several hopes. By adopting the cryotop vitrification method in cycles involving young donors (mean age 26.7 years), Cobo *et al.* (Cobo *et al.*, 2008) achieved an implantation rate per embryo transferred of more than 40%. Nevertheless, when this study is analyzed considering the original number of oocytes used, the implantation rate corresponds to a value of 8.6%. Such a rate is not very dissimilar from the one (7.3%) resulting from a study conducted by a slow cooling method and including patients with a mean age of 33.7 years (Bianchi *et al.*, 2007). Evidence gained from approximately one thousand babies born from cryopreserved oocytes, even though still insufficient and incomplete, has not suggested so far that the process of low temperature storage is associated with an increase in birth abnormalities. Although the number of published studies is still relatively modest, the number of oocyte cryopreservation treatments is dramatically increasing. This will have undoubtedly a significant impact on the practice of human IVF in the near future.



# L28

## EMBRYOS: SLOW FREEZING VERSUS VITRIFICATION - WHAT IS MORE EFFICIENT?

**Etienne Van den Abbeel**

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Fertility preservation is a vital branch of reproductive medicine and involves the preservation of gametes (sperm and oocytes), embryos, and reproductive tissues (ovarian and testicular tissues) used in artificial reproduction. The most common fertility preservation technique is cryopreservation, which involves freezing cells and tissues at cryogenic temperatures. Cryopreserved cells and tissues can endure storage for centuries with almost no change in functionality or genetic information, making this storage method highly attractive. However, developing efficient cryopreservation techniques is challenging, as is known that both freezing and thawing exposes cells to severe stresses, potentially causing cell death. There are two major techniques for cryopreservation: freeze-thaw processes (conventional freezing) and vitrification. The major difference between them is that vitrification is able to avoid lethal intracellular ice formation. Additionally, chilling injuries are considerably reduced. Many variables in the vitrification process exist that can profoundly influence its effectiveness and the potential to improve the survival rates of vitrified cells. In this regard, increasing the speed of thermal conduction and decreasing the concentration of cryoprotectant is an ideal strategy for cryostorage of embryos with vitrification methods. However, the actual rate of heat transfer during vitrification procedures may vary extremely depending on the device used, technical proficiency, and the specific movement at immersion. Vitrification is often performed under non-equilibrium conditions (non-equilibrium vitrification), therefore the term vitrification is often misused since samples are often not really "truly" vitrified but "apparently" vitrified. In addition, it is very important to mention that every cell has its own optimal cooling rate. To date, an official vitrification protocol has yet to be defined. In light of this, it is important for researchers to achieve more consistent results from existing protocols and thereby to establish a standardized vitrification protocol that can be applied for cryopreservation of different developmental stages. Toward this end, it should be noted that vitrification protocols are starting to enter the mainstream of human ART. The convenience of vitrification will push the development of this technique to higher levels of clinical efficiency and use.

It is well known that fully intact cleaved embryos have a higher implantation potential than partially damaged embryos. Therefore it should be the aim of a cryopreservation programme of embryos to have fully intact embryos after thawing. Recent published data clearly demonstrate that after thawing/warming human embryos, the percentage of fully intact embryos is significantly higher after vitrification than after slow controlled-rate freezing. Successful pregnancies and deliveries after using the human vitrification technique at Day 3 and Day 5 using 6 embryos have been reported. Results of recent meta-analysis demonstrate that indeed better results are obtained using vitrification compared to conventional freezing. Despite the lack of properly designed prospective randomized controlled trials comparing vitrification and conventional freezing in Reproductive Medicine, we suspect that the current results obtained with vitrification and the convenience of vitrification will push the development of this technique to higher levels of clinical efficiency and utilization.

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## EMBRYOS: SLOW FREEZING VERSUS VITRIFICATION - PROS AND CONS

### Pedro N. Barri

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Survival rates of young cancer patients have shown a positive trend in recent decades, particularly in case of breast cancer and Hodgkin's disease. Advances in treatment modalities have raised awareness of the need for effective and safe fertility preservation procedures for young women with malignancies. The Ethics Committee of the American Society for Reproductive Medicine (ASRM) and the American Society of Clinical Oncology (ASCO) recommend that physicians advise cancer patients of their fertility-preserving options before cancer treatment is initiated and inform them that sperm and embryo freezing are the only established methods of fertility preservation. Other fertility preservation methods should be considered investigational and should only be performed in centers with the necessary expertise.

The most successful approach is embryo cryopreservation, as this technique yields a pregnancy rate of 20-30 % if at least two embryos are available for replacement. However, there are some drawbacks:

1. The patient must have a male partner or use donor sperm.
2. Ovarian stimulation, oocyte retrieval and IVF cause a delay in the initiation of chemotherapy or radiotherapy, which may not be acceptable in some cases.
3. High estrogen concentrations associated with ovarian stimulation may be contraindicated in women with estrogen-sensitive malignancies.

For young cancer patients, it is important to know the impact of the duration of freezing on survival and pregnancy rates. We analyzed pregnancy rates obtained with frozen-thawed embryos 7 years, 7-9 years and more than 9 years after cryopreservation and did not observe any difference between the three groups, with a child born after 10 years and another after 14 years of freezing. We reported the birth of a healthy boy 4 years after embryo cryopreservation in a patient with a bilateral borderline ovarian tumor.

At present, it appears that:

1. Embryo freezing is the most efficient technique for fertility preservation.
2. Freezing of early-stage embryos (PN and cells) yields better survival and pregnancy rates than blastocyst freezing and thawing.
3. The duration of freezing does not affect embryo survival after thawing of implantation potential.
4. Vitrification will probably serve to improve embryo cryopreservation results in the near future.







# L32

## THE NEXT DEBATE: FUTURE, IS THERE A PLACE FOR OVARIAN TISSUE AND OOCYTE CRYOPRESERVATION FOR SOCIAL REASONS?

**Guido Pennings**

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### **Introduction**

Soon after the first birth from frozen ovarian tissue, the debate started on the possibility to use this technology for healthy women. However, people's fantasies about future scenarios really went wild when vitrification of oocytes produced such excellent results. We face two questions at the moment: should fertility preservation (in whatever form) be offered for non-medical reasons, and if so, which position should be adopted now during the transition period awaiting confirmation or refutation of the results?

### **Results**

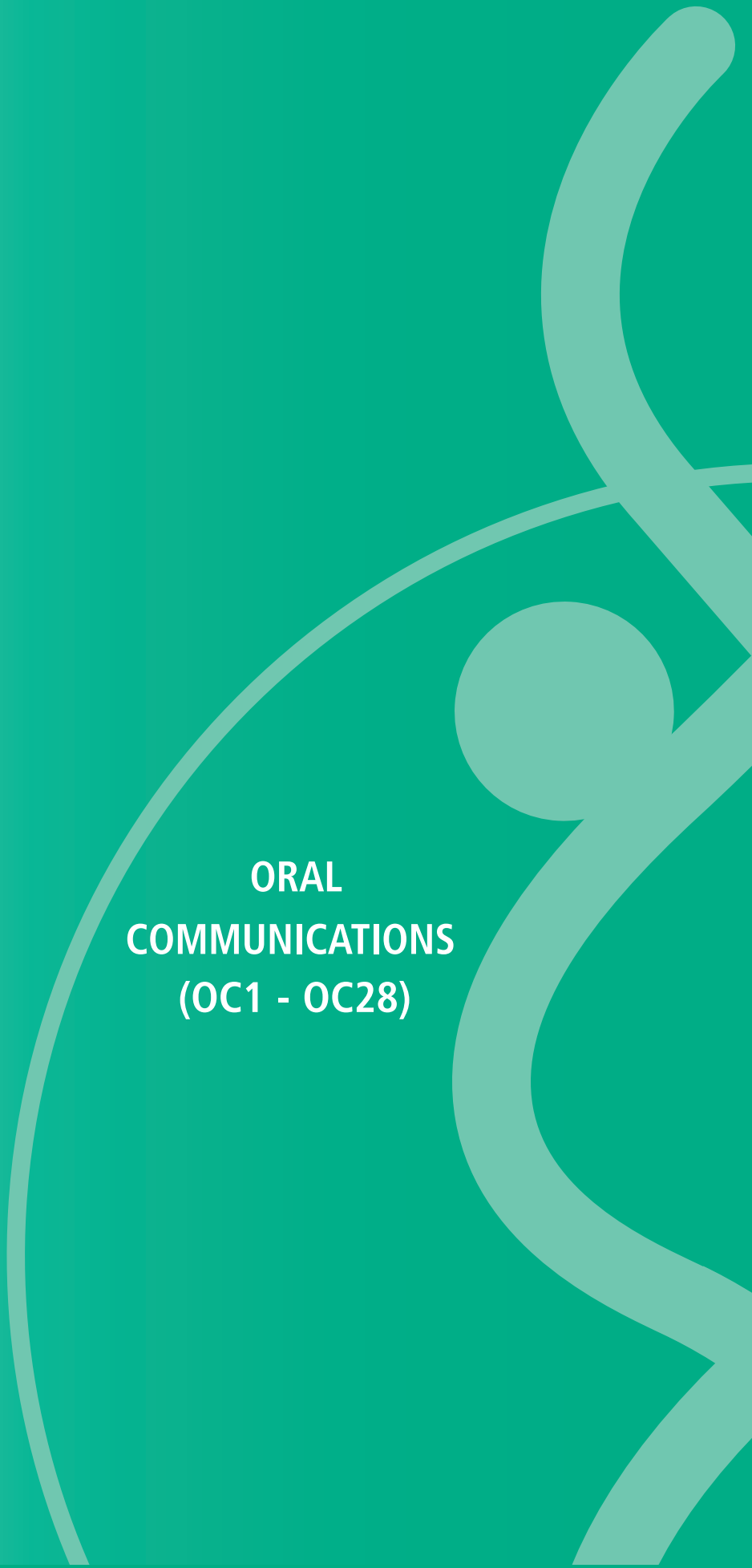
The question regarding the application of medical techniques for non-medical applications has a long history. The distinction between medical and non-medical applications has never been clear-cut. Moreover, analysis of the use of the label reveals that the concept 'medical' is used as a normative concept: it sanctions the use of a technique for certain problems and it justifies reimbursement from public funds. Simultaneously, the 'medical'-'non-medical' tandem frequently overlaps with the need-desire tandem. Desires, contrary to needs, are a matter of personal choice and autonomy. The second question concerns the 'experimental nature' of the procedure and the consequences that follow from this label. This argument, together with the proportionality principle, underlies the cautious position adopted by several professional societies. A key question is whether medical technology (fertility preservation) should be used to cure a health problem (infertility) that is caused by individual behaviour (postponed parenthood) strongly determined by the social and cultural context (career, education). An analogy can be made with medical interventions (e.g., gastric bypasses) for morbidly obese persons. Some argue that one should alter the social and cultural context so that women can and want to have children earlier. This is easier said than done. Moreover, even if we believe that this is the way forward, this does not tell us what we should do in the meantime. Autonomy is a very strong argument to allow women to decide for themselves whether the burden of the intervention is worthwhile in order to preserve the chance of having genetically related children in the future.

### **Conclusion**

Fertility preservation for non-medical reasons is a highly complex issue that touches upon general views on the appropriate use of medicine, beliefs about motherhood and women's role in society, long-term consequences for society etc. The blunt rejection of these applications that dominate the present discussion is too simplistic. We will need stronger arguments than those offered at the moment to make a convincing case for a prohibition for social reasons.



**ORAL  
COMMUNICATIONS  
(OC1 - OC28)**



## ONCOLOGISTS' PRACTICE AND ATTITUDES REGARDING FERTILITY PRESERVATION IN FEMALE CANCER PATIENTS: A PILOT STUDY IN THE NETHERLANDS

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<sup>2</sup> Department of Gynaecology, Reinier de Graaf Hospital, Delft, the Netherlands.

### Introduction

Fertility changes are a common side effect of cancer treatment. The combination of improved survival and advances in reproductive medicine provide some hope for fertility preservation after cancer treatment in women.

The aim of this study was to assess oncologists' practice and attitudes regarding treatment-related infertility and regarding fertility preservation.

### Participants, design and methods

Recruitment letters were mailed to a selected group of 454 oncologists; the letter referred to a link to complete a 7-item questionnaire online.

### Results

Hundred and eighty-eight of the 454 oncologists (41%) responded. Ninety-six of the 188 (51 %) oncologists saw at least 5 women with cancer within the reproductive age group, during the last year. These 96 questionnaires were used for analysis. The sample included 28 (29%) gynaecologists, 22 (23%) medical oncologists, 19 (20%) surgeons, 16 (17%) radiotherapists and 11 (12%) haematologists. Mean number of years of practice was 13.3 years; seventy percent of the sample was male. Nearly 70% of the oncologists reported that at least one of their patients had undergone fertility preservation prior to cancer treatment. The most important reason for not offering fertility preservation was "factors concerning the disease" (61%). About one-third of the oncologists (31.3 %) did not discuss fertility issues. If the possible fertility preservation options were standardized almost half of the oncologists would refer 75-100% of their patients for fertility preservation. No significant differences were found between oncologists in (1) the mean value of raising fertility issues with premenopausal women prior to cancer treatment (5.9 on a 7-point scale) and (2) the mean value of fertility after a malignant disease (5.6 on a 7-point scale)

### Conclusion

Early results indicate that most of the oncologists are willing to discuss fertility issues with premenopausal women prior to the cancer treatment, but currently this is not done by one-third of the oncologists.

## AUTOTRANSPLANTATION OF CRYOPRESERVED OVARIAN TISSUE IN A COHORT OF 12 DANISH WOMEN

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### Introduction

Antineoplastic treatments have a known gonadotoxic effect, which in women can cause premature ovarian failure (POF) and unwanted infertility. Cryopreservation of ovarian tissue has been developed as a means to restore ovarian function and possibly fertility in these women. We report our experience in a cohort of 12 Danish women who had some of their cryopreserved ovarian tissue re-implanted after treatment-induced POF.

### Patients and methods

Among the 12 women, 9 had a malignant and 3 a benign disease. The mean age at cryopreservation was 28.4 years. All had a potentially gonadotoxic treatment between March 2000 and July 2006 and cortical tissue from one ovary cryopreserved prior to treatment. All developed amenorrhea and hot flushes after treatment and POF was confirmed by ultrasonography of the remaining ovary and by menopausal levels of FSH values (mean 74 IU/l). Autotransplantation was performed between 17 and 65 months after end of treatment. Five of the patients returned for a second autotransplantation due to cessation of function in the first transplant or to increase chances of pregnancy. The ovarian tissue was transplanted to both orthotopic and heterotopic sites.

### Results

All women regained menstrual cycles within 8-26 weeks (mean 19 weeks) after transplantation, levels of FSH and LH were normalised, oestradiol levels increased and antral follicles appeared on ultrasonography. Ten of the women underwent a total of 43 in vitro fertilisation (IVF) cycles, 35 oocytes were aspirated, 21 were fertilised and 14 embryos were transferred resulting in five pregnancies: two biochemical, one clinical that miscarried in week seven and two ongoing that resulted in the delivery of two healthy babies born at term to two women. One of these women subsequently conceived spontaneously, which resulted in the delivery of another healthy baby.

### Conclusions

Autotransplantation of cryopreserved ovarian tissue has proven to be an efficient way of restoring ovarian function in women with treatment-induced POF. In our material all women regained ovarian function and four women additionally became pregnant, after IVF or spontaneously, resulting in the delivery of three children to two women. However, IVF in this subgroup of infertility patients is difficult and raises many challenges.

# OC3

## OVARIAN CORTEX CRYOPRESERVATION IN GIRLS UNDER 16 YEARS OF AGE

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### Introduction

In prepubertal and adolescent girls, fertility may be impaired by gonadotoxic treatments, repeat ovarian surgery or genetic disorders. Cryopreservation of ovarian cortex is an existing option to preserve fertility, and pregnancies have been obtained after reimplantation of frozen-thawed ovarian cortex in adults. Our aim was to evaluate the feasibility and safety of ovarian cortex cryopreservation in young girls at risk of premature ovarian failure.

### Materials and Methods

We reviewed data from 58 girls under 16 years of age, who underwent ovarian tissue harvesting and cryopreservation in our institution between May 2001 and May 2009. Patient age ranged from 10 months to 15 years (10.4  $\pm$  4.4 years). Twenty-one girls were under 10 years of age and 38 were prepubertal. Forty-eight patients were suffering from cancer, three from genetic disorders, one from systemic disease requiring chemotherapy, and four from benign ovarian pathologies. Two patients required cryopreservation prior to bone marrow transplantation for benign disease. We analyzed the feasibility and safety of the procedure, and reviewed our indications.

### Results

All procedures were performed by laparoscopy. Unilateral or bilateral cortical biopsies were taken from 38 patients, and unilateral oophorectomy was performed in 20 patients. No complications occurred during surgery. Histological analysis of a small piece of ovarian cortex showed the presence of follicles in all cases, except one patient suffering from galactosemia. In one patient with lymphoma, laparoscopy revealed tumoral infiltration of the ovary by a 10-cm mass, confirmed by histological analysis. No ovarian involvement was observed in any other cases. For all oncological indications, chemotherapy was initiated from day 0 to day 5 after cryopreservation. During subsequent months, seven girls (12%) changed categories from low or medium risk to high risk of premature ovarian failure.

### Conclusions

Our series shows that ovarian tissue cryopreservation is feasible at any age by a laparoscopic approach, without complications and without postponing cancer treatment. We also demonstrate that it is virtually impossible to give the patient or her parents an accurate assessment of the risk to fertility. Although we can define treatment regimens associated with varying degrees of risk, disease evolution is never completely predictable. We therefore believe that fertility preservation options should be discussed with all patients at risk of impaired fertility, even very young girls.

## CRYOPRESERVATION OF OVARIAN TISSUE FOR FERTILITY PRESERVATION IN GIRLS

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### Introduction

Chemo- or radiation therapy treatment in girls may cause unwanted long-term side effects such as absent menarche or early onset of premature ovarian failure (POF) after puberty. Cryopreservation of ovarian tissue prior to gonadotoxic treatment can be offered as a means of preserving fertility in some of these patients. This study describes a cohort of 88 Danish girls with cryopreserved ovarian tissue, which is probably the largest series of its nature available.

### Methods

From 2000 to 2009 a total of 88 girls < 18 years of age had one entire ovary removed and cryopreserved at Rigshospitalet, Copenhagen before bonemarrow transplantation (BMT) or treatment with high-dose alkylating agents for a malignant (N=69) or benign (N=15) disease. Additionally, four girls had one ovary cryopreserved at a young age where follicles were still expected to be present (Turner mosaicism and galactosaemia). A small piece of ovarian tissue was assessed histologically and the follicular density calculated. After treatment a pediatrician examined the girls at regular intervals for pubertal status and ovarian function by means of Tanner staging, menstrual history and measurements of FSH and oestradiol.

