ABOUT THE COVER

The cover depicts fluorescent staining of connexin 43 and transzonal projections in an isolated human pre-antral follicle. The image shows a single confocal plane at the median level of the follicle. Staining was performed using rabbit anti-Cx43 polyclonal antibody and goat anti-rabbit secondary antibody conjugated with FITC to visualize connexin 43, Alexa Fluor™ 568 Phalloidin to visualize transzonal projections which are composed of F-actin and Hoechst for staining nuclei. Images were obtained using a Leica SP8 confocal microscope equipped with a 405 nm Diode laser (to detect the Hoechst nuclear stain in blue), an Argon laser used at 488 nm (to visualize connexins in green) and a DPSS laser used at 561 nm (to visualize transzonal projections in red) with a 60× water immersion (N.A. 1.20) objective.

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Faculty of Biomedical, Pharmaceutical and Veterinary Sciences
Department of Veterinary Sciences

Assessment of the effects of cryostorage on pre-antral follicle survival and preservation of intercellular connections

Beoordeling van de effecten van cryopreservatie op de overleving van pre-antrale follikels en het behoud van intercellulaire contacten

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Sciences (PhD) to be defended by

ANNIEK BUS

Supervisors
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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ART	Assisted reproductive techniques
Bo	Bovine
CPA	Cryoprotective agent
CSF	Controlled slow freezing
CVM	Cryologic vitrification method
Cx	Connexin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DS	Dilution solution
EG	Ethylene glycol
ES	Equilibration solution
EURO-GTP	Good Tissue and cell Practice
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FP	Fertility preservation
GC	Granulosa cell
GJ	Gap junction
Gy	Gray
HE	Hematoxylin-eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic
Hu	Human
IVF	*In vitro* fertilization
IVG	*In vitro* growth
IVM	*In vitro* maturation
LM	Light microscopy
LN_2	Liquid nitrogen
LY	Lucifer Yellow
Mu	Murine
N	Number
NGS	Normal goat serum
NR	Neutral Red
OC	Oocyte cryopreservation
OT	Ovarian tissue
OTC	Ovarian tissue cryopreservation
Ov	Ovine
PAF	Pre-antral follicle
PBS	Phosphate buffered saline
PCNA	Proliferation cell nuclear antigen
PEG	Poly-Ethelene glycol
POI	Premature ovarian insufficiency

PSF	Passive slow freezing
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SF	Slow freezing
SLM	Sterile lyophilized high mannuronate
SRF	Slow rate freezing
TEM	Transmission electron microscopy
TRITC	Tetramethyl rhodamine
TS	Thawing solution
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tx-100	Triton X-100
TZP	Transzonal projection
VEGF	Vascular endothelial growth factor
VS	Vitrification solution
WS	Washing solution

CHAPTER 1
GENERAL INTRODUCTION

Female fertility preservation:

State of the art and problem statement

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In Belgium, 1772 women under 35 years of age were diagnosed with cancer in 2019. In addition, in Belgium, every year, about 340 children (0-14 years) and 180 adolescents (15-19 years) are diagnosed with a malignancy [1]. All malignancies combined, slightly more diagnoses are registered in boys (54%) than in girls (46%). Hematological cancers (leukemias and lymphomas) and brain tumors are the most frequent cancers in children and adolescents followed by carcinomas [1]. Important advances in cancer treatments have led to increased survival rates over the past years. Ten-year survival of children (84%) is very similar to the 10-year survival of adolescents (85%) [1]. Conversely, cancer therapies can result in acute or longer term premature ovarian insufficiency (POI) depending on follicular reserve, age and treatment used (Table 1, 2) [2]. This results from the primordial pool of follicles being formed before birth, such that at birth, the ovary has a fixed amount of oocytes.

Table 1. Chemotherapy agents that can lead to infertility

Risk level	Medication
High Risk (>70%)	Cyclophosphamide[a] Ifosfamide[a] Nitrosoureas[a] Chlorambucil[a] Melphalan[a] Busulfan[a] Procarbazine[a] Temozolomide[a]
Intermediate risk (30%-70%)	Bevacizumab Doxorubicin (as in doxorubicin and cyclophosphamide plus a taxane) FOLFOX regimen (folinic acid, fluorouracil, oxaliplatin[a]) Cisplatin[a]
Low or no risk (<30%)	Methotrexate 5-Fluorouracil Cytarabine Vincristine Bleomycin Doxorubin (without alkylating agents)

[a]Alkylating agents
From: Lee et al. (2006)[3]; Loren et al. (2013)[4]; Peccatori et al. (2013)[5] ; Bedoschi et al. (2016)[6]

Chemotherapy drugs can damage the human primordial follicle reserve by induction of DNA double-strand breaks (DSBs) and resultant apoptotic death, stromal-microvascular damage and follicle activation. Acute exposure to gonadotoxic chemotherapeutics, such as cyclophosphamide or doxorubicin, induce DNA DSBs and triggers apoptotic death of primordial follicle oocytes within 12-24 h, resulting in the massive loss of ovarian reserve. Evidence also indicates that chemotherapeutic agents can cause prenatal loss of oogonia, accelerated activation of primordial follicles (as growing follicles inhibit the recruitment of primordial follicles, the loss of this growing population will lead to

increased activation of primordial follicles and thus loss of the ovarian reserve), direct oocyte damage and microvascular and stromal damage that may induce hypoxia and indirectly affect ovarian reserve (Fig. 1) [7, 8]. Moreover, the impact of estrogen deficiency as a result of loss of ovarian function on quality of life, bone function and cardiovascular and neurological health are also critical aspects of the longer-term effects of chemotherapeutic damage to the ovaries [9, 10].

First-line cancer treatment does not usually compromise the ovarian reserve by more than 10% in girls under 10 years of age, while those aged 11-12 years show an estimated 30% decline in their ovarian reserve [11-13]. There is also a marked association between the intensity of treatment received and the likelihood of POI, even in young girls, but it is impossible to predict exactly who will be affected after aggressive chemotherapy. Alkylating agents are the most toxic. In a review [14], the North American Children's Oncology Group considered the risk of POI to be highest with busulfan administered at a dose of at least 600 mg/m^2 of body surface area, cyclophosphamide at a dose of at least 7.5 g/m^2, and ifosfamide at a dose of at least 60 g/m^2.

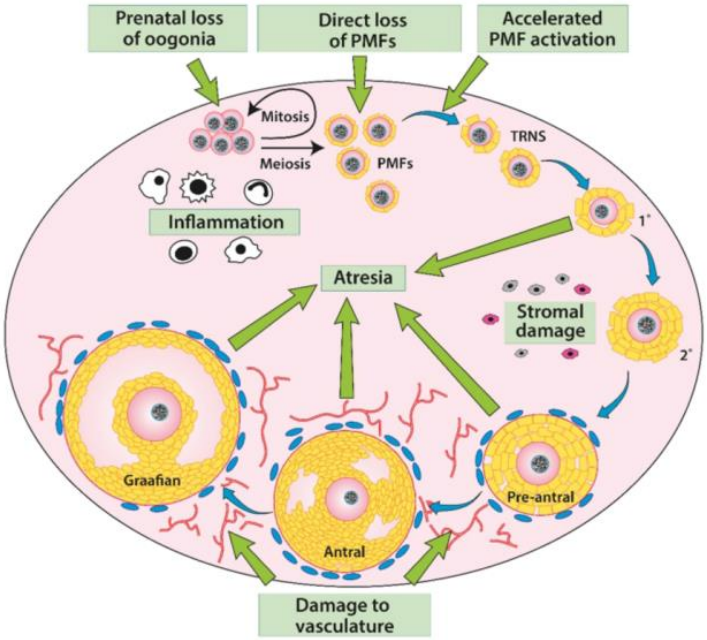

Fig. 1 The damaging effects of chemotherapy drugs on the ovary. Chemotherapy drugs can damage the ovary by inducing prenatal loss of oogonia, direct loss of primordial follicles, accelerated activation of primordial follicles, follicular atresia, stromal tissue damage, damage to the vasculature or inflammation. PMF: Primordial follicle. From Spears et al. (2019) [7].

Radiation therapy may have deleterious effects on future fertility as well. Total-body irradiation, direct pelvic, abdominal, and spinal irradiation or scatter irradiation can have a permanent negative effect on the ovaries by causing the depletion of follicles. Irradiation results in ovarian atrophy and reduced follicle stores [15]. On the cellular level, oocytes show rapid onset of pyknosis, chromosome

condensation, disruption of the nuclear envelope and cytoplasmic vacuolization. The degree and persistence of ovarian damage and suppression of ovarian function is related to the patient's age and the cumulative dose of ionic radiation to the ovaries (Table 2)[15, 16].

Table 2. Effect of radiotherapy on ovarian function

Ovarian dose (cGy)	Risk of ovarian failure	
	Women aged 15-40 years	Women >40 years
<60	No adverse affects	No adverse affects
150	Minimal adverse affects in young women	Some risk of sterilization in women over 40 years
250-500	60% sterilized; remainder may experience transient amenorrhea	100% sterilized
500-800	60%-70% sterilized; remainder may experience transient amenorrhea	100% sterilized
>800	100% sterilized	100% sterilized

From Wallach et al. (1986) [15]

The maintenance of future fertility is considered of great importance by patients. At the time of cancer diagnosis, approximately half of the women are concerned about the potential loss of ovarian function and fertility due to use of cancer treatments [17]. Some benign diseases are also indications for fertility preservation (FP), particularly those requiring chemotherapy (like thalassemia and lupus), recurrent endometriosis and family history of premature menopause. Furthermore, there are also social reasons for FP. It is not only a concern in humans, as the preservation of genetic material from endangered animal species or animals with important genetic traits will also greatly benefit from the development of alternative FP strategies (Table 3).

Table 3. Indications for fertility preservation

A) Malignant diseases most frequently requiring gonadotoxic chemotherapy and/or radiotherapy or bone marrow transplantation: • Hematological diseases (leukemia, Hodgkin's lymphoma, and non-Hodgkin's lymphoma) • Breast cancer • Sarcoma • Some pelvic cancers
B) Benign conditions for which fertility preservation is indicated: 1. Nononcological systematic diseases requiring chemotherapy, radiotherapy, and/or bone marrow transplantation 2. Nonmalignant ovarian diseases: • Bilateral ovarian tumors • Severe and recurrent ovarian endometriosis • Risk of ovarian torsion 3. Risk of premature ovarian insufficiency: • Family history • Turner syndrome
C) Social reasons: • Age • Childbearing postponed to later in life
D) Endangered species, breeds and animals with important genetic traits

Adapted from: Dolmans and Donnez (2021) [18]

Oocyte vitrification has become the standard approach to preserve fertility in women with benign diseases, those seeking FP for personal reasons (also called age-related infertility) and women with cancer if treatment can be safely postponed, given that oocyte cryopreservation requires a longer duration of time (approximately 2–3 weeks) for ovarian stimulation and oocyte retrieval prior to starting chemotherapy [19, 20]. Ovarian tissue cryopreservation (OTC) is specifically indicated for prepubertal girls and women who require immediate cancer treatment (Fig. 2) [19, 21, 22]. Since 2019, the American Society for Reproductive Medicine designated OTC as no longer experimental and an acceptable technique to be offered to patients seeking FP [23, 24]. This technique of ovarian tissue freezing followed by auto-transplantation has already yielded over 200 healthy live births [25]. However, there is great concern about the possible presence of malignant cells in frozen ovarian tissue, although not all cancers have the same risk (Table 4). Potential contamination and reimplantation of malignant cells, and hence recurrence of the primary disease should anyhow be avoided. In a review from Dolmans et al. (2021) [26], three pathologies were considered at particularly high risk of the presence of malignant cells in the ovary: leukemia, Burkitt lymphoma, and neuroblastoma. A safer alternative to allow fertility restoration for these patients could be the isolation, cryopreservation and reintroduction of pre-antral follicles in remaining ovarian tissue *'in situ'* following chemo- or radiotherapy.

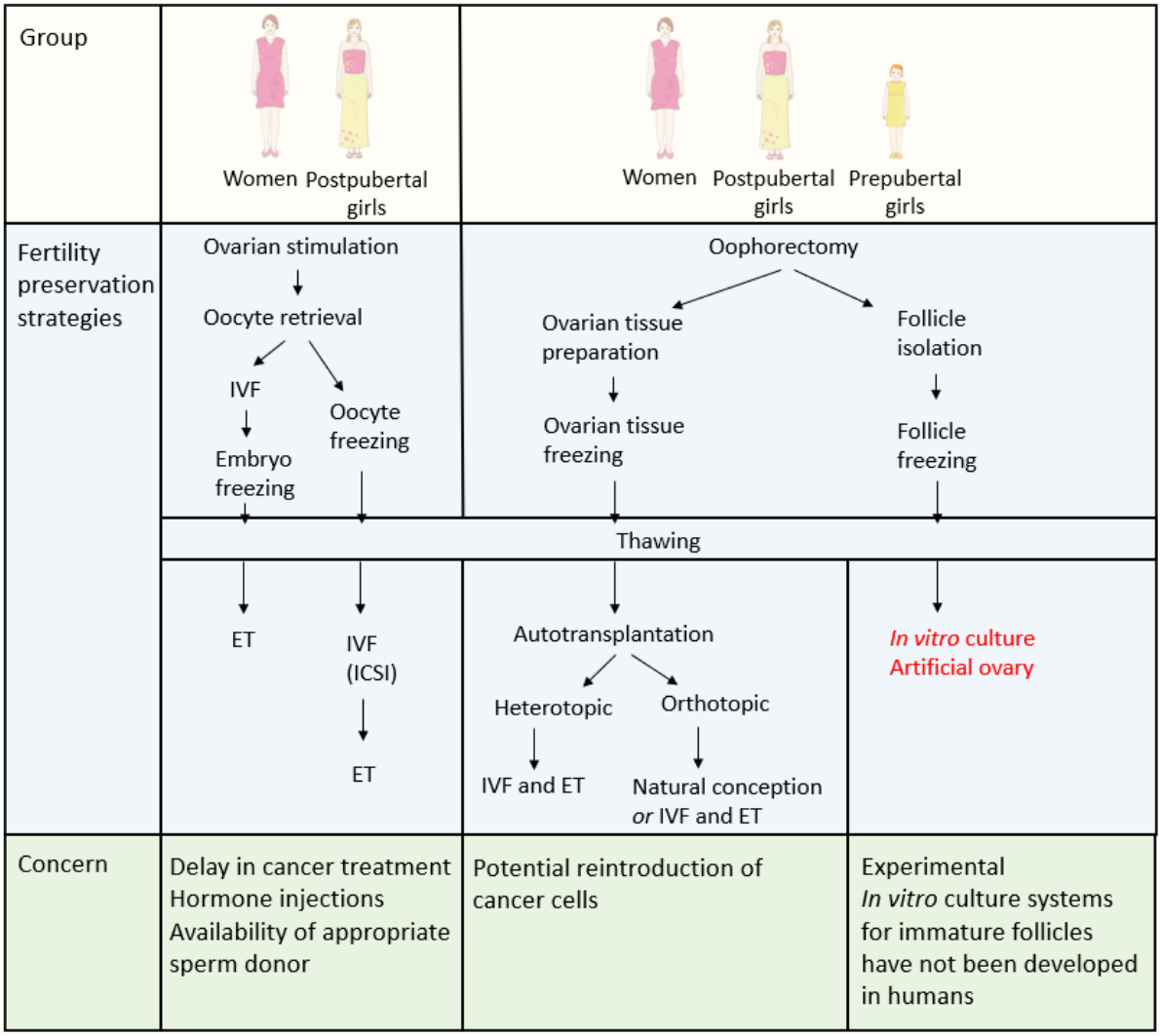

Fig. 2 Strategies to preserve fertility in female cancer patients through freezing

Table 4. Risk of ovarian metastasis according to cancer type

High risk (>10%)	Moderate (2-10%)	Low risk (0-2%)
Leukemia Neuroblastoma Burkitt lymphoma	Breast cancer, advanced stage Colorectal cancer Adenocarcinoma of the cervix Non-Hodgkin lymphoma Ewing sarcoma Ovarian cancer Borderline ovarian tumor	Breast cancer, early stage Squamous cell carcinoma of the cervix Hodgkin lymphoma Rhabdomyosarcomas Soft tissue sarcoma

From: Dolmans 2021 [26]

Based on a prospective cohort study of 1824 women undergoing gonadotoxic treatments, 4.8% of patients undergoing oocyte cryopreservation (OC) (n=1024) and 5.5% of patients undergoing OTC (n=800) returned (after a median follow-up of 5 years) to utilize their respective method of FP to attempt to achieve pregnancy [27]. Another study examining utilization of cryopreserved oocytes among cancer survivors in Milan, Italy, showed that 4.5% patients returned to use their oocytes [28].

It is also important to note that many women are taking the chances of obtaining a pregnancy without use of cryopreserved oocytes (e.g. by natural conception). In a study by Dahhan et al. (2014)[29], most women (71%) intended to conceive with their cryopreserved oocytes as a last resource option. In the former study [27], patients who underwent OC and OTC and desired a pregnancy had a 40.8% and 27.3% probability of having a clinical pregnancy, respectively. Of those who achieved clinical pregnancies, 80% of patients who underwent OC and 67% of patients who underwent OTC achieved live births [27]. in the OTC cohort, no pregnancies were achieved when the tissue was harvested over age 36 years. Recently, data from three major centers (Sheba Medical Center, Israel, Cliniques universitaires Saint Luc, Belgium, and St Louis Infertility Center, USA) involving 60 patients revealed a pregnancy rate of 50% and live birth rate of 41% following OTC [30]. In another study, from 23 women undergoing ovarian tissue reimplantation, the live birth rate was 41% (10 out of 22), yielding a total of 15 live births [19].

At present, many patients do not choose to undergo fertility preservation procedures. This may be explained by the difficult moment they are facing, by the fact that they may not be aware of the potential fertility loss and by the fear of delaying cancer treatment [4]. It has been reported that adequate counseling improves the number of women who choose to undergo fertility preservation treatments [31]. Hence, as endorsed by major international guidelines, it is crucial to counsel prepubertal girls and women in reproductive age who are facing cancer treatment, about the treatment-related loss of fertility and to assist them in taking decisions on fertility preservation [4, 5].

References

1. Cancer in children and adolescents in Belgium 2004-2016, B.C.R., Brussels, 2019.
2. Lambertini, M., et al., *Cancer and fertility preservation: international recommendations from an expert meeting.* BMC medicine, 2016. **14**(1): p. 1-16.
3. Lee, S.J., et al., *American Society of Clinical Oncology recommendations on fertility preservation in cancer patients.* Journal of clinical oncology, 2006. **24**(18): p. 2917-2931.
4. Loren, A.W., et al., *Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update.* Journal of clinical oncology, 2013. **31**(19): p. 2500.
5. Peccatori, F.A., et al., *Cancer, pregnancy and fertility: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Annals of oncology, 2013. **24**: p. vi160-vi170.
6. Bedoschi, G., P.A. Navarro, and K. Oktay, *Chemotherapy-induced damage to ovary: mechanisms and clinical impact.* Future oncology, 2016. **12**(19): p. 2333-2344.
7. Spears, N., et al., *Ovarian damage from chemotherapy and current approaches to its protection.* Human reproduction update, 2019. **25**(6): p. 673-693.
8. Morgan, S., et al., *How do chemotherapeutic agents damage the ovary?* Human reproduction update, 2012. **18**(5): p. 525-535.
9. POI, E.G.G.o., et al., *ESHRE Guideline: management of women with premature ovarian insufficiency.* Human Reproduction, 2016. **31**(5): p. 926-937.
10. Lobo, R.A., *Hormone-replacement therapy: current thinking.* Nature Reviews Endocrinology, 2017. **13**(4): p. 220-231.
11. Wallace, W.H.B., T.W. Kelsey, and R.A. Anderson, *Fertility preservation in pre-pubertal girls with cancer: the role of ovarian tissue cryopreservation.* Fertility and sterility, 2016. **105**(1): p. 6-12.
12. Wallace, W.H.B., et al., *Fertility preservation for girls and young women with cancer: population-based validation of criteria for ovarian tissue cryopreservation.* The Lancet Oncology, 2014. **15**(10): p. 1129-1136.
13. El Issaoui, M., et al., *Effect of first line cancer treatment on the ovarian reserve and follicular density in girls under the age of 18 years.* Fertility and sterility, 2016. **106**(7): p. 1757-1762. e1.
14. Van Dorp, W., et al., *Recommendations for premature ovarian insufficiency surveillance for female survivors of childhood, adolescent, and young adult cancer: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group in collaboration with the PanCareSurFup Consortium.* Journal of Clinical Oncology, 2016. **34**(28): p. 3440.
15. Wallach, E.E., M.D. Damewood, and L.B. Grochow, *Prospects for fertility after chemotherapy or radiation for neoplastic disease.* Fertility and sterility, 1986. **45**(4): p. 443-459.
16. Clough, K.B., et al., *Laparoscopic unilateral ovarian transposition prior to irradiation: prospective study of 20 cases.* Cancer: Interdisciplinary International Journal of the American Cancer Society, 1996. **77**(12): p. 2638-2645.
17. Ruddy, K.J., et al., *Prospective study of fertility concerns and preservation strategies in young women with breast cancer.* Journal of Clinical Oncology, 2014. **32**(11): p. 1151.
18. Dolmans, M.-M. and J. Donnez, *Fertility preservation in women for medical and social reasons: Oocytes vs ovarian tissue.* Best Practice & Research Clinical Obstetrics & Gynaecology, 2021. **70**: p. 63-80.
19. Donnez, J. and M.-M. Dolmans, *Fertility preservation in women.* New England Journal of Medicine, 2017. **377**(17): p. 1657-1665.
20. Cobo, A., et al., *Elective and onco-fertility preservation: factors related to IVF outcomes.* Human Reproduction, 2018. **33**(12): p. 2222-2231.
21. Donnez, J. and M.-M. Dolmans, *Fertility preservation in women.* Nature Reviews Endocrinology, 2013. **9**(12): p. 735-749.
22. Fisch, B. and R. Abir, *Female fertility preservation: past, present and future.* Reproduction, 2018. **156**(1): p. F11-F27.

23. Medicine, P.C.o.t.A.S.f.R., *Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion.* Fertility and sterility, 2019. **112**(6): p. 1022-1033.
24. Donnez, J., et al., *Ovarian cortex transplantation: time to move on from experimental studies to open clinical application.* Fertility and sterility, 2015. **104**(5): p. 1097-1098.
25. Dolmans, M.-M., T. Falcone, and P. Patrizio, *Importance of patient selection to analyze in vitro fertilization outcome with transplanted cryopreserved ovarian tissue.* Fertility and sterility, 2020. **114**(2): p. 279-280.
26. Dolmans, M.-M., *From isolated follicles to the artificial ovary: why and how?* Current Opinion in Endocrine and Metabolic Research, 2021.
27. Diaz-Garcia, C., et al., *Oocyte vitrification versus ovarian cortex transplantation in fertility preservation for adult women undergoing gonadotoxic treatments: a prospective cohort study.* Fertility and sterility, 2018. **109**(3): p. 478-485. e2.
28. Specchia, C., et al., *Oocyte cryopreservation in oncological patients: eighteen years experience of a tertiary care referral center.* Frontiers in endocrinology, 2019. **10**: p. 600.
29. Dahhan, T., et al., *Reproductive choices and outcomes after freezing oocytes for medical reasons: a follow-up study.* Human Reproduction, 2014. **29**(9): p. 1925-1930.
30. Shapira, M., et al., *Evaluation of ovarian tissue transplantation: results from three clinical centers.* Fertility and sterility, 2020. **114**(2): p. 388-397.
31. von Wolff, M., et al., *Fertility-preservation counselling and treatment for medical reasons: data from a multinational network of over 5000 women.* Reproductive biomedicine online, 2015. **31**(5): p. 605-612.

Is the pre-antral ovarian follicle the *'holy grail'* for female fertility preservation?

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TABLE OF CONTENTS

Summary

Fertility preservation is not only a concern for humans with compromised fertility after cancer treatment. The preservation of genetic material from endangered animal species or animals with important genetic traits will also greatly benefit from the development of alternative fertility preservation strategies. In humans, embryo cryopreservation and mature-oocyte cryopreservation are currently the only approved methods for fertility preservation. Ovarian tissue cryopreservation is specifically indicated for prepubertal girls and women whose cancer treatment cannot be postponed. The cryopreservation of pre-antral follicles (PAFs) is a safer alternative for cancer patients who are at risk of the reintroduction of malignant cells. As PAFs account for the vast majority of follicles in the ovarian cortex, they represent an untapped potential, which could be cultivated for reproduction, preservation, or research purposes. Vitrification is being used more and more as it seems to yield better results compared to slow freezing, although protocols still need to be optimized for each specific cell type and species. Several methods can be used to assess follicle quality, ranging from simple viability stains to more complex xenografting procedures. *In vitro* development of PAFs to the pre-ovulatory stage has not yet been achieved in humans and larger animals. However, *in vitro* culture systems for PAFs are under development and are expected to become available in the near future. This review will focus on recent developments in (human) fertility preservation strategies, which are often accomplished by the use of *in vitro* animal models due to ethical considerations and the scarcity of human research material.

1. Introduction

According to recent data, women under 40 years of age have an estimated 2.5% chance of developing cancer [1]. However, due to many improvements in cancer diagnosis and treatment [2], up to 90% of women who are diagnosed with reproductive tract cancer are now long-term survivors [3]. However, as chemo- and radiotherapy often damage ovarian tissue [4, 5], patients are likely to show compromised fertility following treatment [6, 7] up to a level where survivors of childhood cancer have an overall reduction of 19% in the likelihood of ever being pregnant [8]. As a consequence, the interest in fertility preservation (FP) strategies in women has been sparked to the extent that it has now become a key medical sub-discipline [9]. Moreover, fertility preservation is not only a concern in humans, as a parallel need for the preservation of genetic material from endangered animal species or animals with important genetic traits will also greatly benefit from the development of alternative preservation strategies [10]. To further optimize FP strategies, the use of animal *in vitro* models will certainly benefit progress in both human and animal FP research [11]. Recently, there has been an increasing interest for bovine *in vitro* models in human studies on assisted reproductive techniques (ARTs) and FP, as reviewed by Langbeen et al. (2015) [11]. It is well established that a number of similarities exist between bovine and human species with regard to ovarian morphology and function, and that the bovine species could serve as an effective model for human follicular dynamics [12, 13]. Moreover, the easy access to slaughterhouse ovaries guarantees unrestricted availability of study specimens such as PAFs [11]. Also, 'fertility preservation' in case of the individual female patient will often be translated into 'species preservation' when these techniques are applied to endangered animals, thereby elevating (fertility) preservation from the individual to the population level.

Current FP techniques for women comprise ovarian transposition, cryopreservation of embryos and unfertilized oocytes [14-16] and ovarian tissue cryopreservation (OTC) containing pre-antral follicles (PAFs) [9]. In animals, cryopreservation of *in vivo* and *in vitro* produced embryos has become a routinely used technique [17], while freezing of ovarian tissue is still considered to be in its experimental phase. When considering female cancer patients, the most suitable option for a specific patient is based upon different parameters such as the patient's age and relationship status, the type of cancer, and the time available between diagnosis and the onset of treatment [14]. The cryopreservation of embryos and oocytes on the other hand requires the patient to be of pubertal age, have a partner or use donor sperm, and to be able to undergo a cycle of ovarian stimulation. For prepubertal girls and women that cannot delay the start of chemotherapy, cryopreservation of ovarian tissue is the only option available. However, this technique is not advisable for patients with certain types of cancer with medium to high risk of ovarian metastasis, such as leukaemia, as there is a risk of re-introducing malignant cells present in the cryopreserved ovarian tissue following auto-

transplantation [18]. For these patients, a safer alternative to allow fertility restoration could be the isolation of pre-antral follicles or PAFs (before or after cryopreservation) from ovarian tissue for *in vitro* growth, maturation, and fertilization or auto-transplantation of the frozen-thawed isolated PAFs in residual ovarian tissue *in situ* [19]. In animals, (fertility) preservation strategies are often hampered by the urgency that is linked to the procedure. When specimens of endangered animal species die unexpectedly or are found killed under field conditions, the retrieval and cryopreservation of ovarian tissue is often the only rescue option. For animals that already have been dead for a few hours, it might be useful to be able to isolate the PAFs from the surrounding tissue, thereby simultaneously preventing the cryopreservation of ovarian tissue samples that are devoid of follicles. Currently, the main limiting factor is that for many (endangered) species, IVM (*in vitro* maturation) and IVF (*in vitro* fertilization) technologies have not yet been developed. Ovarian tissue xenotransplantation can be applied to maintain the reproductive potential of the donor, but it is still an experimental approach. Using (expensive) rat and mouse immunocompromised hosts, graft rejection has not been a major problem. With regard to species conservation, Snow et al. (2002) [20] harvested mature mouse oocytes from ovarian tissue that was xenotransplanted to a rat recipient that could then subsequently be fertilized and developed into fertile adult mice. Mattiske et al. (2002) [21] observed the development of follicles in grafted cryopreserved ovarian tissue from the tammar wallaby pouch after xenotransplantation into adult mice. Wombat ovarian tissue survived and functioned when grafted into immunocompromised rats [22]. Xenografting has the potential to produce mature oocytes from endangered species for use in assisted reproductive technologies, such as *in vitro* fertilization (IVF). Furthermore, mature oocytes from non-endangered species can be used for nuclear transfer for the preservation of critically endangered species. To encapsulate and protect isolated PAFs, the possibilities of an *in vitro* artificial ovary [23] and a transplantable artificial ovary are being investigated. An artificial ovary is composed of a matrix that encapsulates and protects not only the isolated follicles but also autologous ovarian cells and bioactive factors, which are necessary for follicle survival and development. We position the pre-antral ovarian follicle on the forefront for cryopreservation because it plays a major role in ovarian tissue cryobanking and the establishment of alternative storage techniques for isolated follicles. This review will focus on recent developments in (human) fertility preservation strategies which are often accomplished by the use of *in vitro* animal models. It aims to stimulate researchers in the reproductive field to set goals for improving fertility at the individual and preservation at the population level across species.

2. The pre-antral follicle

The follicular reserve stored in the ovaries determines the length of a woman's reproductive life. In domesticated mammals, that are not limited in their reproductive capacities due to menopause, it merely forms a stock from which a cohort of PAFs is activated with each sexual cycle. About 90% of the total follicular reserve, consisting mainly of resting primordial follicles, is stored in the poorly vascularized outer layer of the ovarian cortex. A primordial follicle consists of an immature, quiescent oocyte, surrounded by a single layer of flattened (pre-)granulosa cells. Activation is accompanied by the proliferation and differentiation of granulosa cells: in a primary follicle, granulosa cells are cuboidal in shape and the number of granulosa cells increases. In the subsequent growth stages, the oocyte undergoes volume expansion and a zona pellucida develops (secondary follicle). The majority of activated follicles evolve to the antral stage, characterized by the formation of a cavity or antrum [24]. Activated follicles migrate towards the more vascularized ovarian medulla [25]. Early PAFs account for the vast majority of follicles in the ovarian cortex. The majority of these (99.9%) will never mature into preovulatory follicles [26], but rather will perish at a premature stage along the developmental path. The stock of primordial and primary follicles thus represents an untapped potential, which could be cultivated for reproduction, preservation, or research purposes. Improvements in the isolation, *in vitro* culture and auto-transplantation of ovarian follicles will therefore greatly enhance current fertility preservation strategies. They provide a safe method for the restoration of fertility in women that are affected by chemo/radiotherapy induced premature ovarian failure. Additionally, they also constitute a genetic pool from which endangered animal populations can be restored when *in vitro* follicle culture will become a mainstream technique.

3. Options for fertility preservation

3.1. Ovarian tissue

Although several dozens of births have already been reported worldwide [27-29], auto-transplantation of frozen-thawed tissue cannot be recommended to cancer patients who are at risk of the reintroduction of malignant cells [30-32]. Dolmans et al. (2010) [30] evaluated the presence of leukemic cells in cryopreserved ovarian tissue from leukemia patients. They found that histological analyses did not identify any malignant cells in the tissue, however, quantitative reverse-transcribed polymerase chain reaction (RT-PCR), demonstrated ovarian contamination by malignant cells in acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML). Immunodeficient mice xenografted for 6 months with ovarian tissue from ALL patients, developed intraperitoneal leukemic masses,

demonstrating that reimplantation of cryopreserved ovarian tissue from patients with ALL puts them at risk of disease recurrence. Another drawback linked to transplantation of ovarian tissue is the extensive loss of PAFs due to delayed and deficient revascularization of the transplanted tissue [32, 33]. From only about 500 to 1,000 primordial follicles present in the transplanted ovarian tissue strip, more than 50% are lost [34]. Several attempts have been made to treat or prepare tissue strips, aiming for a better vascularization following transplantation, such as co-incubation with vascular endothelial growth factor (VEGF) [35]. *In vitro* VEGF exposure of bovine cortical tissue strips prior to transplantation in immunodeficient mice was shown to be beneficial only for short term survival of PAFs, 2 weeks following transplantation (Fig. 1). However, 4 weeks after transplantation, no additional beneficial effects of VEGF on the vascularization of the grafted ovarian tissue could be reported. It was concluded that, although the transplantation process overall negatively influences the number of viable follicles and vascular density, VEGF exposure prior to transplantation can favor follicle survival during a 2 weeks transplantation period.

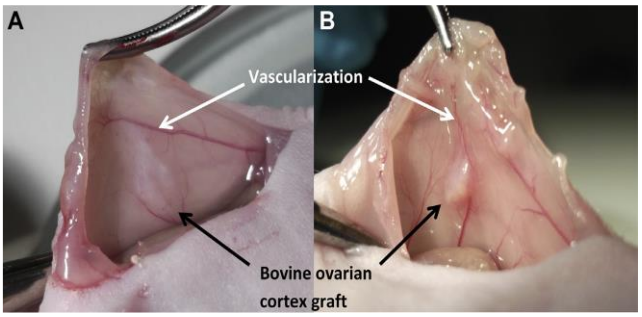

Fig. 1 Transfer of bovine ovarian cortex as a relevant model for human (preimplantation) reproductive research at a retroperitoneal location in an immune deficient mouse serving as an *in vivo* bioincubator. Macroscopic graft aspect after a transplantation period of 2 weeks (A) and 4 weeks (B), with indications of neo-vascularization. (Figure Langbeen et al., 2016) [35]

While chances of restoration of ovarian activity is higher if primordial follicles are present in the transplanted tissue and the chance of live birth after replacement of ovarian tissue is approximately 20% [36], both quantitative and qualitative assessments of the follicle population in ovarian tissue samples are difficult, if not impossible [37-39]. Indeed, PAFs are heterogeneously distributed and clustered throughout the ovarian cortex. Every developmental stage can be present in any given piece of ovarian cortex and many pieces might contain no follicles at all. As a consequence, the risk of freezing tissue samples devoid of (viable) follicles is significant, creating a false idea of preserved fertility for the patient. Although ovarian tissue cryopreservation (OTC) is still considered to be in its experimental phase, many experts believe that there is now sufficient evidence to consider it as a valid and effective technique [40, 41]. However, because laparoscopic oophorectomy and ovarian cortex

collection is an invasive procedure requiring general anesthesia, thorough patient selection is essential [42] with age being one of the most important selection criteria. While first-line cancer treatment does not compromise the ovarian reserve (= the amount of PAFs present in a quiescent state on both ovaries) by more than 10% in girls under 10 years of age [42, 43], girls at the age of 11 or 12 years have an estimated 30% decline in their ovarian reserve. Further selection criteria are a realistic chance of survival for 5 years together with a high risk of premature ovarian insufficiency (>50%) [42, 43]. Finally, in human, there seems to be a correlation between the intensity of cancer therapy and the likelihood of premature ovarian insufficiency, even in young girls [44, 45]. Unfortunately, it is still impossible to predict the effect of aggressive chemotherapy on the ovarian reserve. Due to ethical issues and the scarcity of research material, it is problematic to perform experiments using human tissue. Thus, advances in bovine and sheep ovarian vitrification are relevant, as these have been demonstrated to be anatomically and physiologically similar to the human ovary [46, 47].

3.2. Isolated follicles

PAFs are surrounded by a basement membrane that separates them from the ovarian stroma, blood vessels, white blood cells and innervation [48]. Therefore, malignant cells cannot invade PAFs via the blood stream and as a consequence, cannot be returned back to the patient when isolated PAFs would be auto-transplanted in residual ovarian tissue following cancer treatment. In human reproductive research, two main strategies are being investigated worldwide to allow fertility restoration and to avoid the risk of transplanting malignant cells: the *in vitro* artificial ovary and the 'transplantable' artificial ovary [23]. While the first one aims to achieve the entire folliculogenesis process *ex vivo/ in vitro*, the second aims to transplant isolated pre-antral follicles back to their natural environment, albeit in an artificial scaffold. Primordial follicles are considered to be more resistant to cryo-injury than growing follicles because they only have a small amount of cold sensitive intra-cytoplasmatic lipid droplets, are less differentiated and their metabolism is relatively low, they only have a small amount of organelles and lack a zona pellucida, cortical granules and a meiotic spindle [49, 50]. The small size of primordial follicles (between 30 and 80 micrometers) also greatly facilitates penetration of cryoprotectants [50]. Because these PAFs have been shown to easily maintain normal morphology and ultrastructure during cryopreservation, they are excellent candidates for long term preservation. When it comes to PAF isolation, both mechanical and enzymatic methods [51, 52] are used to separate follicles from the surrounding stromal cells. Enzymatic digestion of small ovarian tissue fragments is reasonably fast, but it might damage the basal membrane, impairing the follicle's viability [51]. Mechanical follicle isolation, on the other hand, is generally considered to be a safe but labor-intensive and time-consuming method [51]. For human primordial follicles, however, mechanical isolation is

difficult due to the fibrous and dense ovarian stroma, and therefore enzymatic digestion with collagenase or liberase has to be used [52]. The originally developed mechanical procedure for isolating PAFs (as used for ruminant ovaries) consists of cutting and pipetting followed by filtration, and yields a large number of follicles [51]. More recently, Langbeen et al. (2015) [53] developed a new mechanical isolation method based on tissue dispersion following cutting and blending small tissue pieces of ovarian cortex. Applied to the ovaries of three ruminant species, this consistently results in the retrieval of viable follicles without visual damage to the basement membrane. In addition, the mechanically isolated follicles can easily be characterized and classified upon retrieval based on generally accepted morphological characteristics, such as follicular diameter and the number of granulosa cells [54, 55]. In animals, ovarian tissue can quite easily be collected post-mortem, and PAF retrieval is also possible using the transvaginal, ultrasound-guided biopsy collection approach [56]. Thus avoiding a laparoscopic intervention, it is possible to repeatedly sample the ovary and collect small pieces of ovarian cortex tissue containing viable PAFs from living donor cows [57]. This method can eventually be developed into a new source of PAFs for *in vitro* maturation of follicles for high genetic merit livestock production as soon as routine *in vitro* follicle culture protocols will be available.