### Results

So far, we have data on pubertal status after treatment on 22 girls >10 years of age: 13 showed signs of ovarian failure (OF) with elevated FSH values >20 IU/l (mean 71 IU/l) and absent menarche or amenorrhea, seven had spontaneous menstruations and in two girls it was too early to assess ovarian function. Nine of the girls with OF had been treated with BMT preconditioned with total body irradiation. Puberty was induced in two girls and hormonal replacement therapy given to 12 of the girls with OF. Data on ovarian function in an additional 24 girls will be presented as well as data on follicular density. As of today, a total of 21 girls in the programme have died from their disease.

### Conclusion

Of the girls so far evaluated for ovarian function, the majority (59%) had OF after treatment. Although still experimental, cryopreservation should be considered in girls at high risk of OF, since no other options for fertility preservation exist in these young patients.



# OC5

## HOW TO PROVIDE A GOOD VASCULARISATION ON OVARIAN TISSUE GRAFTING AND PREGNANCIES

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### Introduction

Since 1995, in France, about 700 children or women have had their ovary cryopreserved for fertility preservation before castrative treatment. In the world, only 5 births have been published after auto-grafting of ovarian cryopreserved tissue. One of the limitations of the technique is the low survival rate of the follicles after grafting. These patients are usually poor responders after ovarian stimulation. The survival rate of the follicles may depend on the quality and the delay of neovascularisation.

Dissen in 1994 and Long in 2007 demonstrated that VEGF secretion of ovarian grafts was maximum at three to seven days after the grafting.

### Materials and Methods

In 1999, a 27 year old woman presented Systemic Vasculitides. She received 3 bolus of 0.850 g of cyclophosphamide, before right ovarian cryopreservation was performed with a slow cooling protocol. During the next two years, the patient received cyclophosphamide (7,65g IV and 58 g per os). In 2003 she presented a clinical and biological menopause, with high levels of FSH and LH. HRT was prescribed during 4 years. In 2007, she wished to be pregnant and asked for her ovarian tissue. The transplantation surgical procedure was the following: during a first laparoscopy, one large fragment of ovarian cortex was rapidly thawed and cut in 10 pieces of 1 to 2 millimetres. We opened a left peritoneal window (LPW) near the uterine vessels, and small pieces of the ovarian cortex were put there. Other small pieces were put in an ovarian window made in the left remaining ovary (LO) by a large biopsy.

A second laparoscopy was performed 3 days later, during the VEGF secretion rise. 8 large fragments of 8 mm<sup>2</sup> were grafted: 2 on the LO, 3 on the LPW, and 3 on a new peritoneal window (RPW) near the right uterus vessels.

### Results

A total of 4 IVF were performed after ovarian stimulation 23 oocytes, 17 M II, 16 embryos, 7 blastocysts and 2 pregnancies were obtained one ectopic and a second ongoing in its third trimester.

The graft on the LPW provided 80% of the oocytes harvested and 90% of the mature oocytes and embryo. All the embryos transferred and both pregnancies came from the LPW graft.

Doppler and Angiography MRI located the origin of the neovascularisation of the LPW on the ombilico-uterin artery. The new vessels are larger than 1 mm.

### Conclusions

We report new pregnancies after cryopreservation of ovarian tissue and auto-grafting, with a new technique using a small piece of ovarian tissue to stimulate angiogenesis factor, providing good neovascularisation for grafting 3 days later. We obtained a large number of oocytes, blastocysts, embryos, and 2 pregnancies in only 4 cycles. One is an ongoing pregnancy in its third trimester. This is the first time that a pregnancy is obtained with ovarian tissue that was frozen for 8 and a half year.

## SYSTEMATIC FOLLOW-UP OF YOUNG PATIENTS UNDERGOING CHEMOTHERAPY IN ORDER TO ASSESS THE DYNAMICS OF FOLLICULAR DEPLETION: WHAT SHOULD WE LEARN? THE EXPERIENCE OF THE LILLE UNIVERSITY HOSPITAL.

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### Introduction

Chemotherapy protocols are ovariotoxic by damaging all follicles. To better characterise the degree and the evolution of follicular depletion in patients treated for lymphoma, we used serial and repeated anti-Müllerian hormone (AMH) measurements and antral follicle counts (AFC).

### Materials and Methods

45 young women were prospectively recruited before the initiation of chemotherapy for Hodgkin's or non-Hodgkin's lymphoma. They were assigned either to an ABVD protocol (ABVD group) or to a protocol that included more ovariotoxic drugs such as cyclophosphamid (non-ABVD group). Serum samples for AMH assays and AFC were performed before and during chemotherapy and every three months after the end of treatment for a period of two years.

### Results

Mean AMH levels fell drastically and rapidly after the start of chemotherapy in a similar fashion between both groups. However, the ovarian recovery phase was significantly different between the 2 groups with an AMH re-increase significantly more pronounced in the ABVD group returning to pre-treatment values twelve months after the end of treatment. Conversely, in the non-ABVD group, AMH values after chemotherapy remained very low or undetectable. The dynamics of the AFC showed the same pattern than AMH in both groups.

### Conclusion

This longitudinal study of AMH levels and AFC during and after chemotherapy well reflects the dynamics of follicular depletion. It highlights differences between protocols that could help in the understanding of the ovarian toxicity and, ultimately, in fertility preservation counselling.

**Key words:** AMH, chemotherapy, lymphoma, follicular depletion, follow-up.

**Topic:** Impact of cancer treatments on gonads.

## OC7

## MICROARRAY APPROACH TO INVESTIGATE GENE EXPRESSION IN HUMAN OVARIAN TISSUE AFTER XENOGRAFTING

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### Introduction

A massive activation of follicular growth and extensive stromal tissue fibrosis is observed after xenotransplantation of cryopreserved human ovarian tissue. This premature follicular activation may be a result of a deregulation of folliculogenesis or a stimulation of follicular cell growth caused by ischemic stress. Our aim was to investigate the impact of avascular human ovarian cortex xenografting on gene expression in order to better understand the mechanisms involved.

### Material and methods

Nude mice were grafted intraperitoneally with frozen-thawed human ovarian tissue from 8 patients. Grafts were recovered after 2 (D2) or 7 (D7) days. Non-grafted tissue was considered as control (D0). Total RNA was extracted using phase separation (Tripure) and its quality was analyzed. Inclusion criterion was  $RIN \geq 6.5$  (RNA integrity number). Differentially expressed transcripts between D0, D2 and D7 (n= 4 biopsies) were identified by Human Genome U133 Plus 2.0 Array (Affymetrix) using a linear analysis model. Main function and gene interactions were highlighted with Ingenuity Pathways Analysis (IPA) software. Transcripts showing a fold change  $>2$  or  $< 0.5$  and p-value  $< 0.01$  were considered to be up- or down-regulated. Taqman RT-PCR with 7 primer/probe sets was used to validate array results (n=8 biopsies).

### Results

Microarray analysis revealed 99 and 326 up-regulated, 321 and 705 down-regulated transcripts on D2 and D7 respectively compared to D0.

**Table:** Selected up- and down-regulated transcripts in xenografted human ovarian tissue.

Genes	TGF $\beta$ 1	CXCR4	MMP14	IL6	IL8	VEGF	PIGF	Inhibi n $\beta$ A	Inhibi n $\beta$ B	Inhibi n $\beta$ C	STAT1	ER1	LIF
Fold change D2 versus D0	5,23	4,32	4,57	5,79	15,44	NA	NA	NA	1,25	NA	0,07	0,21	26,72
Fold change D7 versus D0	NA	5,78	23,86	NA	NA	3,43	5,34	2,0	2,37	4,54	NA	0,13	3,99
Gene function	Hypoxic response			Inflammation		Angiogenesis		Follicular development			Cell cycle		

IPA identified factors linking hypoxic response, inflammation and revascularization. Molecules involved in hypoxia response (TGF $\beta$ 1, MMP14) and inflammation (IL8) stimulate the production of mediators of both angiogenesis (VEGF, PIGF) and follicular development (inhibins). Moreover, STAT1 and ER1, exerting a negative control of cell cycle, are down-regulated while LIF (proliferation inducer) is up-regulated after ovarian tissue grafting. These three molecules may be implied in the activation of follicular proliferation.

### Conclusion

Our study identified, for the first time, the pathways involved in apoptotic and inflammatory processes that are linked to revascularization of human ovarian tissue after grafting. MMP14, IL8 and PIGF involved in these pathways are promising targets to enhance revascularization processes. Over-expression of these molecules may have an implication in the deregulation and subsequent loss of follicles.

**Key words:** Human ovarian transplantation, gene expression.

## IN VITRO MATURATION AND VITRIFICATION OF IMMATURE OOCYTES COMBINED WITH OVARIAN TISSUE CRYOPRESERVATION: A NEW STRATEGY OF FERTILITY PRESERVATION.

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### Introduction

A new attractive combined option to preserve fertility is to vitrify in vitro matured oocytes isolated at the time of ovarian tissue cryopreservation to improve future reproductive potential.

### Materials and Methods

From November 2008 to July 2009, 16/21 patients between 8 and 36 years old underwent the combined procedure. Oocytes-cumulus complexes (OCCs) were retrieved from fresh ovarian cortex after puncture of antral follicles and filtration (Falcon, Cell Strainer 352350, 70 µm Nylon) of discarded material. The OCCs were washed and incubated at 37°C in 5% CO<sub>2</sub> in a commercial medium (Sage, IVM Kit media) supplemented with 75 mIU/ml FSH and 75 mIU/ml LH. After 24 hours, the OCCs were denuded and metaphase II (MII) oocytes were vitrified. Remaining immature oocytes were kept in IVM media for additional 24 hours. All oocytes were vitrified following a standard protocol (Irvine, Vitrification Kit media) using aseptic devices (CryoBiosystem, VHS Kit).

### Results

68 oocytes, 52 germinal vesicles (GV) and 16 metaphases I (MI), were isolated and subjected to IVM. 19 MII oocytes were obtained and vitrified (27.94%), 14 and 5 MII after 24h and 48h respectively. In 4/16 patients, no oocytes were found (25%). Interestingly, we were able to collect and in vitro mature oocytes regardless to menstrual cycle phase or hormonal treatment, while better results were obtained for patients using hormonal treatment (IVM rate: 36.36% vs 23.91% when no hormonal treatment was taken). In one patient 10,000 IU of hCG was administered, 13 GV were retrieved and 5 MII were vitrified.

### Conclusion

These our preliminary experience demonstrates that immature oocytes can be retrieved from the excised ovarian tissue, matured in vitro and vitrified. Combination of both techniques could be an option to improve results of the fertility preservation program. It may be the only option when the risk of auto-transplantation is too high. Oral contraception and hCG priming before collection may increase recovery and maturation rate. Nevertheless, this strategy should currently be regarded as still experimental.

## OC9

## LOCALIZATION OF C-KIT/KIT LIGAND AND ANTI-MÜLLERIAN HORMONE IN OVARIAN FOLLICLES FOLLOWING 28 WEEKS OF HUMAN OVARIAN TISSUE XENOTRANSPLANTATION

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### Introduction

Ovarian tissue cryopreservation and transplantation constitute a promising alternative for safeguarding fertility in cancer patients. However, these procedures influence preantral follicle survival. This may be due to alterations in the expression of important growth and/or inhibitory factors, such as c-kit, kit ligand (KL) and anti-Müllerian hormone (AMH). Our aim was to investigate the effects of freezing and grafting on the expression of these factors in human follicles.

### Materials and Methods

Ovarian biopsies from 8 patients were used for fresh and frozen-thawed xenografting to 13 SCID mice in the intraperitoneal site for a period of 28 weeks, including 2 weeks of gonadotropin stimulation. In the 4 treatment protocols (fresh and frozen-thawed grafted and non-grafted ovarian tissue), expression of c-kit, KL, and AMH were assessed by immunohistochemistry, and follicular proportion, follicular diameters were analyzed.

### Results

The following table shows the percentages of positive-stained follicles for the different factors studied.

**Table:** Follicle percentage (number of positive follicles/total number) stained positive for AMH, c-kit and KL.

	AMH				c-kit				KL			
	Fresh tissue	Frozen tissue	Fresh grafts	Frozen grafts	Fresh tissue	Frozen tissue	Fresh grafts	Frozen grafts	Fresh tissue	Frozen tissue	Fresh grafts	Frozen grafts
Primordial	0% (0/15)	5% (1/18)	6% (2/33)	6% (3/49)	50% <sup>a,b</sup> (4/8)	64% <sup>a</sup> (16/25)	25% <sup>b</sup> (13/52)	37% <sup>b</sup> (26/69)	45% <sup>a</sup> (10/22)	46% <sup>a</sup> (13/28)	17% <sup>b,c</sup> (8/45)	34% <sup>a,c</sup> (19/55)
Primary	55% <sup>a</sup> (10/18)	92% <sup>b</sup> (50/54)	73% <sup>a</sup> (38/52)	61% <sup>a</sup> (35/57)	38% <sup>a</sup> (10/26)	70% <sup>b</sup> (29/41)	38% <sup>a</sup> (21/55)	42% <sup>a</sup> (48/112)	61% <sup>a</sup> (19/31)	94% <sup>b</sup> (37/39)	28% <sup>c</sup> (13/45)	34% <sup>c</sup> (31/89)
Secondary	66% (2/3)	100% (9/9)	89% (41/46)	100% (41/41)	NA	0% <sup>a</sup> (0/4)	47% <sup>a,b</sup> (27/57)	54% <sup>b</sup> (35/64)	NA	100% (3/3)	48% (23/47)	59% (37/62)
Antral	NA	NA	100% (10/10)	100% (10/10)	NA	NA	70% (7/10)	80% (8/10)	NA	NA	90% (9/10)	81% (9/11)

<sup>a-c</sup> Numbers with differing superscripts are significantly different between columns of the same factor (chi-square test,  $p \leq 0.05$ ).

A total of 4090 follicles were identified: 429 from fresh fragments, 761 from grafted fresh tissue, 333 from frozen-thawed tissue, and 2567 from grafted frozen-thawed tissue. Follicle diameters did not differ between fresh and frozen grafted tissue (primordial follicles: 45.87 and 47.93; primary follicles: 53.89 and 54.66; secondary follicles: 121.48 and 137.73). In addition, antral follicles (fresh grafts: 29; frozen-thawed grafts: 24), corpus luteum and corpus albicans were observed.

### Conclusion

Although AMH, c-kit and KL expression appears to be affected by freezing and grafting procedures, follicles can still develop up to the antral stage.

**Keywords:** Cryopreservation, xenografting, antral follicle, c-kit/kil ligand, anti-Müllerian hormone.

## SUCCESSFUL PROPAGATION OF HUMAN SPERMATOGONIAL STEM CELLS IN VITRO

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### Introduction

The recently developed, highly effective, cancer therapy for children allows the majority of them to survive their cancer. One of the major side effects of cancer therapy in male patients is sterility. There are currently no means to preserve reproductive potential in prepubertal boys, which contrasts with adolescents and adults, for whom cryopreservation of semen prior to chemotherapy or radiotherapy is available and widely used. Therefore, establishing a human spermatogonial stem cell (hSSC) culture system to allow successful autotransplantation for young boys diagnosed with cancer is of utmost importance. As the final number of transplantable cells will influence the success rate of this technique, we first focus on the propagation of hSSC in culture.

### Material and Methods

We used testicular tissue from six men undergoing bilateral castration as part of prostate cancer treatment. Testicular cells were isolated with a two steps enzymatic digestion and overnight differential plating. Testicular cells were cultured in supplemented medium. Some formed germline stem cell (GSC) clusters were taken out of the culture and subcultured on human placental laminin coated dishes. The presence of spermatogonia in the cultures was determined by immunohistochemistry and RT-PCR for spermatogonial markers (integrin- $\alpha 6$ , integrin- $\beta 1$ , PLZF). The spermatogonial stem cell transplantation assay was performed using busulphan treated nude mice, as the functional test of stem cell capability. Human cells in recipient mouse testis were detected by Fluorescent In situ hybridization (FISH) using the most common human specific repetitive DNA sequence (COT) as a probe.

### Results

Germline stem cell (GSC) cluster formation was observed in the testicular cell cultures of all six men in testicular cell cultures and in subcultures GSCs. Testicular cells and subcultured GSCs could be cultured for at least 15 and 28 weeks respectively, while expression of spermatogonial cell surface markers integrin- $\alpha 6$  and integrin- $\beta 1$  (on RNA level) and spermatogonial nuclear marker PLZF (on RNA and protein levels) was maintained. The mouse transplantation assay showed successful colonization of cultured testicular cells in 4 out of 6 patients and from the subcultured GSCs in 1 out of 2 patients, indicating the presence of functional spermatogonial stem cells. By determining the number of colonies of transplanted cultured cells from early and later passages of the same culture, we found a more than 50 fold increase of hSSC in 19 days in our testicular cell culture when cultured from day 28 to 47 (passage 2 to 5) and a more than 18000 fold increase in number of hSSC in 64 days in our subcultured GSCs when cultured from day 77 to 141 (passage 7 to 12).

### Conclusions

This report outlines the first successful long term culture and proliferation of hSSC in vitro. This is an important step forward to future clinical application of SSC autotransplantation in prepubertal boys diagnosed with cancer to preserve their fertility.

# OC11

## MORPHOLOGICAL AND ULTRA-STRUCTURAL FEATURES OF CRYOPRESERVED OVINE OVARIAN TISSUE: DELETERIOUS EFFECT OF PROH APPLYING DIFFERENT THAWING PROTOCOLS

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### Introduction

Life after cancer has become a reality for many young women. However, as some cancer treatments pose a threat to reproductive function, fertility preservation methods such as cryopreservation of ovarian tissue (OT) has gained attention. Although efficient cryopreservation protocols can be designed based on the cryoprotectant toxicity and freezing curves, the thawing procedure may interfere in the success of protocols using other cryoprotectants. Sheep serve as optimal model for studies in women OT cryopreservation, with 1,2-propanediol (PrOH) as a frequently used cryoprotectant for human purposes. Aim of the present study was to evaluate the morphology and ultra-structure of preantral follicles and ovarian stroma of the PrOH protocol after applying three different thawing protocols. As a positive control, an EG standard freezing/thawing procedure was applied.

### Material and Methods

Ovine OT fragments were cryopreserved according to a slow-freezing protocol using 1.5 M EG or 1.5 M PrOH. EG-frozen fragments were thawed at 37°C, and cryoprotectant removed in 3-step washes of 5 min each. PrOH thawing protocols varied with respect to temperature (37, 30 or 4 degrees). Thereafter, OT was prepared for light and transmission electron microscopic (TEM) analysis. Preantral follicles and ovarian stroma were evaluated. Data was statistically evaluated by using ANOVA and Tukey's multiple comparison test with  $P < 0.05$ .

### Result

In total, 545 preantral follicles were analysed. Cryopreservation of OT in presence of EG maintained the percentages of morphologically normal preantral follicles similar to control values (72%). Independently of the thawing temperature, OT cryopreservation in presence of PrOH did significantly reduce the percentages of normal follicles when compared to control or with those cryopreserved in presence of EG. Additionally, irreversible stroma damage was observed after OT freezing in presence of PrOH. Although the use of PrOH did reduce the percentages of normal follicles, thawing and removal of cryoprotectant at 30 degrees was significantly less deleterious than thawing at 37 or 4 degrees. All observations were confirmed by ultrastructural analysis.

### Conclusion

The use of PrOH as cryoprotectant may bring stromal and follicular deleterious effects. Although thawing at 30 degrees may minimize this effect, use of other cryoprotective agents, e.g. EG, is indicated in order to provide a safe fertility preservation method in women.



## MINIMAL RESIDUAL DISEASE IN CRYOPRESERVED OVARIAN CORTEX FROM PATIENTS WITH LEUKAEMIA

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### Introduction

Cryopreservation and autotransplantation of ovarian tissue in cancer patients has been justified when the patients had solid tumours and localized disease, as the risk of malignant contamination of the ovarian tissue in these cases was considered low. However in the case of leukaemia, which is a disease of the blood and hence is ubiquitous, the risk of contamination is higher and there is casuistic evidence that malignant cells are actually present in the cryopreserved ovarian cortex. The aim of this study was to systematically search for malignant cells in cryopreserved ovarian cortex from Danish women with leukaemia.

### Materials and Methods

Of the 37 (7 deceased) patients in our program diagnosed with leukemia (AML (Acute Myeloid Leukemia), ALL (Acute Lymphoblastic Leukemia), JMML (Juvenile Myelomonocytic Leukemia and CML (Chronic Myeloid Leukemia)) 26 (87%) agreed to participate after receiving written and oral information.

Results of immunohistochemical and genetic examinations of the blood and bone marrow of the individual patients during active disease were used to guide the investigation of residual malignant cells in the cryopreserved ovarian tissue. In eight cases, a specific chromosomal abnormality could be used for detection of malignant cells by polymerase chain reaction (PCR).

### Results

Of the eight patients with known chromosomal abnormalities in the malignant cells, six patients (75%) had evidence of minimal residual disease by PCR: Four CML-patients, one patient with ALL and one patient with AML. In one case of CML and one case of ALL, PCR did not reveal malignant cells. Histology and immunohistochemistry did not reveal malignant cells in neither the six PCR-positive patients, nor in the remaining 20 patients.

### Conclusion

These In 75% of the cases, where a positive control was available, malignant cells were found in the cryopreserved ovarian cortex. The viability and malignant potential of these cells remains to be disclosed. For the time being however, re-implantation of tissue to leukaemia patients cannot be recommended



# OC13

## From the analysis on 5571 autopsy findings of females under the age of 40 in Japan **ACTIVELY PRESERVE FERTILITY, BUT AUTOTRANSPLANT CAUTIOUSLY**

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### **Introduction**

By July 2009, six babies had been born following ovarian cryopreservation and reimplantation in patients with Hodgkin's lymphoma, non-Hodgkin's lymphoma, Ewing's sarcoma, and sickle cell anaemia. Ovarian cryopreservation and autotransplantation could be of potential value for preservation of fertility in cancer patients; however, minimal residual disease in grafts also poses serious clinical problems in the autotransplantation of stored tissues. The scientific determination of indications is considered very important.

### **Material and Methods**

We examined the percentages of ovarian metastasis in 5571 autopsy files of females under the age under 40 with malignant diseases by reviewing the national autopsy files in Japan from 1981 to 2005.

### **Results**

Total ovarian metastasis was detected in 22.4% (1250/5571) of all the patients with malignancies. In our data, 4.3% (5/115) of patients with Hodgkin's lymphoma and 9.8% (5/51) of patients with non-Hodgkin's lymphoma had ovarian metastasis.

### **Conclusions**

It is strongly suggested that ovarian tissue should be actively cryopreserved for fertility preservation, but we should autotransplant stored tissue with much caution until we establish reliable methods to detect minimal residual disease in grafts in a precise and reproducible manner.

**Keywords:** Malignancy, ovarian cryopreservation, autotransplantation, fertility preservation.

## GLUCOSE/LACTATE METABOLISM AS A GENERAL MARKER FOR OVARIAN TISSUE SURVIVAL AFTER CRYOPRESERVATION OF AN INTACT OVARY

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### **Introduction**

Transplantation of cryopreserved intact ovaries is an option for restoring fertility after sterilizing cancer therapy. Obviously, rigorous optimisation of freeze- and thaw-protocols is a prerequisite for this approach. Thus far, studies have focused mainly on preserving the primordial/primary follicle pool, that is required for restoring fertility after auto-transplantation. However, the optimal survival of stromal tissue, that makes up more than 95% percent of the ovary, should be assured as well. We determined glucose consumption and lactate production as possible quantitative markers for the survival of ovarian tissue before and after cryopreservation.

### **Materials and Methods**

Tissue fragments (cortex, subcortex and medulla) derived from fresh and cryopreserved bovine ovaries were cultured for 4 or 7 days. Glucose consumption and lactate production were determined in culture supernatants, on a conventional blood analyzer.

### **Results**

Our data show that glucose and lactate levels could be readily and reproducibly measured in culture supernatants, with minimal inter-ovary variation. When freezing intact ovaries without any cryoprotectants, only low levels of glucose/lactate metabolism were observed, indicating limited tissue survival. When the ovary was submerged in medium containing DMSO prior to freezing, cortical fragments showed glucose/lactate metabolism levels identical to those observed with fresh tissue fragments. In contrast, subcortex and medulla were not protected. Preliminary experiments indicate limited protection of subcortex and medulla after perfusing the ovary with DMSO prior to freezing.

### **Conclusions**

Our experiments indicate that glucose/lactate metabolism is an indicator of the amount of cryodamage sustained by the entire ovarian tissue mass. Therefore, quantitative analysis of glucose/lactate metabolism is a relatively simple and reproducible method for evaluating cryodamage. As a consequence of the high reproducibility of our assay, optimizing cryopreservation protocols for intact ovaries will require relatively few experiments.

**Keywords:** Intact ovary, cryopreservation, glucose/lactate metabolism.

# OC15

## WHICH ARE THE IDEAL DONOR AND RECIPIENT VESSELS FOR A WHOLE OVARIAN TRANSPLANTATION ?

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### Introduction

Our study was aimed to compare deep circumflex iliac (DCI) and deep inferior epigastric (DIE) pedicles as potential recipient vessels for a whole ovarian microvascular transplantation (WOT).

### Material and Methods

Anatomical dissections were performed on 10 fresh human female cadavers. In 3 of them, vascular injections were carried out with red or bleu colored latex or Altufix P10, in order to emphasize arteries and veins and to compare, after dissection, the morphological characteristics of the three pedicles under study. Fourteen ovaries were harvested on their gonadic (G) vascular pedicle, together with the homolateral DCI and DIE pedicles. Histological analysis was then performed on serial sections performed through all these pedicles in order to measure the diameter of the vessels at regular intervals along their whole length. Calibre values were then compared between donor (DCI or DIE) and recipient (G) pedicles, aiming to determine the optimal size match (1:1 to 1:1,3) between them. Anatomical size match is indeed one of the most prominent technical factor influencing the success rate of any microvascular procedure in tissue transplantation.

### Results

Vascular injections allowed to highlight a very tortuous appearance of the gonadic artery. This arterial morphology contrasted with the venous system which always included 2 or 3 straight veins, one of them usually being wider than the others. We assessed the distance on the lombo-ovarian pedicle where the numerous gonadic vessels converged into a wider artery and vein. This critical point was located in all specimens at about 5 cm from the ovary. Individually, an optimal size match existed between gonadic and DCI arteries and veins sections among 13 out of 14 gonadic pedicles. Finally, we determined the average vascular caliber of the gonadic donor vessels and both potential recipient pedicles. On the gonadic pedicle, between 3 to 6 cm from the ovary, the artery had a constant diameter of about 1 millimeter (SD: 0,2). Mean diameter of the DCI artery was 1,25 mm (SD : 0,1), while this value raised up to 1,51 mm (SD : 0,17), for the DIE artery. Similarly, the medium transverse section of the gonadic vein measured 1,20 mm, closely comparable to the DCI vein diameter which was 1,18 mm.

### Conclusions

This morphological study demonstrates that a safe microsurgical whole ovarian transplantation is feasible if the gonadic pedicle is harvested with a minimal length of 5 cm from the ovary. At this point, the pedicle contains indeed at least one artery and one dominant vein with an average section of 1 mm. Furthermore, the DCI pedicle seems to be show the best size match with the ovarian vessels in order to perform a reliable to a termino-terminal microvascular anastomosis. Additionally, rotation of the DCI pedicle in the pelvis would allow an orthotopic ovarian transplantation. Nevertheless, the DIE pedicle, although somewhat larger, may be an alternative donor site for recipient vessels if the harvest of the DCI pedicle fails or functionally delivers low blood flow vessels.

**Keywords:** Deep circumflex iliac vessels, Ovarian harvesting, Transplantation, Vascular anastomosis, Fertility preservation.

**Topic:** Whole ovary transplantation or artificial ovary: from animal studies to human.

## A DECADE OF EXPERIENCE WITH FERTILITY PRESERVATION AT KAROLINSKA UNIVERSITY HOSPITAL

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### **Introduction**

The fertility preservation program was created in 1998 at the Fertility Unit of Karolinska University Hospital Huddinge. Sperm cryopreservation has been carried out from 1973. Progress in assisted reproduction techniques and ongoing research protocols during these years have made possible to offer both established and experimental fertility preservation options to young patients and children facing gonadal failure.

### **Material and Methods**

All patients who consulted for fertility preservation from 1998 to August 2009 were identified from our clinical database. Indications for fertility preservation, patient groups and interventions are described.

### **Results**

In its early years, the program offered mainly established fertility preservation methods as sperm banking and embryo cryopreservation for adult patients. At present, hundreds of patients had benefited from those methods. The program evolved then rapidly to offer oocyte cryopreservation to single and younger women and ovarian tissue cryopreservation to adult women and both pubertal and pre-pubertal girls. Initially, slow-freezing techniques for embryos, oocytes and ovarian tissue (Hreinsson *et al.* 2003) were used. In 2005 vitrification techniques started to be used systematically for cryopreservation of oocytes and in the most recent years they have been further developed to freeze ovarian and testicular tissue with promising results (Keros *et al.* 2005, 2007 and 2009). We have published several scientific articles from the programme. In addition, we have been developing ovarian follicle maturation methods. At present 170 female patients have frozen ovarian tissue at our unit but only three patients have returned for autotransplantation. 47 out of 67 women counseled to oocyte cryopreservation have frozen oocytes in storage. A pilot experimental testicular tissue freezing protocol, which has included 16 pre-pubertal boys is ongoing. Indications for fertility preservation have extended from malignant diseases to chronic and genetic conditions during this decade. The largest subgroup included girls with Turner's syndrome (Borgström *et al.* 2009). Positive prognostic factors in this subgroup were any sign of spontaneous puberty and mosaicism.

### **Conclusions**

Fertility preservation options include established techniques and experimental methods. In the last ten years indications for fertility preservation have extended to chronic benign conditions and to patients who are genetically predisposed to premature gonadal failure.

**Topic:** Cryopreservation and transplantation of ovarian tissue.

# OC17

## HETEROTOPIC AUTOTRANSPLANTATION OF OVARIAN CORTEX IN CYNOMOLGUS MONKEYS

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### Introduction

Orthotopic autotransplantation of ovarian cortex has advantages such as easy collection of ova and the possibility of spontaneous pregnancy. Although children have been born after successful orthotopic autotransplantation into the residual ovaries, some patients cannot undergo this procedure such as those who need bilateral ovariectomy or pelvic radiation therapy, therefore it is still necessary to investigate suitable heterotopic autotransplantation sites. The present study was performed in primates (cynomolgus monkeys) with the objective of determining the optimum site for heterotopic autotransplantation of ovarian cortex to enhance the clinical application of this method.

### Materials and Methods

The retroperitoneal iliac fossa and omentum were selected as sites for heterotopic autotransplantation. Two cynomolgus monkeys were subjected to laparotomy under anesthesia. After resection of the bilateral adnexae, the ovaries were cut into 0.5 cm cubes that were transplanted. Blood levels of follicle-stimulating hormone, luteinizing hormone, estradiol, and progesterone were monitored, and monkeys with a regular estrus cycle underwent superovulation and egg collection.

### Results

In both monkeys studied, recovery of a regular estrus cycle was confirmed after heterotopic autotransplantation of ovarian tissue. MII phase ova were successfully collected from tissues transplanted into the retroperitoneal iliac fossa or omentum. Development to the early blastocyst stage was confirmed after microfertilization. Collection of ova was still successful at a long time (935 days) after transplantation.

### Conclusions

We established an appropriate method of heterotopic autotransplantation using ovarian cortex into the retroperitoneal iliac fossa or omentum in primates. Our findings may provide useful information for the clinical application of heterotopic autotransplantation in patients without residual ovaries.

## IN VITRO GROWTH OF ISOLATED FOLLICLES IN THREE DIMENSIONAL ALGINATE-COLLAGEN MATRIX

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### Introduction

Recently, many efforts have been done to help young patients to preserve their fertility through the storage of their own germ cells before chemo-and/or radiotherapy partially or completely destroys their follicular reserve. Several cryopreservation protocols have been developed for whole ovary or ovarian cortical strip cryopreservation. Unfortunately, these techniques are associated with the risk to cryopreserve also malignant cells. This risk can be avoided only if the follicles are isolated from the ovarian stroma. On the other hand, the extracellular matrix plays a key role for the development and the fully competence of follicles in vivo. The development of technologies that support the growth and maturation in vitro of oocytes from isolated follicles is attractive for both fertility preservation in oncological patients and human assisted reproduction. Herein we investigated the effectiveness of three dimensional matrix alginate and alginate + collagen to support the in vitro growth of encapsulated isolated primary and secondary follicles in bovine as animal model.

### Material and Methods

Bovine cortical ovarian tissue was dissected in small pieces of 0.5mm x 0.5mm x 1mm, digested with collagenase type 1A, 1mg/mL, and DNase, 0.2 mg/mL, at 37°C for 45 min. Single follicles were collected under the stereomicroscope and encapsulated in alginate 2% or alginate 2% + collagene IV 0,3 mg/mL. The encapsulated follicles were cultured in Medium 199 + 20% FCS in 5% CO<sub>2</sub> atmosphere at 38 °C for ten days and the follicles growth was measured daily with computer assisted image analyzer. The viability was assessed through fluorescent labelling with propidium iodide. Morphology was evaluated under Hoffman modulation contrast and transmission electron microscopy (TEM).

### Results

At the end of the culture period, the average growth and viability of follicles were 57.1% and 44% for 2% alginate and 70.3% and 71,4% for alginate 2% + collagene IV respectively. Moreover TEM analysis showed that the three dimensional follicle architecture was better preserved after alginate + collagen encapsulation.

### Conclusion

The encapsulation in a three dimensional matrix of alginate + collagen better supports the in vitro growth of isolated follicles in a bovine model. These data indicate that compounds of the extracellular matrix play a key role in the modulation of survival, growth and morphological organization of mammalian follicles in vitro and should be taken into account to improve the biotechnology for the in vitro growth of isolated human follicles.

# OC19

## MIXED ORIGIN OF NEOVESSELS AND HOST-GRAFT VASCULAR LINK-UP IN HUMAN OVARIAN XENOGRAPTS

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### Introduction

Ovarian graft behavior depends on the establishment of neovascularization. The aim of the present study was to characterize the angiogenic process leading to vascularization of human ovarian xenografts.

### Materials and Methods

Twenty-eight nude mice were intraperitoneally grafted with frozen-thawed human ovarian tissue obtained from 7 patients. Four time points were analyzed: days 3, 5, 9 and 21 after grafting. Host and graft vascularization were assessed by anti-mouse CD34/ anti-human CD34 double staining, followed by vascular morphometry. Double fluorescence in situ hybridization with species-specific probes, combined with immunostaining, confirmed the species origin of the cells. The endothelial cell proliferation index (EPI) and pericyte coverage index (PCI) of human vessels were assessed by human-specific CD34/Ki67 and human-specific CD34/ $\alpha$ -SMA double staining respectively. Non-grafted ovarian tissue was included as control. The Kruskal Wallis test and  $\chi^2$  test were applied for statistical analysis where appropriate.  $P < 0.05$  was considered statistically significant.

### Results

Murine neovessels appeared on day 3, had significantly increased by day 5 ( $p < 0.05$ ) and persisted until day 21. Human vessels persisted after grafting and a significant increase in human microvascular density was observed on day 21 ( $p < 0.05$ ). From day 5, chimeric vessels composed of both murine and human endothelial cells were observed. These structures persisted until day 21. Human angiogenesis was confirmed by a significant increase in the human EPI on days 5 and 9 (12.8% -13.9%) compared to fresh tissue (1.2%,  $p < 0.001$ ), returning to basal levels by day 21 (1.7%). The PCI of human vessels was shown to have significantly increased on day 9 (29.7 %) and day 21 (50.3 %) compared to fresh ovarian tissue (13.2 %,  $p < 0.001$ ).

### Conclusions

Our results suggest that the mechanism of ovarian graft revascularization is a combination of inosculation (vascular link-up between graft and host vessels) and host and graft angiogenesis. Increased pericyte coverage could be a result of the ovarian graft adapting to the ischemia-reperfusion damage to promote a better blood supply.

**Keywords:** Human ovarian xenotransplantation, angiogenesis, mixed vessels, pericyte.

**Topic choice:** Cryopreservation and transplantation of ovarian tissue. From ovarian tissue to isolated follicles and whole ovary preservation.

## EFFECTS OF IONIZING RADIATION ON OVULATION RATE AND OOCYTE MORPHOLOGY IN MOUSE

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This study aimed to investigate the *in vivo* effects of ionizing radiation on maturation ability and radiosensitivity of oocytes enclosed in preantral and antral follicles, as a result of spontaneous or exogenously stimulated ovulation.