4. Cryopreservation

4.1. Ovarian tissue

Human reproductive researchers reported the first successful pregnancy and childbirth following the auto-transplantation of strips of cryopreserved ovarian cortex in 2004 [34]. Thus far, more than 60 live births have been achieved after re-implantation of cryopreserved ovarian tissue [40]. Most reported live human births were achieved after transplantation of ovarian tissue that had been slow-frozen [36], a technique that is still applied in most FP laboratories and based on a computer-assisted gradual and well controlled cooling down protocol. There are only a few reports of live births from transplantation of vitrified human ovarian tissue [58, 59]. Although rapid advances in vitrification methods - basically avoiding the formation of ice crystals by ultra-rapid cooling and the use of high concentrations of cryoprotectants - have led to successful cryopreservation of embryos and mature oocytes, studies comparing slow freezing and vitrification of ovarian tissue have presented conflicting results [60]. The vitrification of ovarian tissue is difficult because of cellular heterogeneity. The permeation and toxicity of cryoprotectants (CPAs) are specific for each cell type and tissue. The most effective combination and concentration of the CPAs used is therefore a compromise between optimal values for the different cell types (oocyte, granulosa cells, stromal cells, blood vessels). The surrounding ovarian tissue may result in a slower and more complex perfusion process, potentially leading to reduced

oocyte protection during all steps of the cryopreservation procedure. Solid surface vitrification (SSV) uses a metal cube covered with aluminum foil which is pre-cooled to -180 °C by partial submersion in liquid nitrogen (LN_2). Microdrops of vitrification solution, containing the samples, are dropped onto the cold upper surface of the metal cube and are instantaneously vitrified [61]. SSV provides sufficient space for the tissue, maximizes cooling rates, and avoids the generation of the gas phase of LN_2 bubbles and has been successfully applied to both goat and sheep ovarian tissues [46, 62] as well as goat, bovine, porcine and sheep oocytes [46, 63-65]. Kagawa et al. (2007) [66] reported the birth of healthy mouse pups derived from the oocytes of pre-antral follicles obtained from ovarian tissue of adult mice that were vitrified using the Cryotop vitrification technique. The Cryotop method was designed by Kuwayama and consists of a fine, transparent polypropylene film attached to a plastic handle equipped with a cover straw, into which oocytes and embryos can be loaded in very small volumes (~0.1 µl) [67]. Based on this high-efficiency vitrification Cryotop method, a Cryotissue method was developed for the vitrification of ovarian tissue. Bovine and human ovarian tissue have been successfully vitrified using the Cryotissue method. Ovarian tissue was placed in a minimum volume of solution onto a thin metal strip (Cryotissue; Kitazato BioPharma, Fujinomiya, Japan), and submerged directly into sterile LN_2, and subsequently the strip was inserted into a protective container and placed into an LN_2 storage tank. No difference in oocyte viability (>89%) between fresh and vitrified ovarian cortical tissue in either bovine or human samples was found. Auto-transplantation of vitrified–warmed tissue back to cattle-donors also resulted in no loss of oocyte viability [47]. According to some human studies, slow freezing yielded better results in cryopreserving ovarian tissue [68, 69], while others found improved conservation of the ovarian follicular and stromal structures and increased follicle survival rates after vitrification [47, 70-73]. With an increase in the number of children born, data on the efficiency of vitrification and slow freezing of ovarian cortex can be compared and validated in prospective randomised studies, with healthy livebirth rates as the main outcome parameter. Other researchers [74] reported the survival and growth of auto-transplanted murine follicles after cryopreservation of the ovarian cortex by two different methodologies, solid-surface vitrification (SSV) later also known as Cryologic vitrification method (CVM) and slow-rate freezing (SRF). CVM vitrified ovarian fragments were loaded in 3 µL droplets onto the fine hook at the end of a plastic fibreplug, after which the droplets were vitrified by contact with the metal surface. Slow-rate frozen ovarian fragments were cooled using a programmable freezer and plunged in LN_2. Increased follicular proliferation and antral stages were identified after both cryo-treatments. The fraction of secondary and antral follicles was, however, significantly larger after SRF cryo-treatment, suggesting that CVM treated tissue may have suffered a growth disadvantage. Vitrification of pre-antral follicles by the CVM method remains a viable alternative to conventional SRF, but further research is required for

optimizing and standardizing ovarian tissue cryopreservation, with a special focus on follicle quality and viability assessment, which is difficult for follicles *'in situ'*.

4.2. Isolated follicles

Results obtained from studies on the cryopreservation of isolated PAFs have been encouraging. Thus far, studies on slow freezing have been performed in sheep [75-78], goat [79], cat [80], monkey [81] and human isolated follicles [82]. It was demonstrated in a study by Vanacker et al. (2013) [82] that human PAFs can be successfully cryopreserved by slow freezing before or after isolation, without impairing their ability to survive and grow *in vitro*. Vitrification of isolated follicles was investigated in mice [83], rat [84], sheep [46, 76] and cattle [85]. Lunardi et al. (2015) [46] vitrified sheep secondary follicles in the isolated state and within fragments of ovarian tissue. They concluded that both techniques can be used, but isolated follicles displayed a better follicular growth rate and in this group, fewer follicles with a decreased diameter were found after *in vitro* culture. Isolated bovine follicles were successfully vitrified using HSV straws®, showing a high viability (87.5%) post thawing with no significant differences to follicles that were cultured in a 2D culture system [86]. However, considering the labor-intensive procedure and relatively low efficiency as many PAFs are lost during the vitrification procedure, vitrification of embedded follicles, in for example alginate beads (see below) may be the method of choice in the future. Langbeen et al. (2014) [85] demonstrated that isolated PAFs are much easier to quantify and assess for viability, which is a crucial component of a functional fertility preservation strategy (see below).

4.3. Isolated embedded follicles

Bus et al. (2018) [86] studied the effects of encapsulation of bovine isolated PAFs in calcium alginate beads on follicle viability and morphology after vitrification (Fig. 2). Follicles that were vitrified in alginate beads carried in mesh cups showed a low viability post warming (45.9%) and were significantly less viable than non-vitrified PAFs that were cultured directly in beads (88.4%). However, the encapsulation of follicles in beads has important advantages, as their manipulation is much easier and a lot of time is saved because follicles can be vitrified in small groups. While vitrification as such was successfully used for non-embedded PAFs, further optimization for embedded bovine PAFs is necessary and seems advantageous. A first step in this process could be the use of a longer exposure time to the cryoprotectant agents (CPAs), considering the increased volume of embedded follicles which may prolong the CPA diffusion time [87] [88]. In addition, Bian et al. (2013) [87] showed that human PAFs encapsulated in alginate could maintain their normal ultrastructure after vitrification.

Camboni et al. (2013) [89] successfully cryopreserved embedded human primordial/primary follicles using slow freezing. In conclusion, alginate constitutes an easy-to-handle, safe hydrogel matrix to cryopreserve isolated follicles, but further studies are necessary to increase the survival and maintenance of normal morphology after vitrification.

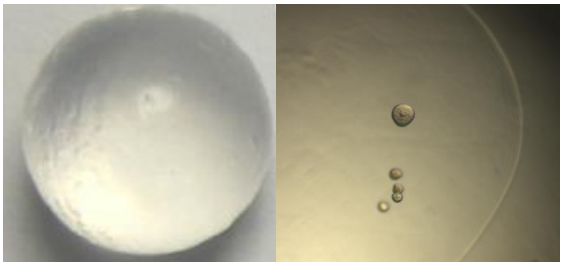

Fig. 2 Left: macroscopic image of an alginate bead. Right: light microscopic image of 5 encapsulated follicles in an alginate bead. (Figure Bus et al., 2018) [86]

5. Follicle quality assessment

5.1. Short term in vitro culture

Survival and follicle growth during short term *in vitro* culture can be used as a non-invasive tool for the evaluation of follicle *in vitro* viability. To generate individual follicle follow-up data, Jorssen et al. (2015) [90] characterized follicular dynamics using an *in vitro* culture system of isolated and individually cultured bovine early pre-antral follicles during 10 days. Individual follicle morphology and growth were evaluated by non-invasive assessment methods that allowed continuous evaluation over time. PAFs were light microscopically evaluated during culture to assess macroscopic cell morphology and follicle diameter. Based on the connection between the oocyte and the surrounding (pre-)granulosa cells and the microscopical integrity of the basal membrane, follicles were subdivided into three categories, with category 1 follicles showing the best morphological features (Fig. 3).

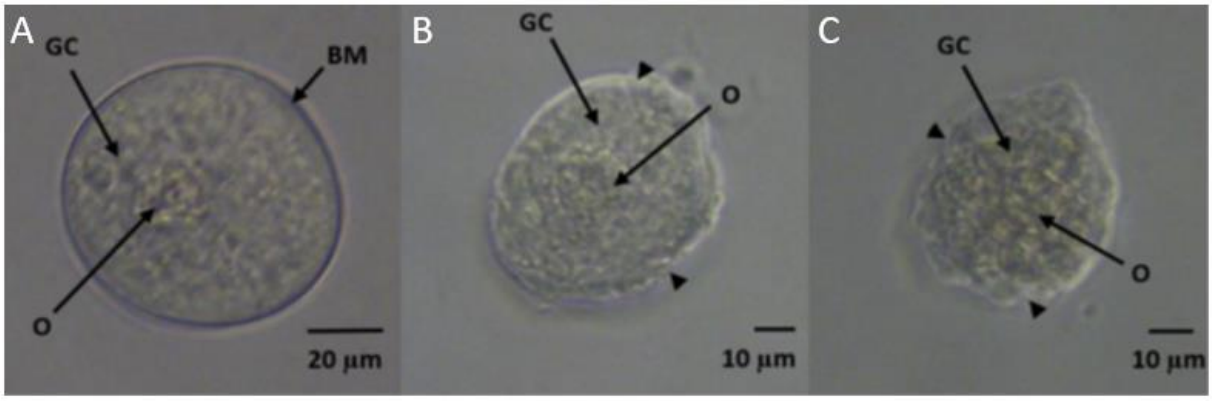

Fig. 3 A) Category 1 includes the follicles with both an intact basal membrane and an intact connection between the oocyte and surrounding granulosa cells. B) Category 2 contains follicles with an intact connection between the oocyte and the surrounding granulosa cells but shows signs of a disrupted basal membrane (arrowhead). C) Category 3 contains the follicles with a disrupted basal membrane (arrowhead) and a disrupted connection between the oocyte and granulosa cells. O: oocyte; GC: granulosa cells; BM; basal membrane (Figure Bus et al., unpublished images)

As shown with Neutral Red staining (see below), nearly 68% of all PAFs (cat. 1, 2 and 3) survived a 10-day *in vitro* culture period, whereby an intact basal membrane and connection between the oocyte and the granulosa cells seem to be a prerequisite for maintaining follicular development. The increase in follicular diameter was found to be correlated with an increase in the total cell number. Follicles maintaining their category 1 morphologic features over time seemed to be of a better quality and showed a higher developmental competence *in vitro* as compared to category 2 and 3 follicles [90].

5.2. Neutral Red staining

Neutral Red (NR) is a water-soluble and nontoxic dye that was proposed as a non-invasive viability assay on the basis of its ability to diffuse through the plasma membrane and concentrate in the lysosomes of viable and metabolically active cells [91, 92]. It can be used to evaluate follicle content and viability in cortical tissue strips or for the staining of isolated follicles (Fig. 4) [93]. Kristensen, Rasmussen [94] validated the use of NR as a vital dye through the application of the carboxyfluorescein diacetate succinimidyl ester as a marker for proliferation after the use of NR, therefore suggesting the absence of (acute) NR toxicity. Langbeen et al. (2014) [85] established a staining protocol for isolated bovine PAFs, indicating that 15 mg/mL of NR, with an exposure time of 30 minutes, was not deleterious when used before and after vitrification. In addition, Jorssen et al. (2014) [39] reported that NR could be used to identify PAFs present in bovine cortical tissue strips and concluded that *in vitro* follicular dynamics were not influenced by oxygen tension or by repeated viability assessments using NR.

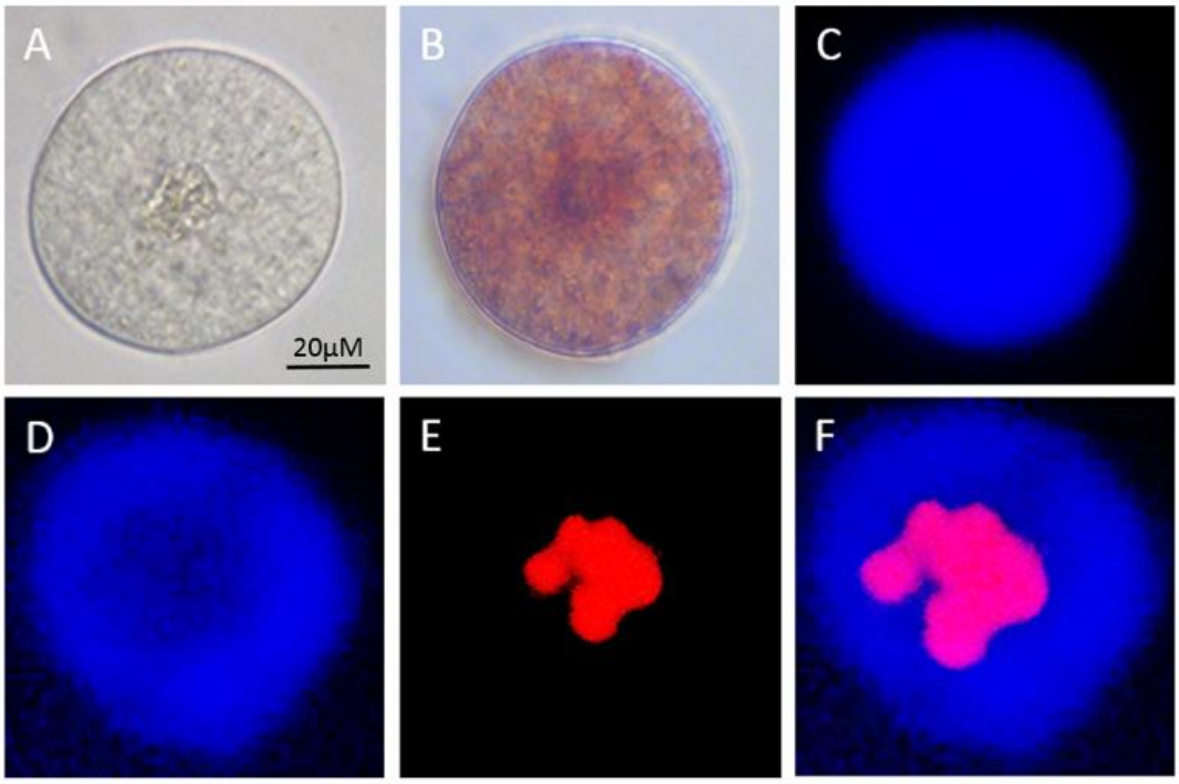

Fig. 4 Follicles stained with Neutral Red (NR) and calcein-AM to indicate the viability of the follicles, propidium iodide shows nuclei of dead cells. A) Light microscopic (LM) image of non-stained follicle. B) LM image of follicle staining positive for NR; viable follicle. C) Follicle staining positive for calcein; viable follicle. D) Follicle of which the oocyte stained negative and the granulosa cells positive for calcein. E) Same follicle as in B, nuclei staining positive for propidium iodide. F) Merged picture of D and E. (Figure Bus et al., 2018) [86]

5.3. Calcein staining

Follicle viability can easily be assessed with the fluorescent probe Calcein (Blue) AM. Through intracellular esterase activity, living cells convert the non-fluorescent cell-permeable Calcein-AM into a fluorescent calcein. The polyanionic dye calcein is retained well in living cells, producing an intense, uniform green or blue (Calcein Blue AM) fluorescence. Green fluorescence can be visualized after exposing the tissue to light with a wavelength of 495 nm (excitation [ex]) and observing the emitted (em) light at a wavelength of 515 nm. Blue fluorescence can be visualized with excitation/emission maxima of 360/449 nm. Staining with Calcein (Blue) AM permits follicle viability to be assessed within 1 hour [95].

5.4. Gap junction identification and assessment of functionality

Gap junctions are intercellular membrane channels directly connecting the cytoplasm of adjacent cells, thus allowing the exchange of ions, second messengers and small metabolites [96], and they are thought to play a crucial role in intercellular communication between different follicular components [97]. A gap junction channel is composed of two hemi-channels (connexons), each of which is composed of six protein subunits (connexins). Among gap junction proteins identified in ovarian follicular cells, the two connexins (Cx) Cx37 and Cx43 seem to be critical at each step of normal folliculogenesis [98]. Investigating Cx expression in the bovine ovary as a prerequisite for follicle quality, Nuttinck et al. (2000) [99] found that Cx43 expression was restricted to granulosa cells, while Cx37 staining was observed in both the oocyte and granulosa cell compartments. Vitrification of follicles can lead to cryoinjuries, which can result in the loss of membrane proteins, such as Cx37 and Cx43. Ortiz-Escribano et al. (2017) [100] showed that vitrification opens hemichannels in bovine blastocysts, which are normally closed, but open in response to stress conditions and concluded that blocking hemichannels can protect embryos during vitrification and warming. After cryopreservation and subcutaneous transplantation of mouse ovarian tissue, proteins forming gap junctions between oocytes and granulosa cells are under-expressed compared with normal controls [101]. The poor retrieval of mature oocytes from such grafts might in part result from the failure of signal transduction and metabolite transmission between granulosa cells and the oocyte. In isolated PAFs, the detection and quantification of immunofluorescently labeled connexins before and after cryopreservation can give an indication about the functional state of follicles post thawing. Functionality of gap junctions can be assessed by injection of the fluorescent dye Lucifer Yellow into the oocyte and by examination of the dye's ability to transfer from the oocyte to the surrounding granulosa cells through intact gap junctions. Barrett et al. (2010) [81] showed that immediately after thawing, mouse follicles were unable to transfer dye into surrounding granulosa cells, indicating that the gap junctions were not functional. However, after 2 days of culture, cryopreserved follicles were able to re-establish gap junctions and transport the dye from the oocyte into the surrounding somatic cells.

5.5. Xenotransplantation

Xenotransplantation of ovarian tissue or isolated PAFs can be used to assess the viability and developmental capacity of PAFs. Massive follicle activation is a typical transplantation effect, but testifies to the survival of cryopreserved follicles [74]. In the study by Aerts et al. (2008)[74], the fraction of primordial follicles in the Cryologic vitrification method (CVM) and slow-rate freezing (SRF) grafts significantly decreased as compared to control tissue, whereas intermediary and primary

follicles significantly increased, indicating massive follicle activation following transplantation. Such massive primordial follicle recruitment typically occurs after grafting, but also during *in vitro* culture experiments. It is hypothesized that the ovary under physiological conditions is dominated by inhibitory mechanisms [102], which are no longer available during culture or transplantation. An alternative explanation was proposed by Cushman et al. (2002) [103] who suggested that the follicle environment *in vitro* or after grafting was richer in oxygen and nutrient levels than the avascular ovarian cortex, which contains the quiescent primordial follicles.

6. Post-thawing applications with isolated follicles

6.1. In vitro culture (2D – 3D culture systems)

Culturing isolated follicles *in vitro* from the primordial stage onwards is potentially an attractive strategy because they represent >90% of the total follicular reserve and show high cryotolerance [104]. However, to date, it has not been possible to grow human and large domestic mammal isolated primordial follicles *in vitro* to the mature oocyte stage [105, 106]. Therefore, further studies are needed to identify factors sustaining follicular growth and maturation and to assess the contribution of stromal cells to these processes. Primary and early secondary follicles are gonadotropin independent and essential factors for their growth and development often require co-culture with various “feeder” cells/follicles [107]. Beyond the two-layer stage, follicle stimulating hormone (FSH) is required for further growth [108]. Isolated single follicles can be cultured in two-dimensional (2D) systems [90, 109], in three-dimensional (3D) systems [110], or in a multistep system [111, 112]. In conventional 2D culture systems, follicles tend to flatten on the bottom of the culture plate, inducing breakdown of the surrounding basal lamina, causing granulosa cells to detach from each other and the oocyte. Follicular flattening interferes with the essential bi-directional communication between oocyte and granulosa cells via gap junctions, which makes it unable to complete maturation. Bovine follicles in 2D culture successfully produced estradiol and formed antral cavities, suggesting that 2D culture is capable of supporting follicular viability and function, but only to a certain level [113]. The *in vivo* development from the primordial to preovulatory follicle stage is a time-consuming event, estimated to be 180 days in the cow [114], 205 days in humans [115] and 20 days in mice [116]. To attain successful *in vitro* follicular growth, more research is necessary to optimize each progressive step in follicular maturation, to more closely mimic the *in vivo* environment. A multi-step *in vitro* culture system has been designed and this is thought to be the only effective method for obtaining good quality oocytes for successful fertilization in humans, non-human primates and large animals [111, 112]. In this approach, the activation of follicle growth is promoted within fragments of the ovarian cortex and followed by

mechanical isolation and individual culture of secondary follicles within a 3D culture system (e.g. alginate hydrogel, see below) [117], as the cortical environment becomes inhibitory for growth to the antral stage. Following formation of the antrum, the oocyte within can also be mechanically extracted from the *in vitro* matured follicle, and subsequently undergo *in vitro* fertilization [112, 118]. Sadr et a;. (2015) [119] found that 3D culture systems are more appropriate than a 2D approach with regard to maintaining the spherical morphology, growth rate, and gene expression patterns associated with normal mouse oocyte development. In 3D culture systems, follicles are embedded in a matrix, maintaining the spherical morphology of the follicle and preserving the cell-cell and cell-matrix interactions within the stromal tissue. This allows the follicle to successfully complete maturation. The composition of the used biomaterials mimic the natural environment to meet the physiologic needs of the follicles. Substances used should be non-cytotoxic and need to have a certain elasticity to allow expansion of the granulosa cells. In addition, these matrices need to allow adequate gas exchange, diffusion of nutrients and removal of cellular waste. Alginate hydrogels are currently the most popular material to embed and culture isolated PAFs [107]. In mice, live offspring were reported from follicles cultured using calcium alginate [120]. Human secondary follicles embedded in calcium alginate gels maintain their spherical structure, survive for up to 30 days, develop into small antral follicles and preserve their zonal projections [121, 122]. The physical characteristics of the 3D-matrix are important to consider, as stiffness and pore size must be carefully adapted to the species and the follicle stage being cultured. As the ovarian stroma of humans and primates is more rigid than other species, especially in early stage follicles, they require a stiffer biomaterial to optimize *in vitro* growth. Murine and caprine models require a less rigid, more flexible scaffold for optimized follicle growth. The combination of alginate matrix with fibrin creates a dynamic environment in which during follicle growth follicular proteases degrade fibrin, decreasing the rigidity of the matrix [123].

6.2. Transplantable artificial ovary

Recently, considerable efforts have been made in the development of an artificial ovary for transplantation, which encompasses a matrix that encapsulates, protects and maintains the 3D structure of follicles and mimics the natural environment of the ovary. The matrix should not only encapsulate cryopreserved-thawed follicles but also autologous ovarian cells and bioactive factors, which are necessary for follicle survival and development [124, 125]. A second biopsy after cancer remission is needed to harvest the required autologous ovarian cells. This avoids the risk of adding malignant cells to the artificial ovary [124]. Moreover, cryopreservation proved harmful to ovarian cells, decreasing their number and viability, while chemotherapy does not seem to have a negative impact on ovarian cells [126]. The artificial ovary would potentially restore both fertility and endocrine

function once transplanted into the patient [127]. The artificial ovary would also be a unique research tool for investigating folliculogenesis, a complex process that is far from being fully understood. Basic features required for an artificial ovary are its non-cytotoxicity, being biodegradable and biocompatible and being able to encapsulate cells, which makes the identification of an optimal matrix a key challenge. Several polymers have been tested for use as an artificial ovary matrix. One of the first studies in the development of the artificial ovary was performed by Gosden (1990) [128]. In this study, pups were obtained after allotransplantation of isolated murine follicles and ovarian cells encapsulated in autologous plasma clots. It was also demonstrated that murine follicles isolated from cryopreserved ovaries encapsulated in plasma clots were able to yield offspring [129]. Plasma clots were also used to transplant human isolated pre-antral follicles to the ovarian bursa of immunodeficient mice. This resulted in the development of secondary follicles [125] and, after five months antral follicles [130]. However, plasma clots have an inconsistent composition and degrade quickly, which can lead to follicle loss and variable results. Vanacker et al. (2012, 2014) [131, 132] were the first to perform studies with alginate as a matrix for a transplantable ovary. In a first study, they autografted successfully isolated mouse ovarian cells encapsulated in a matrix of alginate and matrigel into a pocket in the internal part of the mouse peritoneum. After one week, the matrix was able to degrade, allowed vascularization, and supported cell survival and proliferation and elicited only a moderate inflammatory response [131]. In a second study, isolated mouse pre-antral follicles and ovarian cells were grafted in a 1% sterile lyophilized high mannuronate (SLM) alginate matrix to a peritoneal pocket for one week. The authors recovered 22% of grafted follicles, including antral stage specimens. The alginate matrix was invaded by proliferating cells and vessels were formed, but the degradation rate was slow and vascularization was observed mainly at the periphery of the graft [132]. These studies demonstrate that alginate is a promising candidate for the creation of an artificial ovary. Fibrin is a natural polymer and its physical properties can easily be modulated by varying fibrinogen and thrombin concentrations [23]. Pups were obtained after the transplantation of murine isolated pre-antral follicles to the ovarian bursa when encapsulated in fibrin-VEGF clots [133]. Synthetic polymers can be produced in large uniform quantities and can be combined with bioactive compounds to stimulate cellular adhesion, proliferation and differentiation [134]. Poly-etheleneglycol (PEG) was investigated by Kim et al. (2015) [135], whom encapsulated isolated primordial murine follicles to PEG modified with vinyl sulfone groups, and transplanted them to the ovarian bursa of mice for 30 days, after which antral follicles were found. To overcome the problems associated with the matrix, such as slow vascularization, Aerts et al. (2010) [136] tested xenotransplantation devoid of solid vehicle support, in order to improve follicle survival and growth. A suspension of isolated pre-antral follicles and stroma cells were micro-

injected under the kidney capsule of nude mice (Fig. 5). A massive follicular activation was seen after 14 days of transplantation, indicating that isolated pre-antral follicles were able to survive and grow.

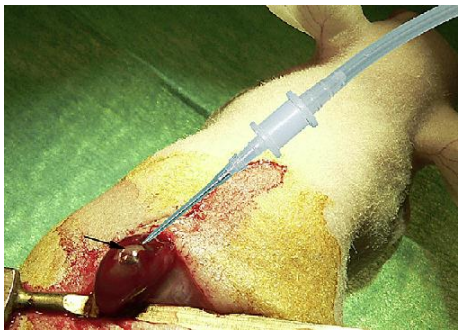

Fig. 5 Xenotransplantation of isolated pre-antral follicles to the kidney capsule of a nude mouse. The pipette tip was introduced through a capsulotomy to deposit the follicular suspension (arrow). (Figure Aerts et al., 2010) [136]

7. Conclusions

The stock of pre-antral ovarian follicles represents an untapped potential, which could be 'unlocked' for reproduction, preservation, or research purposes. Advancements in the *in vitro* culture and auto-transplantation of ovarian follicles will greatly improve current fertility preservation strategies. Pre-antral follicles provide a safe method for the restoration of fertility in women affected by chemo/radiotherapy induced premature ovarian failure and they also constitute a genetic pool out of which endangered animal populations can be restored when *in vitro* follicle culture becomes a mainstream technique. In the near future, it is expected that effective means of avoiding reseeding of malignant cells with ovarian grafts, such as an artificial ovary and an *in vitro* culture system for primordial follicles will become available. With these groundbreaking techniques under development, the pre-antral ovarian follicle may indeed become the *'holy grail'* for female fertility preservation.

References

1. Araujo, V.R., et al., *Long-term in vitro culture of bovine preantral follicles: Effect of base medium and medium replacement methods.* Anim. Reprod. Sci., 2015. **161**: p. 23-31.
2. Detti, L., D.C. Martin, and L.J. Williams, *Applicability of adult techniques for ovarian preservation to childhood cancer patients.* J. Assist. Reprod. Genet., 2012. **29**(9): p. 985-995.
3. Jemal, A., et al., *Cancer statistics, 2008.* CA Cancer J Clin, 2008. **58**(2): p. 71-96.
4. Rodriguez-Wallberg, K.A. and K. Oktay, *Options on fertility preservation in female cancer patients.* Cancer Treat. Rev., 2012. **38**(5): p. 354-361.
5. Hyman, J.H. and T. Tulandi, *Fertility preservation options after gonadotoxic chemotherapy.* Clin. Med. Insights Reprod. Health, 2013. **7**: p. 61-69.
6. Leung, W., et al., *Late effects of treatment in survivors of childhood acute myeloid leukemia.* J. Clin. Oncol., 2000. **18**(18): p. 3273-3279.
7. Lee, S., et al., *American Society of Clinical Oncology: Recommendations on fertility preservation in cancer patients.* J. Clin. Oncol., 2006. **24**: p. 2917-2931.
8. Green, D.M., et al., *Fertility of female survivors of childhood cancer: a report from the childhood cancer survivor study.* J. Clin. Oncol., 2009. **27**(16): p. 2677-2685.
9. Seli, E. and A. Agarwal, *Fertility preservation in females: emerging technologies and clinical applications, first ed.* . 2012: Springer, New York.
10. Comizzoli, P., *Biotechnologies for wildlife fertility preservation.* Animal Frontiers, 2015. **5**(1): p. 73-78.
11. Langbeen, A., et al., *Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies.* Theriogenology, 2015. **84**(4): p. 477-489.
12. Adams, G. and R. Pierson, *Bovine model for study of ovarian follicular dynamics in humans.* Theriogenology, 1995. **43**(1): p. 113-120.
13. Campbell, B., et al., *Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans.* Reproduction, 2003: p. 429-443.
14. Donnez, J., et al., *Ovarian tissue cryopreservation and transplantation: a review.* Hum. Reprod. Update, 2006. **12**(5): p. 519-535.
15. Wallace, W.H.B., *Oncofertility and preservation of reproductive capacity in children and young adults.* Cancer, 2011. **117**(S10): p. 2301-2310.
16. Revelli, A., et al., *Oocyte cryostorage to preserve fertility in oncological patients.* Obstet. Gynecol. Int., 2012. **2012**.
17. Niemann, H. and C. Wrenzycki, eds. *Animal Biotechnology*. Vol. Reproductive biotechnologies. 2018, Springer International Publishing AG.
18. Rosendahl, M., T. Greve, and C.Y. Andersen, *The safety of transplanting cryopreserved ovarian tissue in cancer patients: a review of the literature.* J. Assist. Reprod. Genet. , 2013. **30**(1): p. 11-24.
19. Fisch, B. and R. Abir, *Female fertility preservation: past, present and future.* Reproduction, 2018. **156**(1): p. F11-F27.
20. Snow, M., et al., *Generation of live young from xenografted mouse ovaries.* Science, 2002. **297**(5590): p. 2227-2227.
21. Mattiske, D., G. Shaw, and J. Shaw, *Influence of donor age on development of gonadal tissue from pouch young of the tammar wallaby, Macropus eugenii, after cryopreservation and xenografting into mice.* Reproduction, 2002. **123**(1): p. 143-153.
22. Wolvekamp, M., et al., *Follicular development in cryopreserved common wombat ovarian tissue xenografted to nude rats.* Anim. Reprod. Sci., 2001. **65**(1-2): p. 135-147.
23. Amorim, C.A. and A. Shikanov, *The artificial ovary: current status and future perspectives.* Future Oncol., 2016. **12**(19): p. 2323-2332.
24. Aerts, J. and P. Bols, *Ovarian follicular dynamics: a review with emphasis on the bovine species. Part I: Folliculogenesis and pre-antral follicle development.* Reprod. Domest. Anim. , 2010. **45**(1): p. 171-179.

25. van Wezel, I.L. and R.J. Rodgers, *Morphological characterization of bovine primordial follicles and their environment in vivo.* Biol. Reprod., 1996. **55**(5): p. 1003-1011.
26. Morita, Y. and J.L. Tilly, *Oocyte apoptosis: like sand through an hourglass.* Dev. Biol., 1999. **213**(1): p. 1-17.
27. Donnez, J., et al., *Children born after autotransplantation of cryopreserved ovarian tissue. A review of 13 live births.* Ann. Med., 2011. **43**(6): p. 437-450.
28. Donnez, J., et al., *Live birth after transplantation of frozen-thawed ovarian tissue after bilateral oophorectomy for benign disease.* Fertil. and Steril., 2012. **98**(3): p. 720-725.
29. Jadoul, P., et al., *Efficacy of ovarian tissue cryopreservation for fertility preservation: lessons learned from 454 cases.* Hum. Reprod., 2017. **32**(5): p. 1046-1054.
30. Dolmans, M.-M., et al., *Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe.* Blood, 2010. **116**(16): p. 2908-2914.
31. Rosendahl, M., et al., *Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia.* Fertil. and Steril., 2010. **94**(6): p. 2186-2190.
32. Van Eyck, A.-S., et al., *Both host and graft vessels contribute to revascularization of xenografted human ovarian tissue in a murine model.* Fertil. and Steril., 2010. **93**(5): p. 1676-1685.
33. Martinez-Madrid, B., et al., *Chick embryo chorioallantoic membrane (CAM) model: a useful tool to study short-term transplantation of cryopreserved human ovarian tissue.* Fertil. and Steril., 2009. **91**(1): p. 285-292.
34. Donnez, J., et al., *Livebirth after orthotopic transplantation of cryopreserved ovarian tissue.* The Lancet, 2004. **364**(9443): p. 1405-1410.
35. Langbeen, A., et al., *Morphometrical analysis of preantral follicular survival of VEGF-treated bovine ovarian cortex tissue following xenotransplantation in an immune deficient mouse model.* Anim. Reprod. Sci., 2016. **168**: p. 73-85.
36. Donnez, J., et al., *Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation.* Fertil. and Steril., 2013. **99**(6): p. 1503-1513.
37. Shaw, J., et al., *Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications.* Mol. and Cell. Endocrinol., 2000. **161**(1): p. 103-110.
38. Amorim, C., P. Gonçalves, and J. Figueiredo, *Cryopreservation of oocytes from pre-antral follicles.* Hum. Reprod. Update, 2003. **9**(2): p. 119-129.
39. Jorssen, E.P., et al., *Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension.* Theriogenology, 2014. **82**(3): p. 387-395.
40. Donnez, J. and M.-M. Dolmans, *Ovarian cortex transplantation: 60 reported live births brings the success and worldwide expansion of the technique towards routine clinical practice.* J. Assist. Reprod. Genet., 2015. **32**(8): p. 1167-1170.
41. Donnez, J. and M.-M. Dolmans, *Fertility preservation in women.* N. Eng. J. Med., 2017. **377**(17): p. 1657-1665.
42. Wallace, W.H.B., T.W. Kelsey, and R.A. Anderson, *Fertility preservation in pre-pubertal girls with cancer: the role of ovarian tissue cryopreservation.* Fertil. and Steril., 2016. **105**(1): p. 6-12.
43. Wallace, W.H.B., et al., *Fertility preservation for girls and young women with cancer: population-based validation of criteria for ovarian tissue cryopreservation.* Lancet Oncol., 2014. **15**(10): p. 1129-1136.
44. Salih, S.M., et al., *Evidence to incorporate inclusive reproductive health measures in guidelines for childhood and adolescent cancer survivors.* J. Pediatr. Adol. Gynecol., 2015. **28**(2): p. 95-101.
45. El Issaoui, M., et al., *Effect of first line cancer treatment on the ovarian reserve and follicular density in girls under the age of 18 years.* Fertil. and Steril., 2016. **106**(7): p. 1757-1762. e1.

46. Lunardi, F.O., et al., *Vitrified sheep isolated secondary follicles are able to grow and form antrum after a short period of in vitro culture.* Cell tissue Res., 2015. **362**(1): p. 241-251.
47. Kagawa, N., S. Silber, and M. Kuwayama, *Successful vitrification of bovine and human ovarian tissue.* Reprod. Biomed. Online, 2009. **18**(4): p. 568-577.
48. Rodgers, R.J., H.F. Irving-Rodgers, and D.L. Russell, *Extracellular matrix of the developing ovarian follicle.* Reproduction, 2003. **126**(4): p. 415-424.
49. Hovatta, O., et al., *Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants.* Hum. Reprod., 1996. **11**(6): p. 1268-72.
50. Shaw, J.M., A. Oranratnachai, and A.O. Trounson, *Fundamental cryobiology of mammalian oocytes and ovarian tissue.* Theriogenology, 2000. **53**(1): p. 59-72.
51. Figueiredo, J.R., et al., *Development of a combined new mechanical and enzymatic method for the isolation of intact preantral follicles from fetal, calf and adult bovine ovaries.* Theriogenology, 1993. **40**(4): p. 789-99.
52. Dolmans, M.M., et al., *Evaluation of Liberase, a purified enzyme blend, for the isolation of human primordial and primary ovarian follicles.* Hum. Reprod., 2006. **21**(2): p. 413-20.
53. Langbeen, A., et al., *Characterization of freshly retrieved preantral follicles using a low-invasive, mechanical isolation method extended to different ruminant species.* Zygote, 2015. **23**(5): p. 683-694.
54. Braw-Tal, R. and S. Yossefi, *Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary.* J. Reprod. Fert., 1997. **109**(1): p. 165-171.
55. Rodgers, R.J. and H.F. Irving-Rodgers, *Morphological classification of bovine ovarian follicles.* Reproduction, 2010. **139**(2): p. 309-318.
56. Aerts, J.M., M. Oste, and P.E. Bols, *Development and practical applications of a method for repeated transvaginal, ultrasound-guided biopsy collection of the bovine ovary.* Theriogenology, 2005. **64**(4): p. 947-957.
57. Aerts, J., et al., *Quantification and viability assessment of isolated bovine primordial and primary ovarian follicles retrieved through a standardized biopsy pick-up procedure.* Reprod. Domest. Anim. , 2008. **43**(3): p. 360-366.
58. Kawamura, K., et al., *Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment.* Proc. Natl. Acad. Sci., 2013. **110**(43): p. 17474-17479.
59. Suzuki, N., et al., *Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency.* Hum. Reprod., 2015. **30**(3): p. 608-615.
60. Kim, S.-Y., et al., *Toward precision medicine for preserving fertility in cancer patients: existing and emerging fertility preservation options for women.* J. Gynecol. Oncol., 2015. **27**(2).
61. Dinnyés, A.s., et al., *High Developmental Rates of Vitrified Bovine Oocytes Following Parthenogenetic Activation, In Vitro Fertilization, and Somatic Cell Nuclear Transfer1.* Biol. Reprod. , 2000. **63**(2): p. 513-518.
62. Santos, R., et al., *Vitrification of goat preantral follicles enclosed in ovarian tissue by using conventional and solid-surface vitrification methods.* Cell Tissue Res., 2007. **327**(1): p. 167-176.
63. Begin, I., et al., *Cryopreservation of goat oocytes and in vivo derived 2-to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods.* Theriogenology, 2003. **59**(8): p. 1839-1850.
64. Sripunya, N., et al., *A comparison of cryotop and solid surface vitrification methods for the cryopreservation of in vitro matured bovine oocytes.* J. Reprod. and Develop., 2009: p. 0910060211-0910060211.
65. Somfai, T., et al., *Effect of centrifugation treatment before vitrification on the viability of porcine mature oocytes and zygotes produced in vitro.* J. Reprod. and Develop., 2007: p. 0802200042-0802200042.
66. Kagawa, N., et al., *Production of the first offspring from oocytes derived from fresh and cryopreserved pre-antral follicles of adult mice.* Reprod. Biomed. Online, 2007. **14**(6): p. 693-699.

67. Kuwayama, M., *Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method.* Theriogenology, 2007. **67**(1): p. 73-80.
68. Isachenko, V., et al., *Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation.* Reproduction, 2009. **138**(2): p. 319-327.
69. Isachenko, V., et al., *Human ovarian tissue cryopreservation: quality of follicles as a criteria of effectiveness.* Reprod. Biomed. Online, 2010. **20**(4): p. 441-442.
70. Keros, V., et al., *Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue.* Hum. Reprod., 2009. **24**(7): p. 1670-1683.
71. Herraiz, S., et al., *Improving ovarian tissue cryopreservation for oncologic patients: slow freezing versus vitrification, effect of different procedures and devices.* Fertil. and Steril., 2014. **101**(3): p. 775-784. e1.
72. Amorim, C.A., et al., *Vitrification of human ovarian tissue: effect of different solutions and procedures.* Fertil. and Steril., 2011. **95**(3): p. 1094-1097.
73. Dalman, A., et al., *Slow freezing versus vitrification technique for human ovarian tissue cryopreservation: An evaluation of histological changes, WNT signaling pathway and apoptotic genes expression.* Cryobiology, 2017. **79**: p. 29-36.
74. Aerts, J.M., et al., *Follicle survival and growth to antral stages in short-term murine ovarian cortical transplants after Cryologic solid surface vitrification or slow-rate freezing.* Cryobiology, 2008. **57**(2): p. 163-169.
75. Amorim, C.A., et al., *Isolated ovine primordial follicles cryopreserved in different concentrations of ethylene glycol.* Theriogenology, 2003. **60**(4): p. 735-42.
76. Amorim, C.A., et al., *Cryopreservation of isolated ovine primordial follicles with propylene glycol and glycerol.* Fertil Steril, 2004. **81 Suppl 1**: p. 735-40.
77. Amorim, C.A., et al., *Permeability of ovine primordial follicles to different cryoprotectants.* Fertil Steril, 2006. **85 Suppl 1**: p. 1077-81.
78. Santos, R.R., et al., *Effect of cryopreservation on viability, activation and growth of in situ and isolated ovine early-stage follicles.* Anim Reprod Sci, 2007. **99**(1-2): p. 53-64.
79. Rodrigues, A., et al., *Cryopreservation and short-term culture of isolated caprine primordial follicles.* Small Ruminant Research, 2005. **56**(1-3): p. 103-111.
80. Jewgenow, K., et al., *Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation.* J. Reprod. Fertil., 1998. **112**(1): p. 39-47.
81. Barrett, S.L., L.D. Shea, and T.K. Woodruff, *Noninvasive index of cryorecovery and growth potential for human follicles in vitro.* Biol. Reprod., 2010. **82**(6): p. 1180-1189.
82. Vanacker, J., et al., *Should we isolate human preantral follicles before or after cryopreservation of ovarian tissue?* Fertil. and Steril., 2013. **99**(5): p. 1363-1368. e2.
83. Segino, M., et al., *In vitro culture of mouse GV oocytes and preantral follicles isolated from ovarian tissues cryopreserved by vitrification.* Hum Cell, 2003. **16**(3): p. 109-16.
84. Xing, W., et al., *Solid-surface vitrification is an appropriate and convenient method for cryopreservation of isolated rat follicles.* Reprod Biol Endocrinol, 2010. **8**: p. 42.
85. Langbeen, A., et al., *Effects of neutral red assisted viability assessment on the cryotolerance of isolated bovine preantral follicles.* J. Assist. Reprod. Genet., 2014. **31**(12): p. 1727-1736.
86. Bus, A., et al., *Effects of vitrification on the viability of alginate encapsulated isolated bovine pre-antral follicles.* J. Assist. Reprod. Genet., 2018. **35**(7): p. 1187-1199.
87. Bian, J., et al., *Vitreous cryopreservation of human preantral follicles encapsulated in alginate beads with mini mesh cups.* J. Reprod. Dev., 2013. **59**(3): p. 288-95.
88. Sadeghnia, S., et al., *Development of sheep primordial follicles encapsulated in alginate or in ovarian tissue in fresh and vitrified samples.* Cryobiology, 2016. **72**(2): p. 100-5.
89. Camboni, A., et al., *Alginate beads as a tool to handle, cryopreserve and culture isolated human primordial/primary follicles.* Cryobiology, 2013. **67**(1): p. 64-69.
90. Jorssen, E.P., et al., *Morphologic characterization of isolated bovine early preantral follicles during short-term individual in vitro culture.* Theriogenology, 2015. **84**(2): p. 301-11.

91. Elliott, W.M. and N. Auersperg, *Comparison of the neutral red and methylene blue assays to study cell growth in culture.* Biotech. Histochem., 1993. **68**(1): p. 29-35.
92. Repetto, G., A. Del Peso, and J.L. Zurita, *Neutral red uptake assay for the estimation of cell viability/cytotoxicity.* Nature protocols, 2008. **3**(7): p. 1125.
93. Chambers, E., et al., *In situ identification of follicles in ovarian cortex as a tool for quantifying follicle density, viability and developmental potential in strategies to preserve female fertility.* Hum. Reprod. , 2010. **25**(10): p. 2559-2568.
94. Kristensen, S.G., et al., *Isolation of pre-antral follicles from human ovarian medulla tissue.* Hum. Reprod., 2010. **26**(1): p. 157-166.
95. Cortvrindt, R.G. and J.E. Smitz, *Fluorescent probes allow rapid and precise recording of follicle density and staging in human ovarian cortical biopsy samples.* Fertil. and Steril., 2001. **75**(3): p. 588-93.
96. Larsen, W., *Biological implications of gap junction structure, distribution and composition: a review.* Tissue and Cell, 1983. **15**(5): p. 645-671.
97. Grazul-Bilska, A.T., L.P. Reynolds, and D.A. Redmer, *Gap junctions in the ovaries.* Biol. Reprod., 1997. **57**(5): p. 947-957.
98. Kidder, G.M. and B.C. Vanderhyden, *Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence.* Can. J. Physiol. Pharmacol., 2010. **88**(4): p. 399-413.
99. Nuttinck, F., et al., *Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary.* Mol. Reprod. Dev., 2000. **57**(1): p. 60-6.
100. Ortiz-Escribano, N., et al., *Blocking connexin channels improves embryo development of vitrified bovine blastocysts.* Biol Reprod, 2017. **96**(2): p. 288-301.
101. Lee, R.K.-K., et al., *Abnormally low expression of connexin 37 and connexin 43 in subcutaneously transplanted cryopreserved mouse ovarian tissue.* J. Assist. Reprod. Genet. , 2008. **25**(9-10): p. 489-497.
102. Baird, D., et al., *Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at− 196 C.* Endocrinology, 1999. **140**(1): p. 462-471.
103. Cushman, R., C. Wahl, and J. Fortune, *Bovine ovarian cortical pieces grafted to chick embryonic membranes: a model for studies on the activation of primordial follicles.* Hum. Reprod., 2002. **17**(1): p. 48-54.
104. Smitz, J.E. and R.G. Cortvrindt, *The earliest stages of folliculogenesis in vitro.* Reproduction, 2002. **123**(2): p. 185-202.
105. Abir, R., et al., *Morphological study of fully and partially isolated early human follicles.* Fertil. and steril., 2001. **75**(1): p. 141-146.
106. Torrents, E., et al., *Applications of ovarian tissue transplantation in experimental biology and medicine.* Hum. Reprod. Update, 2003. **9**(5): p. 471-481.
107. Green, L.J. and A. Shikanov, *In vitro culture methods of preantral follicles.* Theriogenology, 2016. **86**(1): p. 229-38.
108. Oktay, K., et al., *Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone.* Hum. Reprod., 1998. **13**(5): p. 1133-1138.
109. Wu, J., L. Zhang, and X. Wang, *In vitro maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes.* Reproduction, 2001. **121**(3): p. 389-393.
110. Pangas, S.A., et al., *Novel approach for the three-dimensional culture of granulosa cell–oocyte complexes.* J. Tissue Eng., 2003. **9**(5): p. 1013-1021.
111. Picton, H., et al., *The in vitro growth and maturation of follicles.* Reproduction, 2008. **136**(6): p. 703-715.
112. Telfer, E.E. and M. McLaughlin. *In vitro development of ovarian follicles*. in *Semin. Reprod. Med.* 2011. © Thieme Medical Publishers.
113. McLaughlin, M., et al., *Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles.* Mol. Hum. reprod., 2010. **16**(9): p. 644-653.

114. Lussier, J., P. Matton, and J. Dufour, *Growth rates of follicles in the ovary of the cow.* Reproduction, 1987. **81**(2): p. 301-307.
115. Gougeon, A., *Regulation of ovarian follicular development in primates: facts and hypotheses.* Endocr. rev., 1996. **17**(2): p. 121-155.
116. Pedersen, T., *Determination of follicle growth rate in the ovary of the immature mouse.* Reproduction, 1970. **21**(1): p. 81-93.
117. McLaughlin, M. and E.E. Telfer, *Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system.* Reproduction, 2010. **139**(6): p. 971-978.
118. Smitz, J., et al., *Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation.* Hum. Reprod. Update, 2010. **16**(4): p. 395-414.
119. Sadr, S.Z., et al., *Mouse preantral follicle development in two-dimensional and three-dimensional culture systems after ovarian tissue vitrification.* Eur. J. of Obstet. Gynecol. Reprod. Biol., 2015. **194**: p. 206-211.
120. Xu, M., et al., *Tissue-engineered follicles produce live, fertile offspring.* Tissue Eng., 2006. **12**(10): p. 2739-2746.
121. Xu, M., et al., *In vitro grown human ovarian follicles from cancer patients support oocyte growth.* Hum. Reprod., 2009. **24**(10): p. 2531-2540.
122. Yin, H., et al., *Survival and growth of isolated pre-antral follicles from human ovarian medulla tissue during long-term 3D culture.* Hum. Reprod., 2016. **31**(7): p. 1531-1539.
123. Shikanov, A., et al., *A method for ovarian follicle encapsulation and culture in a proteolytically degradable 3 dimensional system.* J. Vis. Exp., 2011(49).
124. Amorim, C.A., *Artificial ovary*, in *Gonadal Tissue Cryopreservation in Fertility Preservation*. 2016, Springer. p. 175-192.
125. Dolmans, M.-M., et al., *Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice.* Reproduction, 2007. **134**(2): p. 253-262.
126. Soares, M., et al., *The best source of isolated stromal cells for the artificial ovary: medulla or cortex, cryopreserved or fresh?* Hum. Reprod., 2015. **30**(7): p. 1589-1598.
127. Vanacker, J., et al., *Transplantation of an alginate–matrigel matrix containing isolated ovarian cells: first step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells.* Biomaterials, 2012. **33**(26): p. 6079-6085.
128. Gosden, R., *Restitution of fertility in sterilized mice by transferring primordial ovarian follicles.* Hum. Reprod., 1990. **5**(2): p. 117-122.
129. Carroll, J. and R.G. Gosden, *Physiology: Transplantation of frozen—thawed mouse primordial follicles.* Hum. Reprod., 1993. **8**(8): p. 1163-1167.
130. Dolmans, M.-M., et al., *Development of antral follicles after xenografting of isolated small human preantral follicles.* Reprod. Biomed. Online, 2008. **16**(5): p. 705-711.
131. Vanacker, J., et al., *Transplantation of an alginate-matrigel matrix containing isolated ovarian cells: first step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells.* Biomaterials, 2012. **33**(26): p. 6079-85.
132. Vanacker, J., et al., *First transplantation of isolated murine follicles in alginate.* Regen. Med., 2014. **9**(5): p. 609-19.
133. Kniazeva, E., et al., *Primordial follicle transplantation within designer biomaterial grafts produce live births in a mouse infertility model.* Sci. Rep., 2015. **5**: p. 17709.
134. Yoon, D.M. and J.P. Fisher, *Natural and synthetic polymeric scaffolds*, in *Biomedical Materials*. 2009, Springer. p. 415-442.
135. Kim, J., et al. *Engineering artificial ovarian tissue using multi-arm poly (ethylene glycol) hydrogel*. in *Tissue Engineering Part A*. 2015. Mary Ann Liebert, New Rochelle, NY, USA.
136. Aerts, J.M., et al., *Xenotransplantation by injection of a suspension of isolated preantral ovarian follicles and stroma cells under the kidney capsule of nude mice.* Fertil. and Steril., 2010. **94**(2): p. 708-714.

CHAPTER 2
AIMS OF THE STUDY

The need for fertility preservation is increasing. Due to improvements in cancer diagnosis and treatment, more women who are diagnosed with cancer now become long-term survivors. However, as chemo- and radiotherapy often damage ovarian tissue, patients are likely to show compromised fertility after treatment. Ovarian tissue cryopreservation is specifically indicated for prepubertal girls and women whose cancer treatment cannot be postponed. However, auto-transplantation of frozen-thawed tissue cannot be recommended to cancer patients who are at risk of the reintroduction of malignant cells. For these patients, a safer alternative to allow fertility restoration could be the isolation of pre-antral follicles (before or after cryopreservation) from ovarian tissue for *in vitro* growth, maturation, and fertilization or auto-transplantation of the frozen-thawed isolated pre-antral follicles (PAFs) in an artificial ovary.

This thesis is focusing on the optimization of vitrification protocols for isolated follicles, while *in vitro* culture systems for PAFs and an artificial ovary are making great progress. Vitrification is the most successful preservation method for gametes and embryos. It is advantageous over slow freezing since the fast cooling rates prevent one of the main sources of injuries, namely ice-crystal formation inside the cells. However, a negative consequence of this strategy is the increased probability of injuries caused by exposure of cells to other non-physiological conditions (osmotic, pH and chemical changes) that may impair their further development. Despite the advantages of the vitrification of isolated PAFs, research performed on effects of vitrification on the viability of isolated PAFs remains scarce. An explanation for this could be that the isolation and processing of isolated PAFS remains a huge challenge in terms of follicular retrieval and manipulation. Therefore, the **overall objective** of this thesis was to optimize cryopreservation protocols and facilitate manipulation of isolated PAFs. In the meantime we aimed to more extensively evaluate the quality of PAFs following cryopreservation by assessing different parameters which are crucial for further follicle development. In addition, while making the switch from bovine to human research material, we started by investigating the effects of slow freezing on follicle survival in situ because until now no standard protocols have been established for the use of vitrification with ovarian tissue.

The **specific objectives** of this thesis can therefore be described as follows:

Aim 1. Firstly, to assess the viability and morphology of bovine isolated PAFs after short-term culture in alginate beads. Secondly, to vitrify isolated bovine PAFs on a time and labor efficient way using alginate beads in mesh cups (**Chapter 3**).

Aim 2. To assess the feasibility of passive slow freezing (using Nalgene's Mr. Frosty container) as an alternative to controlled slow rate freezing (using Air Liquide's Freezal™) for human ovarian tissue cryopreservation (**Chapter 4**).

Aim 3. Firstly, to visualize and determine the precise location of Cx43 and transzonal projections using immunocytochemical methods in isolated bovine PAFs before and following vitrification. Secondly, to vitrify non-embedded PAFs in bulk by making use of mini cell strainers (**Chapter 5**).

Aim 4. To assess the survivability of human isolated PAFs upon isolation and following vitrification and warming. In this context, we aimed to visualize Cx43 and TZPs and measure levels of the mRNAs encoding Cx43 to provide a quantitative analysis of this connexin protein (**Chapter 6**).

CHAPTER 3

Effects of vitrification on the viability of alginate encapsulated isolated bovine pre-antral follicles

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Summary

Purpose Individual follicle cryopreservation techniques, without hydrogel support, are labor-intensive and a substantial proportion of isolated follicles are lost during handling and after warming. Therefore, the viability and morphology of isolated bovine (as a model for human) pre-antral follicles after vitrification and warming, when encapsulated in alginate beads, were investigated.

Methods Bovine pre-antral follicles were mechanically isolated and divided into four different groups; 1) culture in 2% alginate beads (3D system) and vitrification in beads using mesh cups (3DVIT), 2) culture in 2% alginate beads (3DCUL), 3) culture in 96-well plates (2D system) and vitrification using High Security Vitrification straws® (2DVIT), 4) culture in a 2D system (2DCUL). The same vitrification and warming protocols were used for embedded (3DVIT) and non-embedded follicles (2DVIT).

Results No differences were observed in follicle viability between group 2DCUL and 3DCUL. Group 3DVIT showed the lowest viability (45.9%) according to calcein and Neutral Red staining among all groups. Group 2DVIT displayed the highest viability (87.5%) and largest percentage of follicles with a well preserved morphology.

Conclusions Our results show that, using a vitrification protocol optimized for non-embedded follicles, 2D culture is more effective in vitrifying isolated follicles. However, embedding in alginate allow to handle follicles more efficiently; i.e. without excessive manipulation and thus less labor-intensive in combination with a reduced loss of follicles during the procedure. Based on the increased work efficiency, but lower viability and higher proportion of follicles showing impaired morphology, we consider it advantageous to optimize the protocol for the vitrification of embedded follicles to increase survival and maintain morphology after vitrification.

1. Introduction

According to recent data, women under 40 years of age have an estimated 2.5% chance of developing cancer [1]. Fortunately, thanks to constant improvements in diagnosis and cancer treatment [2], up to 90% of women who are diagnosed with reproductive tract cancer are now long-term survivors [3]. However, as chemo- and radiotherapy often damage ovarian tissue [4, 5], patients are likely to show compromised fertility [6, 7] up to a level where survivors of childhood cancer, have an overall reduction of 19% in the likelihood of ever being pregnant [8]. As a consequence, the interest in fertility preservation (FP) strategies in women has been sparked to the extent that it has now become a key medical sub discipline [9]. Current FP techniques for women comprise ovarian transposition, cryopreservation of embryos and unfertilized oocytes [10-12] and ovarian tissue cryopreservation (OTC) containing pre-antral follicles (PAFs) [9]. Clearly, the most suitable option for a specific patient is based upon different parameters such as the patient's age and relationship status, the type of cancer and the time available between diagnosis and the onset of treatment [10].

Although a few dozens of births have already been reported worldwide [13-15] several authors have raised concerns associated with OTC, the most important of which being the risk of the reintroduction of malignant cells following auto-transplantation of the frozen-thawed tissue [16-18]. On top of that, transplantation often results in extensive follicular loss due to delayed and deficient revascularisation [19, 20]. Furthermore, both quantitative and qualitative assessment of the follicle population in ovarian tissue samples is difficult if not impossible [21-23]. Consequently the risk of freezing tissue samples devoid of (viable) follicles is significant, creating a false idea for the patient on preserved fertility. On the contrary, cryopreservation of isolated PAFs and subsequent auto-transplantation, grafting [24, 25] or future *in vitro* culture [26] offers interesting new perspectives [27], minimizing the risk of transfer of malignant cells and avoiding banking of ovarian tissue devoid of PAFs. Recently there is an increasing interest for bovine *in vitro* models in human studies on assisted reproductive techniques (ARTs) and FP as reviewed by Langbeen et al. (2015) [28]. This is due to physiological similarities and the easy access to slaughterhouse ovaries which guarantee unrestricted availability of study specimens such as PAFs [28]. Early pre-antral follicles account for the vast majority of follicles in the ovarian cortex and the *in vitro* culture of PAFs could be an excellent strategy to produce fertilizable oocytes. However, the development of a culture system that allows the development of a primordial follicle to a mature fertilizable oocyte remains a challenge in larger mammalian species because of the lengthy period of folliculogenesis and the larger size of the follicle [29]. In bovine species, the growth of PAFs was limited to the stage of antrum formation [30]. Production of live offspring with oocytes from *in vitro* cultured primordial follicles has, so far, only been achieved in mice [31]. Gupta et al.

(2008) [32] reported the successful production of embryos from *in vitro* grown pre-antral follicles from buffalos after long term PAF culture (100 days). Xiao et al. (2015) [33] accomplished for the first time, the development of human follicles until the antral stage and production of meiotically competent MII oocytes, using a two-step culture method. *In vitro* growth of pre-antral follicles is a delicate process because the dynamics of the ovarian environment need to be mimicked.

Three-dimensional (3D) culture systems seem to simulate more effectively the physiologic conditions of the ovary than traditional two-dimensional (2D) systems (monolayer, such as multiwell plates) [26]. According to West et al. (2007) [34] alginate is a suitable matrix for *in vitro* culture of isolated follicles due to its gentle gelling properties and biochemical characteristics. Encapsulation of follicles better preserves the follicular morphology which is imperative to maintain communication between the oocyte and the granulosa cells. Alginate encapsulation is not only a valuable tool for *in vitro* culture of isolated follicles, but also for their cryopreservation. It would protect the cells from direct exposure to cryoprotectants and reduce the impact of cooling and warming. Alginate hydrogels, also known as beads, are already successfully applied for cryopreservation of human isolated follicles [35, 36]. However, the available scientific literature on the possible effects of vitrification on embedded human isolated PAFs is scarce and as far as we know non-existing for bovine isolated PAFs. Encapsulation of follicles is advantageous as one bead can contain several follicles, it simplifies follicle handling and the number of lost follicles during the process can be minimized. To assess the effects of vitrification on follicles after warming, survival during short term culture is an important parameter, next to the use of different other yet more invasive methods such as calcein staining.

Because of the advantages of 3D systems for vitrification and culture, in the current study we aimed to 1) assess viability and morphology of follicles after short term culture in alginate beads using the bovine model, 2) vitrify isolated bovine pre-antral follicles on a time and labor efficient way using alginate beads in mesh cups, 3) assess viability of follicles, that were vitrified either in alginate beads or without encapsulation using High Security Vitrification (HSV) straws®, after warming.

2. Materials and methods

2.1. Collection of ovaries, ovarian follicle isolation and culture

As described earlier (Jorssen et al. (2015)) [37], adult bovine ovaries were collected upon slaughter and transported in warm physiologic saline (0.9% NaCl, Braun) to the laboratory within three hours at 25°C. Following removal of the adnexa, the ovaries were washed in warm (38.5 °C) physiologic solution supplemented with kanamycin (0.25%) and rinsed in alcohol (70%). Active ovaries were selected, free of abundant antral follicles, corpora lutea or scar tissue. The ovarian cortex was cut into pieces of approximately 1 mm^3, using a scalpel. The pieces of ovarian cortex were transferred to isolation

medium: M199, supplemented with hepes (0.04 M), gentamycin (50 µL/ml), bovine serum albumine (10 mg/ml), polyvinylpyrrolidone (4mg/ml) and filtered through a 0.2 µm filter. Ovarian cortex tissue was mixed and dispersed using an Ultra Turrax T18 Basic device (IKA®, VWR, Leuven, Belgium) with a larger plastic dispersing tool (IKA®, S18D-14G-KS) for two minutes and with a smaller one (IKA®, S18D-10G-KS) for one minute. The resulting follicle suspension was subsequently filtered through a 100 µm, a 70 µm filter (BD Falcon®, Corning, NY, USA) and a 20 µm nylon filter (Millipore®, Cork, Ireland). Early PAFs were recovered from the 20 µm mesh filter by rinsing with isolation medium. Follicles were visualized using standard inverted light microscopy (Olympus, Aartselaar, Belgium). PAFs with an oocyte surrounded by one layer of cuboidal granulosa cells and intact basal membrane were selected [38]. They were individually transferred and cultured in 70 µl culture medium in 96-well plates (Greiner Bio-One, Germany) at 38.5 °C and 5% CO_2. The culture medium consisted of equal parts DMEM and Ham's F12 nutrient supplemented with penicillin G (240 U/ml) and streptomycin (240 µg/ml), fungizone (5 µg/ml), fetal calf serum (2.3 (v/v)%) and newborn calf serum (2.3 (v/v)%), bovine serum albumin (0.75 (w/v)%), insulin (0.01 mg/ml), transferrin (0.55 µg/ml) and selenium (6.7 ng/ml). Two plates were used per isolation. Per plate, 30 wells were used for culture. Plates were filled alternately per row to minimize exposure time of follicles to light. The experimental set-up is described below and summarized in a flow chart (Fig. 2). To evaluate follicles, the connection between the oocyte and the surrounding granulosa cells and the integrity of the basal membrane were microscopically evaluated as reported by Jorssen et al. (2015) [37] (Fig. 1). Category 1 contains follicles with both an intact basal membrane and an intact connection between the oocyte and surrounding granulosa cells. Category 2 contains follicles with an intact connection between the oocyte and the surrounding granulosa cells but shows signs of a disrupted basal membrane. Category 3 holds the follicles with a disrupted basal membrane and a disrupted connection between the oocyte and granulosa cells. During follicle isolation (day 0), selection for embedding (day 2) and vitrification (day 3), only category 1 and 2 follicles were selected. Category 3 follicles were not further used.

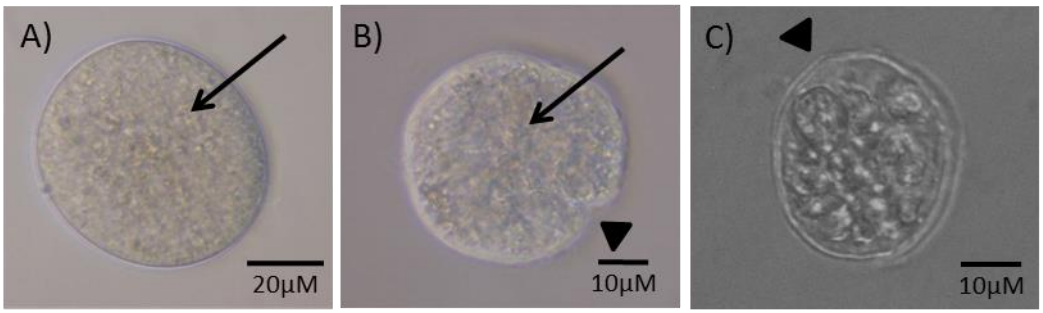

Fig. 1 Follicle morphological categories. A) A category 1 follicle at day 7 of in vitro culture showing an intact basal membrane and connection between the oocyte and surrounding granulosa cells, B) A category 2 follicle at day 4 of in vitro culture showing a tight connection between the oocyte and surrounding granulosa cells but a disrupted follicle basal membrane, C) A category 3 follicle at day 4 of in vitro culture showing a disrupted basal membrane and a disrupted connection between the oocyte and surrounding granulosa cells. Arrows indicate a tight connection between the oocyte and granulosa cells and the arrow heads indicate disruptions in the basal membrane

On day 0, primary follicles were selected and 60 wells from two 96-well plates were filled. On day 2, follicles were divided into two groups within which follicle categories 1 and 2 were represented on an equal basis. Half of all category 1 and 2 follicles were embedded in alginate beads, the other half stayed in culture in the corresponding 96-well plate. On day 3, the two groups were each splitted into two groups in which follicle categories 1 and 2 were again represented on an equal basis. Half of the embedded category 1 and 2 follicles were vitrified using mesh cups. Half of the category 1 and 2 follicles cultured in a 96-well plate were vitrified using HSV straws®.

At each day of evaluation, follicles were assigned to three categories. Follicles were not allocated to a category, when the basement membrane or connection between oocyte and granulosa cells could not be fully defined, for example when a follicle was located to the side of a well. In addition, follicle diameter was determined at each evaluation day. Two perpendicular measures were recorded for each follicle, and the average of the two values was reported as follicular diameter (µm). When the diameter of a follicle could not be measured (when a follicle was located to the side of a well and the basal membrane could not be fully defined), it was reevaluated the next day. At day 4, all follicles were used to evaluate follicle quality by morphology, diameter, Neutral Red staining, calcein, propidium iodide and Hoechst staining and histological assessment. For practical reasons and time constraints to minimally expose follicles to light and low temperatures, follicles were not followed up individually but as a group. Every 48 hours, half of the medium in each well was refreshed. Follicles from two 96-well plates were alternately assessed per row to minimize exposure time of follicles to light.

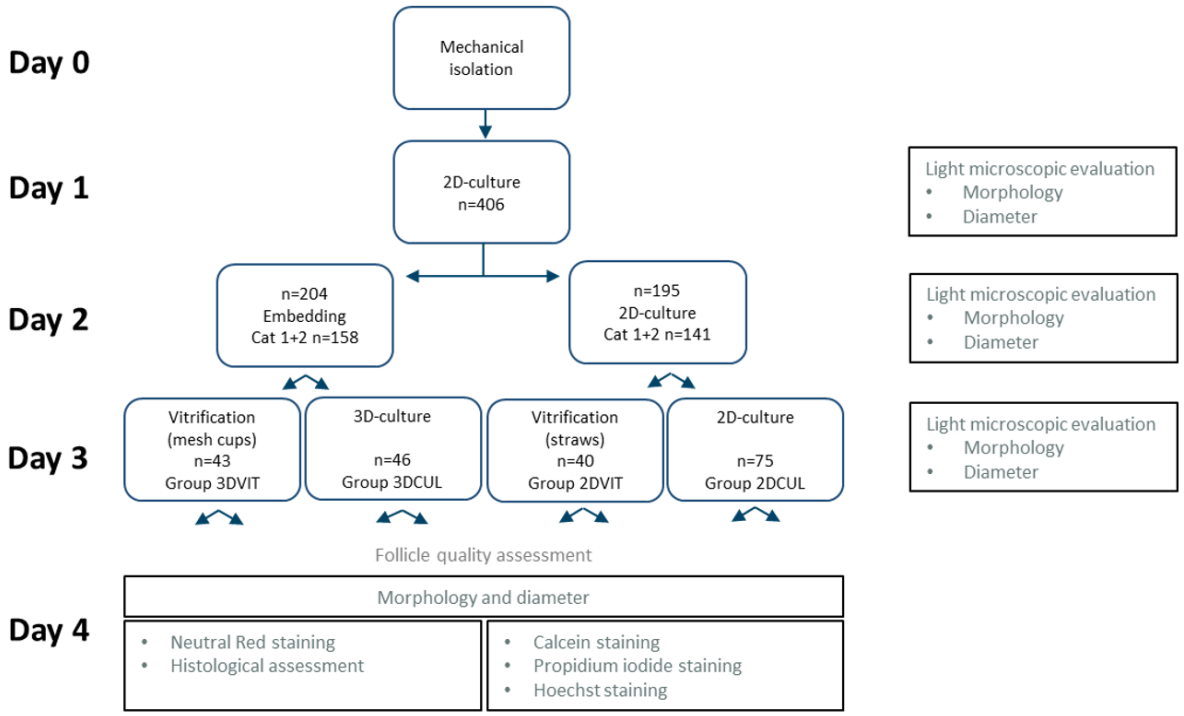

Fig. 2 Experimental design (divided over 6 replicates). On each evaluation day (1, 2, 3, 4), morphology was assessed and follicle diameter measured. On day 4, half of the follicles from each group was stained with Neutral Red (NR) for viability assessment and fixed for histological assessment. The other half was used for calcein, propidium iodide and Hoechst staining. Grey text describes follicle quality assessment methods

2.2. Calcium alginate embedding

2.2.1. Alginate bead size

To determine the optimal bead size, in preliminary experiments, PAFs were embedded in 2.5 µL or in 5 µL alginate beads. At day 2 of culture, category 1 and 2 follicles were selected for embedding. A 2.0% (w/v) solution of sodium alginate (Sigma-Aldrich) in DPBS was prepared and filtered through a 0.2 µm filter. Follicles were transferred to a 20 µL droplet of sodium alginate solution at 38.5 °C. On average 2 follicles in 2.5 µL (n=47) or 5 µL (n=51) alginate solution were released in a small beaker containing CaCl2 (0.1 M) to form beads at 38.5 °C (Fig. 3). After three minutes the beads were removed with a small spoon and washed in culture medium. Alginate beads were incubated individually in a 24-well plate (38.5 °C, 5% CO2). To each well, 500 µL culture medium was added and 10 ml distilled water was added to the plate outside the wells. To assess survivability, 24 hours post embedding, follicle morphology and follicle survival using NR staining were determined. As follicles embedded in smaller (2.5 µL) beads resulted in a higher viability and higher rate of category 1 follicles, in the current experiment follicles were embedded in 2.5 µL alginate beads.

2.2.2. Follicle embedding in alginate beads

At day 2 of culture, category 1 and 2 follicles were selected for embedding. Follicles were embedded in 2.5 µL alginate beads as described above.

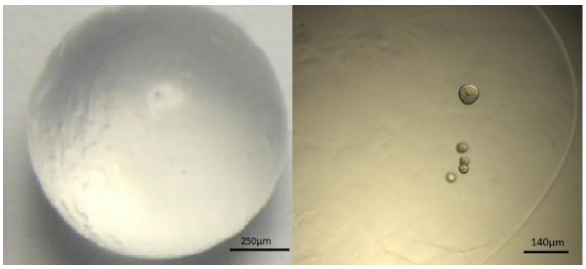

Fig. 3 Left: macroscopic image of an alginate bead. Right: light microscopic image of five encapsulated follicles in an alginate bead

2.3. Freezing and thawing

2.3.1. Encapsulated follicles using mesh cups

Embedded follicles were given 24 hours to recover from the embedding procedures and equilibrate within the alginate bead. One day post embedding (Day 3), half of all embedded follicles were vitrified (group 3DVIT). The other follicles stayed in 3D culture (group 3DCUL). Beads were vitrified using mesh cups [36]. Pieces of metal mesh (mesh size 20 µm; Shijiazhuang Qunkun Metal Products Co., Ltd., Hebei, China) were molded into a cup shape and sterilized (Fig. 4A). Two beads were transferred to a cup immersed in culture medium in a 35 mm petri dish. Cups with beads were transferred with tweezers to equilibration solution consisting of 7.5% DMSO and 7.5% EG in M199 with 10 mg/ml BSA for two minutes at room temperature twice (Fig. 4B). Subsequently, cups were transferred to vitrification solution (15% DMSO, 15% EG, 0.5 M sucrose) at room temperature for 30 seconds and directly plunged into LN_2. To eliminate the media from the metal mesh, the mesh was placed on an absorbent paper towel after each step. The vitrification procedure was carried out at room temperature.

Follicles were cryopreserved for two hours in liquid nitrogen and subsequently warmed. After plunging the cups as fast as possible in thawing solution (1M sucrose in M199 with 10 mg/ml BSA) for one minute at 38.5 °C, cups were transferred to dilution solution (0.5 M sucrose) for four minutes at room temperature (RT). The beads were washed twice in culture medium for four minutes. After removing beads from the cups with a spatula and tweezers, they were cultured individually in a 96-well plate (38.5 °C, 5% CO2).

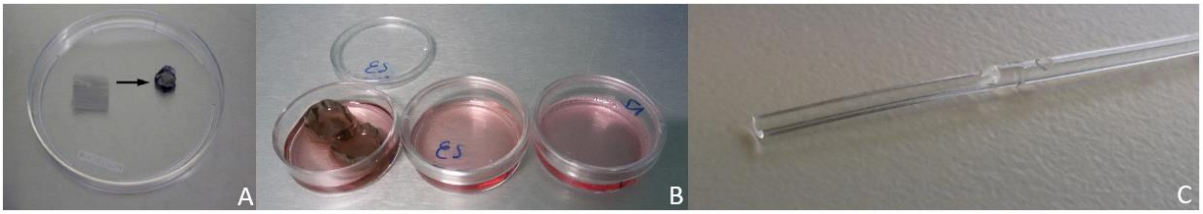

Fig. 4 A: A piece of stainless steel mesh is molded into a cup shape. **B**: Cups with alginate beads are placed in a petri dish with cryoprotectants, cups are transferred with tweezers between different media. Cups can also serve as cryocontainer. **C**: High Security Vitrification straw® with on the left hand side the gutter of the capillary rod where the follicles are deposited

2.3.2. Isolated follicles using HSV straws®

At day 3 of culture, half of all non-embedded follicles, classified as category 1 and 2, were selected for vitrification using HSV Straws® (HSV Kit, Groupe I.M.V.Technologies, Clemenceau, France) (group 2DVIT). The other follicles stayed in 2D culture (group 2DCUL). Follicles were transferred using a Stripper® (Origio). After placement of a follicle in a drop of culture medium on a 90mm petri dish, the drop was merged into the first drop of equilibration solution (7.5% DMSO, 7.5% EG) for two minutes. Then the follicle was transferred to a second drop of equilibration solution for two minutes and subsequently transferred to a drop of vitrification solution (15% DMSO, 15% EG, 0.5M sucrose) for 30 seconds. Follicles were loaded on the gutter of a capillary rod (Fig. 4C) that was placed into a straw and quickly plunged into LN_2.

Follicles were thawed the same day. After merging the capillary rod in a drop of thawing solution (1M sucrose) for one minute at 38.5°C, follicles were transferred to a drop of dilution solution (0.5M sucrose) for four minutes at room temperature. The follicles were washed twice in culture medium. Follicles were cultured individually in a 96-well plate (38.5°C, 5% CO2).

2.4. Follicle quality assessment

2.4.1. Neutral Red (NR) staining

To assess follicle survival and immediate viability, follicles were stained with the non-toxic viability indicator Neutral Red [39]. 5 µL Neutral Red solution (3.3 g/L, Sigma) was added to each well. A follicle was considered to be positively stained when both the oocyte and at least ¾ of the granulosa cells colored red. When the dye is incorporated in the lysosomes of the follicles, they are considered metabolically active and thus viable. Following 20 minutes of incubation (38.5 °C, 5% CO2), follicle staining was evaluated [39].

2.4.2. Follicle mounting and histological assessment

Morphological integrity of the oocyte, the granulosa cells and the basement membrane were investigated by histological examination. Follicles in alginate beads were washed in 0.1M phosphate buffer (PB) and fixed at 4 °C for four hours in 4-well dishes in a solution containing 4% formaldehyde. Follicles were washed and stored in PB until further use. Non-embedded follicles, were fixed in 4% formaldehyde for four hours in 4-well dishes. The follicles were washed and stored in PB until further use.

As stated previously [37] fixed follicles were placed on a glass slide. 30 µl of 80 °C pre-heated Histogel® (Thermofisher, Breda, The Netherlands) was added to the follicles. The mixture was subsequently cooled down at room temperature for three minutes and the Histogel® was cut into a small cube and stored in PB in the fridge. The resulting Histogel® containing the follicles was dehydrated through increasing ethanol solutions and embedded in paraffin. Paraffin blocks were cut in 5 µm thick slices using a microtome and stained with hematoxylin and eosin. All sections were examined and the follicles morphologically evaluated. Follicle morphology was considered to be normal when they contained an oocyte with regular shape and uniform cytoplasm, and organized layers of granulosa cells. Follicles were considered degenerated when the oocyte exhibited a pyknotic nucleus and/or ooplasm shrinkage, granulosa cell layers were disorganized or detached from the basement membrane or when the basement membrane showed irregularities.