Experiments were performed in 6-8 weeks age, Balb/c female mice. Mice received single dose, total body 7.2 Gy gamma radiation at the diestrous-proestrous transition period. Irradiated animals were sacrificed in two subsequent estrous stages following irradiation, corresponding to spontaneous ovulation time. To analyse the ovulation function of the exposed ovaries, irradiated animals were superovulated in the first and second proestrous stages. Ovulated oocytes were collected and ovulation rates were obtained both of spontaneous or exogenously stimulated ovulations.

Spontaneous ovulation rate of the exposed antral follicles was significantly lower than sham-irradiated mice in the first estrous stage following irradiation ( $p<0.01$ ). Furthermore, most of the oocytes were M I stage. Oocyte morphology and ovulation rate of the exposed preantral follicles were similar with sham-irradiated group in the second estrous stage following irradiation. Morphological abnormalities were observed in the oocytes and polar body as well, both in the first and second estrous, after irradiation. Superovulation response of the all irradiated animals were lower than respective control animals. Ovarian response was significantly low particularly in the first ovulation time after irradiation ( $p<0.01$ ). On the other hand, 8 of 16 irradiated animals had longer cycle than pre-irradiation ( $p<0.05$ ).

In conclusion, these *in vivo* results indicate early signs of ionizing radiation-induced premature ovarian failure in mice. Also, in this study, estrous stages dependent applications may contribute to a better understanding of acute effects of ionizing radiation.

**Keywords:** Ionizing radiation, ovulation rate, oocyte, estrous cycle.

*This study received financial support from Trakya University Research Center (project no: 816), Edirne, Turkey.*



# OC21

## RECONSTITUTION OF SPERMATOGENESIS FROM TESTIS FAILURE AFTER TRANSPLANTATION OF GERMINAL CELLS FOR MALE FERTILITY PRESERVATION - A TRANSGENIC MOUSE MODEL

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### Introduction

Fertility preservation for prepubertal male is feasible by the reconstruction of spermatogenesis after spermatogonial stem cells transplantation.

### Material and Methods

#### A. Donor cell preparation

Cells for transplantation are obtained from FVB/N-Tg (*PoIII-luc*) Ltc transgenic mouse testes of mice 5 to 60 days after birth by a two-step enzymatic digestion protocol. The cell volume needed to inject both testes of a mouse ranges from 0,1 to 0,5 ml depending on the injection method. Cell concentrations of up to about  $300 \times 10^6$  cells per ml can be used. The cells are maintained at SCC until the time of loading into an injection pipette, usually 1 to 4 h.

#### B. Transplantation of SCC into recipient mouse

In this procedure, donor cells are harvested from the testes of fertile donor mice that express a reporter transgene, and a single cell suspension of the cells is microinjected into seminiferous tubules of FVB/NJNarl wild type recipient infertile adult mice after busulfan-induced testis failure.

#### C. In vivo tracking of grafted SCC by bioluminescence imaging

Reporter genes can be used to assay for the activity of a particular RNA Polymerase II promoter (*PoIII*) in each viable cell. Reporter gene products are luciferase (enzymes). The enzyme luciferase catalyzes a reaction with a luciferin to produce light which is quantified as quantum. The reporter gene is simply placed under the control of the target promoter (*PoIII*) and the reporter gene product's activity is quantitatively measured. After gonadal tissues or germ cells transplantation, each mouse was imaged every other day for 2 weeks and subsequent every weeks for 2 months. For imaging of mice with gonadal tissue transplants. Mice were anesthetized with isoflurane. A saturating concentration of the substrate D-luciferin was injected intraperitoneally (150 mg/kg). Bioluminescence was quantified the quantum by summing pixel intensities within equal area ROI.

#### D. Fertilization assay

Mating the recipient male to a wild-type female, then progeny is produced by nature fertility.

### Results

Live pup of FVB/N-Tg (*PoIII-luc*) Ltc transgenic mouse were born and imaged by bioluminescence after mating FVB/NJNarl female wild type and male wild type recipient 4-5 month after FVB/N-Tg (*PoIII-luc*) SCC transplantation.

### Conclusions

Spermatogonial stem-cell transplantation could be used to restore fertility in men following chemotherapy or radiation treatment. Development of techniques for the in vitro differentiation of spermatogonial stem cells to functional spermatozoa is a crucial step for the treatment of infertility or germline gene therapy.

**Keywords:** testis, spermatogonia, germinal epithelium, transplantation, bioluminescence.

## EFFECTIVE CRYOPRESERVATION OF PREPUBERTAL MOUSE TESTICULAR TISSUE BY VITRIFICATION

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### Introduction

Cryopreservation of testicular tissue from prepubertal boys with cancer has emerged as a promising approach to preserve fertility prior to gonadotoxic treatment. Our aim was to set up a vitrification protocol and compare this ice-free system with an established freezing protocol in a prepubertal murine model.

### Material and Methods

Testicular tissue from 6-day-old mice was cryopreserved by slow freezing (SF, n=10) [DMSO (0.7 mol/l) + sucrose (0.1 mol/l)] or vitrification (V, n=10) [DMSO (2.8 mol/l) and EG (2.8 mol/l)]. Lactate dehydrogenase (LDH) released from damaged cells was evaluated as a measure of viability. Apoptosis (caspase-3) and proliferation (Ki67) were assessed by IHC as indicators of tissue survival, after 1 day (D1) and 3 or 10 days (D3-D10) respectively of organotypic culture. Germ cell differentiation was evaluated by LM after D10 as a marker of functionality, while tubular cell density, diameter and integrity served as parameters of structural preservation throughout culture. Ultrastructural preservation of spermatogonial, Sertoli and Leydig cells was assessed by TEM on D10. Data were expressed as medians and percentiles [P25 and P75]. The Mann-Whitney U-test was employed for statistical analysis.  $P \leq 0.05$  was considered significant.

### Results

Before D1, specific and transitory damage caused by each treatment was identified. The percentage of released LDH revealed SF to have a more "necrotic" effect than V (54.6 [51.8, 58] vs 26.7 [23.7, 28.6]) ( $p \leq 0.01$ ), while the mean number of caspase-3-positive cells/tubule showed V to have a more "apoptotic" effect than SF (2.13 [1.17, 2.40] vs 0.07 [0.05, 0.09]) ( $p \leq 0.01$ ). On D1, both SF and V groups demonstrated a decrease in tubular cell number compared to fresh cultured controls (FR) ( $p \leq 0.01$ ), but these differences were not subsequently observed. On D3, a marked increase in proliferation was seen for both SF and V groups vs FR ( $p \leq 0.01$ ), which was not subsequently detected. Up to D10, no change in tubular diameter or integrity was noted between the SF, V and FR groups. On D10, pachytene spermatocytes were the most advanced germ cell stage observed and ultrastructure of testicular cells does not seem to be altered in FR as well as SF and V tissues.

### Conclusion

Using an original long-term organotypic culture model, we demonstrated that prepubertal mouse testicular structure and exocrine function up to the pachytene spermatocyte stage are preserved equally well by V and SF. Further investigation should be performed in vivo to assess the yield of this fast, cheap and convenient method in terms of complete germ cell maturation and sperm fertilization capacity before its implementation in human tissue cryopreservation.

**Keywords:** Prepubertal testicular tissue, vitrification, slow-freezing, organotypic culture.

# OC23

## AMIFOSTINE-DOXORUBICIN ASSOCIATION EFFECTS ON PREPUBERTAL RAT TESTES: LONG TERM DAMAGE ON SPERM DNA INTEGRITY AND EARLY EMBRYO DEVELOPMENTAL DELAY

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### Introduction

Amifostine have been applied to reduce unwanted sequelae from toxicities that can occur after chemotherapy protocols, including doxorubicin-induced cardiomyopathy. Over the past 20 years, increasingly survival rates have been reported after neoplastic malignancies occurrence during childhood. Doxorubicin, a potent anticancer anthracycline that is widely used to combat childhood leukemia, is known to produce spermatogenic damage even in low doses. Although some studies have suggested that amifostine does not confer protection to doxorubicin-induced testicular damage, schedules including this cytoprotector have yet been applied to reduce other chemotherapy toxicities in children.

### Methods

Prepubertal rat testes (30-day-old) were analyzed 64 days after treatments, by assessing some microscopic morphometric parameters and sperm structure integrity (SCSA); the fertility status was evaluated during adulthood (from 100 to 130-day-old). Forty-eight male prepubertal rats were equally divided into 4 groups: Doxorubicin (5mg/kg), Amifostine (400mg/kg), Amifostine/Doxorubicin (amifostine 15 minutes before doxorubicin) and Sham Control (0.9% saline solution).

### Results

Adult rats showed diminution of seminiferous epithelium height compared to Control, Amifostine and Amifostine/Doxorubicin group. However, reduction of sperm concentration, fetus implants in utero, fertility index and early embryo developmental delay were observed in both doxorubicin-treated groups, only when compared to Control and Amifostine groups. Conversely, an increase of sperm chromatin fragmentation, as assessed by SCSA, as well as blastomere fragmentation in two different moments of preimplantation embryo development were seen in both doxorubicin-treated group. The rats from Amifostine group also presented some bad effects on fertility, however, only early embryo fragmentation number was statistically increased when compared to Control group.

### Conclusions

These results suggest that although amifostine seemed to promote a reduction of doxorubicin-induced toxicity on the seminiferous epithelium of prepubertal rats, their reproductive success can be compromised, as a consequence of damaged sperm DNA and early embryo loss, after using these two drugs. Further investigation on the heritable effects produced by damaged sperm DNA on its progeny future must be a concern and should be carried out.

**Keywords:** amifostine; doxorubicin; male fertility; prepubertal rat; sperm integrity; embryo.

## HUMAN MEIOTIC SPINDLE ALTERATIONS FOLLOWING SLOW-COOL CRYOPRESERVATION

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### Introduction

Cryopreservation of human oocytes permits detailed investigations into meiotic spindle dynamics. Here we evaluated the dynamic instability of spindle microtubules under cryologic conditions and their reassembly after warming to gain further insight into meiotic spindle dynamics. Meiotic spindles from slow cooled human oocytes thawed and cultured for 0, 1, 2 and 3hrs were processed for confocal microscopy to analyse meiotic spindle organization and chromosome alignment.

### Materials and Methods

Oocytes were obtained from consenting IVF patients. Oocyte cryopreservation involved exposure to 1.5 mol/l propanediol and 0.3 mol/l sucrose. After thawing, cryoprotectant dilution was performed in the presence of 0.3 mol/l sucrose and decreasing concentrations of propanediol. Oocytes were fixed immediately after thawing (time 0; n=22) or following 1 (n=35), 2 (n=19) or 3 (n=22) hrs post thaw culture and labeled for total tubulin and DNA. Single oocytes were analysed using a Zeiss LSM Pascal confocal imaging system. Spindles were classified according to shape, length, polar constriction and chromosomes position.

### Results

Chilling of MII oocytes is known to depolymerise microtubules within the meiotic spindle. Immediately after thawing (time 0), no oocytes displayed a bipolar spindles with aligned chromosomes, where as at 1hr post-thaw, 71.4% of oocytes had recovered bipolarity and chromosomal alignment. Following two and three hours of culture the incidence of bipolar spindles with aligned chromosomes was significantly reduced compared to fresh controls (31.6% and 22.7% respectively). A significant 30.7% decrease in spindle tubulin volume was seen in all oocytes following freezing. In addition to a 13.8% increase in pole-pole length in spindles with mis-aligned chromosomes a positive correlation between the number of displaced chromosomes and spindle length was witnessed in thawed oocytes.

### Conclusions

This study is the first of its kind to trace the biomechanics of human oocyte meiotic spindles following cryopreservation. Directly following thawing disorganisation of the meiotic spindle is rapidly corrected. Oocytes cultured for 1hr post-thaw displayed an ability to reform bipolar spindles that precedes chromosomal alignment along the metaphase plate. We have shown considerable alterations in the physical properties of the spindle following freezing beyond the formation of a bipolar spindle. This study has allowed us to set a baseline for human meiotic spindle dynamics that should be useful in optimizing protocols for cryopreserved human oocytes. Supported by K-INBRE, Hall family Foundation and the ESHE Fund.

# OC25

## ULTRA-RAPID VITRIFICATION SUPPORTED FOLLICLE MORPHOLOGIES OF CYNOMOLGUS MONKEYS AFTER FREEZING COMPARED TO CONVENTIONAL VITRIFICATION AND SLOW FREEZING

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### Introduction

Cryopreservation of preantral follicles in ovarian tissues has been expected to be an effective measure for preserving young women who need to undergo cytotoxic therapy. However, cryopreservation protocol has not yet been well-established. In this study, we assessed the effects of ultra-rapid vitrification (URV), vitrification and slow freezing (SF) on morphologies of cynomolgus-ovarian tissues after freezing.

### Materials and Methods

Ovarian-cortical sections of 7-cynomolgus monkeys were randomly allocated to control and 3 freezing groups. Vitrification solution containing 5.64 M ethyleneglycol (EG) + 5% (w/v) PVP + 0.5 M sucrose was used for vitrification. For URV, ovarian sections loaded on Cryosupport which consist of 3 fine needles were immersed in liquid nitrogen directly. For conventional vitrification, the sections were packaged in 0.25 mL straw and the straws were immersed in LN<sub>2</sub>. The cryoprotectants used in slow freezing were 1.5 M propanediol and 0.1 M sucrose. After warming, morphologies of follicles were analyzed using light microscopy and transmission electron microscopy.

### Results

The proportion of morphologically-normal follicles vitrified ultra-rapidly (93%) was higher ( $P<0.05$ ) than those of follicles vitrified in a straw (63%) and frozen by slow freezing (59%). When the ovarian cortex was vitrified ultra-rapidly, the surface ratio of lysosomes per oocyte cytoplasm (1.3%) was lower ( $P<0.05$ ) than that in slow freezing (2.6%) and similar to that in non-frozen ovaries (1.1%). In the case of conventional vitrification, the surface ratio of lysosomes per oocyte cytoplasm (2.1%) was increased ( $P<0.05$ ) compared with non-frozen ovaries (1.1%).

### Conclusions

Results of the present study indicated that URV can support the morphology of frozen preantral follicles and oocytes compared with conventional vitrification and slow freezing.

**Keywords:** Ultra-rapid vitrification, autophagosome, subcellular organelle, mitochondria, slow freezing.

**Topic choice:** Cryopreservation of ovarian tissue.

## EFFICIENCY OF OOCYTE CRYOPRESERVATION

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### Introduction

Since the legislation on Assisted Reproduction Technology passed in Italy in March 2004, limiting to 3 the maximum number of eggs to be inseminated and banning embryo freezing, cryopreservation of spare oocytes entered the clinical practice. Like other procedure, oocyte cryopreservation can be affected by several factors related to patient's characteristics or to the technique itself. Here we present an analysis of some factors affecting the efficiency of oocyte cryopreservation.

### Material and Methods

Between March 2004 and April 2009, a total of 5543 spare oocytes were cryopreserved in 831 cycles using slow freezing with 1.5M propandiol in 0.3M sucrose. Each oocyte was individually stored. Thawing has been performed in 735 HRT cycles. According to the law, a maximum of three eggs were inseminated. Insemination was always executed by ICSI. The results were analyzed dividing the cycles according to patient age ( $\leq 29$ , 30-34, 35-40,  $> 40$  yrs) and number of frozen oocytes ( $\leq 3$ , 4-6, 7-9,  $\geq 10$ ). The end points considered were: transferred cycles/thawing, clinical Pregnancy Rate (PR) per transfer, Implantation Rate (IR), Ongoing Pregnancy Rate (OPR).

### Results

**Cumulative results.** In the 735 thawed cycles, the survival and fertilization rates were respectively 69% and 74%. The transferred cycles were 604 (82% ), resulting in 15,2% PR (92/604), 9.6% IR (115/1199) and 9.1% OPR (67/735).

**Patient age.** Survival, fertilization and cleavage rates were similar in all age groups. No differences were found in the number of transferred cycles, while PR and OPR showed a continuous decline with age increasing ( PR from 24% to 10% and OPR from 15% to 5%). This is always true in ART, but with cryopreserved oocytes the age factor started to significantly affect results at very early stages, as soon patient was gather than 30 years.

**Number of frozen eggs.** The survival rate was not affected by this variable, but cycles with  $\leq 3$  frozen eggs had significantly lower chance of transferring embryos compared to other groups ( 65% vs 87%,  $p < 0.001$  ). In the transferred cycles, PR and IR were not influenced by the number of frozen eggs, while OPR was significantly higher in patients with more than 10 fozen eggs than the other categories (14% vs 7.5%,  $p < 0.025$ ).

### Conclusions

At the present time, oocyte cryopreservation is beneficial only in optimal conditions: young patients, high number and high quality oocytes. Given the efficacy of this technique, we can consider oocyte cryopreservation effective for preserving fertility in young women.

# OC27

## CLINICAL GRADE VITRIFICATION OF HUMAN OVARIAN TISSUE

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### Introduction

Ovarian tissue cryopreservation helps young women who face premature ovarian failure, due to chemotherapy, radiotherapy or genetic causes.

Orthotopic transplantation of human frozen-thawed ovarian tissue has already resulted in birth of several infants. Maturation of ovarian follicles in vitro is also progressing. To optimise the cryopreservation of ovarian tissue we carried out a study comparing slow rate freezing and vitrification (Keros *et al.* 2009). Now we have developed a closed system for a clinically safe vitrification of human ovarian tissue.

The aim of the study was to evaluate the possibility to perform vitrification according to good manufacturing practice quality system, GMP.

### Material and Methods

Ovarian tissues were donated by women, aged 28-43 years, undergoing Caesarean sections.

The cryoprotectants used for vitrification were a combination of dimethyl sulphoxide (DMSO), PrOH, EG and polyvinylpyrrolidone (PVP) established recently in our laboratory (Keros *et al.* 2009). Nunc Cryo Tube Vials with silicon ring were used as device for vitrification.

The morphology of the pre-antral follicles, granulosa cells and ovarian stroma after vitrification was analysed by light microscopy in semi-thin sections.

### Results

The morphology of the vitrified tissue in cryotubes did not differ from that obtained earlier using a system in which the tissue pieces were in contact with liquid nitrogen.

We observed a good preservation with intact morphology of the stroma and the neighboring stromal cells. A nucleus with no shrinkage of nuclear membranes was found. Granulosa cells had contact with a uniform basement membrane, neighboring granulosa cells and oocytes.