2.4.3. Follicle staining with calcein blue AM, propidium iodide and Hoechst 33342

Calcein is a substrate to determine the enzymatic activity and cell-membrane integrity. In living cells non-polar, non-fluorescent cell-permeant calcein acetomethylester (AM) enters the cells and is hydrolyzed by intracellular esterases. It produces a polar, fluorescent molecule, calcein, which is retained in the cytoplasm for several hours and generates intense and uniform blue fluorescence [40]. Dead and dying cells stain with propidium iodide. 1 µL calcein blue AM (1mM, Molecular probes) and 1 µL propidium iodide solution (1 mg/ml, Sigma) were added to 70 µL culture medium. Follicles were incubated for 20 minutes (38.5 °C, 5% CO2). Follicles were observed under an inverted fluorescence microscope (Olympus IX71) equipped with a DAPI and a TRITC filter. Blue fluorescence was visualized in live cells and red fluorescence in dead cells. Two hours later, 1 µL Hoechst 33342 (10 mg/ml, Invitrogen) was added to the culture medium to determine the total cell number. After 20 minute incubation Hoechst positive nuclei were counted.

2.5. Statistical analysis

Different statistical models were used to assess the difference in follicle diameter, number of dead cells, total cell number, viability, follicle categorization and follicle morphology between treatment

groups. The procedure to isolate the follicles from the ovaries, as described in section 2.1, was carried out 6 times. To account for possible batch effects, the replicate number was entered as a random effect in the subsequent statistical analyses. Treatment was entered as a fixed effect. Interaction between the treatment and the batch was also entered, but if this latter term was not significant, it was omitted from the model.

Within each batch (in total 6), follicles were derived from 10 ovaries, that originated from 10 different cows. The ovaries were selected as described in section 2.1. Within each batch, follicle isolation was carried out as outlined in paragraph 2.1 on all 10 ovaries together. After the isolation procedure, approximately 60 intact follicles were selected for culture. No separate follicle isolations were performed for each separate ovary. As a consequence, there is no information which follicle originated from which ovary. Therefore, all follicles within one replicate are considered independent. All follicles were considered as statistical units.

Dependent continuous variables were follicle diameter, number of dead cells and total cell number. Kolmogorov-Smirnov test was used to check whether the dependent variables were normally distributed. Dependent categorical variables were viability (binary, viable vs. non-viable), follicle categorization (binary, category 1 vs. category 2) and follicle morphology (binary, normal vs. degenerated). Independent categorical variable was treatment group (categorical: 3DVIT, 3DCUL, 2DVIT, 2DCUL).

Potential differences in follicle diameter, number of dead cells and total cell number between treatment groups were analyzed using analysis of variance (ANOVA) with Scheffé's method for post hoc comparison. Prior to ANOVA, data were analyzed for normal distribution and homogeneity of variance by performing Kolmonogrov-Smirnov and Levene's test, respectively. Potential differences in viability, follicle categorization and follicle morphology between treatment groups were analyzed using binary logistic regression.

Differences between treatment groups were considered to be significant when P-value <0.05 [41]. Data are presented as means ± S.E.M. All statistical analyses were performed using IBM SPSS version 24® (New York, USA).

3. Results

3.1. Alginate bead size

One day post embedding, a total of 152 follicles were assessed for microscopic morphology and NR staining (n=47, n=51, n= 54 for 2.5 µL beads group, for 5 µL beads group, for control group, respectively). The percentage of category 1 follicles on the total number of follicles was 40%, 25%, 39% for 2.5 µL beads, 5 µL beads or the control group, respectively. Follicles staining positive for the non-

invasive dye NR were considered viable. According to NR staining, viability differed significantly between the group follicles embedded in 2.5 µL beads and in 5 µL beads (P=0.024) (Fig. 5).

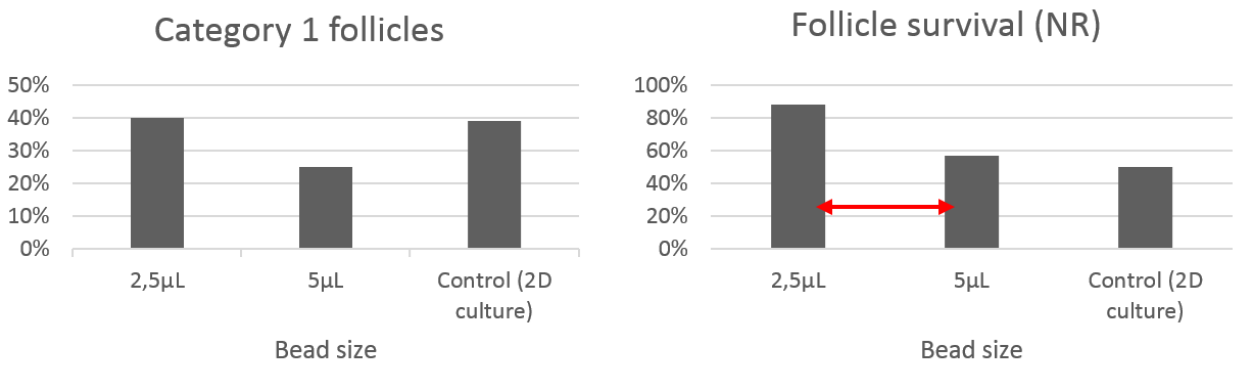

Fig. **5** Left: Percentage category 1 follicles on total number of follicles (category 1, 2 and 3) 24 hours post embedding in 2.5 µL or 5 µL alginate beads or in the control group (2D culture). Right: follicle survival, 24 hours post embedding, assessed after NR staining in 2.5 µL or 5 µL alginate beads or in the control group (2D culture). Red arrow indicates a significant difference between groups

3.2. Follicle quality assessment

3.2.1. Follicle gross morphology

A total of 386 PAFs were evaluated for microscopic morphology on day 1, 406 on day 2, 253 on day 3 and 263 on day 4; divided over 6 replicates. At day 1, most follicles (50.4%) were allocated to category 2 (23.5% to category 1, 20.5% to category 3, 5.6% remained undetermined). Moreover at day 2, a lower percentage of follicles was allocated to category 2, whilst more follicles were allocated to category 1 (29.4% category 1, 43.4% category 2, 24.6% category 3, 2.7% remained undetermined). From day 2 onwards, category 1 and 2 follicles developed until the secondary stage, the outline of the basal membrane was more pronounced and morphological distinction between granulosa cells could no longer be made. Ultimately after warming at day 4, group 3DVIT was the only group showing less category 1 than category 2 follicles. Further at day 4, the percentage of category 1 follicles on the total number of category 1 and 2 follicles was 40.5%, 74.4%, 75%, 58.7% for group 3DVIT, 3DCUL, 2DVIT and 2DCUL, respectively. The numbers of category 1 and 2 follicles were significantly different between groups 3DVIT and 2DVIT (P=0.003) and groups 3DVIT and 3DCUL (P=0.003). Differences were not significant between groups 2DVIT and 2DCUL (P=0.085) and between groups 3DCUL and 2DCUL (P=0.088) (Fig. 6).

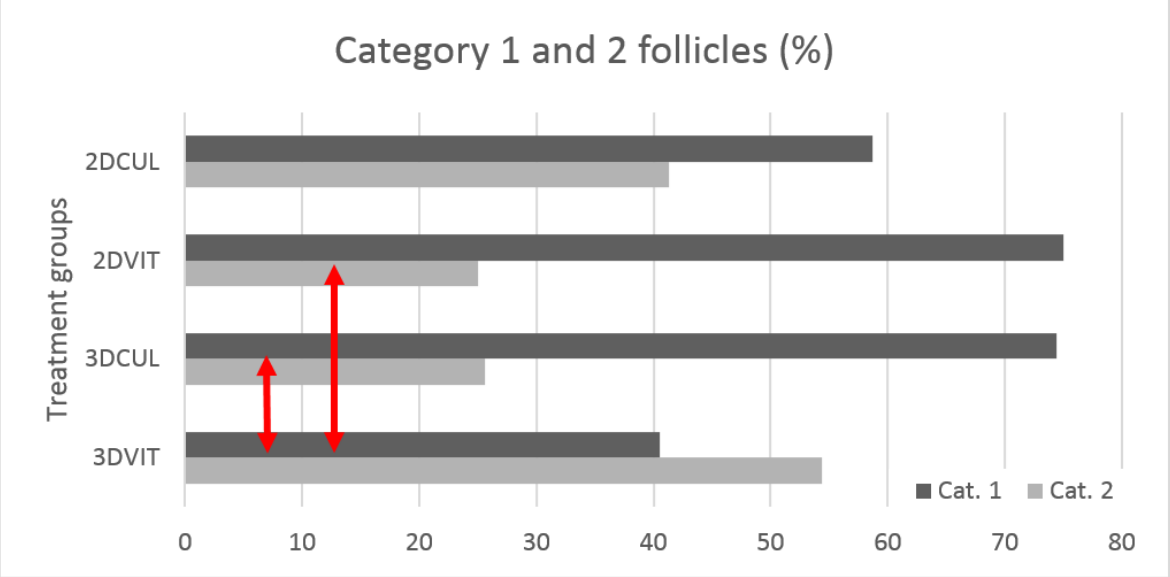

Fig. 6 Percentage category 1 and 2 follicles per treatment group at day 4. Group 3DVIT was the only group showing less category 1 than category 2 follicles. Red arrows indicate a significant difference between groups

3.2.2. Follicle diameter

The follicle diameter of PAFs was measured at day 1 (n=398), day 2 (n=400), day 3 (n=251) and day 4 (n=260) (divided over 6 replicates). The mean follicle diameter ± S.E.M. for category 1 and 2 follicles taken together was 45.92 µm ± 0.42 at day 1 and 47.14 µm ± 0.47 at day 2. At day 3 before vitrification the mean diameter ± S.E.M. for group 3DCUL was 50.48 µm ± 1.21 and 49.77 µm ± 0.84 for group 2DCUL. At day 4 the mean follicle diameter ± S.E.M. was 48.61 µm ± 1.36 for group 3DVIT, 50.45 µm ± 1.48 for group 3DCUL, 56.10 µm ± 1.81 for group 2DVIT and 50.34 µm ± 1.24 for group 2DCUL (Fig. 7). The difference was significant between groups 3DVIT and 2DVIT ($P<0.001$), groups 3DCUL and 2DVIT ($P=0.008$), and groups 2DVIT and 2DCUL ($P<0.001$).

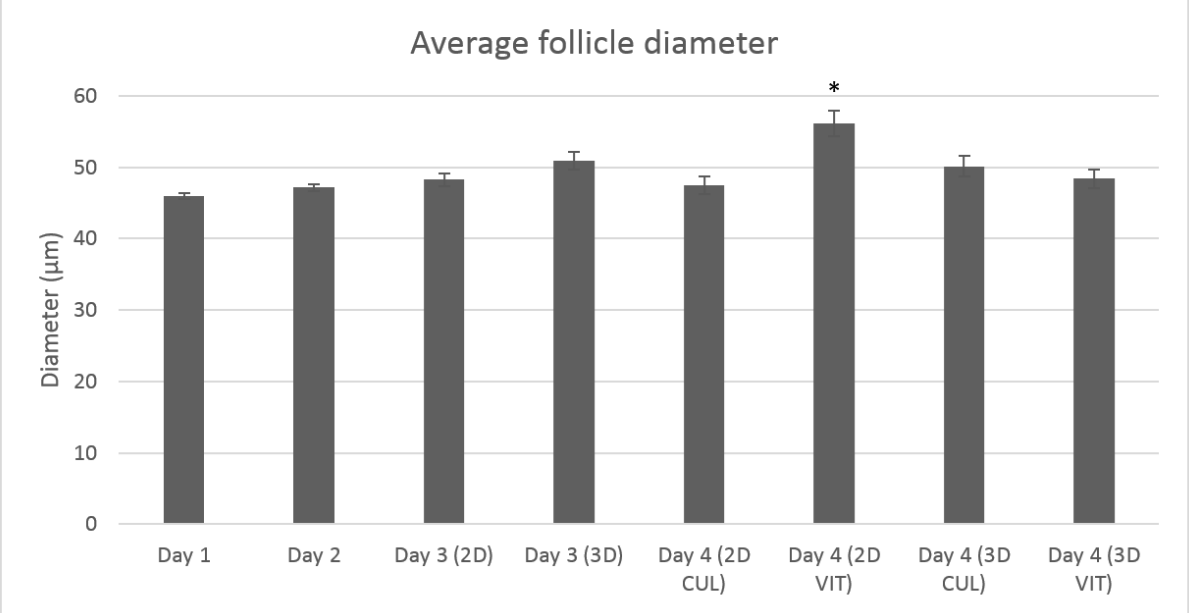

Fig. 7 Mean pre-antral follicle growth ± S.E.M. (follicle diameter of category 1 and 2 PAFs at day 1, 2, 3, 4) linked to treatment group. From day 2 onwards follicles were divided in a 2D and 3D culture group. From day 3 onwards all four treatment groups were formed. Data are presented as mean. *The diameter of group 2DVIT was significantly different from the three other groups

3.2.3. Neutral Red and calcein staining

To evaluate follicle survival, PAFs were stained with NR and calcein at day 4. Only category 1 and 2 follicles were taken into account. Half of the follicles in each group were stained with NR (n=18, n=19, n=18, n=36 for group 3DVIT, 3DCUL, 2DVIT and 2DCUL, respectively) or with calcein (n=19, n=24, n=22, n=37 for group 3DVIT, 3DCUL, 2DVIT and 2DCUL, respectively). Follicles staining positive for the non-invasive dye NR or calcein were considered viable (Fig. 9). According to NR, viability differed significantly between groups 3DVIT and 2DVIT (P=0.015), and between groups 3DVIT and 3DCUL (P=0.002). There was no significant difference between groups 3DCUL and 2DCUL (P=0.998) and between groups 2DVIT and 2DCUL (P=0.167) (Fig. 8). According to calcein staining, viability differed significantly between groups 3DVIT and 2DVIT (P=0.009), and between groups 3DVIT and 3DCUL (P=0.032). There was no significant difference between groups 3DCUL and 2DCUL (P=0.846) and between groups 2DVIT and 2DCUL (P=0.209) (Fig. 8).

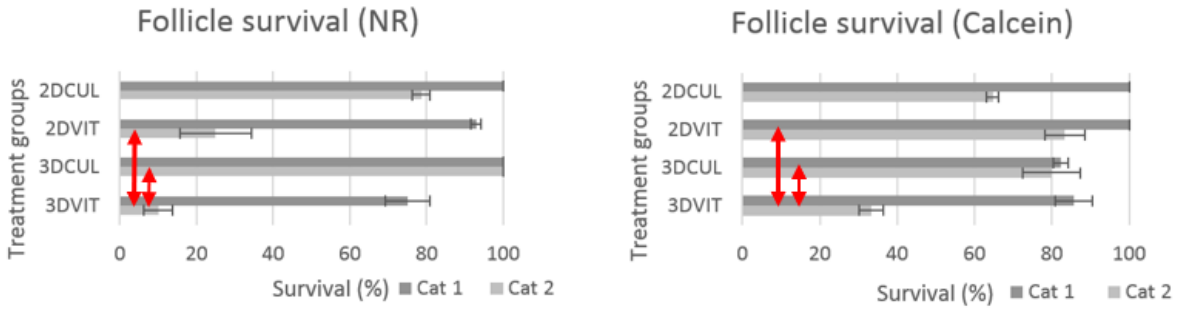

Fig. 8 Follicle survival ± S.E.M. of category 1 and 2 follicles at day 4, assessed after NR staining (left) and after calcein staining (right). Error bars represent the S.E.M. between replicates. Red arrows indicate a significant difference between treatment groups

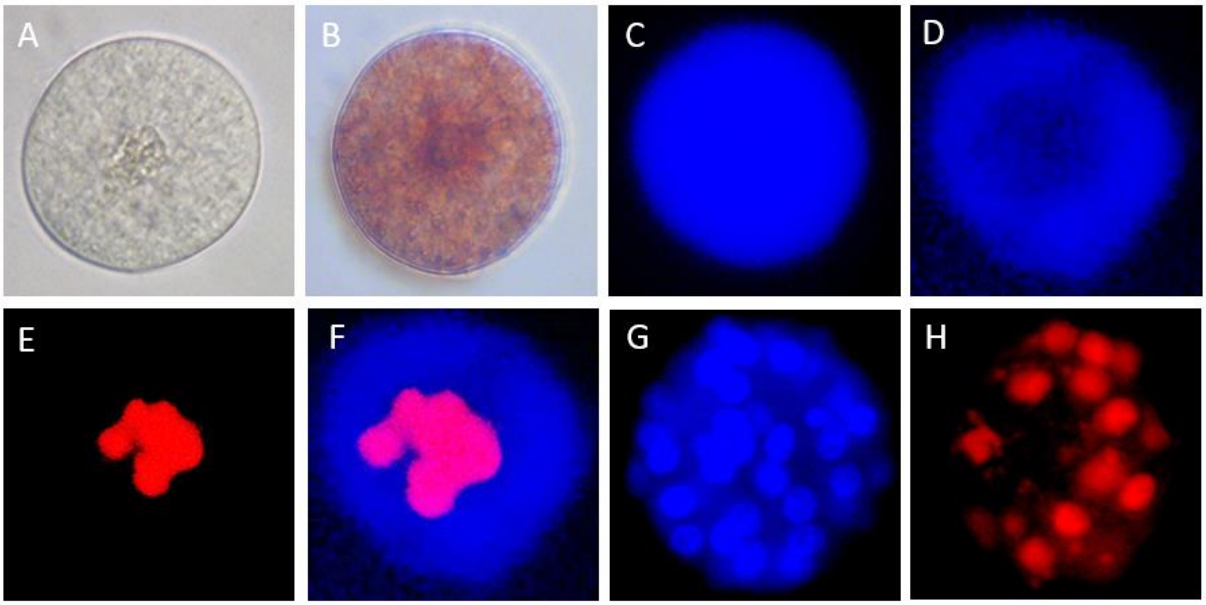

Fig. 9 Follicles were stained with Neutral Red (NR) and calcein-AM to indicate the viability of the follicles, Hoechst was used to assess the total cell number, propidium iodide was used to show nuclei of dead cells. **A**) Light microscopic (LM) image of non-stained follicle. **B**) LM image of follicle staining positive for NR; viable follicle. **C**) Follicle staining positive for calcein; viable follicle. **D**) Follicle of which the oocyte stained negative and the granulosa cells positive for calcein. **E**) Same follicle as in D, nuclei staining positive for propidium iodide. **F**) Merged picture of D and E. **G)** Follicle stained with Hoechst. **H)** Follicle stained with propidium iodide

3.2.4. Hoechst and propidium iodide staining

No significant differences in total cell number and total number of dead cells were found between groups using Hoechst and propidium iodide staining (Fig. 9), respectively. The mean total cell number ± S.E.M. was 14.23 ± 1.70, 17.94 ± 1.46, 20.55 ± 1.43 and 16.88 ± 1.26 for group 3DVIT, 3DCUL, 2DVIT and 2DCUL, respectively (Fig. 10). The mean total number of dead cells ± S.E.M. was 5.12 ± 1.56, 2.59

± 1.10, 3.36 ± 1.57 and 4.36 ± 1.42 for group 3DVIT, 3DCUL, 2DVIT and 2DCUL, respectively. The mean number of dead cells, as a percentage of the total cell number, per follicle per treatment group is shown in Fig. 11.

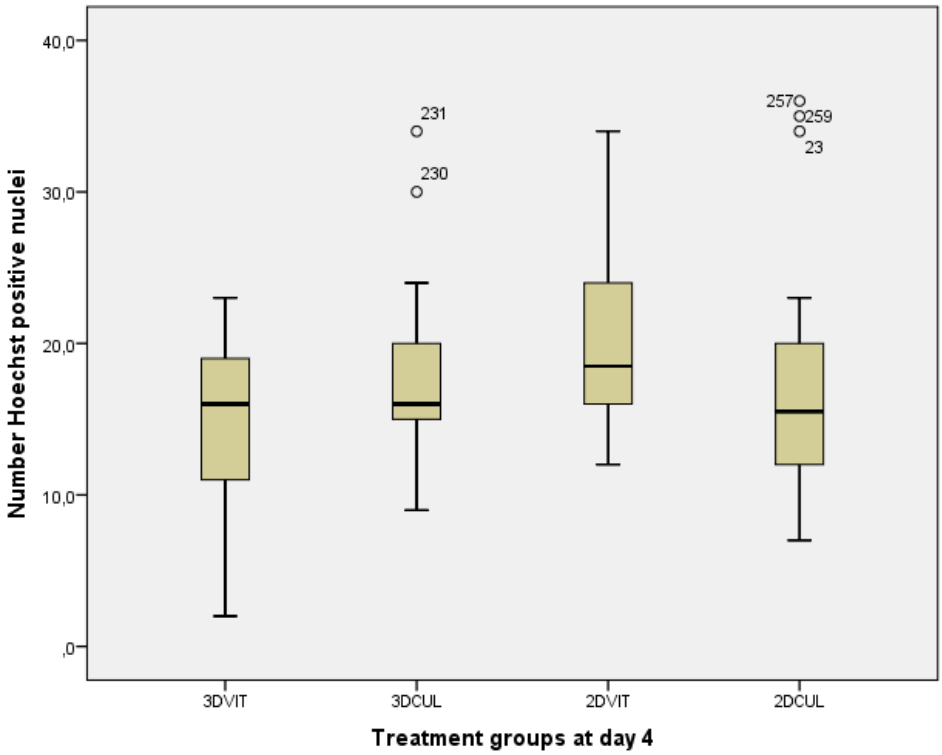

Fig. 10 Number of Hoechst positive nuclei in category 1 and 2 follicles in each treatment group at day 4

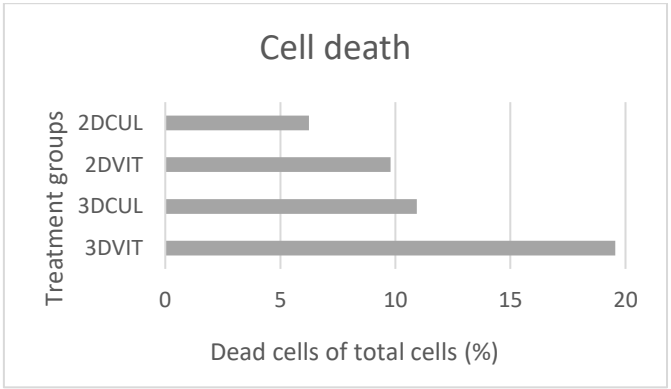

Fig. 11 Mean number of dead cells, as a percentage of the total cell number, per follicle per treatment group for category 1 and 2 follicles at day 4

3.2.5. Histological assessment

A total of 93 sections of pre-antral follicles were examined (14, 25, 18, 36 in group 3DVIT, 3DCUL, 2DVIT and 2DCUL, respectively). In morphologically normal PAFs, granulosa cells were well-organized in layers surrounding the oocyte and a distinguishable intact basement membrane could be observed

(Fig. 12). Degenerated follicles showed a retracted oocyte with or without a pyknotic nucleus. Layers of granulosa cells remained unaltered or became disorganized and were often detached from each other and the basement membrane. Empty spaces or rupture of the basement membrane were also observed. Histological evaluation revealed that the proportion of follicles showing a normal morphology tended to be highest in group 2DVIT (55%) and lowest in group 3DVIT (21%). However, this difference was not statistically significant (P=0.059).

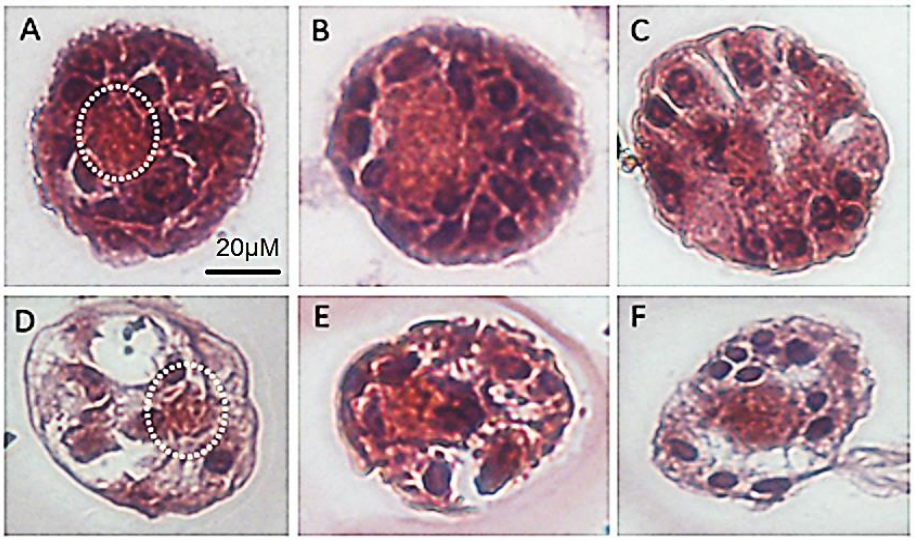

Fig. 12 Histological assessment following follicle embedding in Histogel®. **A, B, C**) PAFs from group 2DVIT, 2DVIT and 3DCUL, respectively showing an oocyte and surrounding granulosa cells. **D**) PAF from group 3DVIT, the oocyte shows signs of degeneration, empty spaces are observed. **E**) PAF from group 2DCUL, basement membrane is ruptured. **F**) PAF from group 2DCUL, empty spaces between oocyte and granulosa cells are visible. The white dotted line indicates the oocyte. PAF: pre-antral follicle

3.3. Work efficiency

Follicle encapsulation limited the handling time and beads were easily transferable. The same number of follicles could be vitrified 2 to 3 times faster using beads in mesh cups instead of straws. A mesh cup can contain several beads, and one bead contained 2-3 follicles which allowed the vitrification of many isolated follicles in a short period of time. Mini mesh cups also served as a cryocontainer, so the laborious loading procedure was omitted. The recovery rate of embedded follicles after warming was 86,3%. From the follicles that were vitrified using straws, 56,9% of the follicles were recovered after warming.

Category 1 follicles from group 2DVIT followed a typical shrink-swell curve, the osmotic equilibrium between intracellular and extracellular solutions occurred within 30 seconds. Category 2 follicles showed less morphological changes, or needed more time to resume their original size, or stayed

shrunken. The malformation and small size make these follicles hard to recognize and easy to lose. After thawing, category 2 follicles were often less viable following the vitrification procedure.

4. Discussion

In this study, isolated bovine PAFs were vitrified on a time and labor efficient way using alginate beads. However, when using the same vitrification protocol, the viability of encapsulated PAFs after vitrification in alginate beads was lower compared to the viability of non-encapsulated PAFs after vitrification when straws were used (45.9% vs. 87.5%). Additionally, we compared 2D and 3D systems for short term culture of isolated bovine pre-antral follicles. We concluded that isolated bovine PAFs which were short term cultured in a 3D alginate bead culture system were of the same quality as compared to a 2D culture system, since no significant differences in viability and morphology were seen.

An important advantage of alginate encapsulation is the quick and safe manipulation of follicles. We observed a high loss of follicles when non-encapsulated isolated follicles were cryopreserved individually. The small size and shrinkage of follicles during exposure to equilibration, vitrification and thawing solutions make it difficult to retrieve follicles as also reported by Rodrigues et al. [42] and Camboni et al. (2013) [35]. Although it is possible to vitrify several follicles at the same time using straws, we chose to vitrify follicles individually because of the small number of follicles and the high risk of not retrieving them.

Cryopreservation of embedded isolated bovine PAFs has, to the best of our knowledge, never been reported so far. Bian et al. (2013) [36] successfully vitrified human PAFs embedded in a 1.5% sodium alginate solution. In preliminary experiments, 1.5% and 2% alginate concentrations were compared to vitrify embedded bovine secondary follicles. As 2% (w/v) sodium alginate solution resulted in highest survival rate of PAFs, it was decided to use a 2% alginate concentration for the experiments. Preliminary experiments revealed that comparisons concerning the bead size showed a significant higher viability of PAFs cultured in beads with a size of 2.5 µL compared to 5 µL. Decreased viability of follicles in larger beads may result from reduced diffusion of nutrients to follicles in the center of the bead. The location of PAFs in the center or in the side of a bead may have an impact on development in larger beads, as nutrients may be in higher concentrations available for follicles located to the side of a bead. However, in our experience it is not possible to control the position of PAFs in the bead.

We observed that non-embedded category 1 follicles followed a typical shrink-swell curve initiated by volume changes caused by exposure to CPAs [43]. When follicles are exposed to CPAs, they shrink as a consequence of a concentration difference between the extracellular and intracellular spaces. Outflow of water caused by the external hyperosmotic solution is faster than the CPA penetration, indicating that the cells are more permeable to water than to CPAs [44]. When the permeable CPAs move in the cell, the follicle swells again. In our experiments the osmotic equilibrium between intracellular and extracellular solutions occurred within 30 seconds for category 1 follicles. However, category 2 follicles did not show a typical shrink-swell curve and were less viable following vitrification.

Survival during short term culture is an important parameter to assess the viability of follicles, for example after vitrification. When 2D culture systems are used for follicle growth and development, long term culture periods can cause disruption in follicle architecture due to flattening because granulosa cells break through the basement membrane and attach to the culture dish [26, 45]. This can cause disruption in the cell-cell connection between the oocyte and granulosa cells, which is critical for oocyte growth and cytoplasmic meiotic competence [46]. 3D culture systems are thus essential to ensure survival and further development of PAFs during long term culture.
During the 4 day culture period in this experiment, or as a previously investigated 10 day [37] culture period, our results showed no follicle attachment to the culture dish, no spreading of granulosa cells or release of the oocyte after in vitro culture of isolated bovine PAFs. However, since we only applied short term culture periods, these features are likely to occur during longer culture periods. Silva et al. [47] reported follicle adherence to the culture plate after 12 days that resulted in loss of normal morphology by day 18 in goats in a 2D culture system.

Although primordial follicles (56.1%) are more abundant than primary follicles in the bovine ovary (32.3%) [48], we chose to select primary follicles at isolation for in vitro culture. Primary category 1 and 2 follicles developed in two days until the secondary stage. Preliminary experiments showed that primordial follicles which did not grow until the secondary stage within two days of culture, were mostly allocated to category 3 and less viable after short term in vitro culture. Factors that regulate follicle activation are most likely paracrine, thus surrounding growing follicles would be necessary to enhance the activation of primordial follicles [49]. Furthermore, it was assessed in a study of Yin et al. [50] that secondary medullar follicles survived and grew to the antral stage, while most primordial and primary follicles died during 3D culture. Hornick et al. (2013) [51] demonstrated that follicles themselves can exert a beneficial coculture effect, as increased growth and survival were seen when primary follicles were cultured in group. However, in our opinion, the choice for multiple follicle culture or single follicle culture from day two onwards, did not have an impact on growth and survival, as the

culture period was too short (only 2 days). Only category 1 and 2 follicles were selected for the experiments. Category 3 follicles showed signs of ongoing degeneration, as reported in earlier experiments category 3 follicles where they lacked significant growth during 10 day in vitro culture [37].

The highest increase of mean follicle diameter at day 4 was observed in the 2DVIT group (vitrification of non-embedded follicles using straws). An increase in follicle diameter can be induced by an increase of total cell number as a result of cell division, by the change of granulosa cell shape from flattened to cuboidal or by cellular swelling due to follicular membrane damage. This increase in follicle diameter is more likely to occur as a consequence of swelling of granulosa cells caused by osmotic influx after thawing. Although cell swelling during removal of CPAs is likely to be more deleterious than cell shrinkage induced by exposure to CPAs [52], follicles vitrified using HSV straws® showed a high viability (87.5%) according to Neutral Red and calcein staining. Group 2DVIT showed the highest mean count of total cell number by Hoechst staining. Differences in total cell number were not significantly different between the 4 groups. Based on the appearance of well-preserved isolated follicles after light microscopic and histological assessment, we can assume that bovine pre-antral follicles can be successfully vitrified using HSV straws without impairing their viability.

As demonstrated by Santos et al. (2006) [53] a 24 hour in vitro culture period after thawing is essential to evaluate the cryopreservation process. Survival was assessed using Neutral Red and calcein staining, which resulted in comparable results after statistical analysis. This indicates that both are reliable evaluation tools to assess viability. The reversibility of NR is beneficial when further growth and development need to be assessed or when other follicle assessment methods will be applied [39].
In non-embedded follicles, NR uptake in lysosomes is reversible. However, in embedded follicles NR was not eliminated when subsequently cultured in NR free medium. This demonstrates the ability of NR to spread throughout the alginate bead, but also the inability to diffuse from the bead into a surrounding solution devoid of the substrate. The low molecular weight of NR should not hinder the diffusion into and from the bead because of the high porosity range of alginate beads. Resistance to diffuse into the beads occurs for larger proteins from a molecular weight larger than 2 × 104, diffusion from the bead is not hindered until the molecular weight approaches 3 × 104 [54]. The mechanism behind this process needs still to be elucidated. The vitrification of PAFs using HSV straws resulted in a significantly higher viability (87.5%; group 2DVIT) than the vitrification of encapsulated PAFs using mini mesh cups (45.9%; group 3DVIT). The same CPA concentrations and exposure times were used for vitrification in both groups. These results illustrate that the protocol for the vitrification of encapsulated follicles needs to be adjusted. There was no significant difference in viability between

the vitrified follicles using HSV straws (87.5%; group 2DVIT) and those cultured in a 2D culture system without cryopreservation at day four (87.7%; group 2DCUL).

Follicles that were vitrified using mini mesh cups (45.9%; group 3DVIT) were significantly less viable than PAFs that were cultured in beads (88.4%; group 3DCUL). These findings were consistent with Sadeghnia et al. (2016) [55]. In contrary, Bian et al. (2013) [36] showed that human pre-antral follicles encapsulated in alginate could maintain their normal ultra-structure after vitrification. Camboni et al. (2013) [35] successfully cryopreserved embedded human primordial/primary follicles using slow freezing.

In conclusion, we studied the effects of encapsulation on follicle viability and morphology after vitrification and short term culture. No differences were observed between follicle viability and morphology in short term 2D and 3D culture systems. Vitrification of non-embedded follicles displayed the highest viability and largest percentage of follicles with a well preserved morphology. Follicles that were vitrified in alginate beads using mesh cups showed the lowest viability (45.9%) according to calcein and Neutral Red staining among all treatment groups. However, the encapsulation of follicles in beads has important advantages as manipulation is much easier and a lot of time is saved because follicles are vitrified in group. The vitrification protocol was successfully used for non-embedded PAFs, however further optimization for embedded bovine PAFs is necessary and in our opinion advantageous. A first step in this process could be the use of a longer exposure time to CPAs, considering the increased volume of embedded follicles which may prolong the diffusion time of CPAs [36]. Follicles vitrified using HSV straws® showed a high viability (87.5%) with no significant difference from follicles that were cultured in a 2D culture system. Considering the labor intensive procedure and low efficiency since many follicles are lost during the vitrification procedure, vitrification in beads may be the method of choice in the future.

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References

1. Howlader, N., et al., *SEER cancer statistics review, 1975–2010.* Bethesda, MD: National Cancer Institute, 2013. **9**.
2. Detti, L., D.C. Martin, and L.J. Williams, *Applicability of adult techniques for ovarian preservation to childhood cancer patients.* J. Assist. Reprod. Genet., 2012. **29**(9): p. 985-995.
3. Jemal, A., et al., *Cancer statistics, 2008.* CA Cancer J Clin, 2008. **58**(2): p. 71-96.
4. Rodriguez-Wallberg, K.A. and K. Oktay, *Options on fertility preservation in female cancer patients.* Cancer Treat. Rev., 2012. **38**(5): p. 354-361.
5. Hyman, J.H. and T. Tulandi, *Fertility preservation options after gonadotoxic chemotherapy.* Clin. Med. Insights Reprod. Health, 2013. **7**: p. 61-69.
6. Leung, W., et al., *Late effects of treatment in survivors of childhood acute myeloid leukemia.* J. Clin. Oncol., 2000. **18**(18): p. 3273-3279.
7. Lee, S., et al., *American Society of Clinical Oncology: Recommendations on fertility preservation in cancer patients.* J. Clin. Oncol., 2006. **24**: p. 2917-2931.
8. Green, D.M., et al., *Fertility of female survivors of childhood cancer: a report from the childhood cancer survivor study.* J. Clin. Oncol., 2009. **27**(16): p. 2677-2685.
9. Seli, E. and A. Agarwal, *Fertility preservation in females: emerging technologies and clinical applications, first ed.* . 2012: Springer, New York.
10. Donnez, J., et al., *Ovarian tissue cryopreservation and transplantation: a review.* Hum. Reprod. Update, 2006. **12**(5): p. 519-535.
11. Wallace, W.H.B., *Oncofertility and preservation of reproductive capacity in children and young adults.* Cancer, 2011. **117**(S10): p. 2301-2310.
12. Revelli, A., et al., *Oocyte cryostorage to preserve fertility in oncological patients.* Obstet. Gynecol. Int., 2012. **2012**.
13. Donnez, J., et al., *Children born after autotransplantation of cryopreserved ovarian tissue. A review of 13 live births.* Ann. Med., 2011. **43**(6): p. 437-450.
14. Donnez, J., et al., *Live birth after transplantation of frozen-thawed ovarian tissue after bilateral oophorectomy for benign disease.* Fertil. and Steril., 2012. **98**(3): p. 720-725.
15. Jadoul, P., et al., *Efficacy of ovarian tissue cryopreservation for fertility preservation: lessons learned from 454 cases.* Human Reproduction, 2017. **32**(5): p. 1046-1054.
16. Dolmans, M.-M., et al., *Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe.* Blood, 2010. **116**(16): p. 2908-2914.
17. Rosendahl, M., et al., *Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia.* Fertil. and Steril., 2010. **94**(6): p. 2186-2190.
18. Donnez, J. and M.-M. Dolmans, *Fertility preservation in women.* Nature Reviews Endocrinology, 2013. **9**(12): p. 735-749.
19. Martinez-Madrid, B., et al., *Chick embryo chorioallantoic membrane (CAM) model: a useful tool to study short-term transplantation of cryopreserved human ovarian tissue.* Fertil. and Steril., 2009. **91**(1): p. 285-292.
20. Van Eyck, A.-S., et al., *Both host and graft vessels contribute to revascularization of xenografted human ovarian tissue in a murine model.* Fertil. and Steril., 2010. **93**(5): p. 1676-1685.
21. Shaw, J., et al., *Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications.* Mol. and Cell. Endocrinol., 2000. **161**(1): p. 103-110.
22. Amorim, C., P. Gonçalves, and J. Figueiredo, *Cryopreservation of oocytes from pre-antral follicles.* Hum. Reprod. Update, 2003. **9**(2): p. 119-129.
23. Jorssen, E.P., et al., *Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension.* Theriogenology, 2014. **82**(3): p. 387-395.