### Conclusions

Vitrification is an advanced alternative for cryopreservation of ovarian tissue. Based on the results from this study the procedure can be carried out according to good manufacturing practice quality system. No direct contact with liquid nitrogen is necessary to achieve good preservation of the vitrified tissues. The tissues can be safely stored for the future, in closed cryotubes immersed in liquid nitrogen.

Transmission electron microscopy will be done and the results will be presented.



## VITRIFIED HUMAN OVARIES HARBOR LESS PRIMORDIAL FOLLICLES AND PRODUCE LESS ANTIMULLERIAN HORMONE (AMH) THAN SLOW FROZEN OVARIES

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### Introduction

Slow freezing is the most commonly used cryopreservation method for human ovarian tissue. Even though vitrification is being widely used in oocyte and embryo freezing, data on its applicability in human ovarian tissue freezing is limited. Therefore we aimed in this study to compare slow freezing and vitrification for human ovarian tissue freezing.

### Design

A controlled in vitro study

### Materials and Methods

1.0 x 0.5 cm samples of healthy ovarian cortical tissue were obtained from 15 patients (mean age  $\pm$  [SE]  $32.2 \pm 0.8$ ) undergoing ovarian cystectomy for benign indications. Informed consent was obtained from all subjects. After dividing the tissue into 6 equal pieces two pieces were allocated into three groups (fresh, slow freezing and vitrification). A DMSO based freezing solution and a propanediol and ethylene glycol based one were used for slow freezing and vitrification, respectively. Frozen samples were thawed 24 hrs later. One piece from each group was fixed for follicle count and the other one was cultured in alpha-MEM medium with 100 mIU/mL recombinant FSH for three days. Half of the culture medium was refreshed daily and assayed for estradiol (E2 pg/mL) by DPC and AMH (ng/mL) by ELISA. Ovarian histology, primordial follicle counts and in vitro estradiol and AMH levels were compared among fresh, slow-frozen and vitrified ovarian samples.

### Results

The structure of the follicles and the stroma were preserved better in slow frozen samples compared to vitrified ones. Compared to fresh and slow frozen samples vitrified ovaries contained significantly less primordial follicles ( $0.97 \pm 0.1$  vs.  $1.95 \pm 0.2$   $p < 0.05$ ;  $0.97 \pm 0.01$  vs.  $1.27 \pm 0.1$   $p < 0.05$ ; respectively); and produced significantly less AMH in vitro ( $0.07 \pm 0.02$  vs.  $0.47 \pm 0.2$   $p < 0.05$ ;  $0.07 \pm 0.02$  vs.  $0.21 \pm 0.1$   $p < 0.05$ ; respectively). AMH production between fresh and slow frozen samples were comparable ( $0.47 \pm 0.2$  vs.  $0.21 \pm 0.1$   $p > 0.05$ ). E2 production from slow frozen and vitrified ovaries were similar ( $1578 \pm 270$  vs.  $2120 \pm 303$   $p > 0.05$ ), but they were significantly lower than fresh cultured samples ( $1578 \pm 270$  vs.  $3706 \pm 943$   $p < 0.05$  and  $2120 \pm 303$  vs.  $3706 \pm 943$   $p < 0.05$ ).

### Conclusions

Perturbed ovarian histology, more primordial follicle loss and impaired AMH production after vitrification may suggest that it may not be a suitable cryopreservation method for human ovarian tissue banking.





**POSTER  
PRESENTATIONS  
(P1 - P34)**



P1

## EXPRESSION OF GENES HEAT SHOCK PROTEIN 70 AND MNSOD DURING VITRIFICATION OF MOUSE MII OOCYTE FOLLOWING CRYOTOP METHOD

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**Introduction:** The aim of this study was to investigate the effect of two vitrification protocols on mouse MII oocytes and evaluate its effects on expression of genes heat shock protein 70 and MnSOD.

**Methods and materials:** The oocytes were collected and vitrified with 10 % (v/v) EG + 10 % (v/v) DMSO + 0.5M sucrose in group A(VSI) and 14.5% (v/v) EG + 14.5% PROH +0.5M sucrose in group B(VSII), respectively. For thawing vitrified oocytes were put into 1M sucrose for 1 min and two diluted solution for 3 min. After thawing the oocytes were fertilized and cultured in vitro to develop in two cells. Survival rate and two cell embryos were evaluated and gene expression (HSP 70, MnSOD and  $\beta$  actin) was examined by reverse transcription polymerase chain reaction (RT\_PCR).

**Results:** Survival rate of mouse oocytes after warming was lower in two groups (VSI:  $91.2\% \pm 1.7$ , VSII:  $89.2\% \pm 1.5$ ) compared to control ( $100.0\% \pm 0.01$ ). The rate of IVF were significantly ( $p \leq 0.05$ ) reduced in vitrified-warmed (VSI:  $39\% \pm 5.8$ ; VSII:  $34\% \pm 5.7$ ) oocytes compared to control ( $88.36\% \pm 2.3$ ). We analyzed the expression of specific gene such as Hsp70, and MnSOD. The abundance of mRNA was generally reduced in oocytes related to vitrification procedures, but expression of MnSOD increased in vitrified-warmed compared to control oocytes. We also detected Hsp70 only in control and VSI group.

**Conclusion:** Our results demonstrated that increasing HSP 70 activity in VSI might have to the good developmental competence of vitrified oocyte.

**Keywords:** Cryotop, gene expression, oocyte, vitrification.

P2

## SLOW COOLING PROTOCOL: RESULTS IN OOCYTES CRYOPRESERVATION

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**Objective:** The study evaluated success rates derived from the use of cryopreserved oocytes by using a slow freezing / rapid thawing protocol.

**Materials and Methods:** Since January 2007, a total of 126 oocyte freezing cycles were performed. No restriction in the selection of the patients. According to morphologic features, good quality oocytes were frozen using slow cooling protocol which make use of PrOH (1.5M) and sucrose (0.2M) as cryoprotectants. Oocytes cryopreservation was carried out between 1 and 2 hours from oocytes retrieval. Oocytes were thawed by a rapid thawing protocol, and cryoprotectants were removed by step-wise dilutions. After 150-180 minutes, survived oocytes were microinjected.

**Results:** 52 thawing cycles were performed and a total of 289 oocytes were thawed.

The survival rate was 63%. Fertilization and cleavage rates were 66.2% and 91.3% respectively. A total of 91 embryos were transferred in 44 embryo transfers.

11 pregnancies were achieved and the pregnancy rate per transfer was 25%.

14 gestational sacs were observed at ultrasound examination and the implantation rate was 15.4%. 3 patients aborted with a rate of 27.3%. 6 babies were born and 3 pregnancies, 2 singleton and 1 twin, were still ongoing.

**Conclusions:** Good results in IVF cycles performed using frozen / thawed oocytes, allow to consider oocytes cryopresevation in addition to conventional IVF. Oocyte cryopreservation represents a strategy to improve chances of success per patients, without recurring to ovarian stimulation; furthermore it represents an efficient methodology used to preserve female fertility for patients at risk of loosing ovarian function.

**Keywords:** cryopreservation, oocytes, slow freezing.

**Topic choice:** oocytes cryopreservation.

P3

## OOCYTE RETRIEVAL AT THE TIME OF LAPAROTOMY FOR OOPHORECTOMY

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**Introduction:** We present two cases of egg retrieval at the time of laparotomy after controlled ovarian stimulation with subsequent fertilisation of oocytes. Both women had a diagnosis of carcinoma, one a borderline tumour of the ovary with previous unilateral oophorectomy, the other metastatic endometrial cancer requiring bilateral oophorectomy post hysterectomy. In both cases transvaginal oocyte retrieval risked pelvic spread of disease and was contraindicated. Although collection of immature oocytes and subsequent in vitro maturation after oophorectomy has previously been described this is the first report of mature oocyte collection prior to oophorectomy at the time of laparotomy.

**Materials and Methods:** An antagonist treatment cycle was utilised for both patients. FSH stimulation was commenced on day 2 of the menstrual cycle and LHRH antagonist on day 5 of FSH stimulation as per protocol. FSH and LHRH antagonist were continued until the day of hCG injection. Laparoscopic oocyte retrieval was timed for 36 hours post hCG injection. The abdomen was opened by a Pfannenstiel incision and the ovarie(s) isolated. Before occluding their blood supply oocyte aspiration was performed using a double channel needle with direct puncture of the follicles and flushing with warmed Hartmann's solution if required. The ovaries were examined post oophorectomy and aspirated to ensure all follicles had been punctured. An ICSI procedure was then undertaken to achieve fertilisation.

**Results:** On the day of hCG instruction patient DT had one follicle >17mm, patient TW 7 oocytes >17mm. Two oocytes were retrieved from DT of which one fertilised and a single two cell embryo was transferred two days after oocyte retrieval. Subsequently serum hCG fourteen days post oocyte retrieval was negative. Ten oocytes were retrieved from patient TW of which 6 fertilised including two oocytes which were retrieved post oophorectomy. All embryos were frozen at the PN stage for later use in a surrogate.

**Conclusions:** Controlled ovarian stimulation and subsequent oocyte retrieval at the time of laparotomy provides a novel method of fertility preservation in those women faced with oophorectomy and for whom transvaginal oocyte retrieval is contraindicated. Oocyte retrieval and fertilisation rates appear comparable with transvaginal oocyte retrieval.



# P4

## OVARIAN PROTECTION BY GnRH AGONIST FROM GONADOTOXIC CHEMOTHERAPY - A MOUSE ANIMAL MODEL

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**Introduction:** To demonstrate ovarian protective effect of GnRH agonist administered before gonadotoxic chemotherapy.

### Materials and methods:

**A. Experimental design** - 24 female sex mature FVB mice (8 weeks old) were used for this study and divided equally into four groups. The control group was prepared without administration of drugs. The Busulfan group underwent injection of Busulfan at dose of 50mg/kg and was sacrificed four weeks later for histological examination of ovary. GnRH agonist (Triptorelin) was injected subcutaneously in the Triptorelin (low dose, 3.8mg/kg) + Busulfan group and the 10Triptorelin (high dose, 38mg/kg) + Busulfan group, followed by injection of Busulfan four weeks later. They were also sacrificed another four weeks later as the Busulfan group. First we compared the total number of follicles and numbers of primordial, primary, secondary, and antral follicles between the Busulfan group and the control group to see the impact of chemotherapy on ovaries. Secondly, the protective efficacy of different dosages of GnRH agonist was evaluated by comparison of the numbers of follicles the Busulfan + Triptorelin groups with the Busulfan group.

**B. Histological analysis and follicle counting** - The excised ovaries were fixed in formalin and then embedded in paraffin. Six- $\mu$ m serial sections were taken with a microtome and stained by hematoxylin and eosin. The numbers of primordial, primary, secondary and antral follicles containing an oocyte were counted by an experienced examiner blind to condition of the animal.

**C. Statistical analysis** - Data were presented as mean  $\pm$  SEM and median. The significance was determined by the Tukey Method. The 95% confidence interval not including 0 meant the difference between those 2 groups was statistically significant. All the statistics was processed by STATA 8.0.

**Results:** Significant depletion of primordial, primary and secondary follicles was found in the Busulfan group compared with the control group with a statistical difference. We also observed obvious destruction of the ovarian tissue under the microscope. Both have demonstrated the gonadotoxicity of Busulfan. To examine if GnRH agonist could provide any protective effect on the ovaries, we used different dose of single Triptorelin injection four weeks before administration of Busulfan. In the Triptorelin + Busulfan group, larger primordial follicle count was found compared with Busulfan group without significant difference, and so was primary follicle count. In the 10Triptorelin + Busulfan group, the numbers of primordial follicles and primary follicles were significantly higher compared with the Busulfan group. Although secondary follicle count increased, the difference was not significant. Because of the pool of primordial follicles and primary follicles making up more than 90% of follicle population, ovarian reserve preservation has been demonstrated when Triptorelin was added. The reason why the data of the Triptorelin + Busulfan group was not statistically significant might attribute to insufficient dose of Triptorelin. A larger number of primordial follicles and primordial follicles were preserved in the 10Triptorelin + Busulfan group than in the Triptorelin + Busulfan group. If there is any dose scale for protective effect of GnRH agonist on the ovary need to be tested in the future.

**Conclusions:** Our animal study indicates GnRH agonist as an ovarian protection method against gonadotoxicity by chemotherapy. It also provides the first quantitative histological evidence suggesting a dose-dependent protective effect on the ovarian reserve. The number of primordial follicles is still the most direct marker for evaluating fertility and the effect of protection, but developing a more applicable surrogate is urgent. Based on the results of this study and the recent published literature, GnRH agonist co-treatment is not only a non-invasive strategy but a rational option for fertility preservation. It should be considered in all patients during their reproductive age before receiving chemotherapy.

**Keywords:** GnRH agonist, ovarian protection.

P5

## XENOTRANSPLANTATION OF HUMAN OVARIAN TISSUE TO NUDE MICE: COMPARISON BETWEEN FOUR GRAFTING SITES

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**Introduction:** Transplantation of human ovarian tissue to nude mice is a useful experimental model to assess ovarian function and developmental potential of ovarian follicles after grafting. However, there is currently no consensus in the literature as to the optimal grafting site. This study was designed to assess the impact of different ovarian tissue transplantation sites on the follicular pool and ovarian tissue integrity after short-term grafting.

**Material and methods:** Frozen-thawed ovarian tissue from eight patients was grafted for one or three weeks to the peritoneum, inside the ovarian bursa, under the skin, and into the muscle of sixteen nude mice. Assessment of follicular density and follicle classification were carried out by histological analysis. Proliferative activity was evidenced by immunostaining with anti-Ki-67 antibodies, and fibrotic areas were analyzed by morphometry on histological slides. Statistical analyses were performed using the Kruskal-Wallis test, Mann-Whitney test and chi-square test where appropriate.

**Results:** No significant differences in follicular density were evidenced between sites or days post-grafting. One week post-transplantation, the proportion of Ki-67-positive primordial follicles was higher (20-42%) than in controls (1.7%) ( $p < 0.05$ ), demonstrating follicular activation in all four sites. Three weeks post-grafting, activation was lower and most primordial follicles (34.1 to 66.9% of the follicle population, depending on the grafting site) were quiescent. Cryopreservation and grafting resulted in extensive fibrosis in the stroma. This fibrosis was significantly less pronounced in intramuscular grafts, representing 18.8% of the surface, versus 44.7-60.5% for other sites, after three weeks' grafting.

**Conclusions:** All four grafting sites equally supported early follicular growth and maintained a pool of quiescent follicles after short-term frozen-thawed human ovarian tissue transplantation. The extensive fibrosis observed does not appear to have a major impact on early follicle development, but its long-term effects must be investigated. The graft environment may be implicated in the preservation of the stroma, as suggested by a lower degree of fibrosis in the intramuscular site.

**Keywords:** Xenografting, ovarian tissue, follicular activation, transplantation site, stromal fibrosis.

**Topic choice:** Cryopreservation and transplantation of ovarian tissue.

P6

## SUPPRESSION OF FOLLICULAR DEVELOPMENT BETWEEN OVARIAN GRAFTS AFTER AUTO-TRANSPLANTATION AT DIFFERENT SITES IN HUMANS

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**Introduction:** Cancer treatment in young women has greatly enhanced their life expectancy, but these treatments often cause infertility due to a massive destruction of the ovarian reserve resulting in premature ovarian failure. One possibility to overcome sterility is to cryopreserve ovarian tissue before oncological treatment and later to transplant the tissue back into the patient. However, many unanswered questions remain regarding the development of follicles in the transplanted tissue as well as what will happen with the tissue when the hypergonadotropic condition is attained after the follicular pool is exhausted. In this study we histologically examined ovarian grafts at different transplantation sites after LH and FSH serum levels increased again.

**Material and methods:** Cryopreserved ovarian tissue was retransplanted orthotopically by laparoscopy in different peritoneal pockets after a period of cancer remission. One site was near the fallopian tube, the other in the pelvic wall. 11 months after transplantation, when several cycles had passed the grafted tissue was removed because LH and FSH serum levels increased to menopausal levels. The grafts were fixed in formalin. After routine paraffin embedding, the entire samples were serially sectioned and the number of follicles as well as the presence of atypical cell formation were examined.

**Results:** At one site of transplantation no follicles were found, however in the other site (near the fallopian tube) primordial as well as antral follicles were found. No atypical mitosis were found in any of the grafts. There was an abundance of blood vessels and a normal stromal tissue.

**Conclusions:** It is known that the transplantation of small grafts can only restore fertility for a short period of time. In this case it seems to be evident that the grafts at one transplantation site suppress the growth of follicles in the other transplantation site, since follicle growth started only after the reserve of primordial follicles was exhausted at the other site. The factors responsible for this growth inhibition are unknown and should be further investigated.

**Keywords:** Cryopreservation, ovarian tissue, fertility preservation, cancer.



P7

## IN-VITRO GROWTH OF LARGE VERSUS SMALL PREANTRAL FOLLICLES AND OOCYTE MATURATION FROM MOUSE OVARIES

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**Introduction:** The ability to develop mature oocytes yielded from immature preantral ovarian follicles is a key success in female gamete preservation. The purpose of this study was to describe the in-vitro growth (IVG) of preantral follicles and in-vitro maturation (IVM) of oocytes taken from mouse ovarian tissue.

**Material and Methods:** The preantral follicles were mechanically isolated from fresh ovaries of 7-weeks-old specific-pathogen-free, female (C57/BL/6xDBA/2) F1 mice (Japan SLC Inc. Shizuoka, Japan). Those preantral follicles were cultured on IVG medium for 8-11 days then transferred to IVM medium for 16-18 hours. The maturation stage of oocytes was evaluated after mechanical denudation.

**Results:** The preantral follicles in this study were grouped into large preantral follicles and small preantral follicles based on the growth and the expansion of granulosa cells. The antral follicles formation was found 85% in large follicles group and 33% in small follicles group. The growth of antral follicles was more likely to happen in the large preantral follicles group. This finding may be explained by the expansion of granulosa cells on day 2 IVG in large follicles which increased the probability of large follicles to develop into antral follicles. All antral follicles were matured into metaphase II (MII) oocytes (22%) and metaphase I (MI) oocytes (78%). There was no germinal vesicle oocyte found at the end of maturation process. This point showed potency of antral follicles as the yielded source of MII and MI oocytes.

**Conclusion:** Mature oocytes could be yielded from preantral mouse ovarian follicles. Large pre-antral follicles at the earlier day of IVG were more likely to become antral follicle compared to small preantral follicles.