24. Aerts, J.M., et al., *Xenotransplantation by injection of a suspension of isolated preantral ovarian follicles and stroma cells under the kidney capsule of nude mice.* Fertility and sterility, 2010. **94**(2): p. 708-714.
25. Chiti, M., et al., *Influence of follicle stage on artificial ovary outcome using fibrin as a matrix.* Human Reproduction, 2015. **31**: p. 427-435.
26. Green, L.J. and A. Shikanov, *In vitro culture methods of preantral follicles.* Theriogenology, 2016. **86**(1): p. 229-238.
27. Vanacker, J., et al., *Should we isolate human preantral follicles before or after cryopreservation of ovarian tissue?* Fertility and sterility, 2013. **99**(5): p. 1363-1368.
28. Langbeen, A., et al., *Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies.* Theriogenology, 2015. **84**(4): p. 477-489.
29. Araujo, V.R., et al., *Long-term in vitro culture of bovine preantral follicles: Effect of base medium and medium replacement methods.* Anim. Reprod. Sci., 2015. **161**: p. 23-31.
30. Gutierrez, C.G., et al., *Growth and antrum formation of bovine preantral follicles in long-term culture in vitro.* Biol Reprod, 2000. **62**(5): p. 1322-1328.
31. O'Brien, M.J., J.K. Pendola, and J.J. Eppig, *A Revised Protocol for In Vitro Development of Mouse Oocytes from Primordial Follicles Dramatically Improves Their Developmental Competence 1.* Biology of reproduction, 2003. **68**(5): p. 1682-1686.
32. Gupta, P., et al., *Production of buffalo embryos using oocytes from in vitro grown preantral follicles.* Zygote, 2008. **16**(01): p. 57-63.
33. Xiao, S., et al., *In vitro follicle growth supports human oocyte meiotic maturation.* Sci Rep, 2015. **5**: p. 17323.
34. West, E.R., et al., *Physical properties of alginate hydrogels and their effects on in vitro follicle development.* Biomaterials, 2007. **28**(30): p. 4439-4448.
35. Camboni, A., et al., *Alginate beads as a tool to handle, cryopreserve and culture isolated human primordial/primary follicles.* Cryobiology, 2013. **67**(1): p. 64-69.
36. Bian, J., et al., *Vitreous cryopreservation of human preantral follicles encapsulated in alginate beads with mini mesh cups.* J Reprod Dev, 2013. **59**(3): p. 288-95.
37. Jorssen, E.P., et al., *Morphologic characterization of isolated bovine early preantral follicles during short-term individual in vitro culture.* Theriogenology, 2015. **84**(2): p. 301-11.
38. Braw-Tal, R. and S. Yossefi, *Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary.* J Reprod Fertil, 1997. **109**(1): p. 165-171.
39. Langbeen, A., et al., *Effects of neutral red assisted viability assessment on the cryotolerance of isolated bovine preantral follicles.* J Assist Reprod Genet, 2014. **31**(12): p. 1727-1736.
40. De Clerck, L.S., et al., *Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function.* Journal of immunological methods, 1994. **172**(1): p. 115-124.
41. Dohoo, I., S. Martin, and H. Stryhn, *Veterinary Epidemiological Research. Second ed. VER inc.* Charlottetown, 2009.
42. Rodrigues, A., et al., *Cryopreservation and short-term culture of isolated caprine primordial follicles.* Small Ruminant Research, 2005. **56**(1): p. 103-111.
43. Amorim, C.A., et al., *Permeability of ovine primordial follicles to different cryoprotectants.* Fertility and sterility, 2006. **85**: p. 1077-1081.
44. Paynter, S., et al., *Permeability characteristics of human oocytes in the presence of the cryoprotectant dimethylsulphoxide.* Human Reproduction, 1999. **14**(9): p. 2338-2342.
45. Cortvrindt, R., J. Smitz, and A. Van Steirteghem, *Ovary and ovulation: In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system.* Human Reproduction, 1996. **11**(12): p. 2656-2666.
46. Carabatsos, M.J., et al., *Oocyte–granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence.* Developmental biology, 2000. **226**(2): p. 167-179.

47. Silva, G.M., et al., *In vitro development of secondary follicles from pre-pubertal and adult goats cultured in two-dimensional or three-dimensional systems.* Zygote, 2015. **23**(4): p. 475-484.
48. Choi, M.H., et al., *Morphological criteria of bovine ovaries for predicting retrieval efficiency of preantral follicles.* Asian australasian journal of animal sciences, 2006. **19**(12): p. 1711-1715.
49. Da Silva-Buttkus, P., et al., *Inferring biological mechanisms from spatial analysis: prediction of a local inhibitor in the ovary.* Proceedings of the National Academy of Sciences, 2009. **106**(2): p. 456-461.
50. Yin, H., et al., *Survival and growth of isolated pre-antral follicles from human ovarian medulla tissue during long-term 3D culture.* Hum Reprod, 2016. **31**(7): p. 1531-1539.
51. Hornick, J.E., et al., *Multiple follicle culture supports primary follicle growth through paracrine-acting signals.* Reproduction, 2013. **145**(1): p. 19-32.
52. Armitage, W., B. Juss, and D. Easty, *Differing effects of various cryoprotectants on intercellular junctions of epithelial (MDCK) cells.* Cryobiology, 1995. **32**(1): p. 52-59.
53. Santos, R.R., et al., *Preservation of caprine preantral follicle viability after cryopreservation in sucrose and ethylene glycol.* Cell and tissue research, 2006. **325**(3): p. 523-531.
54. Smidsrød, O. and G. Skja, *Alginate as immobilization matrix for cells.* Trends in biotechnology, 1990. **8**: p. 71-78.
55. Sadeghnia, S., et al., *Development of sheep primordial follicles encapsulated in alginate or in ovarian tissue in fresh and vitrified samples.* Cryobiology, 2016. **72**(2): p. 100-105.

CHAPTER 4

Passive slow freezing is an efficacious and cost-effective alternative to controlled slow freezing for ovarian tissue cryopreservation

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Summary

We aimed to assess the feasibility of passive slow freezing (PSF using Mr. Frosty container, Nalgene) as an alternative to controlled slow rate freezing (CSF using (Freezal™, Air liquide)) for human ovarian tissue (OT) cryopreservation. Validation studies needed were determined after assessing the risk associated (EuroGTP-II ART tool) and were conducted in 66 OT samples from 10 transgender men aged 23.4 ± 5.1 y. Folliculogenesis was assessed *in vitro* (after 2 h and 2 days of culture) and *in vivo* (2, 4 and 6 weeks xenotransplantation in Balbc/nude mice) by haematoxilin-eosin staining. Fibrosis was assessed by Masson's trichrome staining. Immunohistochemistry was used to study cell proliferation (PCNA and Ki-67) and apoptosis (caspase-3 and TUNEL). Differences in percentages were estimated using a generalized estimated equations method. After 2 days of *in vitro* culture, higher odds of primordial follicles (PF) (OR 1.626; 95%CI (1.162–2.266); P = 0.004) and lower odds of growing follicles (GF) (OR 0.616; 95%CI (0.441–0.861); P = 0.004) were associated with the established CSF technique. No statistical differences were found in the mean estimated proportion of proliferating (Ki-67+ or PCNA+) or apoptotic (caspase-3+ or Tunel+) follicles. Two and 6 weeks after xenotransplantation, respectively lower odds of GF (OR 0.419; 95%CI (0.217–0.809); P = 0.010) and secondary follicles (OR 0.135; 95%CI (0.071–0.255); P < 0.001) were associated with CSF. Proportion of fibrosis was similar. This validation study shows a higher follicle activation after 2 days *in vitro* and after 2 weeks following xenotransplantation in mice using PSF. PSF may be an easy, cost-effective low-risk alternative to CSF for cryopreservation of human OT.

1. Introduction

Ovarian tissue (OT) cryopreservation is a technique for the preservation of fertility in women that allows restoration of ovarian structure and function, which may benefit women in different situations [17]. Moreover, it is currently the only available option for prepubertal patients and those with hormone-dependent diseases [12,15]. OT cryopreservation has the advantage over other traditional methods such as embryo or oocyte cryopreservation of requiring neither sperm donors nor ovarian stimulation, thus avoiding any delay in cancer treatment [17]. By using this method, a number of follicles can be preserved, which can then be used either for autologous transplantation or for *in vitro* growth, maturation and fertilization [15]. Although experience with OT cryopreservation and transplantation is growing, this technique is still considered innovative, mainly due to the variable rate of success. A meta-analysis conducted in 2017, which included 309 cases of OT cryopreservation and transplantation, revealed cumulative live-birth and ongoing pregnancy rates of 57.5% and 37.5%, respectively, and an endocrine restoration rate of 63.9% [20]. However, some experts consider that OT cryopreservation may already fulfil the criteria for an "established method" [8].

The survival rate of follicles is largely affected by cryoinjury and ischemic damage [15]. Different cryopreservation techniques have been developed to minimize these risks, among which slow freezing (SF) is the one most frequently used. SF is performed at regular freezing rates (i. e., controlled, CSF) thanks to the use of programmable biofreezers. However, this equipment requires making a great investment and maintenance effort, they take up nitrogen consumption and there is a risk linked to gas leakage. Conversely to CSF, passive slow freezing (PSF) is an inexpensive technique that uses insulated containers filled with isopropyl alcohol where cryovials are left overnight (- 80 ◦C) and finally transferred into liquid nitrogen at - 196 ◦C. The validity of passive slow freezing for the cryopreservation of testicular tissue has been proven in several experimental studies [19]. However, there is a lack of experience with OT.

As with other innovative technologies and practices in assisted reproduction treatment (ART), the delay in obtaining solid clinical data on effectiveness and safety requires assessing the risk/benefit ratio before any of them can be implemented in the clinical practice [4,9]. A risk assessment tool (EuroGTP-II) has been recently developed, which provides a systematic way to identify and quantify the risks to individuals of clinical application of novel *Tissue and Cellular Therapies and Products* (TCTPs) as well as providing advice on the extent of studies and/or follow-up needed to assure their safety and efficacy [29]. An ART-specific EuroGTP-II tool aimed at assessing the potential impact of novel ART on embryo development and child health has been recently published [30].

Taking advantage of this new tool, we have identified and quantified the potential risks associated with the implementation of PSF for OT cryopreservation. Two pre-clinical studies were conducted with the aim of mitigating these risks by providing evidence of follicle proliferation and apoptosis rates after thawing (*in vitro* study) and follicle growth and fibrosis generation after xenotransplantation in mice (*in vivo* study). The impact on a subsequent risk assessment is also reported.

2. Materials and methods

2.1. Risk assessments

The EuroGTP-II risk-assessment tool and its ART-specific version have been described elsewhere [10,29,30]. Briefly, it consists of three steps. Step 1 consists of 7 questions assessing the novelty. When the answer to all questions is "Yes", the technique or procedure is not considered a novelty and risk assessment is therefore not needed. Step 2 identifies potential risks and associated risk consequences through a questionnaire where their probability (P) of the risk (rare, unlikely, possible, likely, almost certain), the severity (S) of the consequences (non-serious, serious, and life-threatening), and the probability of the risk consequence being detected (D) (very high, moderately high, low, very low and cannot be detected) are individually scored. A percentage of risk reduction is calculated on the basis of relevant sources of evidence. A combined risk value and a final risk score is calculated upon which a series of risk reduction strategies are proposed as well as extent studies to ensure the safety and efficacy of the ART are proposed. Two risk assessments were performed: one for the initial evaluation, and another one after having conducted the pre-clinical validation studies. These assessments were conducted with the interactive tool available at https://tool.goodtissuepractices.site. Pre-clinical studies to address the specific risks are recommended in the EuroGTP-II guidelines [10].

2.2. Pre-clinical validation studies

2.2.1. Experimental design

Two studies, one *in vitro* and another one *in vivo*, were conducted where the validity of PSF to freeze OT was assessed and compared to that of CSF. Folliculogenesis was assessed by means of the evaluation of follicle activation/apoptosis index in the former, and follicle growth/ fibrosis index in the latter. A summary of the design of the studies is available in Figs. 1 and 2 Suppl. material.

2.2.2. Ovarian tissue procurement

Human OT was obtained from 10 female-to-male transgender persons who provided informed consent before participating in the study. Mean age at the time of transition surgery was 23.4 ± 5.1 years. The

ovaries were bisected and the medulla was carefully removed, resulting in a remaining ovarian cortex of at least 1 mm thickness. Cortical tissue was cut into ~5 × 5 mm pieces under sterile conditions and were transferred to freezing medium containing Leibovitz L-15 medium (Life Technologies) supplemented with 0.45% human serum albumin (HSA, Red Cross, Belgium) and 1.5 M dimethylsulphoxide (DMSO, Sigma). The tissue was transferred to 1.8 mL cryogenic vials containing 800 µL of freezing medium and incubated for 20 min at 4 °C.

2.2.3. Freezing procedures and tissue preparation after warming

A programmable freezer (Freezal™, Air Liquide) was used for CSF. The freezing protocol was as follows: lowering temperature at an initial rate of 2 °C/min between 4 °C and – 9 °C, manual seeding at – 9 °C, cooling at a rate of – 0.3 °C/min to – 40 °C and finally to – 140 °C at a rate of 10 °C/min. Vials were afterwards transferred to liquid nitrogen for long-term storage. For the PSF, the cryogenic vials were placed in an isopropyl alcohol container ('Mr. Frosty™', Nalgene) and stored overnight in a – 80 °C freezer, which allows a cooling rate ≈ - 1 °C/min. Vials were subsequently transferred to liquid nitrogen for long-term storage. Samples were thawed in a 37 °C water bath for 2 min. Tissue fragments were washed 3 times (5 min each) in Leibovitz L-15* medium (Life Technologies) supplemented with 0.45% HSA at room temperature. OT tissue samples were cut in 2 equal pieces for the *in vitro* experiment and were stretched out mechanically using tweezers. A superficial 'waffle'- like structure of partially interconnected 1 mm^3 cubes was made [28]. Tissue pieces were cultured individually as described below. For the *in vivo* experiment, OT tissue samples were cut in 2 equal pieces and incubated individually during 2 h in growth medium as described below. Because half of the original OT pieces was too large for transplantation, 1/3 was transplanted and 2/3 were used as non-transplanted control. Two tissue pieces originating from the same patient that were frozen by either CSF or PSF were transplanted into the same mouse (Fig. 3 Suppl. Material).

2.2.4. In vitro culture of human cortical strips

The cortical tissue strips were cultured individually in 24-well culture plates containing 300 µL of McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM, 0.1% HSA, 3 mM glutamine (Thermo Scientific), 0.1 mg/mL penicillin G–streptomycin (Thermo Scientific), 2.5 µg/mL transferrin, 4 ng/mL selenium, 10 ng/mL insulin and 50 µg/mL ascorbic acid. All products were purchased from Sigma- Aldrich unless otherwise stated. For each patient, 2 tissue strips were cultured for 2 h and 2 strips for 2 days at 37 ◦C in humidified air with 5% CO2 and 6% O2. The 2 strips were afterwards fixed separately in 4% buffered formalin and embedded in paraffin. Each pair of incised strips cultured per condition was considered as 1 cortical fragment for the analysis of the downstream parameters.

2.2.5. Granulosa cell proliferation by immunohistochemistry

In the *in vitro* experiment, follicle activation was assessed by the presence of the cellular marker for proliferation Ki-67 and the proliferation cell nuclear antigen (PCNA) [3]. For this purpose, 10 sections per tissue piece were subjected to Ki-67 immunostaining and 10 sections to PCNA immunostaining. Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 (Sigma) for 30 min at room temperature. After incubation in citrate buffer (pH 6) for 75 min at 98 °C, nonspecific binding sites were blocked by incubation with normal goat serum for 30 min. The sections were then incubated overnight at 4 °C with primary antibodies: mouse monoclonal anti-human Ki-67 antibody (M7240, 1:100 dilution, Dako) or mouse monoclonal anti-human PCNA antibody (13–3900, 1:100 dilution, Thermo Fisher Scientific). The slides for Ki-67 detection were incubated with rabbit anti-mouse secondary antibody (E0413, 1:200 dilution, Dako) for 30 min at room temperature followed by 30 min incubation with horseradish peroxidase (HRP, P0397, 1:200 dilution, Dako). The PCNA slides were incubated for 30 min with rabbit anti-mouse IgG secondary antibody (PA1-28568, 1:200 dilution, Thermo Fisher Scientific). Diaminobenzidine (DAB, D5637, Sigma) was used as a chromogen and haematoxylin/eosin as a counterstain. The proliferation index was calculated as the percentage of Ki-67 positive or PCNA -positive primordial (PF) or growing follicles (GF) with respect to the total amount of PF or GF. Follicles with at least one Ki-67 positive stained nucleus in the GF or PCNA positive stained cytoplasm were considered positive. Follicles containing an oocyte with a positive Ki-67 stained nucleus or a positive PCNA stained cytoplasm were considered as positive. Negative controls were slides incubated without the primary antibody and human brain tumour was used as positive control for Ki-67 and mouse liver as positive control for PCNA staining.

2.2.6. Follicle atresia by immunohistochemistry

Early apoptosis was assessed by the active caspase-3 assay; the late apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL, G7130, Promega). Each method was used in 10 sections. For the active caspase-3 assay, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 (Sigma) for 30 min at room temperature. After demasking in citrate buffer (pH 6) for 75 min at 98 °C, sections were incubated overnight at 4 °C with a primary mouse monoclonal anti-human caspase-3 antibody (43–7800, 1:20 dilution, Thermo Fisher Scientific). The slides were then incubated for 30 min with rabbit anti-mouse IgG secondary antibody (PA1-28568, 1:200 dilution, Thermo Fisher Scientific). After the TUNEL assay, sections were deparaffinized and rehydrated. Diaminobenzidine (DAB, Sigma) was used as a chromogen and haematoxylin/eosine as a counterstain. The specificity of the primary antibody was confirmed by negative controls based on the absence of a signal in the section incubated without primary antibody. The apoptosis index was calculated as the percentage of PF or GF with TUNEL- or caspase-3 positive cells on the total number

of PF or GF. Follicles with at least one caspase-3 positive stained cytoplasm in the granulosa cells or with TUNEL positive stained nuclei in the granulosa cells were considered as apoptotic. An oocyte stained with either caspase-3 in the cytoplasm or a positive TUNEL stained nucleus was considered apoptotic. Early- (caspase-3) and late (TUNEL) apoptosis were assessed in 200 × 2 sections per method. Negative controls were slides incubated without the primary antibody and DNAse treated ovarian was used a positive control for both TUNEL and caspase-3 staining.

2.2.7. Xenotransplantation in nude mice

Experimental animals were handled according to the Ethical guidelines of the European Union (Directive 2010/63/EU) and the Belgian legislation (Royal Decree of May 2013). Twenty-seven female BALB/c-nu mice (Janvier Labs, France) aged 6–8 weeks were housed individually under controlled temperature (25–27 °C), air (HEPA-filter) and lighting (12 h light/12 h dark) conditions. Food and water were given *ad libitum*. An adaptation period of one week was implemented prior to xenotransplantation. The transplantation technique has been previously described [16]. Briefly, anesthetized animals were placed in a dorsal recumbency, a midline laparotomy was performed and both ovaries were removed to create a supporting environment for developing pre-antral follicles by eliminating the natural negative feedback on gonadotropin-releasing hormone (GnRH) release [18]. A pouch was created between the abdominal internal oblique muscle and the peritoneum on both sides of the abdominal incision at the linea alba. In the right-hand side pouch of the mid-line incision, a PSF frozen/thawed ovarian tissue fragment was transplanted. On the opposite side, a similar pouch was filled with a CSF frozen/thawed fragment of the same patient (Fig. 3 Suppl. material). Xenotransplantation was performed in duplo for each patient. Two, 4 and 6 weeks after transplantation, mice were euthanized by cervical dislocation. Grafts were extracted, fixed in 4% buffered formalin and embedded in paraffin (Fig. 4 Suppl. material).

2.2.8. Follicle classification

Follicles were classified according Gougeon's classification [13]. Classification was based on the cross section containing the oocyte's nucleus to avoid double counting. Follicles were further classified as PF or GF follicles, these latter being the sum of intermediate, primary, secondary and antral follicles. Their percentage was calculated on the basis of total follicle count at each time point (2 h and 2 days).

2.2.9. Assessment of follicle growth after xenotransplantation

Xenografts were collected and fixed for paraffin sectioning before haematoxylin/eosin staining. Follicles were analysed using an inverted microscope (Primo Star, Zeiss, Belgium) with a 40× magnification. The follicle count was performed according to Gougeon's classification [13]. The total

number of sections per xenograft was counted. For the corresponding non-transplanted part of each tissue sample, half of the tissue was sectioned. The non-transplanted piece was twice the size of the corresponding xenograft, allowing follicle counts and comparisons made on equal sizes of tissue. Follicles were classified further as PF or GF. Their percentage was calculated on basis of total follicle count per each time point after transplantation (0, 2, 4 and 6 weeks).

2.2.10. Assessment of fibrosis after xenotransplantation

Masson's trichrome staining method was used to assess fibrosis in tissue paraffined sections. Pictures were recorded at 20× magnification and analysed. Quantification of collagen was performed on 5 sections (5 fields/section, 25 fields) per graft, and were expressed as the percentage of the total tissue graft. All measurements and analysis were performed with Image J software (http://rsb.info.nih.gov//ij/). This evaluation was performed in the ovarian tissue grafts of both study groups and in their corresponding non-transplanted parts of the tissue sample. The percentage of fibrosis was determined by taking the mean of the 25 fields of the blue-green area/total area × 100.

2.3. Statistical analysis

The mean estimated proportion was calculated (95%CI) and differences in percentages of types of follicles or positively stained follicles for apoptosis or proliferation markers, between both methods, were estimated using GEE (Generalized estimating equations) with correlation structure "exchangeable" and patient id defined as "subject variable". Odd ratios are given including the 95%CI. The statistical model was mostly based on cases with valid data for all variables in the model. If one or more cases were equal to zero, than the cases were not used in the model. In the event where too many cases were equal to zero, the effect size could not be calculated and this is shown as 'n\a' in the result tables. For the fibrosis measurement, all percentages measured were pooled per group and time points of transplantation. An unpaired *t*-test was performed. Values of $P < 0.05$ were considered statistically significant. Analyses were carried out using SPSS v23.0.

2.4. Ethical issues

This study was approved by the Ethical Committee of Ghent University Hospital (UZ Ghent Reference: 2015/0124 – B670201523543). The study protocol for animal experiments was approved by the Ethical Committee on Animal Experimentation from the University of Ghent (approval number ECD 18/12).

3. Results

3.1. Initial risk assessment

The initial assessment indicated that the proposed freezing method was an innovation (Table 1) with a moderate risk (combined risk of 7, see Table 2).

Table 1. Assessment of novelty of the proposed freezing technique (EuroGTP-II ART tool Step 1)*.

	Yes	No	NA
A. Has this type of TCTP previously been prepared and issued for clinical use by your establishment? *Ghent University hospital has been cryopreserving ovarian tissue since 1998 and in 2019, 2 patients requested transplantation of tissue. Both transplantation procedures were successful in restoring the endocrine function in the patient.*	X		
B. Will the starting material used to prepare this TCTP be obtained from the same donor population previously used by your establishment for this type of TCTP? *There is no difference in patient population between the 2 freezing methods*	X		
C. Will the starting material for this TCTP be procured using a procedure used previously by your establishment for this type of TCTP?	X		
D. Will this TCTP be prepared by a procedure (processing, decontamination and preservation) used previously in your establishment for this type of TCTP? *The processing of the tissue is the same in CSF or PSF method, however the freezing protocol is different. The cooling rates are different and there is no seeding step in the PSF method.*		X	
E. Will this TCTP be packaged and stored using a protocol and materials used previously in your establishment for this type of TCTP?	X		
F. Will this type of TCTP provided by your establishment be applied clinically using an implantation method used previously?	X		
G. Has your establishment provided this type of TCTP for transplantation into the intended anatomical site before? (this question will only appear if gonadic tissue is chosen)	X		

Adapted from the EuroGTP-II guidelines [10]

*Justifications for the evaluation of novelty of the procedure are provided when needed

NA, not applicable; CSF, controlled slow freezing; PSF, passive slow freezing; TCTP, Tissue and cellular therapies and products.

Table 2. Risk identification and assessment according to the EuroGTP-II ART tool before and after (shown **in bold**) pre-clinical studies to address identified specific risks*

	Risk factors	Does it apply ?	Comments	Risk consequences*	P	S	D	PR	RR (%)	Risk
Donation	**Donor Characteristics / Source of material**	No	There is no difference in the donor population between the CSF and PSF procedure.	-	-	-	-	-	-	-
Procurement	**Recovery/ Procurement process and environment**	No	There are no differences in the procurement of the tissue.	-	-	-	-	-	-	-
Processing/ storing /transport	**Preparation process and environment**	Yes	There are no differences in the processing of the tissue. The media used and the manipulation of the tissue are the same and it takes place in the same environment. The cooling rates during the cryopreservation protocol are different, which may have an impact. Cryoprotectant is the same as in CSF. It is highly unlikely that changes in cooling rate would result in toxicity.	Implant failure/ pregnancy loss	3	2	2	12	0 / **25†**	12 / **12**
				Toxicity/ Carcinogenicity	1	2	1	2	0	2 / **2**

	Reagents	No	The same media are used in the CSF and PSF procedure	-	-	-	-	-	-	-
	Storage Conditions	Yes	There is indeed a difference in storage conditions. In the PSF method, the vials are stored overnight in -80°C, while the CSF program ends the same day and the vials are plunged in the liquid nitrogen.	Implant failure/ pregnancy loss	3	2	2	12	0 / **25†**	12 / **9**
				Disease transmission	2	2	1	4	0 / **25†**	4 / **3**
				Toxicity/ Carcinogenicity	1	2	1	2	0 / **25†**	2 / **1.5**
	Transport Conditions	No	Transport conditions are the same.	-	-	-	-	-	-	-
Product	**Loss of viability and or functionality**	Yes	There is a possibility that the cooling rates during PSF lead to loss of follicles and thus lead to a reduction in outcome after transplantation.	Implant failure/ pregnancy loss	3	2	2	12	0 / **25†**	12 / **9**
Clinical application procedure	**Complexity of the preparation/ application method**	No	There is no difference in pre-implantation preparation, the same thawing procedure will have to be used before application.	-	-	-	-	-	-	-
Total risk score = 7 / **6**										

Adapted from the EuroGTP-II guidelines [10]

CSF, controlled slow freezing; D, detectability; P, probability; PR, potential risk; PSF, passive slow freezing; RR, risk reduction; S, severity;

*Only risks consequences identified among those exposed in the EuroGTP-II tool when a given risk factor is identified are shown.

†Pre-clinical experiments conducted have proven follicle activation despite these differences vs. CSF, which translates into a 25% RR

3.2. *In vitro* pre-clinical validation experiment

Fifty OT strips from 10 patients were assessed: two strips/patient were randomly assigned to each group (CSF and PSF, total 20 OT strips per group). Additionally, one strip/patient in each group (total 10 pieces) was used as a control immediately after thawing.

Follicular morphology was assessed on two thousand sections. On day 2, the mean estimated proportion of PF was significantly lower in the PSF group (16% with 95%CI (11%–23%) vs. 23% with 95%CI (15%– 34%)) and the proportion of GF was higher (84% with 95%CI (77%– 89%) vs. 77% with 95%CI (66%–85%)). The proportion of primary follicles was also higher in PSF (57% with 95%CI (49%–63%) vs. 48% with 95% CI (41%–56%)). After 2 days of *in vitro* culture, higher odds of primordial follicles (PF) (OR 1.626; 95%CI (1.162–2.266); P = 0.004) and lower odds of growing follicles (GF) (OR 0.616; 95%CI (0.441–0861); P = 0.004) were thus associated with the established CSF technique (Table 3). Assessment of cell proliferation was carried out on 200 × 2 PCNA and 200 × 2 Ki-67 stained sections. After 2 h and 2 days of culture, there was no statistical difference in the estimated proportion of PCNA positive PF and GF in PSF (Fig. 1, Suppl. Table 1). After 2 h and 2 days of culture, no differences were observed between CSF and PSF with regard to the estimated proportion of Ki-67 positive PF and GF (Fig. 1, Suppl. Table 1). Differences between CSF and PSF in early- (caspase-3) and late (TUNEL) apoptosis were not observed after two days of culture; there was no statistical difference between the estimated proportion of caspase-3-positive or TUNEL positive PF and GF in the PSF vs. the CSF group (Fig. 2, Suppl. Table 1).

Table 3. Total number of follicles counted and estimated proportion in the CSF and PSF groups after 2h and 2 days of *in vitro* culture.

	CSF N	Estimated proportion % (95%CI)	PSF N	Estimated proportion % (95%CI)	effect size OR (95%CI), p-value
2 h					
Total (PF + GF)	6940		7185		
PF	3559	52% (44%–60%)	3661	51% (46%–56%)	1.055 (0.768–1.450), P = 0.740
GF	3381	48% (40%–56%)	3524	49% (44%–54%)	0.948 (0.690–1.302), P = 0.740
I	1471	21% (19%–23%)	1577	22% (20%–24%)	0.931 (0.835–1.039), P = 0.202
P	1759	25% (19%–31%)	1808	25% (22%–28%)	0.978 (0.695–1.377), P = 0.900
S	151	2% (1%–3%)	137	2% (1%–3%)	1.145 (0.615–2.134), P = 0.669
A	0	0% (0%–0%)	2	0% (0%–0%)	n/a
2 days					
Total (PF + GF)	4551		5478		
PF	1041	23% (15%–34%)	850	16% (11%–23%)	1.623 (1.162–2.266), P = 0.004*
GF	3510	77% (66%–85%)	4628	84% (77%–89%)	0.616 (0.441–0.861), P = 0.004*

	CSF N	Estimated proportion % (95%CI)	PSF N	Estimated proportion % (95%CI)	effect size OR (95%CI), p-value
I	1064	23% (21%–25%)	1170	21% (17%–27%)	1.122 (0.853–1.477), P = 0.410
P	2194	48% (41%–56%)	3087	57% (49%–63%)	0.715 (0.587–0.871), P = 0.001*
S	248	5% (4%–8%)	370	6% (4%–11%)	0.816 (0.564–1.181), P = 0.282
A	4	0% (0%–0%)	1	0% (0%–0%)	4.793 (0.353–65.115), P = 0.239

CSF, control slow-rate freezing; PSF, passive slow-rate freezing; PF, primordial follicle; GF, growing follicle; I, intermediate; P, primary; S, secondary; A, antral; N, number based on the sum of all follicles counted; Estimated proportion and OR odds ratio based on GEE model; CI, confidence interval; *statistical significance $p < 0.05$.

n/a, not applicable: no OR could be calculated due to absence follicles leading to too many cases equal to zero.

3.3. *In vivo* pre-clinical validation experiment

OT-strips were selected at random from 4 of the 10 patients. Twenty-seven female Balb/c nude mice were used for xenotransplantation. During the first xenotransplantation experiment, 3 mice were lost within 1–2 days post-transplantation. These were replaced by another 3 mice. The *in vivo* study was therefore conducted in 24 Balb/c nude mice and used 48 OT-strips, 24 of which were previously frozen with CSF and 24 with PSF.

The total recovery rate was 95.8% (46/48 grafts). The recovery rate 2, 4 and 6 weeks after xenografting was 87.5% (7/8), 100% and 87.5% for PSF and 100% (8/8), 100% and 87.5% in the CSF group. Neovascularization was observed in 89.1% (41/46) of grafts (Fig. 4 Suppl. Material).

Two weeks after xenotransplantation the estimated proportion of PF was significantly lower in the PSF group (14% with 95%CI (8%–22%) vs. 28% with 95%CI(26%–29%)) while the proportion of GF was significantly higher (86% with 95%CI(78%–92%) vs. 72% with 95%CI (71%–74%)), largely due to the higher proportion of primary follicles (45% with 95%CI (42%–48%) vs. 35% with 95%CI (30%–41%)). Two weeks after xenotransplantation, significantly higher odds of PF (OR 2.386; 95%CI (1.237–4.604); P = 0.010) and lower odds of GF (OR 0.419; 95%CI (0.217–0.809); P = 0.010), especially for primary follicles (OR 0.671; 95%CI (0.556–0.808); P < 0.001) were associated with the established CSF technique.

After 4 weeks, the proportion of intermediate follicles was significantly lower in the PSF group (5% with 95%CI of 2%–12%) vs. (27% with 95%CI of 5%–72%), while the proportion of secondary follicles was higher (38% with 95%CI of 31%–46%) vs. (8% with 95%CI (4%–15%)). After 4 weeks of xenotransplantation, significantly higher odds of intermediate follicles (OR 7.001; 95%CI (1.899–25.819); P = 0.003) and significantly lower odds of secondary follicles (OR 0.135; 95%CI (0.071–0.255); P < 0.001) were associated with the established CSF technique. After 6 weeks, the proportion of secondary follicles did not differ significantly between the two methods (Table 4, Fig. 3).

The rate of fibrosis in the non-grafted tissue vs. the grafted tissue in CSF and PSF is shown in Table 5 and Fig. 4. Differences in the distribution of fibrosis (%) were only observed 6 weeks after xenografting, being higher in PSF: 13.6% vs. 11.6% in CSF; P = 0.003.

3.4. Final risk assessment

The final assessment showed a reduction in the combined risk to 6, which is indicative of low risk (Table 2, numbers in bold).

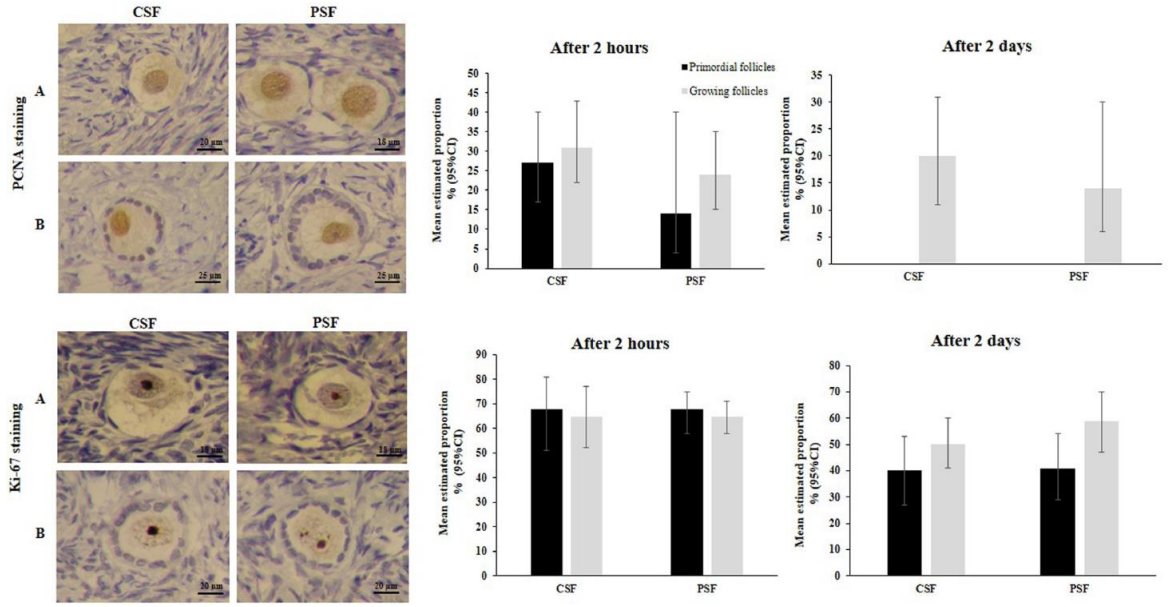

Fig. 1 Estimated proportion of follicular proliferation measured by positive PCNA or Ki-67 staining in primordial and growing follicles in tissue cultured *in vitro* for 2 h and 2 days. (A) Primordial follicle and (B) growing follicle and the mean estimated proportion together with 95%CI is shown

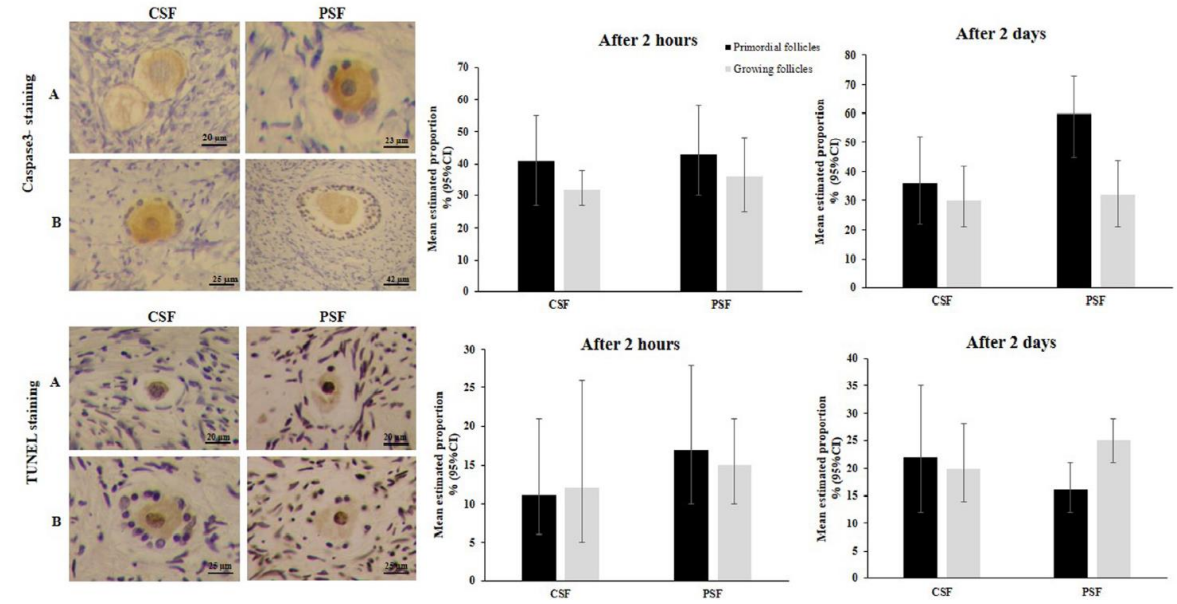

Fig. 2 Estimated proportion of follicular apoptosis measured by positive caspase-3 or Tunel staining in primordial and growing follicles in tissue cultured *in vitro* for 2 h and 2 days. (A) Primordial follicle and (B) growing follicle and the mean estimated proportion together with 95%CI is shown

Table 4. Total number of follicles counted and estimated proportion in the CSF and PSF groups for 0, 2, 4 and 6 weeks after xenografting

	CSF N	Estimated proportion % (95%CI)	PSF N	Estimated proportion % (95%CI)	Effect size OR (95%CI), p-value
0 weeks					
Total (PF + GF)	2393		3188		
PF	1309	55% (51%–59%)	1690	54% (48%–59%)	1.065 (0.862–1.315), P = 0.562
GF	1084	45% (41%–49%)	1498	46% (41%–52%)	0.939 (0.761–1.160), P = 0.562
I	528	22% (20%–24%)	692	22% (21%–23%)	0.995 (0.866–1.143), P = 0.940
P	507	21% (19%–24%)	741	23% (18%–30%)	0.890 (0.644–1.230), P = 0.481
S	48	2% (1%–3%)	64	2% (1%–3%)	0.992 (0.519–1.896), P = 0.980
A	1	0% (0%–0%)	1	0% (0%–0%)	1.316 (0.078–22.214), P = 0.849
2 weeks					
Total (PF + GF)	396		340		
PF	108	28% (26%–29%)	50	14% (8%–22%)	2.386 (1.237–4.604), P = 0.010*
GF	288	72% (71%–74%)	290	86% (78%–92%)	0.419 (0.217–0.809), P = 0.010*
I	130	33% (25%–42%)	122	35% (30%–42%)	0.889 (0.528–1.359), P = 0.588
P	140	35% (30%–41%)	151	45% (42%–48%)	0.671 (0.556–0.808), P < 0.001*
S	18	4% (1%–9%)	17	4% (1%–16%)	0.924 (0.221–3.854), P = 0.913
A	0	n/a	0	n/a	n/a

	CSF N	Estimated proportion % (95%CI)	PSF N	Estimated proportion % (95%CI)	Effect size OR (95%CI), p-value
4 weeks					
Total (PF + GF)	59		173		
PF	0	n/a	0	n/a	n/a
GF	59	n/a	173	n/a	n/a
I	15	27% (5%–72%)	8	5% (2%–12%)	7.001 (1.899–25.819), P = 0.003*
P	40	67% (28%–91%)	100	57% (48%–66%)	1.481 (0.340–6.454), P = 0.601
S	4	8% (4%–15%)	65	38% (31%–46%)	0.135 (0.071–0.255), P < 0.001*
A	0	n/a	0	n/a	n/a
6 weeks					
Total (PF + GF)	40		39		
PF	0	0% (0%–0%)	1	3% (0%–14%)	n/a
GF		40	1% (100%–100%)	38	97% (86%–100%)
I	1	0% (0%–0%)	1	92% (0%–100%)	n/a
P	20	58% (44%–70%)	21	53% (30%–74%)	1.229 (0.824–1.833), P = 0.312
S	19	61% (48%–73%)	15	61% (39%–80%)	1.000 (0.675–1.482), P = 1.000
A	0	0% (0%–0%)	1	3% (1%–8%)	n/a

CSF, control slow-rate freezing; PSF, passive slow-rate freezing; PF, primordial follicle; GF, growing follicle; I, intermediate; P, primary; S, secondary; A, antral; N, number based on the sum of all follicles counted; Estimated proportion and OR odds ratio based on GEE model; CI, confidence interval; *statistical significance $p < 0.05$.

n/a, not applicable: no OR could be calculated due to absence follicles leading to too many cases equal to zero.