P8

## THE OPTIMAL TIMING OF CRYOPROTECTANT AGENT EXPOSURE BASED ON MORPHOLOGICAL ALTERATION IN PRIMARY FOLLICLES FROM VITRIFIED SHEEP OVARIAN CORTEX

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**Introduction:** Vitrification of ovarian tissue has been known as the best method in female gamete cryopreservation. The optimal technique and timing of cryoprotectant agent (CPA) exposure in vitrification should result in no alteration or minimal alteration in order to ensure the viability of gametes after cryopreservation. This study is purposed to find the optimum duration of sheep ovarian cortex exposure to CPA based on the morphological alteration in primary follicles after vitrification.

**Material and Methods:** The ovaries from five months old sheep were collected at the slaughterhouse and transported to the laboratory within 2 hours of collection. The ovarian cortex fragments were divided into control group (no CPA exposure), 15 minutes CPA exposure, 30 minutes CPA exposure, 45 minutes CPA exposure and 60 minutes CPA exposure. The morphological alteration including disconnection among granulosa cells of primary follicles, oocytes cytoplasm, zona pellucida, and oocytes shape were analyzed.

**Result:** The percentage of normal follicles in those groups after vitrification were 0% in no CPA group, 58% in 15 minutes CPA, 95% in 30 minutes CPA, 1% in 45 minutes CPA, and 0% in 60 minutes CPA. The percentage of intact oocytes were 0% in no CPA group, 75% in 15 minutes CPA, 100% in 30 minutes CPA, 21.5% in 45 minutes CPA, and 12% in 60 minutes CPA. It indicated that the various timing with CPA exposure results in various outcome so that determining the best optimum timing of CPA was really important in vitrification. From that result, the best timing of CPA exposure before vitrification was 30 minutes.

**Conclusion:** The optimal timing of CPA exposure is influential in vitrification process. From this study 30 minutes CPA exposure gave the best result.



P9

## FERTILITY PRESERVATION IN A YOUNG WOMAN UNDERGOING CHEMOTHERAPY: A NEW APPROACH

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Chemotherapy in young patients with breast cancer usually causes premature ovarian failure and infertility. Fertility preservation in such cases is one of the main challenges for specialists in human reproduction. Oocyte vitrification have been proposed as a technique to preserve fertility in young women. Unfortunately, since breast cancer proliferation and dissemination can be triggered by estrogen, conventional ovarian stimulation regimens are considered by many oncologists to be contraindicated in these patients. We report here the possibility of a successful oocyte cryopreservation benefiting of the protocols usually prescribed by the oncologist for the treatment of breast carcinoma. A 31 year old patient (G0P0) with an intraductal breast carcinoma T1 N0 Grade 3 Stage I, positive for estrogen receptors (80 %)(p ER) , positive for progesterone receptors (20%) and positive for HER-2/neu underwent left superior quadrantectomy and sentinel nodule resection. Following surgery, and two weeks before starting with chemotherapy, in order to try to preserve her menstrual function by ovarian suppression she began the administration of Goserelin depot. Utilizing the "flare up" effect of the GnRh analogue she was monitored daily by transvaginal ultrasound (US) and at the third day, when the follicles reached 10 mm diameter, 5000 I.U of hCG were injected and 35 hours later US guided transvaginal oocyte retrieval was carried out: 6 immature oocytes were obtained. Three of them matured in vitro after 48 hours and vitrified. The adjuvant chemotherapy was: 4 cycles of Doxorubicin 60 mg./m<sup>2</sup> with Cyclophosphamide 600 mg./m<sup>2</sup> every 3 weeks followed by Paclitaxel 80 mg/m<sup>2</sup> weekly with concomitant administration of Trastuzumab, which was continued for one year. At the end of the administration of Paclitaxel and Goserelin the patient initiated Tamoxifen 20 mg/day for five months. Following the discontinuation of Tamoxifen the patient was monitored by US and two follicles of 15 mm were observed; after 35 hours of hCG 5000 UI administration two metaphase II oocytes were harvested and vitrified.

**Conclusion:** A young woman with breast carcinoma ( pER) who desired to preserve her fertility underwent two ovarian retrievals in order to store oocytes benefiting by the use of two drugs included in the oncological protocols: Goserelin and Tamoxifen. As a result, five mature oocytes have been cryopreserved for a potential future use. For many investigators the use GnRh analogues in parallel with chemotherapy in cancer patients has demonstrated a significant decrease in the rate of premature ovarian failure than in controls. In addition, in those patients with breast cancer the use of GnRh analogues was associated with less recurrence and improved survival rate. Tamoxifen is broadly used in premenopausal pER breast cancer patients. The possibilities to preserve fertility in women exposed to chemotherapy are limited, because none of the methods is ideal. Combination of several methods such as proposed in this report should be considered.

P10

## ASSESSMENT OF OVARIAN RESERVE AFTER RADIO-CHEMOTHERAPY TREATMENTS IN CANCER PATIENTS WHO UNDERWENT FERTILITY PRESERVATION

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**Introduction:** Chemo-radiotherapy treatments are potentially gonadotoxic, thus necessitating fertility preservation. This study was aimed to assess ovarian reserve after chemo-radiotherapy in cancer patients who underwent fertility preservation, in order to establish a "tailored" approach for preserving fertility.

**Materials and Methods:** A retrospective study including 63 cancer patients who had undergone fertility preservation procedures. Indications for treatment included: breast cancer (13 patients, 21%), hematological malignancies (27 patients, 43%), sarcoma (9 patients, 14%), colon cancer (5 patients, 8%), cervical cancer (5 patients, 8%) and miscellaneous cancers (4 patients, 6%). Mean age was 29+1 years. 71% had no children. 48% underwent OTCP (ovarian tissue cryopreservation), 62% were treated with GnRH analogs, 30% underwent IVF with embryos cryopreservation (of them 20% underwent also oocytes cryopreservation) and 10% underwent ovarian transposition. In 47% of patients a combination of several techniques was applied.

**Results:** 1year after chemo-radiotherapy breast cancer patients mean FSH level was 9.6+7 mIU/L; mean antral follicles count (AFC) 2.7+2 and mean Anti Mullerian Hormone (AMH) level 5.2+4 ng/ml. 12 pregnancies and 10 live births were achieved. Hematological malignancies patients mean FSH level was 36+24 mIU/L, mean AFC 5+3 and mean AMH 9+6 ng/dl. 8 pregnancies and 7 live births were achieved. Sarcoma patients mean FSH level was 14+7 mIU/L, mean AFC 1.8+1 and mean AMH 6+4 ng/dl. 3 pregnancies and 1 live birth were achieved. Colon cancer patients had normal ovarian reserve while cervical cancer patients showed premature ovarian failure parameters.

**Conclusions:** Following chemotherapy, breast cancer patients have preserved ovarian reserve; thus, procedures like OTCP seem to be unjustified, while sarcoma patients have low post treatment ovarian reserve, hence OTCP and other procedures are justified. Hematological malignancies patients show heterogeneous results with diminished ovarian reserve parameters but good recovery as expressed by a relatively high rate of pregnancies and live births. In this sub-group of patients OTCP seems to be unjustified and GnRH analog treatment should be considered.



P11

## OVARIAN TISSUE CRYOPRESERVATION (OTCP) FOR FERTILITY PRESERVATION IN CANCER PATIENTS - IS IT JUSTIFIED IN ADVANCED STAGES?

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**Introduction:** Ovarian tissue cryopreservation (OTCP) has been developed to sustain reproductive function of women who face sterilizing chemotherapy, radiotherapy or radical surgery. This study was aimed to assess the impact of advanced cancer stage among a cohort of cancer patients who underwent OTCP for fertility preservation.

**Materials & Methods:** Retrospective study including 54 cancer patients who underwent OTCP for fertility preservation in our institution. Indications for treatment were: breast cancer (n=16), sarcoma (n=14), Hodgkin's disease (n=10), cervical cancer (n=3), colon cancer (n=2), miscellaneous cancers (n=9). Co-treatment with monthly injections of GnRH agonist during chemotherapy was also administered.

**Results:** Mean age of patients was  $26 \pm 6$  years. Based on history, physical examination, imaging and diagnostic work up, 68% of patients were in early stages of disease (up to stage II) when OTCP was performed. Twelve patients (21%) passed away during follow up: six patients died from osteosarcoma (6/14, 43%), 3 of them were staged as local disease (up to stage II) at diagnosis. Two patients died from breast cancer (2/16, 12.5%), both had metastasis at diagnosis. One patient died from vulvar cancer (1/1, 100%), found to be stage IV at surgery at which OTCP was also performed. One patient died from acute leukemia (1/1, 100%) and one patient died from cervical cancer (1/3, 33%) which was found to involve lymph nodes at surgery. One patient died from colon cancer (1/2, 50%), initially diagnosed as stage I but found to be stage III at surgery. All together, 4 patients of the 12 patients who died (25%) were initially diagnosed as having local disease at the time OTCP was performed.

**Discussion:** Thus far, studies regarding OTCP focused on the negative impact of radio/chemotherapy on future sterility, without taking into account the expected prognosis of patients. The fact that 75% of patients who died were at advanced stages of disease when diagnosed, and that the other 25% had local disease but still died raises two major issues: First, the limitations of pretreatment evaluation and secondly an ethical question regarding proper selection of candidate patients for OTCP. Indeed, if fertility is to be preserved, one should first be as sure as possible that life is preserved in the first square. We believe, thus, that the expected prognosis of the basic cancer disease is an important component in the decision whether to perform OTCP and that patients with unfavorable prognosis are not candidates for this procedure. It seems that when prognosis is poor, minimal intervention to preserve fertility may be more appropriate, and OTCP may be unjustified.

P12

## MELATONIN PROMOTES THE CUMULUS-OOCYTE COMPLEXES QUALITY OF VITRIFIED-THAWED MURINE OVARIES; WITH INCREASED MEAN NUMBER OF FOLLICLES SURVIVAL AND OVARY SIZE FOLLOWING HETEROTOPIC TRANSPLANTATION

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**Introduction:** We have tested the protective effect of melatonin on neonate murine ovarian tissue after vitrification, thawing and heterotopic transplantation into ovariectomized recipient mice.

**Methods and materials:** Vitrified ovaries from neonate (CBA×C57Bl/6) F1 hybrid mice were thawed under standard condition with or without the addition of 100  $\mu$ M melatonin. Following transplantation, melatonin (20 mg/kg/day) or saline solution (physiological saline) was injected i.p. to the treated and non-treated groups for 48h respectively. Follicle survival and development, together with ovary size followed. Also, vaginal cytology was carried out for monitoring restored puberty.

**Results:** Histological and immunohistochemical studies showed that melatonin could promote the quality of the cumulus-oocyte complexes with uniform distribution of granulosa and stromal cells in the ovarian grafts. Furthermore, the mean follicles survival was improved and the ovary size increased ( $P=0.001$ ). The overall mean number of follicles entering the next maturation stage dramatically increased. However, the revascularization and restoration of puberty of ovarian grafts were similar between melatonin-treated and control groups.

**Conclusion:** melatonin as a protection from ischemic injury and a reduce oxidative stress, was shown beneficial during the early days of transplantation.

**Keywords:** Melatonin, Vitrification, Ovary, Transplantation, Heterotopic, Oxidative stress, Follicular survival and growth.



P13

## EFFECT OF ANTIFREEZE PROTEIN ON OVARIAN TISSUE CRYOPRESERVATION: A PRELIMINARY STUDY

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**Introduction:** Antifreeze proteins (AFPs) are a class of polypeptides produced by certain animals such as antarctic fish which permit their survival in subzero environments. Recently, several studies on protective effect of AFP for cryopreservation of animal cells and organs were reported. However there has been no study on antifreeze protein for ovarian cryopreservation. The purpose of this study was to investigate the effect of AFP supplementation during ovarian vitrification procedure.

**Materials and Methods:** Ovaries from 4 week-aged ICR mice were vitrified using a two-step exposure to equilibrium and vitrification solutions. Equilibrium solution was 20% ethylene glycol (EG) and vitrification solution was composed of 40% EG, 18% Ficoll and 0.3M sucrose. All solutions were based on Dulbecco's phosphate buffered saline containing 20% fetal bovine serum. Ovaries were divided into two groups (AFP and control) according to whether AFP III (5mg/mL) was supplemented to the solutions. After exposure to solutions, ovaries were transferred to cryovials and plunged into liquid nitrogen. Two weeks later, the ovaries were thawed and follicular morphology and apoptosis were analyzed and compared to control group. Morphology was graded into three (grade I, II, III) and apoptosis was assessed using TUNEL assay.

**Results:** A total of 487 and 588 follicles from 22 ovaries (10 from control 12 from AFP group) were analyzed for morphologic and TUNEL assay, respectively. The rate of follicle with grade I morphology was significantly higher in AFP group (41.4% (99/239) vs. 28.6% (71/248),  $p=0.003$ ). Apoptotic follicle (TUNEL-positive) rate was significantly lower in AFP group compared to control group (19.3% (59/305) vs. 27.6% (78/283),  $p=0.019$ ).

**Conclusions:** Our data suggest that AFP supplementation could improve tissue survival after ovarian vitrification.

**Keywords:** antifreeze protein, ovary, cryopreservation, vitrification.

**Support:** Grant of the Korea Healthcare Technology R&D Project (A080126).

P14

## MANAGEMENT OF FERTILITY-PRESERVATION IN BREAST-CANCER-PATIENTS UNDER 40 YEARS IN A LARGE BREAST CANCER CENTER

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**Introduction:** The increase of breast cancer in young women under 40 years and the increasing age of women at the time of the birth of their first child underlines the importance to implement counseling for fertility-preserving strategies in the management of breast cancer care. We present the fertility preserving procedures performed after routine counseling for primary breast cancer patients in a large breast cancer center (approx. 600 primary cases per year).

**Material and methods:** Between November 2006 and June 2009 42 patients under 40 years with histologically confirmed breast cancer were counselled for the fertility-preserving possibilities before breast surgery and chemotherapy in the fertility-center of the university womens hospital in Tuebingen.

**Results:** In 32 months 42 primary breast cancer patients were counselled. The majority of the patients ( $n = 17$ ) decided for ovarian tissue cryopreservation as fertility preserving method. The laparoscopic approach was normally combined with the oncological - surgical procedure. GnRH-protection was performed in 10 patients. In 9 patients an ovarian stimulation protocol was initiated to cryopreservate fertilized or unfertilized oocytes. A combination of different fertility-preserving methods was performed in 9 patients.

**Conclusions:** The majority of the young primary breast cancer patients in our study were hormone receptor positive. Because of this special tumor entity the possibilities to perform fertility-preserving methods is very limited especially in those patients, who have to undergo neoadjuvant chemotherapy. The only therapeutic option for fertility preserving methods in this patient group is the ovarian tissue cryopreservation.

**Keywords:** breast cancer under 40 years - hormone receptor positive tumor - neoadjuvant chemotherapy.



P15

## PATIENT EXPERIENCES WITH FERTILITY PRESERVATION IN CANCER: SATISFACTION IN GENERAL BUT UNMET INFORMATION NEEDS

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**Introduction:** Interest in fertility preserving therapy (FPT) in female cancer patients has grown since the birth of a healthy baby girl after transplantation of cryopreserved ovarian tissue. Limited knowledge is available on the decision-making process regarding fertility preservation therapy. The aim was to examine perceptions of the decision-making process of young female cancer patients who were informed about female fertility preserving options, and to assess patients' information needs.

**Design and Methods:** We conducted retrospective semi-structured interviews with women with cancer (18+) who had had at least one face-to-face or telephone consultation about FPT with a gynaecologist in the Leiden University Medical Center (Leiden) or the Reinier de Graaf Hospital (Delft). Themes were communication about infertility and FPT, experience with the consultation on FPT, the decision making process, experience with FPT, feelings about possible infertility, and reflection on the local procedures.

**Results:** Of 46 eligible women, 12 (26%) declined. Of the 34 participating women, 21 had undergone FPT. Of the women who had had face-to-face consultations 47% underwent FPT vs. none of those who had had a telephone consultation. In retrospect, one in four women were dissatisfied with the timing of the consultation, but 85% were satisfied with the consultation. One-third indicated having felt a lack of clarity about the FPT options. Of those who underwent FPT, 75% were very satisfied with the procedures.

**Conclusion:** Patients were generally satisfied with the procedures, but experience a difficult choice in which not all options appear to be clear. As centralization of FPT limits the options for face-to-face consultation, we have decided to develop a web-based decision-aid for FPT decision-making.

P16

## FERTILITY PRESERVATION OPTIONS FOR FEMALE PATIENTS: TWO YEARS OF EXPERIENCE

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**Introduction:** The improvement in the survival of children and young women with cancer and another non oncologic conditions treated with gonadotoxic agents, has led to consideration of long-term side-effects of treatments. One of these effects is premature ovarian failure. Since 2005 patients were referred to our Unit from oncologist for aGnRH treatment. In 2007 we initiated a program of fertility cryopreservation.

**Material and methods:** 57 patients were referred to the fertility cryopreservation program from different departments of our hospital (Oncology, Haematology and Autoimmune Diseases). A first evaluation included the following factors: age, pathology, type of chemotherapy, prognostic, parity, marital status and time available before the initiation therapy. After that, patients were informed about available strategies for fertility preservation: ovarian tissue freezing and embryo/oocyte cryopreservation. After conformity, we performed a gynaecological and ultrasound control, blood analyse and contact with the oncologist.

**Results:** A total of 57 patients were referred to our program. From this, 21 have been diagnosed with breast cancer, 15 with lymphoma, 7 with leukemia, 2 ovarian cancer, 7 with other types of cancer and 5 with autoimmune disorders. 7 women declined and 28 were excluded for different factors. The most frequent excluding factors were impossibility to delay cancer treatment, advanced age or health status. We performed 17 laparoscopies for cryopreservation of ovarian tissue, following biopsy (12) or unilateral oophorectomy (5). And 8 ovarian stimulated cycles were started for oocyte or embryo cryopreservation.

**Conclusions:** The different fertility preservation treatments were well accepted for the patients (87%). Our results show the need for multidisciplinary treatment plan between gynaecologist and oncologist to increase patient acceptance and to choose the best option.

P17

## CRYOPRESERVATION OF THE WHOLE SHEEP OVARY - EVALUATION OF THE CRYOPROTECTANT DMSO BY IN VITRO TESTS

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**Introduction:** Whole ovary cryopreservation and retransplantation of the ovary by vascular anastomosis has been suggested as a way to bypass the dramatic loss of follicles that occurs after avascular transplantation of cryopreserved ovarian tissue. The aim of the present study was to evaluate the effectiveness of the cryoprotectant dimethylsulphoxide (DMSO) to preserve the sheep ovary during freezing.