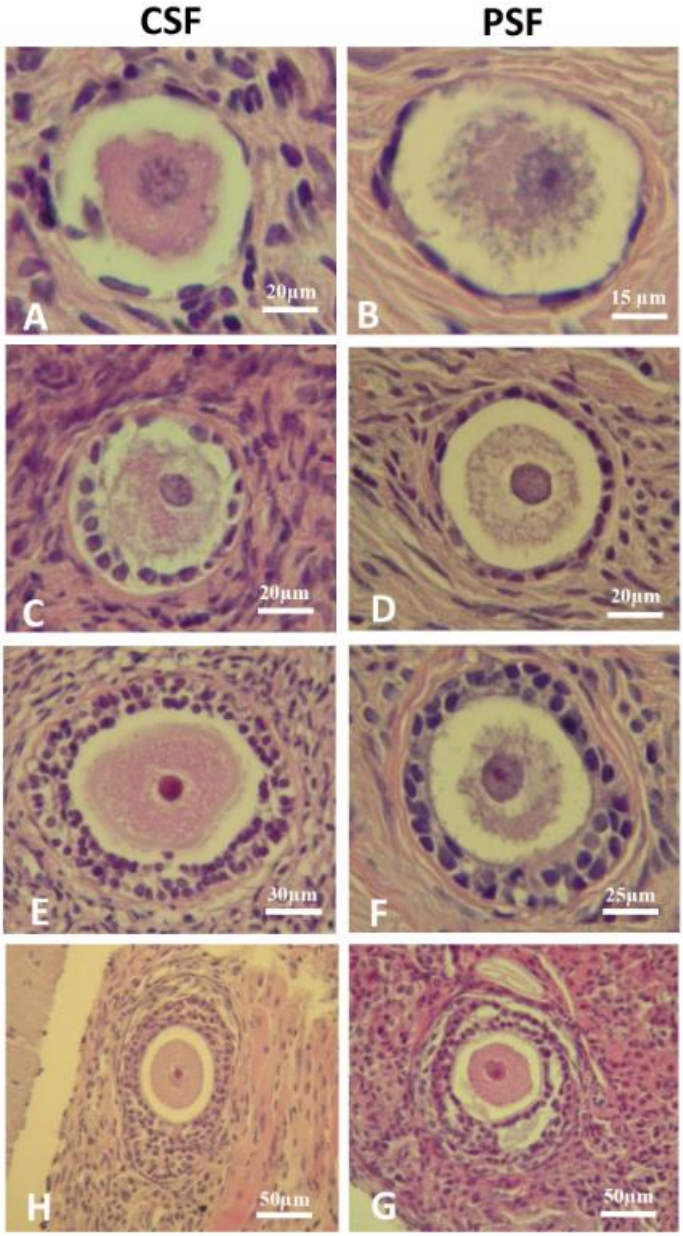

Fig. 3 Follicles stained with haematoxylin-eosin from controlled slow rate freezing (CSF) and passive slow freezing (PSF) tissue after being xenografted into Balb/c nude mice. Primordial follicles (A) and (B) after PSF and CSF. Primary follicles (C) and (D) after PSF and CSF. Secondary and late secondary follicles (E) and (F, H) after PSF and CSF. Follicle with antrum formation (G) from the PSF group after 6 weeks of xenotransplantation

Table 5. Fibrosis distribution (%) in the transplanted tissue 2, 4 and 6 weeks after xenotransplantation in CSF and PSF groups.

Duration time	CSF (%)	PSF (%)	P-value
2 weeks	10.3	10.7	0.941
4 weeks	10.0	9.4	0.371
6 weeks	11.6	13.6	**0.003**

Differences were analyzed by the unpaired t-test.

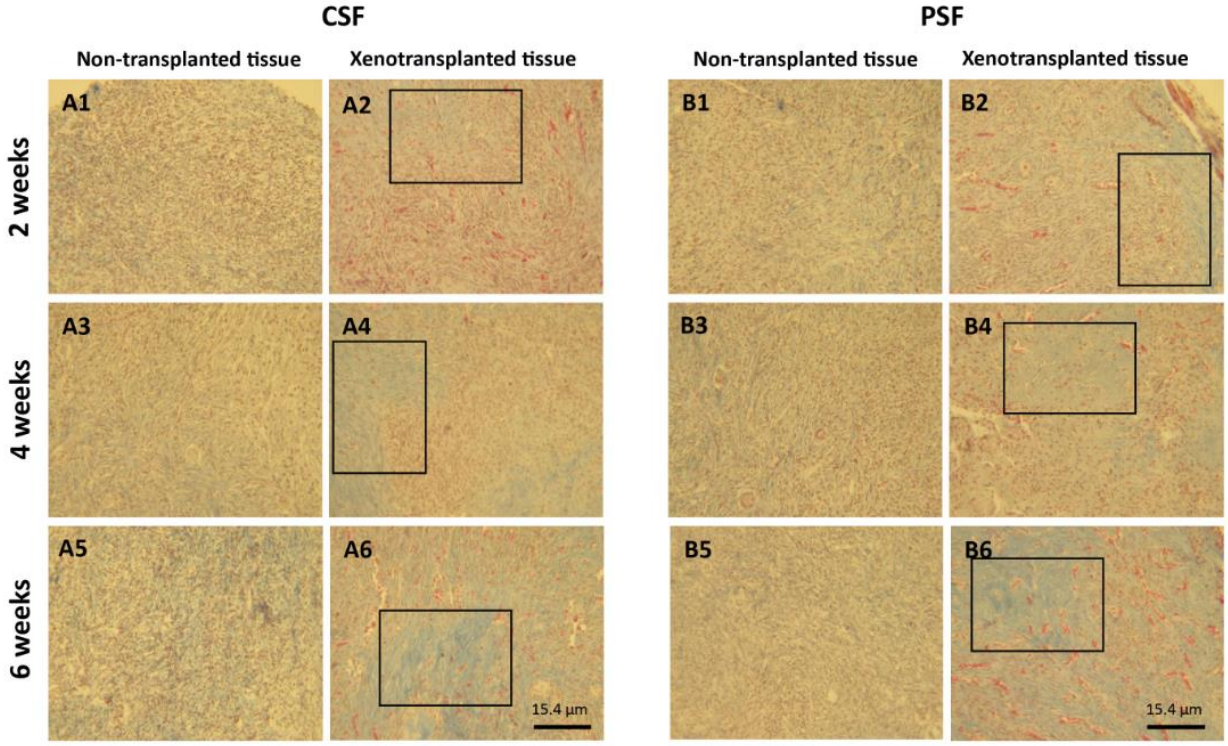

Fig. 4 Evaluation of fibrosis by haematoxylin-eosin and Masson trichrome staining. Ovarian tissue section after CSF and thawing (A1-6) or PSF (B1-6). For the analysis of fibrosis, the transplanted tissues preserved with CSF (A2, A4, A6) were linked to the leftover tissue that was not transplanted (A1, A3, A5) based on time since transplantation. Likewise, the transplanted tissues preserved with PSF (B2, B4, B6) and the leftover linked tissue (B1, B3, B5) during the transplantation time of 2, 4 and 6 weeks. Masson's trichrome stain was used to identify fibrotic areas, which were stained blue given the high number of collagen fibers present among the stromal cells (see squares in pictures). These pictures show that 2 weeks after transplantation (A2 and B2). There was less than 25% of fibrosis in the tissue area. Four weeks after transplantation (A4 and B4) approximately 25% of the area showed fibrosis and 6 weeks after transplantation (A6 and B6) there was more than 25% fibrosis

4. Discussion

On the one hand, our study highlights the value of systematic risk assessment as provided by the EuroGTP-II ART tool for identifying potential risks associated with implementing novel technologies in clinical practice –in this case OT cryopreservation based on PSF- and on the other hand, the viability of PSF as an alternative to traditional CSF for OT cryopreservation as shown by the similar rates of follicle activation in both *in vitro* and *in vivo* experiments. The evidence showed a reduction of risk from moderate to low.

Reproductive medicine is a highly dynamic field where, unfortunately, the rapid pace at which novel technologies and treatment options are developed and implemented in clinical practice contrasts with the delay in obtaining solid clinical data on effectiveness and safety [7; 31]. Harper et al. (2012) [14] described an ideal paradigm of hypothesis-driven research that includes the use of animal models followed by studies on human gametes/embryos donated for research, small-scale clinical studies and finally well-designed randomized clinical trials; the evidence would be further complemented by data from long-term follow-up studies. Unfortunately, time is of essence in this highly-demanding field [2; 24] and, what is more, conducting proper pre-clinical and clinical trials in ART is hampered by several limitations that involve difficult and highly costly solutions [11]. This situation, which fails to meet the standards of evidence-based medicine, is even more conflicted when it is considered that the safety concerns extend to children conceived with these methods and probably to their offspring as well. The initial risk assessment performed by our study prior to conducting the experimental studies responds to the recognized need to validate new technologies and practices in ART so that the potential risk/benefit ratio can be calculated before these innovations are brought into clinical practice [4; 14; 26]. The GTP-II ART provides systematic assessment by means of identifying risks associated to every step in the ART process (from donation to procurement, testing, processing, storage and distribution), the final product and the clinical application procedure. Risk assessment of OT cryopreservation using PSF was undertaken after considering the existing pre-clinical studies with testicular tissue (reviewed by Onofre et al. 2016) [19] and a case report providing a proof-of-principle in which OT from an 18-year old woman was urgently cryopreserved using this technique given the unavailability of a biofreezer at the closest cryopreservation center. After 10 years, the transplanted OT was capable of oocyte maturation and fertilization [21]. The overall moderate risk obtained when the risk was first assessed was consistent with this evidence. However, enhanced validation is still needed and should include pre-clinical studies aimed at assessing the impact of the implemented changes on OT.

The validation studies conducted showed follicle activation both *in vitro* and *in vivo*. The morphological assessment performed after 2 days of culture showed a lower proportion of PF and greater of GF (especially primary follicles).

Immunostaining with Ki-67 and PCNA after 2 days of culture showed no differences between the two techniques. Similarly, no statistical differences were observed in the apoptosis rate using caspase-3 (early) or TUNEL (late). In the animal model, similar results were obtained with regard to follicle activation between PSF and CSF. A clear activation shift was observed after 2 weeks of xenotransplantation. This was especially relevant for the proportion of primary follicles, which was much higher at week 2 for the PSF strips (45% vs. 35% in the CSF group). The difference in maturation in CSF sustained after 4 weeks at the expense of lower intermediate follicles (5% vs. 27%), resulting in a higher amount of secondary follicles (38% vs. 8%), a difference that disappeared after 6 weeks of transplantation. The explanation for this is unknown, and may point to different maturation speeds which reach similar outcomes 6 weeks after xenotransplantation.

These results are of interest given the focus placed on vitrification as an alternative to CSF instead of looking for more accessible and technically simpler cryopreservation techniques suitable for OT. Conversely to slow freezing, vitrification is based on ultrafast cooling rates combined with a high concentration of cryoprotectants. Given that vitrification is a quick and simple procedure with proven experience in embryo and oocyte cryopreservation, several experimental studies have tested this technique for human and animal OT. Generally, vitrification shows similar results as with CSF [1; 23; 32]. The benefit of reduced crystallization with the fast cooling rate may be counterbalanced by the need for a higher concentration of cryoprotectants which could lead to more damaged follicles and higher expression of apoptotic biomarkers [5]. Although a common strategy is to use a mixture of two or more cryoprotectants to lessen toxicity associated with high concentrations of each one [33], this technique still needs to be further optimized [22]. Vitrification is also attractive because it does not require special and expensive equipment. However, no standard protocols have been established for the use of this approach with OT, resulting in limited use and experience [22]. Only two live births have been reported using vitrification of human OT [27]. PSF is also a quick and simple technique for cryopreservation that, contrary to vitrification, does not have the above-mentioned risks associated with ultra-rapid cooling or the use of high concentrations of cryoprotectants. Smaller sized samples can be vitrified to optimize the penetration of the cryoprotectants and to optimize the temperature transfer thus lowering the probability of ice formation [22]. This size limitation, which is applicable to vitrification, does not exist in PSF.

This new evidence leads to a decrease in the level of risk to "low" upon reassessment of the risk in implementing this new freezing technique. The quality of evidence to minimize the risk was the reason for applying a 25% reduction in risk. It must be noted that the source of OT used in our study were the ovaries of transgender men before female-to-male reassignment surgery since prolonged testosterone treatment has been shown to present increased cortical stiffness in the most outer part of the ovarian cortex compared to oncological patients [6]. Whether the stiffness has an effect on resistance to cryoprotectant toxicity is unknown, and therefore extrapolating these findings to other patient populations must be performed with caution. On the other hand, the case report discussed (or noted) above [21] supports the viability of PSF for OT cryopreservation in cisgender women, including oncological patients. The results of our study are sometimes conflicting, especially when comparing different tests for the same analysis (i.e. follicle proliferation or apoptosis). Rather than trying to find differences between CSF and PSF, our results point to similar outcomes being achieved with both freezing methods. It is likely that prolonged follow-up would have shown more similarities than the ones observed although it cannot be ruled out that some differences may also appear. Despite these encouraging results, these are pre-clinical and it should be taken into account that data from animal models may not always be applicable to humans although reproductive processes seem to be highly conserved across mammals [14; 25].

5. Conclusion

The application of PSF to OT cryopreservation has shown that follicle proliferation *in vitro* and follicle growth in mouse models is similar to that achieved with CSF. The proliferation markers, the apoptosis rate and fibrosis were acceptable in both techniques. Extended follow up is likely to show greater similarity between both methods. This pre-clinical evidence resulted in a reduction of the level of risk level for this technique from moderate to low, as assessed by the EuroGTP-II ART and shows that PSF can be an interesting alternative type of slow freezing for OT.

Authors' roles

K.T., S.L. and A.B. designed the study, performed all experiments, acquired data, interpreted data and wrote the manuscript. S.A. and P.E.J.B. assisted in the transplantation *in vivo* experiment. E.T. gave guidance on the risk assessment exercise. All authors contributed to the interpretation of the results, revised and approved the manuscript.

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Conflicts of interest

The authors have no conflicts of interest to declare.

References

1 C.A. Amorim, M. Curaba, A. Van Langendonckt, M.M. Dolmans, and J. Donnez, *Vitrification as an alternative means of cryopreserving ovarian tissue*. Reprod Biomed Online 23 (2011) 160-186.

2 D. Bijlenga, E. Birnie, B.W. Mol, and G.J. Bonsel, *Obstetrical outcome valuations by patients, professionals, and laypersons: differences within and between groups using three valuation methods.* BMC Pregnancy Childbirth 11 (2011) 93.

3 R. Bologna-Molina, A. Mosqueda-Taylor, N. Molina-Frechero, A.D. Mori-Estevez, and G. Sanchez-Acuna, *Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors.* Med Oral Patol Oral Cir Bucal 18 (2013) e174- e179.

4 D.R. Brison, S.A. Roberts, and S.J. Kimber, *How should we assess the safety of IVF technologies?* Reprod Biomed Online 27 (2013) 710-721.

5 A. Dalman, N.S. Deheshkar Gooneh Farahani, M. Totonchi, R. Pirjani, B. Ebrahimi, and M. Rezazadeh Valojerdi, *Slow freezing versus vitrification technique for human ovarian tissue cryopreservation: An evaluation of histological changes, WNT signaling pathway and apoptotic genes expression.* Cryobiology 79 (2017) 29-36.

6 C. De Roo, K. Tilleman, C. Vercruysse, H. Declercq, G. T'Sjoen, S. Weyers, and P. De Sutter, *Texture profile analysis reveals a stiffer ovarian cortex after testosterone therapy: a pilot study.* J Assist Reprod Genet 36 (2019) 1837-1843.

7 W. Dondorp, and G. de Wert, *Innovative reproductive technologies: 546 risks and responsibilities.* Hum Reprod 26 (2011) 1604-1608.

8 J. Donnez, and M.M. Dolmans, *Fertility Preservation in Women*. N Engl J Med 377 (2017) 1657-1665.

9 M. Ethics Committee of American Society for Reproductive, *Moving innovation to practice: a committee opinion.* Fertil Steril 104 (2015) 39-42.

10 *EuroGTP-II, Good Practices for evaluating quality, safety and efficacy of novel tissue and cellular therapies and products*, EuroGTP II Good Tissue and Cell Practices, 2019. Available from: http://www.goodtissuepractices.site/docs/EuroGTP_II_Guide.pdf. [Last accessed 25th September 2020].

11 J.L. Evers, *The wobbly evidence base of reproductive medicine*. Reprod Biomed Online 27 (2013) 742-746.

12 B. Fisch, and R. Abir, *Female fertility preservation: past, present and future.* Reproduction 156 (2018) F11-F27.

13 A. Gougeon, *Dynamics of follicular growth in the human: a model from preliminary results*. Hum Reprod 1 (1986) 81-87.

14 J. Harper, M.C. Magli, K. Lundin, C.L. Barratt, and D. Brison, *When and how should new technology be introduced into the IVF laboratory?* Hum Reprod 27 (2012) 303-313.

15 S.Y. Kim, S.K. Kim, J.R. Lee, and T.K. Woodruff, *Toward precision 565 medicine for preserving fertility in cancer patients: existing and emerging fertility preservation options for women.* J. Gynecol. Oncol. 27 (2016) e22.

16 A. Langbeen, C.V. Ginneken, E. Fransen, E. Bosmans, J. Leroy, and P.E.J. Bols, *Morphometrical analysis of preantral follicular survival of VEGF-treated bovine ovarian cortex tissue following xenotransplantation in an immune deficient mouse model.* Anim Reprod Sci 168 (2016) 73-85.

17 F. Martinez, *Update on fertility preservation from the Barcelona International Society for Fertility Preservation-ESHRE-ASRM 2015 expert meeting: indications, results and future perspectives.* Hum Reprod 32 (2017) 1802-1811.

18 E.A. McGee, E. Perlas, P.S. LaPolt, A. Tsafriri, and A.J. Hsueh, *Follicle-stimulating hormone enhances the development of preantral follicles in juvenile rats.* Biol Reprod 57 (1997) 990-998.

19 J. Onofre, Y. Baert, K. Faes, and E. Goossens, *Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation.* Hum Reprod Update 22 (2016) 744-761.

20 F. Pacheco, and K. Oktay, *Current Success and Efficiency of Autologous Ovarian Transplantation: A Meta-Analysis.* Reprod Sci 24 (2017) 1111-1120.

21 A. Povoa, P. Xavier, L. Calejo, S. Soares, M. Sousa, J. Silva, S. Sousa, 583 A. Barros, and J.Beires, *First transplantation of cryopreserved ovarian tissue in Portugal, stored for 10 years: an unexpected indication.* Reprod Biomed Online 32 (2016) 334-336.

22 E.C. Rivas Leonel, C.M. Lucci, and C.A. Amorim, *Cryopreservation of Human Ovarian Tissue: A Review*. Transfus Med Hemother 46 (2019) 173-181.

23 S. Sanfilippo, M. Canis, J. Smitz, B. Sion, C. Darcha, L. Janny, and F. Brugnon, *Vitrification of human ovarian tissue: a practical and relevant alternative to slow freezing.* Reprod Biol Endocrinol 13 (2015) 67.

24 G.S. Scotland, P. McNamee, V.L. Peddie, and S. Bhattacharya, *Safety versus success in elective single embryo transfer: women's preferences for outcomes of in vitro fertilisation*. BJOG 114 (2007) 977-983.

25 R.M. Sharpe, *Of mice and men: long-term safety of assisted reproduction treatments*. Hum Reprod 33 (2018) 793-796.

26 V. Steirteghem, *What next for assisted reproductive technology? A plea for an evidence based approach.* Hum Reprod 23 (2008) 2615-2616.

27 N. Suzuki, N. Yoshioka, S. Takae, Y. Sugishita, M. Tamura, S. Hashimoto, Y. Morimoto, and K. Kawamura, *Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency.* Hum Reprod 30 (2015) 608-615.

28 E.E. Telfer, M. McLaughlin, C. Ding, and K.J. Thong, *A two-step 602 serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin*. Hum Reprod 23 (2008) 1151-1158.

29 E. Trias, R. Lomas, J. Tabera, A.R. Piteira, K. Tilleman, R.P. Casaroli-Marano, A. Chandrasekar, and G.T.P.I.I.S.G*. Euro, EuroGTP II: a tool to assess risk, safety and efficacy of substances of human origin.* Int J Qual Health Care 32 (2020) 80-84.

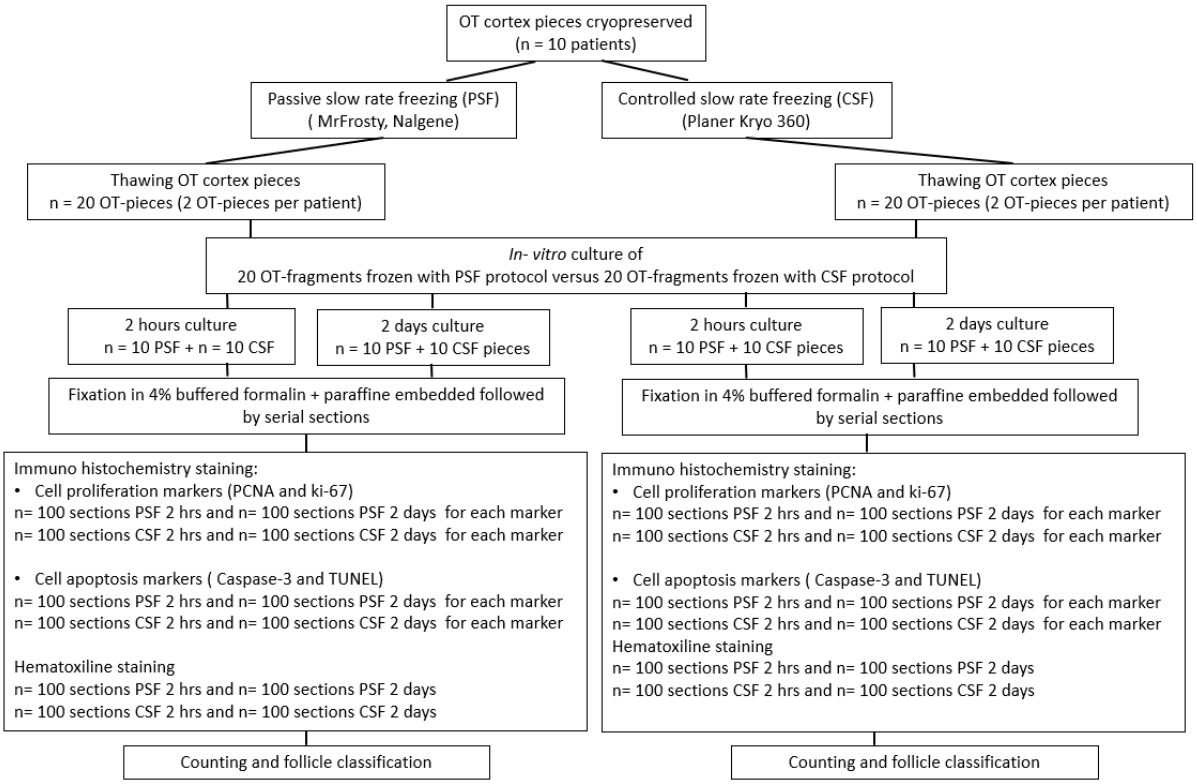
30 E. Trias, M. Nijs, I.A. Rugescu, F. Lombardo, G. Nikolov, V. Provoost, A. Tolpe, N. Vermeulen, Z. Veleva, R. Piteira, R.P. Casaroli-Marano, and K. Tilleman, *Evaluating risk, safety and efficacy of novel reproductive techniques and therapies through the EuroGTP II risk assessment tool.* Hum Reprod 32 (2020) 1821-1838.

31 R. Vassena, , the EBART Group. Evidence-based medicine in ART. Hum Reprod 32 (2017) 256.

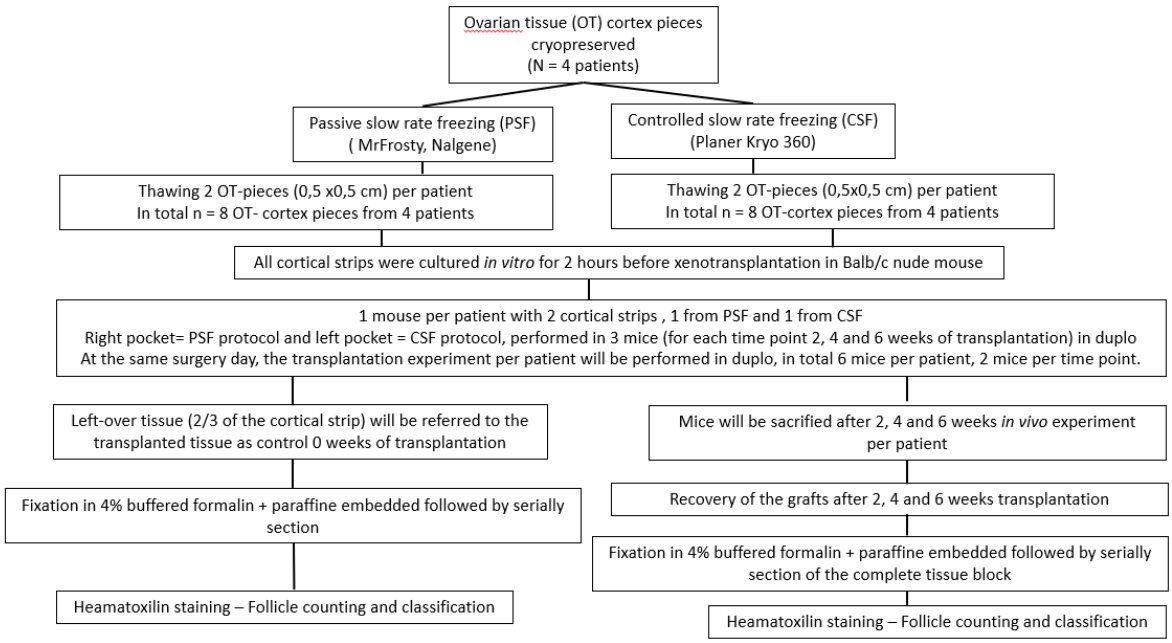
32 Z. Yan, Q. Li, L. Zhang, B. Kang, W. Fan, T. Deng, J. Zhu, and Y. Wang, *The growth and development conditions in mouse offspring derived from ovarian tissue cryopreservation and orthotopic transplantation.* J Assist Reprod Genet 37 (2020) 923- 932.

33 K.W. Yong, L. Laouar, J.A.W. Elliott, and N.M. Jomha, *Review of non-permeating cryoprotectants as supplements for vitrification of mammalian tissues.* Cryobiology (2020)

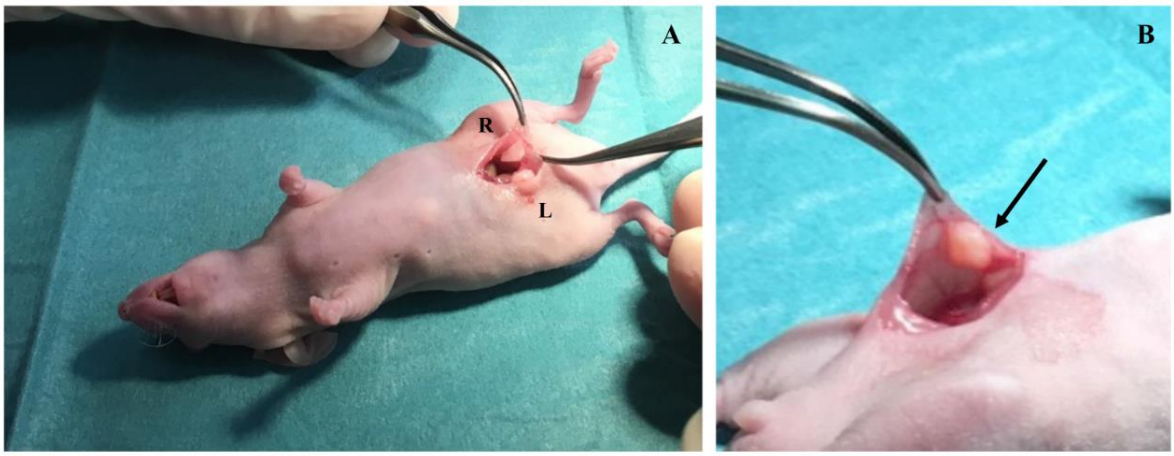
Supplementary materials



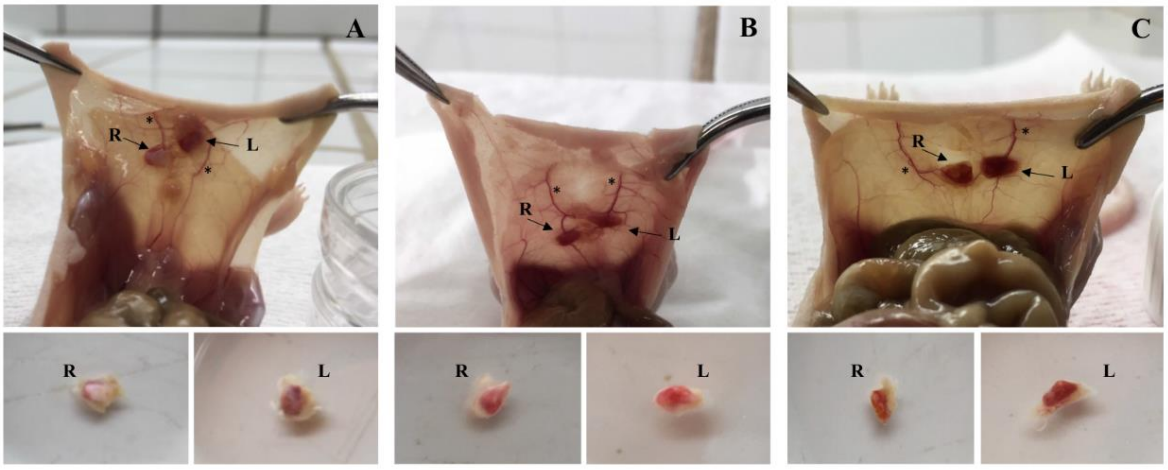
Supplementary Fig. 1. Design of the *in vitro* culture experiment



Supplementary Fig. 2. Design of the *in vivo*, xenotransplantation experiment



Supplementary Fig. 3. Xenotransplantation of human ovarian tissue in Balb/c nude mice. (A) A pouch was created on either side of the abdominal incision at the linea alba. An ovarian tissue fragment that had been subjected to PSF was transplanted in the pouch on the right-hand side (R) of the mid-line incision. On the opposite side, the left-hand side (L), a similar pouch was filled with a fragment that had been subjected to CSF from the same patient. (B) Ovarian tissue fixed in the pouch between the abdominal internal oblique muscle and the peritoneum on both sides of the abdominal incision at the linea alba (see arrow)



Supplementary Fig. 4. Recovery of grafted tissue after transplantation. (A) Recovery of ovarian tissue 2 weeks after transplantation in the Balb/c nude mice. On the right-hand side (R) of the mid-line incision, an ovarian tissue fragment that had been subjected to PSF and on the opposite side, left-hand side (L), a fragment that had been subjected to CSF from the same patient, both sides showed indication of neovascularization (*). (B) Recovery 4 weeks after transplantation and (C) recovery 6 weeks after transplantation

Supplementary Table 1. Total number of follicles counted and estimated proportion of positive stained follicles for proliferation and apoptosis in CSF and PSF

	CSF		**PSF**		**Effect size**
	N	Estimated proportion % (95%CI)	N	Estimated proportion % (95%CI)	OR (95%CI), p-value
2 hours					
PCNA (+)					
Total counted follicles	1789		1387		
PF (PCNA +)	202	27% (17%-40%)	57	14% (4%-40%)	2.257 (0.851 – 5.981), P=0.102
GF (PCNA +)	305	31% (22%-43%)	179	24% (15%-35%)	1.484 (0.910 – 2.419), P=0.114
Ki67 (+)					
Total counted follicles	1233		1697		
PF (Ki67 +)	272	68% (51%-81%)	417	68% (58%-75%)	1.021 (0.546 – 1.907), P=0.949
GF (Ki67 +)	533	65% (52%-77%)	685	65% (58%-71%)	1.031 (0.652 – 1.630), P=0.896
Caspase-3 (+)					
Total counted follicles	1235		1294		
PF (Caspase-3 +)	263	41% (27%-55%)	278	43% (30%-58%)	0.889 (0.433 – 1.825), P=0.749
GF (Caspase-3 +)	188	32% (27%-38%)	238	36% (25%-48%)	0.850 (0.465 – 1.555), P=0.598
TUNEL (+)					
Total counted follicles	969		824		

PF (TUNEL +)	59	11% (6%-21%)	85	17% (10%-28%)	0.635 (0.311 – 1.298), P=0.213
GF (TUNEL +)	56	12% (5%-26%)	58	15% (10%-21%)	0.755 (0.332 – 1.720), P=0.504
2 days					
PCNA (+)					
Total counted follicles	909		931		
PF (PCNA +)	43	n/a	15	n/a	n/a
GF (PCNA +)	138	20% (11%-33%)	111	14% (6%-30%)	1.482 (0.378 – 5.819), P=0.573
Ki67 (+)					
Total counted follicles	780		995		
PF (Ki67 +)	61	40% (27%-53%)	33	41% (29%-54%)	0.952 (0.402 – 2.254), P=0.910
GF (Ki67 +)	331	50% (41%-60%)	0	59% (47%-70%)	0.701 (0.330 – 1.487), P=0.355
Caspase-3 (+)					
Total counted follicles	384		815		
PF (Caspase-3 +)	68	36% (22%-52%)	114	60% (45%-73%)	0.378 (0.122 – 1.173), P=0.092
GF (Caspase-3 +)	163	30% (21%-42%)	273	32% (21%-44%)	0.933 (0.355 – 2.447), P=0.887
TUNEL (+)					
Total counted follicles	873		1038		
PF (TUNEL +)	51	22% (12%-35%)	36	16% (12%-21%)	1.442 (0.593 – 3.507), P=0.419

GF (TUNEL +)	128	20% (14%-28%)	204	25% (21%-29%)	0.765 (0.492 – 1.191), P=0.236

CSF, control slow-rate freezing; PSF, passive slow-rate freezing; PF, primordial follicle; GF, growing follicle; N, number based on the sum of all follicles counted; Estimated proportion and OR odds ratio based on GEE model; CI, confidence interval
n/a, not applicable: no OR could be calculated due to absence of positive stained follicles leading to too many cases equal to zero.

CHAPTER 5

Preservation of connexin 43 and transzonal projections in isolated bovine pre-antral follicles before and following vitrification

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Summary

Purpose Gap junctions and transzonal projections play a crucial role in intercellular communication between different follicular components and are necessary for follicle development. We aimed to demonstrate gap junction protein connexin 43 (Cx43) and transzonal projections (TZPs) in viable, category 1, isolated bovine pre-antral follicles (PAFs) during short term culture and after vitrification and warming.

Methods This study involved four experimental groups: *fresh control*, *2 day culture*, *4 day culture* and *vitrified* secondary PAFs. Isolated PAFs were vitrified using a simple and efficient cryopreservation method by means of mini cell strainers.

Results Cx43 and TZPs were detected in pre-antral follicles of all stages, as well as in every experimental group. The group fresh follicles, showed a higher percentage of follicles that were positive for Cx43 (91.7%) than the follicles that were vitrified (77.4%). All follicles that were cultured for 2 days were Cx43 positive (100%). Follicles cultured for 4 days (65.8%) (P=0.002) showed the lowest percentage of follicles that were Cx43 positive. The percentages of the presence or (partial) absence of the TZP network were shown to be very heterogenous between follicles in different treatment groups.

Conclusions These results suggest the maintenance of communication between the oocyte and the somatic companion cells after vitrification and warming. The varying percentages of the expression of the TZP network within groups suggests that it will be of interest to investigate whether this is truly due to variability in TZP integrity and follicle quality or due to methodological limitations.

1. Introduction

It has been estimated that the number of cancer survivors diagnosed during childhood and adolescence in Europe is between 300 and 500 000, with approximately one in every 640 young adults being a survivor of childhood cancer [1]. Although surviving remains the main objective, quality of life in the increasing population of young female survivors has become an equally important consideration. To varying degrees, these former patients experience a wide range of long-term adverse health effects [2]. Chemo- and radiotherapy often damage ovarian tissue [3, 4] which might render patients infertile. A Childhood Cancer Survivor Study showed that, compared to their siblings, the relative risk for survivors of ever being pregnant following radiotherapy with 5-10 Gy is 0.56, with a decrease to 0.18 when more than 10 Gy is used to a radiation field including the ovaries [5]. As a consequence, the interest in fertility preservation (FP) strategies in women has been sparked to the extent that it has now become a key medical sub- discipline [6]. Prior to cancer therapy, current fertility-preserving options for women include oocyte and embryo cryopreservation. However, for prepubertal girls and women that cannot delay the start of chemotherapy, ovarian tissue cryopreservation (OTC) is offered as an experimental option [7] (while the experimental status of OTC is still under debate [8-11]). While more than 130 births have already been reported worldwide [12, 13], this technique is not advisable for patients with certain types of cancer with medium to high risk of ovarian metastasis, such as leukaemia, as there is a considerable risk of re-introducing malignant cells contained in the cryopreserved tissue following auto-transplantation [14, 15]. For these patients, a safer alternative to allow fertility restoration could be the isolation, cryopreservation and reintroduction of pre-antral follicles or PAFs in remaining ovarian tissue *'in situ'* following chemo- or radiotherapy. In addition, it is expected that effective means of avoiding reseeding of malignant cells with ovarian grafts, such as an artificial ovary and an *in vitro* culture system for primordial follicles will become available in the near future [16-21]. Thus far, individual follicle cryopreservation techniques are labor-intensive, time-consuming and a substantial proportion of isolated follicles is lost during handling and after warming [22]. A vitrification protocol, successfully used for non-embedded isolated PAFs, resulted in higher efficiency, but lower viability when PAFs were vitrified encapsulated in alginate beads [23]. A recent meta-analysis comparing vitrification and slow freezing suggests that vitrification is the best strategy for oocyte cryopreservation in terms of clinical outcomes [24]. However, cell vitrification bares the risk of cell damage, while cell-cell connections are crucial for subsequent follicle survival and developmental capacity [25-27]. Therefore, the possible effects of follicle vitrification on cell-cell contacts need to be thoroughly investigated. Firstly, cell-cell interactions need to be morphologically visualized before and after vitrification [28]. The presence of transzonal projections (TZPs) and gap junctions are predicted to be good biomarkers of follicle health, because they are necessary for

bidirectional communication between the oocyte and granulosa cells. TZPs extend through the glycoprotein-rich zona pellucida and connect via gap junctions on the oocyte membrane [29-32]. Starting in the primordial follicle stage, TZPs form initial, simple cell-cell gap junctions that are continuously remodeled and elongated during the formation of the zona pellucida and de course of oocyte growth [29]. In humans [33] and bovines [34, 35], the zona pellucida starts to develop from the secondary follicle stage onwards. TZPs are essential for the bidirectional transport of molecules such as sugars, amino acids and nucleotides [36-38], which are essential for oocyte growth. Gap junctions are intercellular membrane channels directly connecting the cytoplasm of adjacent cells, thus allowing the exchange of ions, second messengers and small metabolites [39]. They play a crucial role in intercellular communication between different follicular components [40]. A single gap junction channel is composed of two hemi-channels (connexons), each of which is composed of six protein subunits called connexins. Among gap junction proteins identified in ovarian follicular cells, two connexins (Cx, Cx37 and Cx43) seem to be critical at each step of normal folliculogenesis [41]. Investigating Cx expression in the bovine ovary, Nuttinck et al. (2000) [42] found that Cx43 expression was restricted to granulosa cells, while Cx37 staining was observed in both the oocyte and granulosa cell compartments. Vitrification of follicles can lead to cryoinjuries, resulting in the loss of membrane proteins, such as Cx37 and Cx43 [43].

TZPs are composed of F-actin and microtubules [44, 38, 32], which are very sensitive to changes in temperature and sheer stress and as a consequence, vulnerable during cryopreservation [45]. To date, the literature on the visualization of cell-cell connections such as Cxs and TZPs in isolated PAFs is very scarce. In addition, to the best of our knowledge the influence of vitrification on the preservation of cell-cell connections in isolated PAFs has never been investigated. Recently, there has been an increasing interest for bovine *in vitro* models in human studies on assisted reproductive techniques (ARTs) and FP, as reviewed by Langbeen et al. (2015) [46]. It is well established that a number of similarities exist between bovine and human species with regard to ovarian morphology and function, and that the bovine species could serve as an effective model for human follicular dynamics [47, 48]. Moreover, the easy access to slaughterhouse ovaries guarantees unrestricted availability of study specimens such as PAFs [46]. Because of the importance of the presence of intact cell-cell contacts for further development of PAFs after warming and the possible advantages of vitrification of PAFs, the current study aimed to 1) visualize Cx43 and TZPs using immunohistochemical methods in follicles upon isolation and in follicles that were cultured *in vitro* for two and four days, respectively; and 2) assess the presence of Cx43 and TZPs in isolated PAFs following vitrification and warming, as a prerequisite for future follicle developmental capacity.

2. Materials and methods

2.1. Collection of ovaries, ovarian follicle isolation and culture

As described earlier [49], adult bovine ovaries were collected upon slaughter and transported in warm physiologic saline (0.9% NaCl, Braun) to the laboratory within three hours at 25°C. The procedure to isolate the follicles from the ovaries was carried out 36 times. For each isolation procedure, follicles were derived from 10 ovaries that originated from 10 different cows. Follicle isolation was carried out on all 10 ovaries together. Two isolation procedures were performed subsequently on different ovaries, from the same slaughterhouse batch. No separate follicle isolations were performed for each separate ovary. As a consequence, there is no specific information on which follicle originated from which ovary. Following removal of the adnexa, the ovaries were washed in a warm (38.5 °C) physiological solution supplemented with kanamycin (0.25%) and rinsed in alcohol (70%). Active ovaries were selected, free of abundant antral follicles, corpora lutea or scar tissue. The ovarian cortex was cut into pieces of approximately 1 mm^3, using a scalpel. The pieces of ovarian cortex were transferred to isolation medium: M199, supplemented with HEPES (0.04M), gentamycin (50 µL/ml), bovine serum albumin (10 mg/ml), polyvinylpyrrolidone (4 mg/ml) and filtered through a 0.2 µm filter. Ovarian cortex tissue was mixed and dispersed using an Ultra Turrax T18 Basic device (IKA®, VWR, Leuven, Belgium) with a medium-sized plastic dispersing tool (IKA®, S18D-14G-KS) for two min and with a smaller size (IKA®, S18D-10G-KS) for one min. The resulting follicle suspension was subsequently filtered through a 100 µm, a 70 µm filter (BD Falcon®, Corning, NY, USA) and a 20 µm nylon filter (Millipore®, Cork, Ireland). Early PAFs were recovered from the 20 µm mesh filter by rinsing with isolation medium. Follicles were visualized using standard inverted light microscopy (Olympus, Aartselaar, Belgium). Primary PAFs with an oocyte surrounded by one layer of cuboidal granulosa cells and intact basal membrane were selected on day 0 [50]. They were individually transferred and cultured in 70 µl culture medium in 96-well plates (Greiner Bio-One, Germany) at 38.5 °C and 5% CO_2. The culture medium consisted of equal parts DMEM and Ham's F12 nutrient supplemented with penicillin G (240 U/ml) and streptomycin (240 µg/ml), fungizone (5 µg/ml), fetal calf serum (2.3 (v/v)%) and newborn calf serum (2.3 (v/v)%), bovine serum albumin (0.75 (w/v)%), insulin (0.01 mg/ml), transferrin (0.55 µg/ml) and selenium (6.7 ng/ml). Two plates were used per isolation. Per plate, 40 wells were used for culture. The two plates were filled alternately per row to shorten follicle light exposure time and preserve the equilibration of the culture medium. Follicles were cultured for 2 days to recover from the isolation procedure and grow to secondary stage follicles. To evaluate follicle morphology from day 2 onwards, follicles were microscopically evaluated and assigned to three categories based on the morphological assessment of the connection between the oocyte and the surrounding granulosa cells and the integrity of the basal membrane as reported by Jorssen et al. [49].

From day 2 onwards, only secondary category 1 follicles were selected for further use, having the right size range (40-80 μm). Category 2 and 3 follicles were not further used.

2.2. Study design

The experimental design is described and summarized in a flow chart (Fig. 1). Follicles were fixed in 4 different groups: immediately upon isolation (‘*fresh control*’), following 2 days of culture (‘*2 day culture’*), following vitrification, warming and a 24h recovery period on day 3 (‘*vitrified’*) and following 4 days of culture (‘*4 day culture’*). On day 2 and 4 and day 3 for vitrified follicles, follicles were assigned to three categories based on follicle morphology only when the basement membrane or connection between the oocyte and granulosa cells could be completely assessed. In addition, the follicle diameter was determined on day 2 and 4 and day 3 for vitrified follicles. Two perpendicular measures were recorded for each follicle, and the average of the two values was reported as follicular diameter (μm). When the diameter of a follicle could not be measured (when a follicle was located to the side of a well and the basal membrane could not be fully defined), it was reevaluated on the next evaluation day. The fresh follicles fixed on day 0 were primary PAFs with an oocyte surrounded by one layer of cuboidal granulosa cells and intact basal membrane, they were also analyzed for diameter immediately upon isolation. To minimize exposure to light and low temperatures and enhance follicle survival, all other follicles were cultured as soon as possible to equilibrate in the incubator. Follicles were also evaluated for follicle viability by Neutral Red staining before fixation [51]. Cx43 and TZP fluorescent staining were carried out to analyze the presence of cell-cell connections. Hoechst staining was carried out to visualize cell nuclei. For practical reasons, time constraints and to minimize exposure to light and low temperatures, follicles were not followed up individually but as a group.

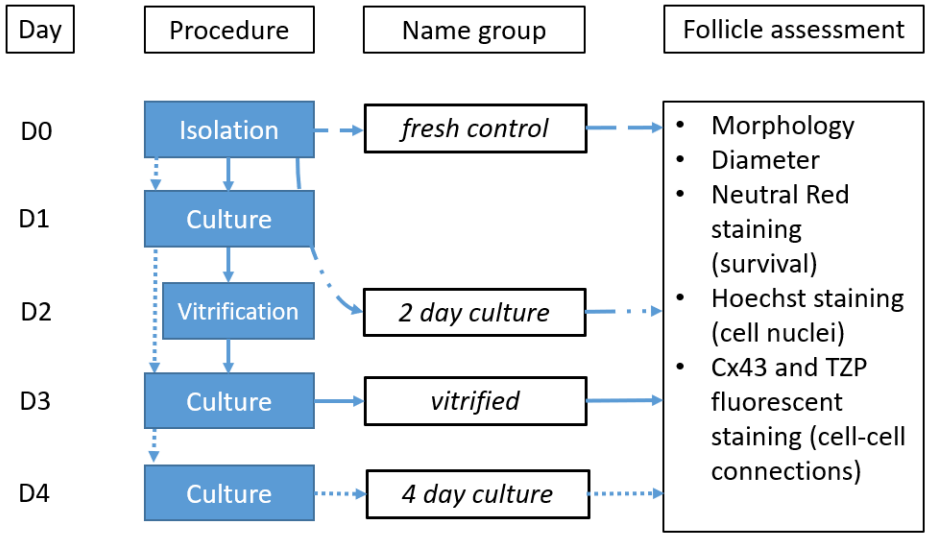

Fig. 1 Experimental design. Follicles were fixed immediately following isolation (‘*fresh control*’), following 2 days of culture (‘*2 day culture*’), following vitrification, warming and a 24h recovery period on day 3 (‘*vitrified*’) or following 4 days of culture (‘*4 day culture*’). Before fixation, follicles were assessed for morphology, diameter and viability. After fixation, the follicles were stained to visualize Cx43 and TZPs and cell nuclei with Hoechst staining

2.3. Freezing and warming

The IrvineScientific® Vitrification Kit for human oocytes and embryos (Alere Health BV. Tilburg, NL) was used for vitrification and warming following slight adaptations to the manufacturer’s guidelines. On day 2, category 1 follicles were transferred to a mini cell strainer (25 µm, nylon, Funakoshi, Japan), immersed in culture medium (Fig. 2). Subsequently, the mini cell strainer with PAFs was first transferred to equilibration solution 1 and 2 (ES1 and ES2; 7.5% DMSO, 7.5% EG) for 1 min each, then to vitrification solution (VS; 15% DMSO, 15% EG, 0.5 M sucrose) for 30 seconds and then directly plunged into liquid nitrogen (LN_2). To eliminate the remaining solution from the mini cell strainer, it was briefly placed on an absorbent paper towel after each step. The vitrification procedure was carried out at room temperature (RT). Follicles were warmed the same day. After plunging the mini cell strainer immediately in a large volume (10ml) of warming solution (38.5 °C; 1 M sucrose) for 1 min, mini cell strainers were transferred to dilution solution (0.5 M sucrose) for 4 min at RT. Next, PAFs were washed in the washing medium twice during 4 min. PAFs stayed in the mini cell strainer and were immersed in fresh culture medium in a 35mm petri dish (38.5 °C, 5% CO_2). Follicles were finally evaluated following a 24h recovery period.

Fig. 2 PAFs are placed in a mini cell strainer immersed in cryoprotective agents (ES and VS) in a 4-well plate. Mini cell strainers are transferred with tweezers between different media and equally serve as cryo-container when they are plunged in LN_2

2.4. Follicle quality and viability assessment

2.4.1. Neutral Red (NR) staining

To assess follicle survival and immediate viability, follicles were stained with the non-toxic viability indicator Neutral Red [51]. 100 µL Neutral Red solution (3.3 g/L, Sigma) was added to the culture medium. A follicle was considered to be positively stained when both the oocyte and at least ¾ of the granulosa cells colored red. When the dye is incorporated in the lysosomes of the follicles, they are considered metabolically active and thus viable. Following 20 min of incubation (38.5 °C, 5% CO_2), follicle staining was evaluated [51].

2.4.2. Cx43 and TZP staining

The protocol for immunostaining of connexins was adapted from Ortiz-Escribano et al. [52]. Follicles were fixed in 4% paraformaldehyde (PFA) for 20 min at RT. They were washed in PBS + 1 mg/ml PVP and permeabilized with 1% Triton X-100 (T8787, Sigma) and 0.05% Tween 20 (P1379, Sigma) in PBS for 20 min at RT. Subsequently, follicles were blocked with a solution consisting of 10% normal goat serum and 0.05% Tween 20 prepared in PBS, at 4 °C for 1 h. Follicles were then incubated with rabbit anti-Cx43 polyclonal antibody (1:500, C6219, Sigma) diluted in blocking solution at 4 °C overnight. Subsequently, follicles were washed and incubated with goat anti-rabbit secondary antibody conjugated with FITC (1:250, A16105, Invitrogen) for 2h at RT. Next, to visualize filamentous actin (F-actin) and consequently TZPs because they are composed of F-actin, follicles were washed and incubated with Alexa Fluor™ 568 Phalloidin, (1:40, A12380, Thermo Fisher Scientific) for 1 h at RT. Follicles were counterstained with Hoechst 10 µg/ml for 10 min, washed and mounted with Antifade mounting medium (Life Technologies) on microscope slides. Images were obtained using a Leica SP8 confocal microscope equipped with a 405 nm Diode laser (to detect the Hoechst nuclear stain; blue) and a white laser source (Leica WLL) used at 488 nm (connexins; green) and at 577 nm (to visualize TZP staining; red). For Cx43 and TZP analysis, a 60× water immersion (N.A. 1.20) objective was used and image acquisition settings were kept constant for the recordings of all follicles. Z-stacks were taken

with a 0.36 μM interval through the complete follicle. The expression of Cx43 was classified as positive (present) or negative (absent) (Fig. 3). The follicles that showed connexin expression in at least ¾ of GCs through the complete follicle were evaluated as Cx43 positive. The organization of the TZPs was classified as 0: total absence (no physical connections between the oocyte and granulosa cells [no TZPs visible]); 1: partial absence (gaps between the oocyte and granulosa cells [no dense staining of F-actin]); 2: complete (no contact loss between oocyte and granulosa cells [i.e. dense F-actin staining]) (categorization of TZPs was adapted from [53]) (Fig. 4). To avoid that the quantification of the expression of Cx43 and TZPs could be attributed to differences in granulosa cell number, the expression of Cx43 and TZPs was qualified and not quantified. Negative controls were obtained by omission of the primary antibodies for Cx43 and omission of TZP staining.

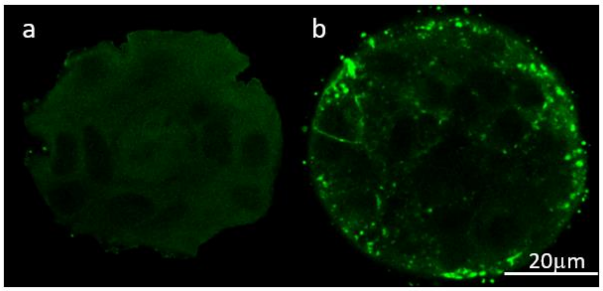

Fig. 3 Follicle staining negative (a) and positive (b) for Cx43

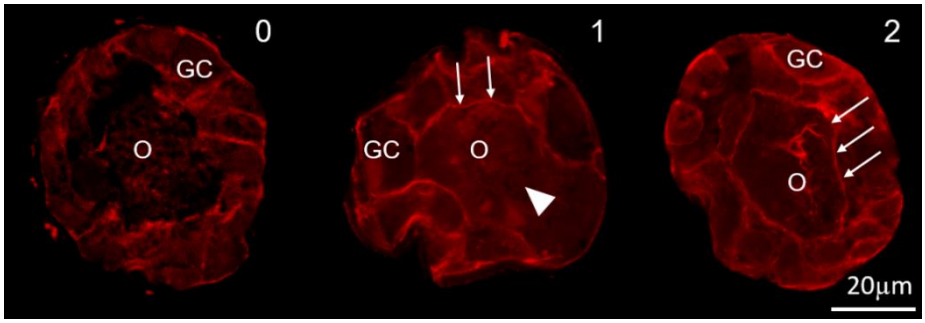

Fig. 4 The organization of the TZPs was classified as 0: total absence (no physical connections between the oocyte and granulosa cells [no TZPs visible]); 1: partial absence (gaps between the oocyte and granulosa cells [no dense staining of F-actin]); 2: complete (no contact loss between oocyte and granulosa cells [i.e. dense F-actin staining]) (categorization of TZPs was adapted from [195]) O: Oocyte, GC: granulosa cell, TZPs are indicated with arrows, interrupted TZPs are indicated with an arrow head

2.5. Statistical analysis

Different statistical models were used to assess the differences in follicle diameter and the presence of Cx43 between groups. The procedure to isolate the follicles from the ovaries, as described in section 2.1, was carried out 36 times. For each isolation procedure, follicles were derived from 10 ovaries that originated from 10 different cows. The ovaries were selected as described in section 2.1. Follicle

isolation was carried out as outlined in paragraph 2.1 on all 10 ovaries together. Two isolation procedures were performed subsequently on different ovaries, from the same slaughterhouse batch. After two isolation procedures, approximately 160 intact follicles were selected for culture. No separate follicle isolations were performed for each separate ovary. As a consequence, there is no specific information on which follicle originated from which ovary. Therefore, all follicles within one replicate are considered independent statistical units. To account for possible replicate effects, the replicate number was entered as a random effect in the subsequent statistical analysis. Treatment was entered as a fixed effect. Interaction between the treatment and replicate was also entered, but if this latter term was not significant, it was omitted from the model. Follicle diameter was considered a dependent continuous variable. Cx43 expression (binary, present vs. absent) was considered as a dependent categorical variable. Treatment group was considered as an independent categorical variable (categorical: fresh control, 2 day culture, 4 day culture, vitrified). Potential differences in follicle diameter between groups were analyzed using analysis of variance (ANOVA). Prior to ANOVA, data were analyzed for normal distribution and homogeneity of variance by performing the Kolmonogrov-Smirnov and Levene's tests, respectively. Potential differences in Cx43 expression between groups were analyzed using a Fisher's Exact Test. Differences between groups were considered to be significant when the *P*-value <0.05 [54]. Data are presented as means ± standard deviation (S.D.). All statistical analyses were performed using IBM SPSS version 24® (New York, USA).

3. Results

3.1. Follicle gross morphology

In total, about 2200 primary follicles were cultured, of which it usually took good quality follicles two days to develop until the secondary stage. From day 2 of culture onwards, the outline of the basal membrane was more pronounced and the morphological distinction between the granulosa cells could no longer clearly be made (Fig. 5). At day 2 of culture, 244 follicles were classified as category 1, meaning that only 11% of all follicles were suitable to use for the experiments according to their gross morphology. Follicles were collected from 36 isolation procedures. However, 5 replicates contained no category 1 follicles following 2 days of culture and these replicates were not taken into account for further analyses.

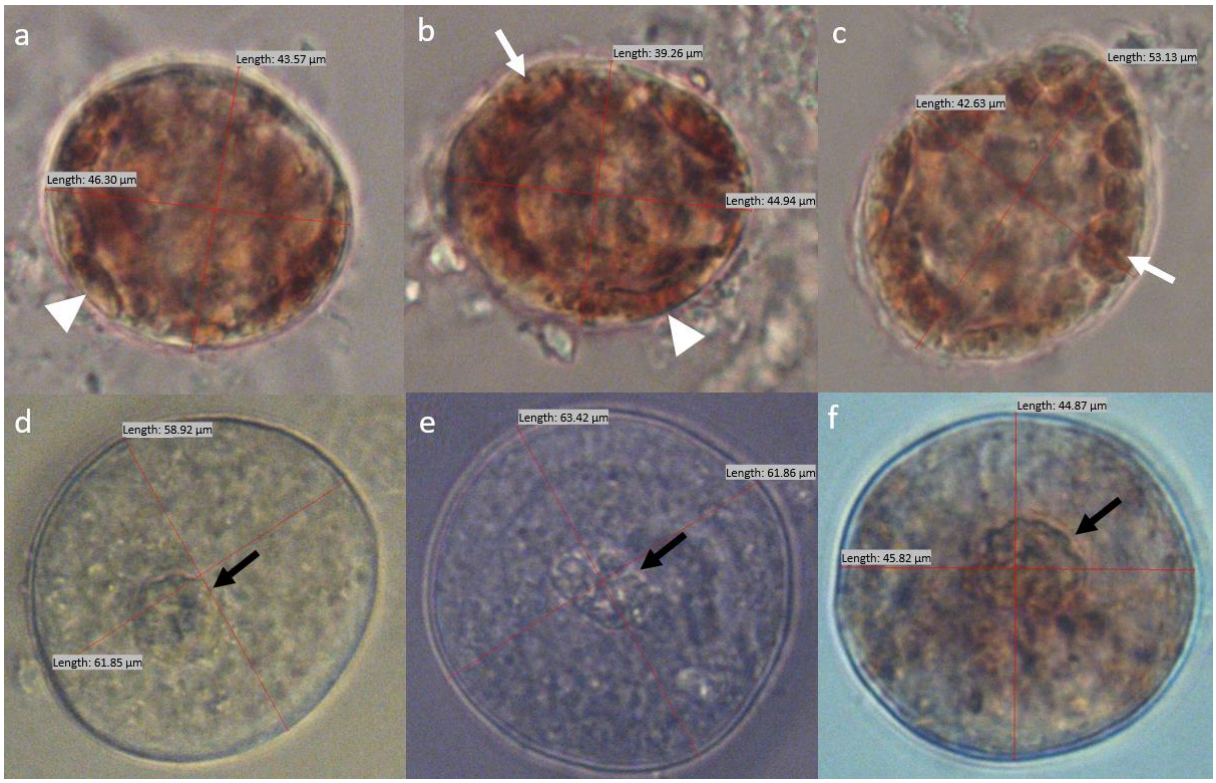

Fig. 5 Light microscopic morphological aspects of different follicle stages upon isolation, on 2 and 4 days of culture and following vitrification. a: Primordial follicle upon isolation (NR positive, Ø: 45 µm); b: transitory follicle upon isolation (NR positive, Ø: 42 µm); c: primary follicle upon isolation (NR positive, Ø: 48 µm); d: secondary follicle on day 2 of culture (non-stained, Ø: 60 µm); e: secondary follicle on day 4 of culture (non-stained, Ø: 63 µm); f: vitrified follicle one day post warming (NR positive, Ø: 45 µm). White arrow heads indicate flattened granulosa cells, white arrows indicate cuboidal granulosa cells, black arrows indicate the oocyte. NR: Neutral Red. Ø: Follicular diameter, reported as an average of two perpendicular measures. Magnification: 400x

3.2. Follicle diameter

The follicle diameter of PAFs was measured on day 0 (n=60), day 2 (n=201), day 3 (post vitrification; n=40) and day 4 (n=89). The mean follicle diameter (µm) ± S.D. for category 1 follicles pooled over all replicates was 46.8 ± 4.6 upon isolation, 48.5 ± 7.0 on day 2 and 50.5 ± 7.7 on day 4 (Fig. 6). On day 3, one day post vitrification, the mean follicle diameter (µm) ± S.D. was 51.0 ± 7.7. Mean follicular diameters were significantly different between day 0 and day 2 (P=0.041) and between day 0 and day 4 (P=0.001).

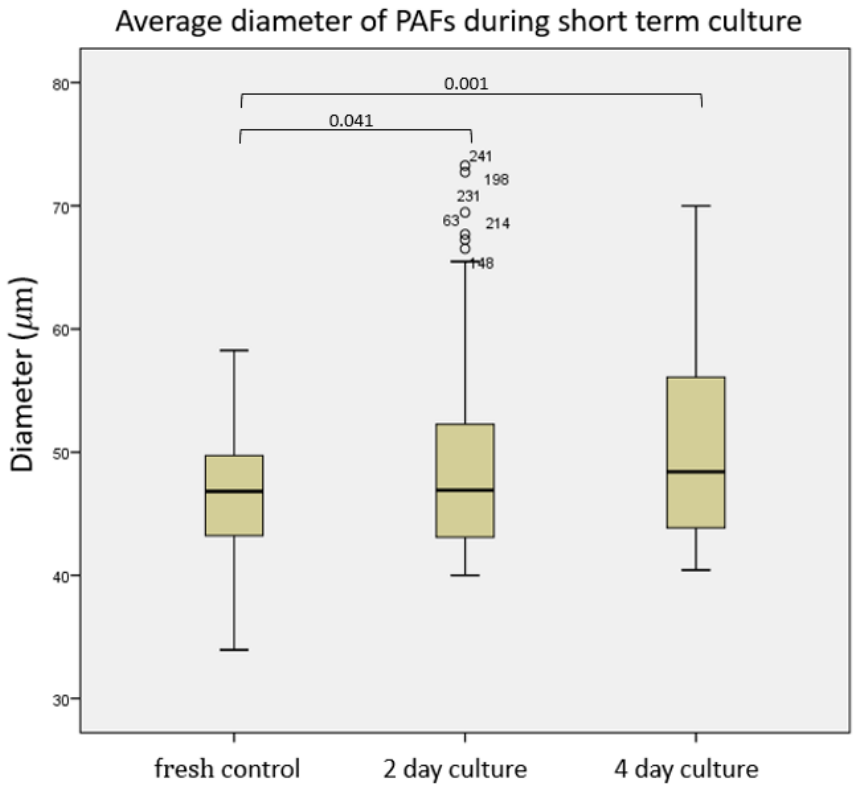

Fig. 6 Boxplot showing average follicular diameter (µm) upon isolation (n=60), and following 2 (n=201), respectively 4 days of culture (n=89)

3.3. Neutral Red (NR) staining

To evaluate immediate follicle survival and instant viability, PAFs were stained with NR. Upon isolation and on day 2 and 4 of culture, only category 1 follicles were taken into account. Follicles staining positive for the vital dye NR were considered viable. 96% of all category 1 follicles on day 2 and day 4 of culture stained Neutral Red positive. Out of 96 follicles that were vitrified, 48 were successfully retrieved and 70.8% (n=34) of them stained NR positive after warming and 24h culture (Fig. 7).

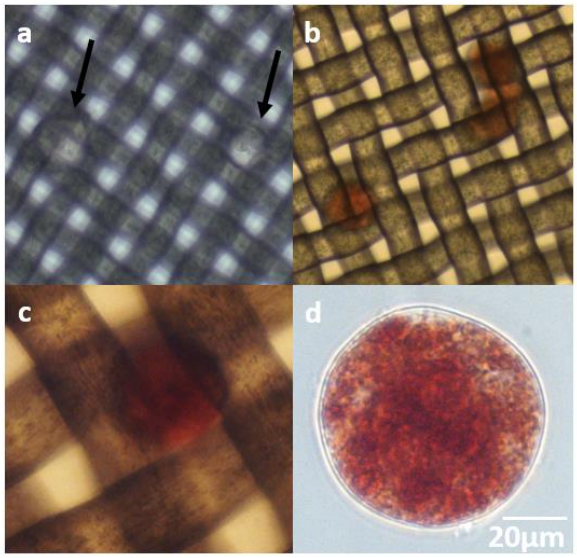

Fig. 7 a: Light microscopic (LM) image of two non-stained follicles (black arrows) on the mesh of the mini cell strainer; b: LM image of NR positive follicles on the mesh of the mini cell strainer; c: detailed LM image of NR positive follicle; d: NR positive follicle removed from the mesh

3.4. Presence of Cx43 and TZPs in different follicle stages

Cx43 was detected in granulosa cells of all pre-antral follicle stages, at the site of TZPs (Fig. 8). Cx43 staining started with distinct spots in resting primordial follicles. As seen in figure 8, the Cx43 positive dots per granulosa cell, and hence the number of gap junctions, increased notably with follicle development.

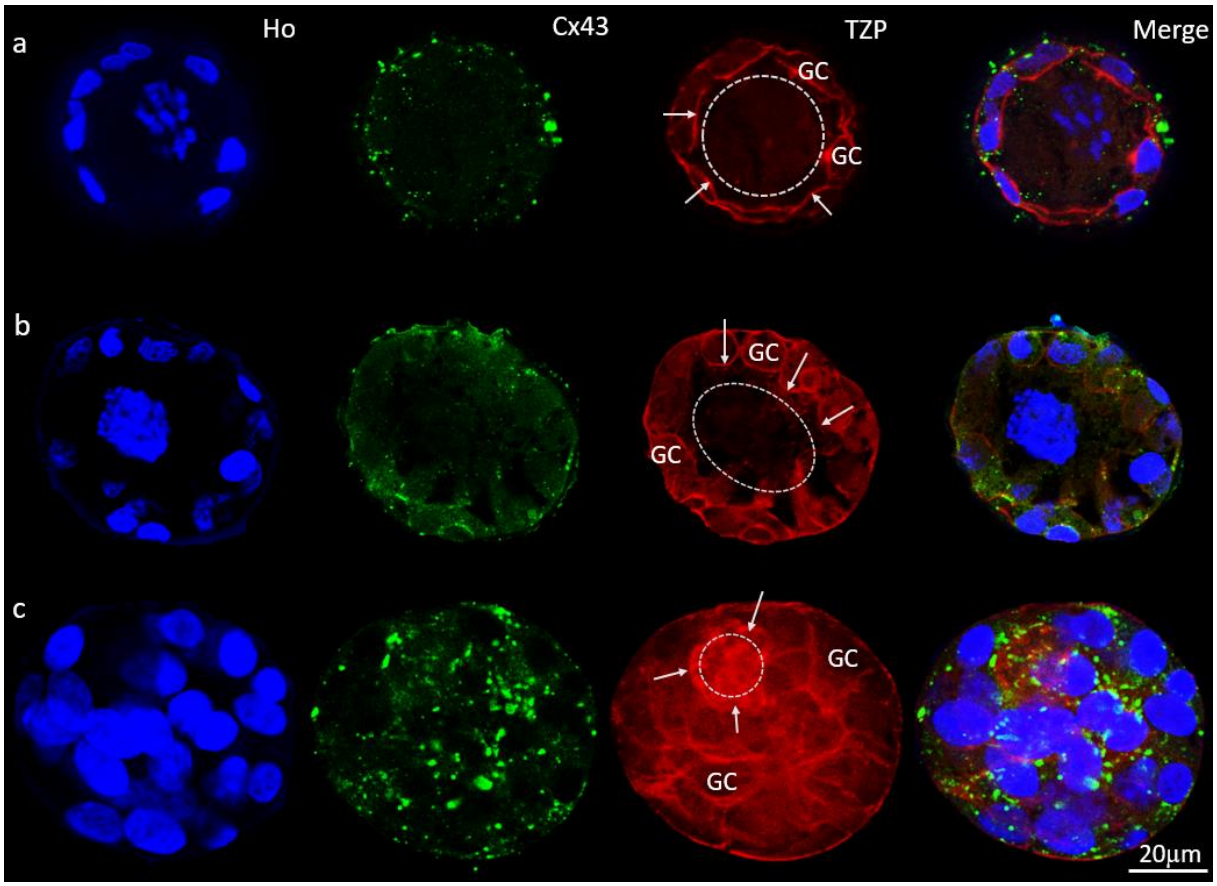

Fig. 8 Confocal images (single confocal plane) of expression and localization of Cx43 and TZPs in PAFs in different stages. Images show a single confocal plane at the median level of the follicle. a: primordial follicle upon isolation: oocyte surrounded by one layer of flattened granulosa cells. b: primary follicle upon isolation: oocyte surrounded by one layer of cuboidal granulosa cells. c: secondary follicle after 2 day culture: oocyte surrounded by several layers of cuboidal granulosa cells. Hoechst nuclear staining in blue, Cx43 staining in green, Alexa Fluor™ 568 Phalloidin staining actin-rich TZPs in red, merged picture shows an overlay of the three stains. White dotted line indicates the oocyte, GC: granulosa cells, TZPs are indicated by arrows

3.5. Cx43 and TZPs before and following vitrification

Cx43 and TZPs could be detected in PAFs in all groups (Fig. 9), before and following vitrification. In figure 9, from each group, one follicle was depicted as representative for the whole group.

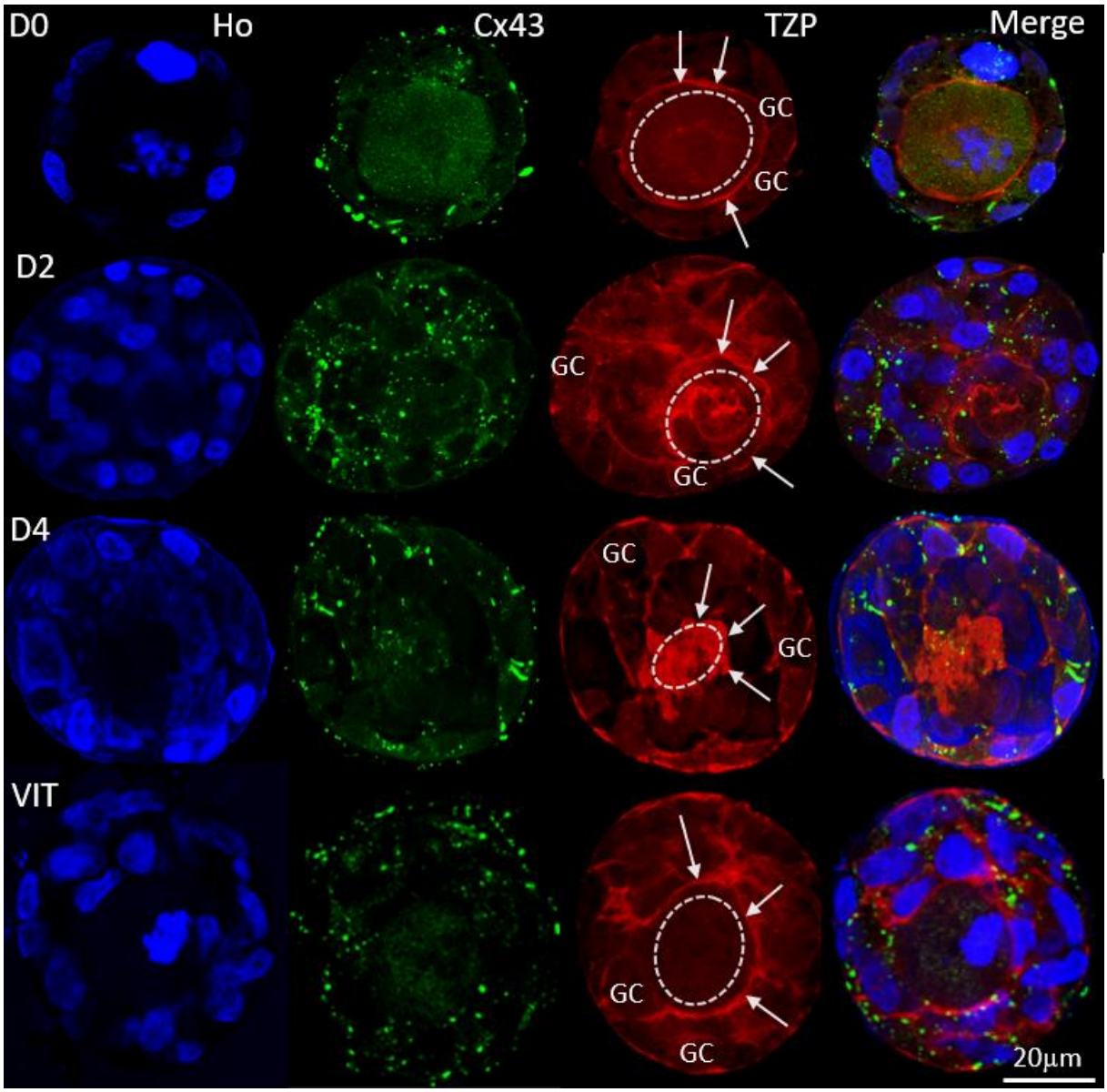

Fig. 9 Confocal images (single confocal plane) of expression and localization of Cx43 and TZPs in PAFs in all treatment groups. D0: primary follicle fixed upon isolation; D2: follicle following 2 days of culture; D4: follicle following 4 days of culture; VIT: follicle fixed after vitrification and warming and a 24h recovery period in culture. Hoechst nuclear staining in blue, Cx43 staining in green, Alexa Fluor™ 568 Phalloidin staining actin rich TZPs in red, merged picture shows an overlay of the three stains. White dotted line indicates the oocyte, GC: granulosa cells, TZPs are indicated by arrows

From the NR positive follicles that were fixed immediately upon isolation and stained for Cx43 (n=48), 91.7% (n=44) stained positive for Cx43. From the NR positive follicles that were stained for TZPs (n=48), 16.7% (n=8) showed a complete TZP network. 8.3% (n=4) showed a complete absence of TZPs and 75% (n=36) showed a partial absence of TZPs (Fig. 11). In 16.7% (n=8) of cases, both TZPs and Cx43 were preserved. From NR positive follicles that were fixed following 2 days of culture, 23 follicles were stained for Cx43 and all of them (100%) stained positive for Cx43. From the NR positive follicles that were stained for TZPs (n=23), 30.4% (n=7) showed a complete TZP network. 52.2% (n=12) showed a

total absence of TZPs and 17.4% (n=4) showed a partial absence of TZPs. In 30.4% (n=7) of cases, both TZPs and Cx43 were preserved. From the NR positive follicles that were fixed after 4 days of culture and were stained for Cx43 (n=38), 65.8% (n=25) stained positive for Cx43. This is significantly different from follicles assessed after isolation and on day 2 (P=0.002) (Fig. 10). From the NR positive follicles that were stained for TZPs (n=38), 10.5% (n=4) showed a complete TZP network. 42.1% (n=16) showed a total absence of TZPs and 47.4% (n=18) showed a partial absence of TZPs. In none of the cases, both TZPs and Cx43 were preserved. From the vitrified NR positive follicles that were stained for Cx43 (n=31), 77.4% (n=24) stained positive for Cx43. From the NR positive follicles that were stained for TZPs (n=31), 35.5% (n=11) showed a complete TZP network. 29% (n=9) showed a total absence of TZPs and 35.5% (n=11) showed a partial absence of TZPs. In 19.4% (n=6) of cases, vitrification resulted in the preservation of both TZPs and Cx43.