**Materials & Methods:** Ovaries of sheep, 1-2 years of age, were primed with vaginal medroxyprogesterone for 12 days followed by equine CG (500 IU) 24 h before surgery to synchronize into follicular phase. During anaesthesia, a laparotomy was performed to isolate the ovary and a short vascular pedicle that included the ovarian artery and veins. The ovary was submerged in cold Ringer-Acetate, while the ovarian artery was cannulated with a 22/24 G over-the-needle Teflon catheter. Blood was flushed out from the ovary and either cryoprotectant solution (1.5 M DMSO, 0.1 M sucrose, 2% human serum albumin) or non-cryoprotectant solution (Ringer-Acetate, 0.1 M sucrose, 2% human serum albumin) was used for flushing (30 min at 80 mmHg pressure). Passive slow cooling was used and the ovaries were stored in liquid nitrogen. After thawing, ovaries were assessed with various test of viability (histology, in vitro perfusion, cultures of ovarian cells, live/dead assay).

**Results:** During in vitro perfusion DMSO-frozen ovaries responded with slightly higher progesterone and cAMP production than noncryoprotectant-frozen ovaries. In cell cultures DMSO-frozen ovaries showed higher progesterone production and this group also showed higher % of live cells both before and after perfusion. Histology indicated better tissue preservation after DMSO.

**Conclusion:** This study demonstrates that several in vitro techniques can be used to assess the viability of an ovary that has been cryopreserved. The increased viability of the ovarian cells and the increased progesterone secretion from cells isolated from whole ovaries that have been cryopreserved in DMSO as compared to cryopreservation in ordinary Ringer Acetate points to the beneficial effects of cryoprotectants for whole ovary cryopreservation. Future studies will be aimed towards optimizing concentrations of DMSO, as well as freezing and thawing rates.

P18

## THE SAFETY OF THE VITRIFICATION TECHNIQUE USING CRYOTOP AT OUR CLINIC

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**Introduction:** We did follow-ups on babies born following vitrification method using cryotop.

**Material and Methods:**

- 1) Cryopreservation methods were slow freezing (straw) and vitrification (cryotop).
- 2) One case received two vitrified-warmed blastocysts produced by ICSI using vitrified-warmed oocytes and frozen-thawed sperm were transferred.
- 3) We compared ordinary slow freezing using straw (straw group: 86 singletons) and vitrification using cryotop (top group: 257 singletons).in terms of the following measurements: the length of gestation periods, average birth weight, average height, average girths of chest and head, premature births, low birth-weight rates, stillborn rates, birth defect rates, and physical development up to the age of 6.

**Results:** We achieved a successful birth of a healthy boy following transfer of two vitrified-warmed blastocysts produced by ICSI using vitrified-warmed oocytes and frozen-thawed sperm in one case. The length of gestation period (week), average birth weight (g), average height (cm), average girths of chest and head (cm) in straw group vs cryotop group were all within the normal range. Premature births rates, low birth-weight rates, stillborn rates, and birth defect rates (straw group vs cryotop group) were 8.1% (17/86) vs 9.3% (24/257) , 16.2% (14/86) vs 9.3% (24/257), 0% (0/86) vs 0.8% (2/257) , and 2.2% (2/86) vs 1.2% (3/287), respectively. Physical development from birth to the age of 6 was within the normal range.

**Conclusions:** In both straw and cryotop groups, physical development from birth to 6 years of age was normal. This suggests that the vitrification method using cryotop is simple, easy and safe, but further studies are still needed.

**Key words:** vitrification, Cryotop, safety, follow-up, children



## P19

### SPERM CRYOPRESERVATION FOR PATIENTS WITH MALIGNANT OR NON-MALIGNANT DISEASES: THE OUTCOMES OF 27 CASES

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**Introduction:** We reviewed cases of sperm cryopreservation in malignant and non-malignant diseases.

**Material and Methods:** The clinical records of 27 cases from January 1997 to February 2008 were reviewed. The age at cryopreservation, marital status, diseases, timing of cryopreservation, semen quality, utility, duration of banking, and results were analyzed.

**Results:** The age at cryopreservation ranged from 18 to 51 years old. At the time of cryopreservation, 14 patients were married, 3 were engaged, and 10 were single. Their original diseases were testicular cancer in 13 patients, other solid tumors in 5, leukemia in 7, and collagen disease in 2. Eighteen patients had their sperm cryopreserved before gonadotoxic therapies and 9 after the therapies. Semen analysis results of 7 patients were within the normal range, and those of 19 were not, including 2 cases with azoospermia. The ones with abnormal semen results included 5 with testicular cancer, 5 with leukemia, and 3 with solid tumors. 6 patients used frozen/thawed sperm in ART, and 5 achieved pregnancies. Healthy babies were born in 4 cases. Two patients died. The periods of sperm banking ranged from 1 to 111 months.

**Conclusions:** The candidates for sperm banking in this study varied compared to the previous reports. It is ideal to have sperm cryopreserved before spermatogenesis shows any decline after gonadotoxic therapy. Thus, it is very important to educate doctors in other fields as well as the public about these findings. Long term storage of sperm is extremely difficult in private clinics. We hope to get our government's cooperation on management, control, supervision, charge, and other care regarding sperm banking.

**Keywords:** sperm freezing, malignancy, azoospermia, pregnancy.

## P20

### FOUR SUCCESSFUL PREGNANCIES FOLLOWING ASSISTED REPRODUCTIVE TECHNOLOGY

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**Introduction:** We report 4 successful pregnancies of 4 women who had overcome their malignant diseases.

**Material and Methods:** The subjects were four female patients with acute lymphoblastic leukemia, aplastic anemia, cervical carcinoma in situ, and breast cancer. The main measurements were their marital ages, disease onsets, the length of treatments and infertility periods, the number of retrieved oocytes, insemination technique, the number, and grade of transferred embryos, and whether there was any male factor.

**Results:** In case 1, the measurements were 36 years old, 37 years old, 3 years, 4 years, 2 oocytes, ICSI, 2 ETs, 1 embryo (D3, fresh 8 cells), and with male factor, respectively. In case 2, the measurements were 20 years old, 17 years old, 10 months, 5 years, 7 oocytes, ICSI, 2 ETs, 1 embryo (D5 frozen-thawed, 4BB), and with male factor, respectively. In case 3, the measurements were 28 years old, 31 and 33 years old, 14 months and 2 years, 9 years and 5 months, 7 oocytes, IVF, 2 ETs, 1 embryo (D5 frozen-thawed, 5BB), and without male factor, respectively. Finally, in case 4, the measurements were 31 years old, 39 years old, 2 years, 4 years, 4 oocytes, ICSI, 2 ETs, 1 embryo (D5 frozen-thawed, 4BC), and without male factor, respectively.

**Conclusions:** We achieved successful pregnancy considering ovarian reserve after chemotherapy and male factors. It is very crucial to educate oncologists on relationships between aging and other factors such as ovarian reserve, pregnancy rate, and miscarriage rate. The fact that half of the infertility is caused by male factors, and pregnancy rate depends on types of cancer treatments. It is advisable to see fertility doctors and receive ART as soon as possible.

**Keywords:** malignancy, pregnancy, chemotherapy, ART, fertility preservation.

P21

## MELATONIN EFFECTS ON VITRIFIED-WARMED TESTIS AFTER GRAFTED INTO CASTRETED MATURE MICE

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**Introduction:** Melatonin besides action on different physiological processes is an antioxidant and powerful direct free radical scavenger. This study assessed different concentration of melatonin on vitrified testis grafted into castrated mature mice.

**Materials and Methods:** Testes from neonate (Balb/c) mice were vitrified-warmed and transplanted heterotopically into host. Following transplantation, melatonin (20, 50, 100, 200 mg/kg/day or saline) were applied via gavage to separate groups. Donor-specific IgM and IgG subtype antibodies, Th1 (IL-2 and IFN- $\gamma$ ) and Th2 cytokines (IL-4 and IL-10) and melatonin in the serum of recipients were measured using ELISA analyses. The subsequent survival (days 1-8 and 42) of the grafted testes were scored morphologically. Indices of cell proliferation and apoptosis within spermatogonia and interstitial cells of tissue grafts were determined by BrdU and TUNEL assay respectively.

**Results:** This study showed that with increasing doses of melatonin, the graft lifespan was significantly prolonged ( $P \leq 0.05$ ). Our results also, indicated that apoptosis in the spermatogonia was dramatically increased ( $P \leq 0.05$ ). Allospecific IgM and also IgG2a of recipients were reduced with increasing doses of melatonin ( $P \leq 0.05$ ). The average level of Th1 cytokines was significantly reduced in 200 mg/kg/day of melatonin ( $P \leq 0.05$ ). No significant differences in Th2 cytokines were observed with increasing melatonin. Also, the variable regimens of melatonin caused higher peak melatonin levels in serum after transplantation.

**Conclusion:** These findings indicate a novel therapeutic approach, based on modulation of the immune and E/R through melatonin as a possible future immunosuppressant and antioxidant in organ transplantation.

**Keywords:** Melatonin, Testis transplantation, Vitrification, Mice.

P22

## OOCYTE VITRIFICATION FOR FERTILITY PRESERVATION IN YOUNG WOMEN SUFFERING FROM CANCER

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Fundación IVI, Spain

**Purpose:** Cancer survival rates have improved significantly over the last years. Consequences of cancer treatment involve ovarian failure and infertility. Several strategies have been proposed to develop a program for patients who wish to preserve their fertility before chemotherapy. The efficacy of vitrification in clinical practice has been demonstrated in the past few years. Oocyte cryo-banking with the Cryotop vitrification method represents a viable option for healthy women, producing excellent survival rates and clinical outcome similar to those obtained with fresh oocytes. We present our experience with this method as useful tool for fertility preservation in cancer patients.

**Methods:** During the last two years, fertility preservation was offered to 96 patients after their oncologist's permission. Breast cancer was the most frequent tumor (n=58; 60.4%), followed by Hodgkin lymphoma (n=13; 13.5%). Among these patients, 10.54% had previous children. Oocyte vitrification (Cryotop method) was recommended on 79 of these patients after obtaining a written consent. The other options were ovarian tissue cryopreservation (n=2; 2%) or no treatment (n=15; 15.6%). In the oocyte vitrification group, ovarian stimulation was performed with a combination of letrozole 5mg and mild stimulation (150 IU rFSH) under a GnRH antagonist protocol, and GnRH agonist to trigger final oocyte maturation when patients were diagnosed of breast cancer. For no hormonal-dependent cancers, stimulations were performed only with gonadotropins. Ovarian stimulation was started on the second or third day of the cycle or when serum E2 levels were under 60 pg/ml following a 3mg GnRH antagonist injection. Letrozole was maintained until the return of menses.

**Results:** Mean age was  $30 \pm 8.9$  years old. A total of 719 oocytes were retrieved (mean  $9.4 \pm 6.7$ ). After evaluating nuclear maturity 536 (74.5%) mature oocytes were vitrified (mean  $7.05 \pm 5.08$ ). The mean E2 level ( $262.5 \pm 190$  pg/mL) in the letrozole group significantly lower when compared with the gonadotropins group ( $1107 \pm 743$  pg/mL):  $p < 0.05$ .

**Conclusion:**

Oocyte vitrification for fertility preservation can be performed in people undergoing cancer treatment. Ovarian response in a mild stimulation cycle in these young patients seems to yield a good response and offers them the possibility of attempt a pregnancy with their own oocytes once cancer is cured.





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## COMPARISON BETWEEN TWO TECHNIQUES OF FOLLICULAR VIABILITY ASSESSMENT FOR EVALUATION OF A SLOW FREEZING PROCEDURE ON HUMAN OVARIAN TISSUE

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**Introduction:** Human ovarian tissue cryopreservation is the best option for fertility preservation for patients undergoing anticancer treatment or suffering from pathologies potentially leading to premature ovarian failure. Follicular viability assessment allows the efficiency of the freezing/thawing procedure and the potential of viable follicles to be checked before grafting. The aim of our study was to adopt the more accurate test to evaluate human follicular viability, comparing the trypan blue and calcein AM/ethidium homodimer staining methods which are currently used in clinical research.

**Materials and Methods:** Ovarian cortical slices were obtained from 10 consenting patients (mean  $29 \pm 1.8$  SEM years) undergoing endoscopic surgery for benign cysts. Small follicles ( $<70 \mu\text{m}$ ) were enzymatically isolated from fresh and frozen ovarian tissue by an original slow cooling protocol previously developed in our lab (Schubert *et al.*, 2005). Dead and live follicles were identified with trypan versus calcein AM/ethidium homodimer (live/death<sup>®</sup> kit, Interchim, France) staining. Viability of at least 100 follicles was assessed per staining process using a light microscope for blue trypan staining or epifluorescent one for calcein AM/ethidium homodimer staining. The viability of each sample was assessed by 2 independent observers. Statistical analysis was carried out using a signedrank test for comparative analysis ( $p$  value  $<0.05$ , significance level). Reliability was evaluated using intraclass correlation coefficient (ICC).

**Results:** Interobserver reliability assessed on fresh and thawed ovarian tissue was good for each staining method (Trypan blue:  $p=0.12$ , ICC=0.59; Calcein AM/ethidium homodimer:  $p=0.27$ , ICC=0.46). A significant systematic higher proportion of viable follicles was observed by using calcein AM/ethidium homodimer ( $+6.5 \pm 1.9$ , mean  $\pm$  SEM) compared with trypan blue ( $p=0.001$ ). The percentages of viable follicles before and after thawing did not differ statistically whether after trypan blue ( $18 \pm 2.6\%$  vs.  $19 \pm 2.5\%$ ;  $p=0.70$ ) or after calcein AM/ethidium homodimer staining ( $32 \pm 3.7\%$  vs.  $25.2 \pm 2.8\%$ ;  $p=0.64$ ).

**Conclusions:** Our results suggest that blue trypan and calcein AM/ethidium homodimer staining are fast and appropriate methods for appreciation of ovarian quality in case of therapeutic cryopreservation. However, the systematic higher percentage of viable follicles after staining by calcein AM/ethidium homodimer compared with trypan blue meant that only data measured using the same method could be compared. The difference in staining specificities between these two methods could explain this systematic difference. The original slow freezing/thawing procedure used in this study appears to preserve follicular viability well, but other markers are needed to better assess the morphological and functional quality of ovarian tissues before grafting.

P24

## THE MARKETING OF 'FERTILITY INSURANCE' IN THE UNITED STATES AND THE NEED FOR GREATER REGULATION OF THE ASSISTED REPRODUCTIVE TECHNOLOGY INDUSTRY

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The multi-billion dollar Assisted Reproductive Technology ("ART") industry in the United States is largely unregulated and privately funded. The lack of regulation of this arena has resulted in women being exploited financially and emotionally by a self serving industry. This paper/presentation will explore the United States' dark history of exploitation in the medical research arena (examples: Tuskegee, Willowbrook, Kennedy Krieger). Given this backdrop and the financial conflicts of interests facing fertility clinics and specialists, it is overly optimistic and naïve to expect the ART industry to act in the best interests of their clients. This paper/presentation examines the example of oocyte cryopreservation, commonly known as egg freezing, to demonstrate the need for greater regulation in the ART arena. In the past, only female cancer patients who had to undergo chemotherapy and therefore risk future infertility have had access to egg freezing. However, in the United States, this procedure is increasingly being offered to healthy women in their late twenties and early thirties as a method of extending their fertility. Theoretically, this technology sounds promising as a means for allowing women to have greater control over their biological clocks. Unfortunately, there are only a few examples in the scientific literature to support the contention that egg freezing will result in a successful pregnancy or live birth. This paper/presentation examines how the unregulated marketing of this largely untested procedure to a vulnerable population, women in their thirties who have strong feelings about fertility, has great potential for exploitation. Although many have argued that this lack of regulation has many benefits (allowed for greater technological innovations in the area of assisted reproduction and allowed women to have more freedom over their reproductive choices), this paper will suggest how regulation may actually increase autonomy.

P25

## PROTECTIVE EFFECTS OF MELATONIN ON HETEROTOPICAL TRANSPLANTED VITRIFIED-WARMED MICE TESTIS TISSUE

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**Introduction:** Cancer therapy applied to young patients most often has severe effects upon male fertility. Present study investigated the protective role of melatonin on neonate mouse testis tissue during vitrification-thawing and heterotopic transplantation into castrated recipient mice.

**Materials and methods:** Vitrified testes from neonate inbred mice, candidates for transplantation were thawed under standard conditions with or without the addition of 100 µM melatonin, respectively. Following transplantation, melatonin (20mg/kg/day) or saline solution was applied as gavage to the treated and the non-treated groups (control) respectively. In 1-8 and 42 days Melatonin, gonadotropins and steroids concentrations were evaluated by ELISA. Spermatogonia survival and spermatogenesis were also assessed by TUNEL and Brdu respectively.

**Results:** Histological and immunohistochemical studies showed that melatonin could improve the spermatogonia quality in the testis graft. Plasma LH and FSH levels were significantly (P<#8804;0.05) higher in the castrated host than intact mice (P<#8804;0.05) at before grafting. However, the melatonin administration reduced these hormones into nearly similar concentrations to those in intact mice. The correlation coefficients between gonadotropins and melatonin concentrations during the days of transplantation of the testis grafts were significantly different from zero (P<#8804;0.05). However, testosterone secretions were not affected by melatonin treatment significantly.

**Conclusions:** The results of present study suggest that melatonin has a positive effect on testis tissue after vitrification, thawing and heterotopic transplantation. Also, it could be beneficial on hypothalamic pituitary-testis axis of the recipient castrated mice.

**Keywords:** Melatonin, Castrated recipient mice, Sex hormones, Transplantation, Testis.

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## PRESERVATION OF OVARIAN FUNCTION FOLLOWING OVARIAN CRYOPRESERVATION WITH GnRH ANALOGUE CO-TREATMENT

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**Introduction:** In recent years, the number of young women with uterine cancer or breast cancer has been increasing. For these patients who underwent surgery or performed chemotherapy, it is very important to preserve ovarian function and keep the quality of life as one woman. Recently it has been published that the techniques of cryopreservation and transplantation of the ovarian cortex, and children have been born after successful orthotopic autotransplantation into the residual ovaries. One of the methods for preventing ovarian damage is the administration of GnRH analogue during or prior to chemotherapy. In this study, we compare the ovarian function with the GnRH analogue administration before cryopreservation and transplantation of the ovarian cortex.

**Materials and Methods:** Six to eight weeks of Wister rats were divided into 3 groups such as control, vitrification group, and slow-freezing group. After cryopreservation and transplantation, we confirmed the day of the recovery of estrous cycle. Then, we examined the same 3 groups which were administrated with GnRH analogue (3.75 mg/kg) before cryopreservation and transplantation, and confirmed the recovery of estrous cycle days.

**Results:** Ovarian function of all groups was preserved, and ovarian function of all the other groups even using GnRH analogue was preserved.

**Conclusions:** Vitrification method is more cheap and convenient rather than slow-freezing method. Therefore, vitrification method might be standard method of cryopreservation and transplantation of the ovary. Since the ovarian function was also preserved by administrating GnRH analogue, this method should be effective for the young cancer patients who already performed chemotherapy.



P27

## REOXYGENATION AND REVASCULARIZATION PROCESSES OF HUMAN OVARIAN XENOTRANSPLANTS

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**Introduction:** The success of ovarian tissue transplantation appears to be limited by ischemia-reperfusion damage caused by an avascular procedure. Our aim was to characterize the oxygen microenvironment and revascularization process ovarian xenograft in the early post-grafting period.

**Material and methods:** Nude mice were intraperitoneally grafted with frozen-thawed human ovarian tissue obtained from 6 patients. Graft reoxygenation was assessed by electron paramagnetic resonance (EPR), which allowed non-invasive and repeated measurement of partial oxygen pressure ( $pO_2$ ). The  $pO_2$  was monitored on post-grafting days 3, 5, 7, 10, 14, 17 and 21 (n mice=18) and graft perfusion was spatially evaluated by Hoechst 33342 uptake on days 3, 5 and 10 (n mice=18). Murine and human vascularization was analyzed by CD31 and von Willebrand factor double staining. ANOVA or t-tests were applied for statistical analysis where appropriate. Results were expressed as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant.

**Results:** Before day 5, a period of hypoxia was identified (day 3  $pO_2$ :  $13.3 \pm 7.4$  mmHg). Fragments were largely non-perfused and occasional murine neovessels were located at the periphery. From day 5, gradual but significant reoxygenation was observed over the subsequent 5 days (day 5  $pO_2$ :  $22.3 \pm 11.6$  mmHg,  $p < 0.001$ ; reoxygenation speed: slope  $^{D5-D10} = 2.4-0.4$  mmHg/d,  $p < 0.001$ ). Murine neovessels progressively penetrated from the periphery and were colocalized with perfused areas. By day 10, the increase in perfusion and murine vascularization was significant. The center of the fragments was perfused and a significant increase in human vasculature was observed. On day 21,  $pO_2$  values were found to have stabilized and remained higher than the initial hypoxic value (day 21  $pO_2$ :  $26.2-7.3$  mmHg;  $p < 0.001$ ).

**Conclusions:** After an initial hypoxic and avascular period, murine angiogenesis initiated reoxygenation and reperfusion of the graft from day 5 onwards. By day 10, human vessels participated in graft revascularization. Host and graft vessels contributed sequentially to graft revascularization and are therefore potential targets to reduce the ischemic period after grafting.

**Keywords:** cryopreservation, human ovarian xenotransplantation, angiogenesis, EPR oxymetry.

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## L-CARNITINE IMPROVES FERTILIZATION RATE FROM MOUSE VITRIFICATED-THAWED OOCYTES

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**Introduction:** L-Carnitine (LC), an important water-soluble molecular in fat metabolism, can protect cell membrane and DNA from free oxygen radicals damage by removing superoxide anion and inhibiting lipid peroxidation. Previous studies showed that embryo culture medium supplementation with L-Carnitine may improve embryogenesis of cultured embryos. This study is to evaluate the effect of L-Carnitine supplement on embryo development of vitrified-thawed mouse oocytes.

**Material and Methods:** This is an animal experimental study in reproductive research center at a tertiary medical center. There are four groups in the study: Group A, 44 fresh oocytes cultured in medium without LC; Group B, 49 fresh oocytes cultured with 0.3 mg/mL LC; Group C, 117 vitrified oocytes cultured without LC; Group D; 69 vitrified oocytes cultured with 0.3 mg/mL LC. Vitrified oocytes were cryopreserved by vitrification using CryoTop®, and were thawed 1 week later. All oocytes were fertilized in vitro with sperms from male mice in Quinn's Advantage® Fertilization Medium. Embryos were assessed every day after insemination. On the day after insemination, fertilized embryos will develop to two-cell stage. Proportional variables were compared using the Fisher's exact test. All statistical tests were based on two-tailed probability. P value  $< 0.05$  was considered statistically significant.

**Results:** Vitrified oocytes had low fertilization rate compared with fresh oocytes [59% (69/117) vs 96% (42/44); odds ratio 0.07 (0.01-0.29), p-value  $< 0.05$ ]. When cultured with L-Carnitine, the difference was not statistically different between vitrified oocytes and fresh oocytes [81% (50/62) vs 86% (42/49); odds ratio 0.69 (0.21-2.13), p-value  $> 0.05$ ]. From vitrified oocytes, the add of L-Carnitine in culture medium improved fertilization rate [81% (50/62) vs 59% (69/117); odds ratio 2.9 (1.34-6.6), p-value  $< 0.05$ ].

**Discussions:** In our study, the supplement of L-Carnitine in culture medium can improve fertilization rate in the vitrified-thawed oocytes. The supplement of L-Carnitine in culture medium may decrease oxidative stress, therefore may increase fertilization rate. However, it needs more experimental studies to prove its effect.

**Keywords:** L-Carnitine, vitrification, fertilization.



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## ANTI-MULLERIAN HORMONE (AMH) REFLECTS OVARIAN RESERVE OVER ONE-YEAR AFTER INTRAOVARIAN INFUSION OF SPHINGOSINE-1-PHOSPHATE (S1P) AND S1P AGONIST, FTY720 (FTY), PRIOR TO OVARIAN X-IRRADIATION IN MACAQUES

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**Introduction:** Intraovarian delivery of the anti-apoptotic agents, S1P and FTY, can protect a cohort of ovarian follicles from radiation-induced damage as well as allow fertility in macaques, and offers a novel option for fertility preservation. The duration for fertility potential following ovarian protection in primates is uncertain. This study determined whether circulating AMH levels reflect ovarian reserve after in vivo X-irradiation of ovaries pre-treated S1P or FTY.

**Materials and methods:** Rhesus monkeys received intraovarian infusion via osmotic minipumps with vehicle (V; n=9), S1P (n=9) or FTY (n=6) into both ovaries for 7 days. Animals a) underwent unilateral ovariectomy (ovx) prior to ovarian sham (n=3, V+Osh) or X-irradiation (15 Gy; n=3, V+OXI; n=6, S1P+OXI; n=3, FTY+OXI) of the other ovary which was removed up to 12 mo later; or b) received bilateral V+Osh (n=3), S1P+OXI (n=3) or FTY+OXI (n=3) with bilateral ovx 21 mo later. Serum levels of AMH were measured in pre- and post-treatment cycles using ELISA (Diagnostic Systems Laboratories, Webster, Texas, USA).

**Results:** AMH levels were similar in all groups before treatment (range 2.0 to 3.5 ng/ml), and were maintained in V+Osh during the 21-mo post-treatment interval. AMH levels were nondetectable in V+OXI post-treatment, wherein preantral follicles were absent. In macaques with 1 ovary, AMH levels were < 0.5 mg/ml up to 12 mo post-S1P+OXI, and slowly increased in animals with 2 ovaries from  $0.8 \pm 0.2$  to  $1.4 \pm 0.2$  ng/ml between 12 and 21 mos, respectively. AMH levels were consistently greater ( $P < 0.05$ ) in FTY+OXI relative to S1P+OXI; levels in animals with 1 ovary were  $0.4 \pm 0.1$  1ng/ml. In FTY+OXI with 2 ovaries, AMH levels ( $1.8 \pm 0.2$  ng/ml) did not change between 12-21 mos post-radiation, but remained lower ( $P < 0.05$ ) than V+Osh. AMH levels correlated with the cohort of primordial follicles remaining after V+Osh ( $85 \pm 2\%$ ) and FTY+OXI ( $22 \pm 9\%$ ) which was greater ( $P < 0.05$ ) than S1P+OXI ( $1 \pm 0.3\%$ ).

**Conclusions:** Circulating levels of AMH correlated with the remaining cohort of primordial follicles that were protected from radiation-induced damage by intraovarian infusion of S1P and FTY. Thus, AMH can be a reliable indicator of ovarian reserve up to 21 mos after in vivo X-irradiation of ovaries pre-treated with anti-apoptotic agents. Whether ovarian reserve and fertility are maintained for longer intervals post-radiation of ovaries protected with anti-apoptotic agents in vivo remains to be determined in primates.

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## CHILDHOOD CANCER TREATMENT CAUSES LESS SEVERE GONADOTOXICITY THAN ADULT CANCER TREATMENT: A RETROSPECTIVE STUDY

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**Introduction:** During the last three decades the survival rate of young women undergoing chemotherapy or radiation because of cancer has raised in response to the improvement of the chemotherapeutic regimes. Nevertheless, little attention has been paid to the effect of cytotoxic therapy on these patients in the past. Objective of our study is to examine the effects of cytotoxic therapy on fertility preservation regarding patients' age during treatment.

**Material and methods:** 75 patients of a current age between 18 and 50 years, who have undergone chemotherapy and/or radiation due to leukemia, sarcomas or lymphomas in the last 15 years in our university clinic, were included in the retrospective study. 30 patients were under 18 years during treatment (group A). All patients of the group A received chemotherapy, while 14 patients (46,66%) were treated with both chemotherapy and radiation. 45 patients were adults during treatment (group B). All patients of the group B received chemotherapy, while 23 patients (51,2%) were treated with both chemotherapy and radiation. Amenorrhea was defined as absence of menstruation for at least 12 months after end of treatment. Anti-müllerian hormone (AMH), as a marker of ovarian reserve, gonadotropins, hot flushes without contraceptive pills or hormonal replacement therapy, fertility preservation methods and pregnancies after treatment were evaluated.

**Results:** Group A had a significantly higher AMH level ( $3.33 \pm 3.26$   $\mu\text{g/l}$  vs.  $1.07 \pm 0.89$   $\mu\text{g/l}$ ,  $p < 0,05$ ). Follicle stimulating hormone (FSH) was significantly lower in the group A ( $5.69 \pm 3,2$  IU/l vs.  $56,25 \pm 1,63$  IU/l,  $p < 0,05$ ), indicating premenopausal status. The rate of amenorrhea without contraceptive pills or hormonal replacement therapy was significantly higher in the group A (23,1% vs. 80,0%,  $p = 0,03$ ), while the rate of patients with hot flushes (8,3 %, vs. 64,3%,  $p < 0,05$ ) was significantly lower in the group A. Although none of the patients of the group A experienced cryopreservation of ovarian tissue or treatment with GnRH analogues as fertility preservation methods compared with the group B, in which 26,7% of the patients was treated with GnRH analogues and 22,2% of the patients underwent a cryopreservation of ovarian tissue, 13,3% of the patients in the group A conceived after cytotoxic treatment compared with 4,5% of the patients in the group B ( $p = 0.39$ ).

**Conclusions:** Our study provides evidence that childhood cancer treatment causes less severe gonadotoxicity than adult cancer treatment. Patients with cancer need to be informed about the effects of cytotoxic therapy on their fertility and be offered all possibilities to preserve their ovarian function.



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## POPULATION STUDY ON FREQUENCY AND OUTCOME OF PREGNANCY IN WOMEN AFFECTED BY NEOPLASIA IN THE VENETO REGION (North-East Italy)

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**Introduction:** To evaluate the frequency and the outcome of pregnancy in women affected by cancer we carried out a descriptive analysis using the data base of the Veneto Tumour Registry (RTV) which has been active since 1987 and covers about half of the population of Veneto region (North East Italy, 4.527,694 inhabitants at the 2001 census).

**Material and methods:** We have extracted from the database of the RTV the women aged 15-49 years with diagnosis of cancer between 1997 and 2001; we have considered all sites of tumour, except non melanoma skin cancer. To retrieve cases with a pregnancy, we have used the hospital discharges for the period 1997-2005, with the International Classification of Diseases codes, ninth edition (ICD IX), in the principal diagnosis: pregnancy, puerperium, delivery. As a results of this cross-linkage we have obtained a cohort of 189 women, 15-49 year old, who represents the study population we report.

**Results:** In the period 1997 - 2001, 3872 women aged 15 - 49 in the population covered by RTV have had a diagnosis of malignant tumour, 14% of all ages. In this cohort, 189 (5%) had at least one pregnancy after their neoplasia diagnosis and before 31st December 2005. The median age was 31 (interquartile range 25 - 34 ). In this population we observed 186 deliveries, 29 spontaneous abortion and 28 volunteer miscarriages. One hundred thirty seven women out of 189 have had only pregnancies; 31 have had only miscarriage; 21 have had both pregnancies and miscarriages.. In addition, 48 women have had more than one pregnancy and 28 women have had more than one delivery. No stillbirth have been reported. In the first year the fertility rate for women under study is 0.4 versus a constant rate of 1.2 in the general population. In the following years it grows steadily and becomes quite constant after the 6° year with a level of 0.6 that is one half of that of the general population.

**Conclusions:** The TFI calculated from our study population demonstrates that pregnancy effectively occurs in many cases after neoplasia's diagnosis and treatment, but in the same time we have to question us if the small value of TFI after ten years may be considered, at least in some patients, a failure of our efforts to preserve fertility in our patients.

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## OVERCOMING INFERTILITY BY ZIFT AND IVF: A CLINICAL CASE STUDY

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IVF and ZIFT are two methods for achieving successful pregnancy. We employed these methods, respectively, to study over 120 infertile women from June 2008 to January 2009. We divided our patients into two different age groups, 77 patients below the age of 35 (group A), and 43 of them above 35 years old (group B). ZIFT was used for 82 patients, among them 55 were belong to group A and 27 patients were belong to group B. The results indicate 63.3% of success in patients below 35 years old, while we succeeded around 52% of pregnancy in patients above 35 years old. IVF is also used for process of conceiving in 38 patients, 22 patients below 35 years old and the rest above, respectively. The younger group shows 45.5% successful pregnancy, whereas only 37.5% positive response has achieved in patients above 35. The above data suggests that at our clinic patients who have had multiple failed IVF cycles or who have patent tubes and they are older should consider ZIFT as a treatment option. Estradiol valerate was prescribed for 74 patients in groups A and B, in 62 patients who used estradiol valerate (1-2 mg) in their first and second period of cycle (days 1-7 and days 7-12) in group A, 63.6% pregnancy was achieved; whereas 58.6% positive response was found in group B. In another case in 12 patients from group A who were prescribed estradiol valerate in their second period of cycle, we achieved 50% success. These results indicate that patients who used estradiol valerate in their first and second cycles show a better results compared to the patients who received the same dose in their second cycle.

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## PRESERVATION OF MEN'S FERTILITY IN PATIENTS WITH LYMPHOMA

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There are 2.6 millions cancer patients in the Russian Federation. Among them there are about 50% reproductive-aged men and women. The cancer treatment (heavy chemotherapy and/or radiotherapy) may reduce fertility by damage of ovaries and testicles. For these patients, there are several options that may help to preserve fertility before and after cancer treatment. If the patient has a partner or accepts donor sperm, embryo cryopreservation should be considered first. If not for preserving men's fertility can be used cryopreservation of sperm or testicles tissue. For the women of reproductive age with cancer there are special options like ovarian tissue and eggs cryopreservation, also ovarian transposition out of the irradiation field before radiotherapy. Because of the variations in type and dose of chemotherapy, the type of cancer, the time available prior to onset of treatment, the patient's age and the partner status, each case is unique and requires a different strategy of fertility preservation.

We collected the semen samples of 21 patients (from 16 to 34 years of age) with Hodgkin's and non-Hodgkin's lymphoma before starting chemotherapy or radiation therapy and frozen at sperm bank. Semen samples were cryopreserved as follows: each aliquot was diluted in the same volume of cryoprotectant medium consisting of 15% glycerin, 2% sucrose, 0.4% human serum albumin in aqueous solution. Cryoprotectant medium was added sequentially drop by drop, with constant mixing. Each aliquot was then placed in cryotubes, frozen and transferred to a liquid nitrogen tank, until use. Samples can be stored for years and used later for insemination.

A total of 21 sperms from patients with Hodgkin's and non-Hodgkin's disease were collected, analyzed and frozen. Our results indicate that in 16/21 cases (76,19%) were astenozoospermia, in 12/21 cases (57,14%) were olygozoospermia and only 5/21 cases (23,8%) were normozoospermia. We are establishing the first human sperm bank for cancer patients in the Russian Federation.

Fertility preservation should be an integral part of improving the quality of live in cancer survivors; however, it is neither possible nor ethical to recommend the same recipe for every cancer patient. The first goal is to cure the cancer, even if the treatment causes sterility.

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## CHANGES IN OOCYTE CHROMATIN AND CYTOSKELETON FOLLOWING CPA EXPOSURE

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**Introduction:** Cryoprotective agents (CPAs) are cytotoxic agents that target multiple organelles during cryopreservation. The impact of CPA exposure without cooling on cytoskeletal and chromatin integrity was evaluated in mouse germinal vesicle stage oocytes using milrinone to block meiotic progression and stabilise transzonal projections (TZPs). We tested the hypothesis that stabilising TZPs would promote retention of cytoskeletal chromatin interactions subject to perturbation by CPAs.

**Material and methods:** GV cumulus masses (COCs) were collected from adult CBA F1 mice. COCs were dehydrated using standard vitrification protocols; samples were fixed prior to or after rehydration in the presence or absence of milrinone. Oocytes were stained for total tubulin, F-actin and DNA and analysed by confocal microscopy.

**Results:** An increase in the number and density of TZPs was observed in oocytes cultured with milrinone for 1 hr. Dehydration of COCs caused the formation tubulin asters on the nuclear membrane and TZP density remained elevated in the presence, but not absence, of milrinone. Upon rehydration, COCs undergo fragmentation of cytoplasmic MTs and TZP loss independent of the use of milrinone. Whilst MTs were fragmented in rehydrated COCs, the degree of MT acetylation, was unchanged compared to dehydrated COCs. Commensurate with the differential loss of TZPs and MT integrity, significant changes in chromatin structure were evident after CPA exposure. Precocious condensation, in the absence of cell cycle progression occurred in addition to an increased association of chromatin with the nuclear envelope.

**Conclusions:** Together, these findings indicate that CPA exposure disengages the TZP/cytoskeleton/nuclear interaction in immature mouse oocytes predisposing the germinal vesicle and its contents to modifications that are likely to impact both the reinitiation and progression of meiosis after thawing and rehydration. We propose that future efforts to optimize immature oocyte cryopreservation should focus on the factors that integrate chromatin structure with the cortical cytoskeleton. Supported by K-INBRE, Hall family Foundation and the ESHE Fund.



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