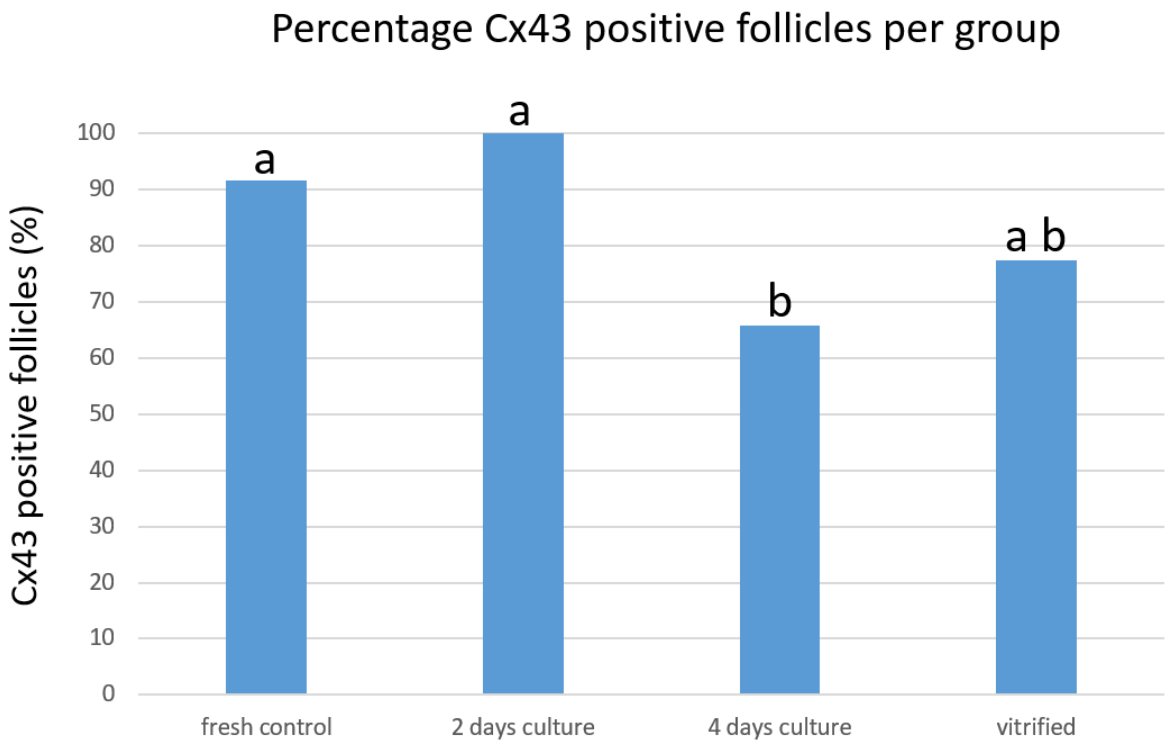

Fig. 10 Percentage of Cx43 positive follicles per group. Columns with the same letters do not differ significantly from each other at the P<0.05 level

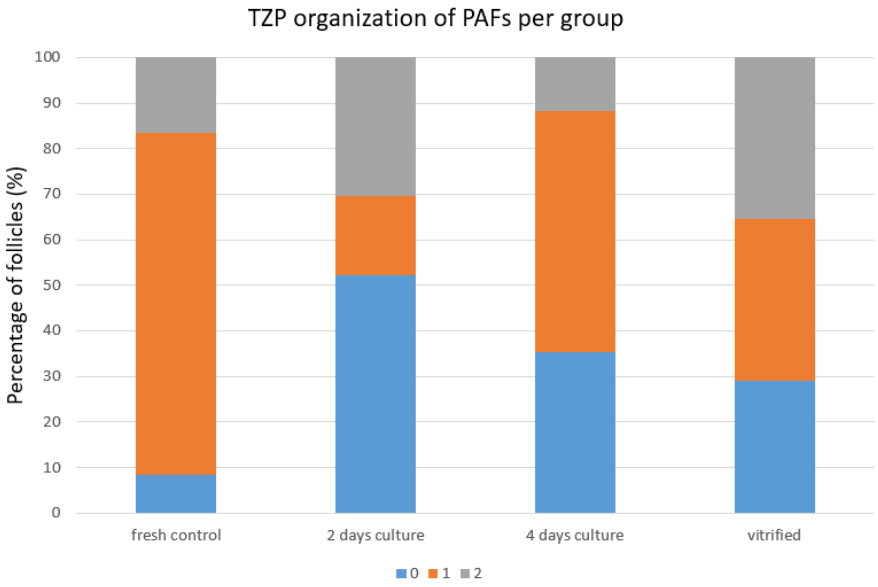

Fig. 11 The percentage of pre-antral follicles allocated to a particular category of TZP organization per group. 0: total absence (no physical connections between the oocyte and granulosa cells [no TZPs visible]); 1: partial absence (gaps between the oocyte and granulosa cells [no dense staining of F-actin]); 2: complete (no contact lost between oocyte and granulosa cells [i.e. dense staining of F-actin])

4. Discussion

These data are the first to demonstrate that it is possible to visualize Cx43 and TZPs in isolated bovine PAFs before and following vitrification and warming, using a simplified and more efficient cryopreservation protocol while using mini cell strainers. The detection and descriptive analysis of immunofluorescent labeled connexins and TZPs can provide an indication about their future developmental capacity post warming. Immunofluorescent staining of connexins in ovine isolated follicles was performed by da Silva et al. (2016) [43], on follicles embedded in paraffin. We were not able to detect Cx37 protein expression in isolated PAFs, potentially due to the absence of Cx37 or the deficiency of reactivity of the antibody for bovine Cx37. As follicles shrink after fixation, the even smaller size hampers follicle manipulation. Due to many transfer steps between different media, a considerable loss of follicles was difficult to prevent. A vital Neutral Red staining was used to improve follicle visibility for handling and assessment.

For the first aim of this study, we demonstrated Cx43 and TZPs in category 1 freshly fixed and cultured follicles. These membrane proteins constitute cell-cell connections and mediate intercellular communication. The identification of Cx43 and TZPs indicate the presence of these proteins which are essential for follicle development. Freshly fixed follicles (91.7%) showed a slightly decreased percentage of Cx43 positive follicles compared to follicles that were cultured for 2 days (100%).

Follicles that were cultured for 2 days showed a higher percentage of Cx43 positive follicles, this may indicate that follicles need a recovery period after the isolation procedure. Or, as the selection of good quality follicles based on morphology is more adequate on day 2 than immediately upon isolation, the batch of follicles selected on Day 0 may contain follicles that were incorrectly qualified as category 1. Follicles that were cultured for 4 days showed the lowest percentage of Cx43 positive follicles (65.8%), which may indicate that current culture conditions were not optimal for follicle development. In each group and pre-antral follicle stage, we saw follicles with a complete TZP network. However, the percentages of the presence or (partial) absence of the TZP network varied between follicles in different treatment groups. This may be due to variability in follicle quality, although when examining morphology and viability, the follicles appeared to be quite similar (category 1 and Neutral Red positive). In this perspective, we suggest that gross morphology can predict immediate individual cell viability [49] but not the presence of cell-cell connections. To start the experiment with a homogenous group of follicles on day 2 of culture, only category 1 secondary follicles at the right size range (40-80 µm) were selected for future use. In our opinion, only when these good quality follicles are used, an experiment can succeed and be reproducible. On the downside, this strict use of category 1 follicles makes it more difficult to obtain a sufficient number of follicles to complete a full experiment.

Secondly, we aimed to visualize Cx43 and TZPs in PAFs following vitrification and warming. Several studies have been conducted on the expression of connexins and TZPs and most of them demonstrated that cryopreservation might cause damage to these membrane proteins, although the results are not consistent. A reason for cryodamage may be that hemichannels ((HC) consist of six Cx proteins and 2 HCs create gap junctions between adjacent cells), are normally closed but open in response to stress conditions such as cryopreservation. Excessive HC opening is detrimental for cell function and may lead to cell death [52]. Our results show that Cx43 expression in vitrified isolated follicles (77.4%) was slightly lower when compared to follicles that were freshly fixed (91.7%) or cultured for 2 days (100%). 35.5% showed a complete TZP network after vitrification, the highest percentage from all groups. Also Tanpradit et al. (2015) [55] showed a reduced Cx43 expression pattern after both slow freezing and vitrification of feline PAFs enclosed in ovarian tissue. In a study of da Silva et al. (2016) [43], it was shown that Cx43 was reduced in ovine secondary follicles after vitrification and in vitro culture of ovarian tissue. In contrast, Donfack et al. (2018) [28] found that in vitro culture of the vitrified goat ovarian cortex did not alter the expression of Cx43. Barrett et al. (2010) [31] showed that immediately after slow freezing and thawing, mouse isolated follicles were unable to transfer Lucifer Yellow into surrounding granulosa cells, indicating that the gap junctions were not functional. However, after 2 days of culture, cryopreserved follicles were able to re-establish gap junctions and transport the dye from the oocyte into the surrounding somatic cells. Trapphoff et al. (2010) [56] also reported the

recovery of contacts by TZPs between mouse oocytes and granulosa cells from vitrified pre-antral follicles during the first 4 days of culture. They suggest that the thick cortical actin layer in oocytes is maintained and may contribute to the rapid reestablishment of membrane organization at the cortex and restoration of contacts between somatic cells and the oocyte. Thus, cryopreservation of follicles does not necessarily damage cell-cell connections in the long term when a sufficient recovery period is provided. However, most studies were performed using slow freezing and ovarian tissue, and data on the survival of cell-cell connections in isolated PAFs after vitrification is very scarce.

Processing isolated PAFS remains a huge challenge in terms of follicular retrieval and manipulation, therefore during this study, we tried to optimize the vitrification protocol and increase the efficiency of follicle recovery using mini cell strainers. In a previous study [23], we reported that bovine PAFs can be successfully cryopreserved by a simple two-step vitrification method using HSV straws. However, transferring individual PAFs between droplets using Stripper tips is time consuming while these manipulations can cause osmotic damage to the follicles. The disadvantage of straws and cryovials is that they only permit relatively low cooling and warming rates, while the ability to survive vitrification is also highly dependent on the rate of warming [57]. Vitrification of alginate embedded PAFs in 20µm metal mesh cell strainers resulted in a higher efficiency, but lower viability [23]. Therefore, in this study we aimed for better permeation of cryoprotectants combined with presupposed higher cooling and warming rates and used a mini cell strainer to freeze non-embedded PAFs. Multiple follicles can be vitrified at the same time, as a mini cell strainer can hold many more follicles, whereas only a few follicles can be loaded into one straw. It was a much easier, quicker and simpler way to freeze PAFs. The mini cell strainer has a small handle that can easily be held by tweezers. The step-by-step transfer of the cell strainer loaded with PAFs between freezing and warming media and the passage through LN_2 can be carried out with tweezers. After being submerged in LN_2, the cells cool down very quickly. The visibility of non-stained PAFs on the mesh of the cell strainer is, however, limited and hampers follicle retrieval. Positive staining with Neutral Red improves the visibility and makes the retrieval of PAFs from the cell strainer easier. Another difficulty is that PAFs tend to settle on the rim of the cell strainer, which makes it impossible to visualize them. Therefore, it is necessary to carefully search for them using a hand pipette with glass capillary along the whole rim of the cell strainer. When visible follicles were retrieved, the cell strainer was rinsed with culture medium in a 35mm petri dish, always yielding extra follicles. In view of the relative high follicle loss (the recovery rate varied between 33.3% and 69.2%), we think it is beneficial to use cell strainers with an even smaller mesh size, taking into account follicle shrinkage.

Martino et al. (1996) [58] were the first to use EM grids for the cryopreservation of bovine oocytes and reported a significantly higher survival rate for vitrified-warmed oocytes using grids as compared to

oocytes frozen in straws. These results may be explained by the physical characteristics such as the ultra-thermo-conductivity of the metal grid that results in about threefold higher cooling rates than that obtained with straws [59]. In addition, Kim et al. (2001) [60] showed that the developmental capacity of immature bovine oocytes vitrified and warmed using EM grids was not hampered. Park et al. (1999) [61] successfully cryopreserved in vitro produced bovine blastocysts using EM grids. More than a decennium later, Nakashima et al. (2010) [62] showed that vitrification using a nylon mesh container for human embryo ultrarapid vitrification is an easy and inexpensive method that improves the reliability of human embryo cryopreservation. Due to the small mesh size and convenient size of the cell strainer, we chose to work with mini cell strainers. A commercially available vitrification kit for human oocytes and embryos was used in this experiment with a slightly modified protocol. We reduced the number of transfer steps and shortened the exposure time as it did not hamper the survival rate of isolated bovine pre-antral follicles. We found this modification beneficial because of the reduced exposure time of PAFs to cryoprotectants, the reduced risk of losing follicles and decreased working time.

In conclusion, we visualized Cx43 and TZPs in isolated bovine PAFs during short term culture and following vitrification and warming, suggesting the maintenance of communication between the oocyte and the somatic companion cells. The aim of this study was limited to the visualization of cell-cell connections in isolated PAFs. Future research should further confirm the presence and functionality of these cell-cell connections at the gene transcription and protein levels, using real-time-quantitative PCR and western blot analyses. In addition, these extra insights will help to gain a greater understanding of the variations in expression of the TZP network, that were observed within our study groups. It is also important to consider that in early pre-antral follicles, TZPs have only just started to develop and they are continuously remodeling during the formation of the zona pellucida, which starts to develop form the secondary stage onwards in bovines and humans. Furthermore, in view of the challenges that still need to be overcome regarding follicular retrieval and manipulation, we were able to use a simpler and much more workable cryopreservation method, by means of mini cell strainers. Vitrification in cell strainers has important advantages, as manipulation is much easier and time is saved because follicles are vitrified in group.

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All (co-)authors state that the funding of this research was provided by the independent Operational Costs of the University of Antwerp. There is nothing to disclose and there are no conflicts of interest, financially nor personal, for none of the (co-)authors.

References

1. Hewitt M, Weiner SL, Simone JV. *Childhood cancer survivorship: improving care and quality of life.* National Academies Press; 2003.
2. Robison LL, Hudson MM. *Survivors of childhood and adolescent cancer: life-long risks and responsibilities*. Nat Rev Cancer. 2014;14(1):61.
3. Rodriguez-Wallberg KA, Oktay K. *Options on fertility preservation in female cancer patients*. Cancer Treat Rev. 2012;38(5):354-61.
4. Hyman JH, Tulandi T. *Fertility preservation options after gonadotoxic chemotherapy*. Clin Med Insights Reprod Health. 2013;7:61-9.
5. Green DM, Kawashima T, Stovall M, Leisenring W, Sklar CA, Mertens AC et al. *Fertility of female survivors of childhood cancer: a report from the childhood cancer survivor study*. J Clin Oncol. 2009;27(16):2677.
6. Seli E, Agarwal A. *Fertility preservation in females: emerging technologies and clinical applications*, first ed. Springer, New York; 2012.
7. Jeruss JS, Woodruff TK. *Preservation of fertility in patients with cancer*. N Engl J Med. 2009;360(9):902-11.
8. Donnez J, Dolmans M-M, Diaz C, Pellicer A. *Ovarian cortex transplantation: time to move on from experimental studies to open clinical application*. Fertil steril. 2015;104(5):1097-8.
9. Forman EJ*. Ovarian tissue cryopreservation: still experimental?* Fertil Steril. 2018;109(3):443-4.
10. Silber S*. Ovarian tissue cryopreservation and transplantation: scientific implications*. J Assist Reprod Gen. 2016;33(12):1595-603.
11. von Wolff M, Sänger N, Liebenthron J. *Is Ovarian Tissue Cryopreservation and Transplantation Still Experimental? It Is a Matter of Female Age and Type of Cancer*. J clin oncol. 2018;36(33):JCO1800425.
12. Jadoul P, Guilmain A, Squifflet J-L, Luyckx M, Votino R, Wyns C et al. *Efficacy of ovarian tissue cryopreservation for fertility preservation: lessons learned from 454 cases*. Hum Reprod. 2017;32(5):1046-54.
13. Donnez J, Manavella DD, Dolmans M-M. *Techniques for ovarian tissue transplantation and results*. Minerva Ginecol. 2018;70(4):424-31.
14. Rosendahl M, Greve T, Andersen CY. *The safety of transplanting cryopreserved ovarian tissue in cancer patients: a review of the literature*. J Assist Reprod Gen 2013;30(1):11-24.
15. Dolmans M-M, Masciangelo R. *Risk of transplanting malignant cells in cryopreserved ovarian tissue.* Minerva Ginecol. 2018;70(4):436-43.
16. Fisch B, Abir R. *Female fertility preservation: past, present and future*. Reprod. 2018;156(1):F11-F27.
17. Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K et al. *Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation.* Hum Reprod Update. 2010;16(4):395-414.
18. Telfer EE, McLaughlin M. *In vitro development of ovarian follicles*. Semin Reprod Med. 2011;29(1):15-23.
19. Telfer EE, Zelinski MB. *Ovarian follicle culture: advances and challenges for human and nonhuman primates.* Fertil Steril. 2013;99(6):1523-33.
20. Vanacker J, Dolmans MM, Luyckx V, Donnez J, Amorim CA. *First transplantation of isolated murine follicles in alginate.* Regen Med. 2014;9(5):609-19.
21. Luyckx V, Dolmans MM, Vanacker J, Legat C, Fortuno Moya C, Donnez J et al. *A new step toward the artificial ovary: survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold.* Fertil Steril. 2014;101(4):1149-56.

22. Bus A, Langbeen A, Martin B, Leroy J, Bols P. *Is the pre-antral ovarian follicle the 'holy grail' for female fertility preservation?* Anim Reprod Sci. 2019;207:119-130.
23. Bus A, van Hoeck V, Langbeen A, Leroy J, Bols PEJ. *Effects of vitrification on the viability of alginate encapsulated isolated bovine pre-antral follicles.* J Assist Reprod Gen. 2018;35(7):1187-99.
24. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM et al. *Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance*. Hum Reprod update. 2017;23(2):139-55.
25. Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF. *Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence*. Dev Biol. 2000;226(2):167-79.
26. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. *Intercellular communication in the mammalian ovary: oocytes carry the conversation*. Science. 2002;296(5576):2178-80.
27. Gilchrist RB, Lane M, Thompson JG. *Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality.* Hum Reprod Update. 2008;14(2):159-77.
28. Donfack NJ, Alves KA, Alves BG, Rocha RMP, Bruno JB, Bertolini M et al. *Stroma cell-derived factor 1 and connexins (37 and 43) are preserved after vitrification and in vitro culture of goat ovarian cortex.* Theriogenology. 2018;116:83-8.
29. Anderson E, Albertini DF. *Gap junctions between the oocyte and companion follicle cells in the mammalian ovary*. J Cell Biol. 1976;71(2):680-6.
30. Kidder GM, Mhawi AA*. Gap junctions and ovarian folliculogenesis*. Reprod. 2002;123(5):613-20.
31. Barrett SL, Shea LD, Woodruff TK. *Noninvasive index of cryorecovery and growth potential for human follicles in vitro.* Biol Reprod. 2010;82(6):1180-9.
32. Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. *Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice*. Dev Biol. 1998;204(2):373-84.
33. Motta PM, Makabe S, Naguro T, Correr S. *Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy.* Arc Histol Cytol. 1994;57(4):369-94.
34. Fair T, Hulshof S, Hyttel P, Greve T, Boland M. *Oocyte ultrastructure in bovine primordial to early tertiary follicles.* Anat Embryol. 1997;195(4):327-36.
35. Armstrong D, Baxter G, Hogg C, Woad K. *Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles.* Reproduction-Cambridge. 2002;123(6):789-97.
36. Eppig JJ. *A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro*. J Exp Zool. 1979;209(2):345-53.
37. Brower PT, Schultz RM. *Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth*. Dev Biol. 1982;90(1):144-53.
38. Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. *Cellular basis for paracrine regulation of ovarian follicle development*. Reproduction-Cambridge. 2001;121(5):647-53.
39. Larsen W. *Biological implications of gap junction structure, distribution and composition: a review.* Tissue and Cell. 1983;15(5):645-71.
40. Grazul-Bilska AT, Reynolds LP, Redmer DA. *Gap junctions in the ovaries*. Biol Reprod. 1997;57(5):947-57.
41. Kidder GM, Vanderhyden BC. *Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence*. Can J Physiol Pharmacol. 2010;88(4):399-413.
42. Nuttinck F, Peynot N, Humblot P, Massip A, Dessy F, Flechon JE. *Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary*. Mol Reprod Dev. 2000;57(1):60-6.

43. da Silva AMS, Bruno JB, de Lima LF, de Sá NAR, Lunardi FO, Ferreira ACA et al. *Connexin 37 and 43 gene and protein expression and developmental competence of isolated ovine secondary follicles cultured in vitro after vitrification of ovarian tissue.* Theriogenology. 2016;85(8):1457-67.
44. Albertini DF, Rider V. *Patterns of intercellular connectivity in the mammalian cumulus-oocyte complex.* Microsc Res Techniq. 1994;27(2):125-33.
45. Vanhoutte L, Cortvrindt R, Nogueira D, Smitz J. *Effects of chilling on structural aspects of early preantral mouse follicles.* Biol Reprod. 2004;70(4):1041-8.
46. Langbeen A, Bartholomeus E, Leroy JL, Bols PE. *Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies*. Theriogenology. 2015;84(4):477-89.
47. Adams G, Pierson R. *Bovine model for study of ovarian follicular dynamics in humans.* Theriogenology. 1995;43(1):113-20.
48. Campbell B, Souza C, Gong J, Webb R, Kendall N, Marsters P et al. *Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans*. Reprod. 2003:429-43.
49. Jorssen EP, Langbeen A, Marei WF, Fransen E, De porte HF, Leroy JL et al. *Morphologic characterization of isolated bovine early preantral follicles during short-term individual in vitro culture.* Theriogenology. 2015;84(2):301-11.
50. Braw-Tal R, Yossefi S. *Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary.* J Reprod Fertil. 1997;109(1):165-71.
51. Langbeen A, Jorssen EP, Granata N, Fransen E, Leroy JL, Bols PE. *Effects of neutral red assisted viability assessment on the cryotolerance of isolated bovine preantral follicles*. J Assist Reprod Gen. 2014;31(12):1727-36.
52. Ortiz-Escribano N, Szymanska KJ, Bol M, Vandenberghe L, Decrock E, Van Poucke M et al. *Blocking connexin channels improves embryo development of vitrified bovine blastocysts.* Biol Reprod. 2017;96(2):288-301.
53. Lierman S, Tilleman K, Cornelissen M, De Vos WH, Weyers S, T'Sjoen G et al. *Follicles of various maturation stages react differently to enzymatic isolation: a comparison of different isolation protocols*. Reprod Biomed online. 2015;30(2):181-90.
54. Dohoo I, Martin S, Stryhn H. Vet Epidemiol Res. Second ed. VER inc. Charlottetown. 2009.
55. Tanpradit N, Comizzoli P, Srisuwatanasagul S, Chatdarong K. *Positive impact of sucrose supplementation during slow freezing of cat ovarian tissues on cellular viability, follicle morphology, and DNA integrity.* Theriogenology. 2015;83(9):1553-61.
56. Trapphoff T, El Hajj N, Zechner U, Haaf T, Eichenlaub-Ritter U. *DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles.* Hum Reprod. 2010;25(12):3025-42.
57. Jin B, Mazur P. *High survival of mouse oocytes/embryos after vitrification without permeating cryoprotectants followed by ultra-rapid warming with an IR laser pulse*. Sci Rep. 2015;5:9271.
58. Martino A, Songsasen N, Leibo S. *Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling*. Biol Reprod. 1996;54(5):1059-69.
59. Steponkus P, Myers S, Lynch D, Gardner L, Bronshteyn V, Leibo S et al. *Cryopreservation of Drosophila melanogaster embryos.* Nature. 1990;345(6271):170-2.
60. Kim E-Y, Kim N-H, Yi B, Yoon S, Park S, Chung K et al. *Developmental Capacity of Bovine Follicular Oocytes after Ultra-Rapid Freezing by Electron Microscope Grid I. Cryopreservation of Bovine Immature Oocytes.* Korean J Fertil Steril. 2001;25(1):71-6.
61. Park S-P, Kim EY, Kim DI, Park NH, Won YS, Yoon SH et al. *Simple, efficient and successful vitrification of bovine blastocysts using electron microscope grids*. Hum Reprod. 1999;14(11):2838-43.
62. Nakashima A, Ino N, Kusumi M, Ohgi S, Ito M, Horikawa T et al. *Optimization of a novel nylon mesh container for human embryo ultrarapid vitrification.* Fertil Steril. 2010;93(7):2405-10.

CHAPTER 6

Preservation of connexin 43 and transzonal projections in isolated human pre-antral follicles before and following vitrification

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Article in preparation

Summary

Purpose We aimed to vitrify human isolated follicles using established protocols for human oocyte vitrification. Because of the importance of the presence of intact cell-cell contacts for further development of PAFs post warming, the current study aimed to visualize the precise location of Cx43 and TZPs using immunocytochemical methods and we aimed to measure mRNA expression levels of gap junction protein alpha 1 (Gja1, Cx43).

Methods This study involved two experimental groups: an *'untreated control',* analyzed upon isolation and a *'treated group'* which was subjected to a vitrification/warming cycle. Cx43 and TZPs were demonstrated by immunochemistry and mRNA expression levels of Cx43 were measured by qPCR. Isolated PAFs were vitrified using Cryotop®.

Results The overall post warming survival rate was 73.7%. Cx43 and TZPs were detected in PAFs of all stages, as well as in both experimental groups. Our results show that Cx43 expression (immunostaining) in vitrified isolated follicles (88%) was lower compared to follicles that were fixed upon isolation (99.2%) (P=0.00). Vitrified follicles showed a complete TZP network in 98.4% of cases, compared with 96.2% in follicles fixed upon isolation (P=0.157). There was no difference (P=0.9) in Cx43 mRNA expression between untreated and vitrified follicles.

Conclusions These results suggest the maintenance of communication between the oocyte and the somatic companion cells following enzymatic isolation and following vitrification and warming. Isolated human PAFs were successfully vitrified and warmed using Cryotop®. However, it is necessary to develop a high throughput system that can be used for clinical purposes. In addition, effects of enzymatic isolation and vitrification, warming and culture should be investigated on the long-term to assure the production of developmentally competent and epigenetically normal oocytes.

1. Introduction

Constant improvements in diagnosis and cancer treatment [1] result in an increasing number of patients now being long term survivors. However, as chemo and radiotherapy often damage ovarian tissue [2, 3], survivors are likely to face compromised fertility [4, 5], therefore, preserving fertility before cancer treatment is necessary for many of these patients [5, 6]. In humans, embryo and mature-oocyte cryopreservation are currently the only approved methods for fertility preservation in many countries. However, a growing number of countries designated ovarian tissue cryopreservation (OTC) as no longer experimental and an acceptable technique to be offered to patients seeking fertility preservation [7, 8]. Ovarian tissue cryopreservation is specifically indicated for prepubertal girls and women whose cancer treatment cannot be postponed. The cryopreservation of isolated pre-antral follicles (PAFs) is a safer alternative for cancer patients who are at risk of the reintroduction of malignant cells. As PAFs account for the vast majority of follicles in the ovarian cortex, they represent an untapped potential, which could be cultivated for reproduction, preservation, or research purposes. *In vitro* development of PAFs to the pre-ovulatory stage has not yet been achieved in humans. So far, the best results were reported from mice with the production of live offspring from primordial follicles cultured *in vitro*. Live birth has been obtained after the *in vitro* culture of bovine early antral follicles. In goat, buffalo and sheep, these results have been limited to the production of a variable number of mature oocytes and low percentages of embryos after *in vitro* culture of isolated secondary pre-antral follicles [9]. However, *in vitro* culture systems for PAFs and an artificial ovary have made great progress and are expected to become available in the near future [10, 11].

Oocytes develop in continuous communication with granulosa cells. The growing early PAFs generate a thick extracellular matrix, termed the zona pellucida, which physically separates the oocyte from the surrounding granulosa cells [12]. Most of this cell-cell communication is established through gap junctions intercellular channels composed of connexins (Cx) that carry small molecules from one cell to the other [13]. Experiments in connexin knockout mice showed that folliculogenesis proceeds normally until the late pre-antral stage in mice lacking Cx37 following targeted disruption of the *Gja4* gene [14]. However, these animals never developed normal mature Graafian follicles. More specifically, Cx37 and Cx43 showed to be crucial during the entire process of folliculogenesis in the human ovary [15]. Connexin expression studies and quantification on immunofluorescently labeled ovary sections revealed that Cx37 was predominantly localized at preantral stages. While Cx37 could be located in both oocyte and granulosa cell compartments, Cx43 seemed to be restricted to the granulosa cell interfaces [16-18]. Narrow cytoplasmic extensions of the granulosa cells, termed transzonal projections (TZPs), traverse the zona to reach the oocyte plasma membrane [19]. These

TZPs enable the growing oocyte and granulosa cells to remain in physical contact. Gap junctions become assembled at the tips of the TZPs, where they contact the oocyte plasma membrane [19, 20]. These enable the oocyte to remain coupled to the surrounding granulosa cells throughout its growth. When this intercellular communication is disrupted, oocytes do not develop into mature gametes, capable of participating in fertilization.

As cell vitrification bares the risk of cell damage [21-24], the possible detrimental effects of follicle vitrification on cell-cell contacts need to be thoroughly investigated. The presence of TZPs and gap junctions are predicted to be good biomarkers of follicle health, considering that their maintenance is crucial for further development of follicles. While connexin expression is described earlier, no reports were published on connexin expression in combination with vitrification of isolated human PAFs *in vitro*.

Because of the importance of the presence of intact cell-cell contacts for further development of PAFs post warming and the possible advantages of vitrification of isolated PAFs, the current study aims to 1) assess the preservation of gross morphology of human PAFs upon isolation and following vitrification and warming, 2) visualize and observe the precise location of Cx43 and TZPs using immunocytochemical methods, 3) measure mRNA expression levels of gap junction protein alpha 1 (Gja1, also known as connexin 43), as a prerequisite for future follicle developmental capacity.

2. Material and methods

2.1. Experimental design

The experimental design is described and summarized in a flow chart (Fig. 1). Following enzymatic isolation, PAFs were classified based on gross morphology. Only follicles showing an intact basement membrane and adherent cuboidal pre-granulosa cells, ranging from primordial to early secondary staged follicles, were included for the experiments. They were randomly divided in two groups, one of which was analyzed immediately upon isolation to serve as 'untreated control' before vitrification. The other group was subjected to a vitrification/warming cycle and analyzed immediately following warming and constitute the 'treated group'. By comparing results from both groups, net effects of the vitrification procedure were assessed. Follicles isolated from 80 ovarian tissue strips derived from 11 patients were used in the study. Confocal microscopy analysis for the presence of Cx43 and TZPs was carried out for each group. The presence of these cell-cell connections was further confirmed and quantified by analysis of mRNA expression levels of Gap junction protein alpha 1 (Cx43) by using real-time quantitative PCR.

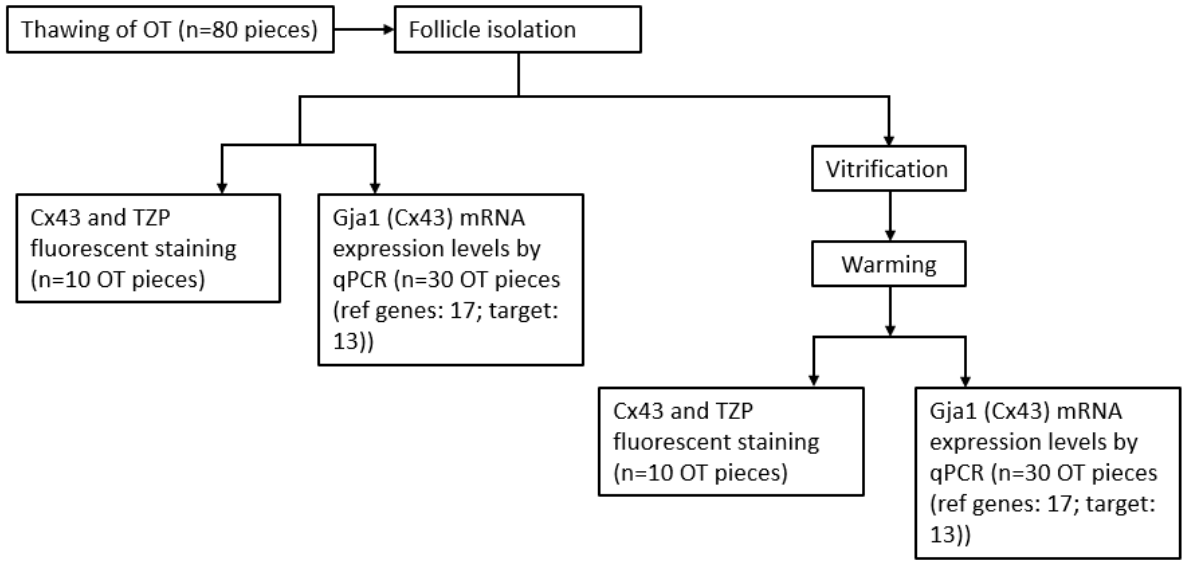

Fig. 1 Experimental design. Follicles were fixed immediately following isolation (untreated group, control); or following a vitrification warming cycle (treated group). Before fixation, follicles were assessed for morphology. After fixation, the follicles were stained to visualize Cx43 and TZPs and cell nuclei with Hoechst staining. In addition, mRNA expression levels of Gap junction protein alpha 1 (Cx43) were analyzed by using real-time quantitative PCR.

Patients

Human ovarian tissue was obtained from 11 consenting female-to-male transgender persons (mean age: 22.8 ± 5.3 years) at the time of gender confirming surgery.

This study was approved by the Ethical Committee of Ghent University Hospital (UZ Ghent Reference: BC-08955).

2.2. Human ovarian tissue

For immediate cryopreservation upon retrieval, the ovaries were bisected and the medulla was carefully removed, resulting in a remaining ovarian cortex of at least 1 mm thickness. Cortical tissue was cut into ~5 × 5 mm pieces under sterile conditions and were transferred to freezing medium containing Leibovitz L-15 medium (Life technologies, Belgium) supplemented with 0.45% human serum albumin (HSA, Red Cross, Belgium) and 1.5 M dimethylsulphoxide (DMSO, Sigma). The tissue was transferred to 1.8 mL cryogenic vials containing 800 µL of freezing medium and incubated for 20 min at 4 °C. For freezing we used the controlled slow rate freezing. The cryogenic vials were placed in

a programmable freezer (Freezal TP60, Air Liquide) by lowering the temperature at 2 °C/min from 4 °C tot -9 °C; manual seeding at -9 °C; cooling at - 0.3 °C/min tot -40 °C; and a final step to -140 °C at a rate of 10° C/min. After programmed slow rate freezing, the vials were transferred to liquid nitrogen and kept in these conditions for long-term storage. For thawing, the vials containing the cortical fragments were placed in a warm water bath at 37 °C for 2 minutes followed by three wash steps in Leibovitz L-15 medium supplemented with 0.45% HSA for 5 minutes each at room temperature. Each piece of ovarian tissue was cut into small pieces (approx. 1-2 mm^3) before it was placed in enzyme solution.

2.3. Isolation of follicles

Ovarian strips (n= 80) were enzymatically digested in a mixture of Liberase TM (0.04 mg/ml) (Roche, Belgium) and DNase (16KUnits/ml) (Sigma) in PBS (Ca^{++}/Mg^{++}) (Gibco). Ovarian strips were incubated for 75-90 min at 37 °C in a water bath. The strips were agitated every 15 min by gentle pipetting. Enzymatic digestion was terminated by the addition of an equal volume of cold (4 °C) PBS (Ca^{++}/Mg^{++}) supplemented with 10% FBS (Sigma). Then, the resulting suspension was transferred to a Petri dish for follicle selection using a stereomicroscope. Based on their morphology, good quality follicles were selected for the experiments. They were collected in gamete buffer (Cook Ireland Ltd., Ireland) under oil for embryo culture (Irvine Scientific, USA) at room temperature, until the process of follicular retrieval was completed (approx. 30 min.).

2.4. Vitrification of isolated follicles

Vitrification was carried out using the Irvine Scientific Vitrification Freeze Kit containing (i) an equilibration solution (ES) (7.5% (*v*/v) ethylene glycol (EG) + 7.5% (*v*/v) DMSO in a M-199 HEPES-buffered medium supplemented with 20% dextran serum supplement (DSS) and 35 µg/ml Gentamycin) and (ii) a vitrification solution (VS) (15% (*v*/v) EG + 15% (*v*/v) DMSO + 0.5 M sucrose in a M-199 HEPES-buffered medium supplemented with 20% DSS and 35 µg/ml Gentamycin). Briefly, the isolated follicles were placed in a 25 µl droplet gamete buffer at room temperature, which was then merged with an adjacent droplet of 25 µl ES and kept at room temperature. After 3 min, it was merged with a second, new adjacent droplet of 25 µl ES and kept at room temperature for 3 min. Then, the follicles were placed in 25 µl ES for 6 min at room temperature. Finally, they were incubated in four drops of 25 µl vitrification solution for less than 1 min at room temperature, followed by loading the follicles into a Cryotop® straw (Kitazato, Japan) using minimal volume. The top was plunged into liquid nitrogen. Moving the Cryotop® in the plastic straw was done in the liquid nitrogen.

2.5. Warming of isolated follicles

For warming, the Irvine Scientific Vitrification Thaw Kit was used with some modifications. The kit contained (i) a thawing solution (TS) (1 mol/l sucrose in a M-199 HEPES-buffered medium supplemented with 20% DSS), (ii) a dilution solution (DS) (0.5 mol/l sucrose in a M-199 HEPES-buffered medium supplemented with 20% DSS) and (iii) a washing solution (WS) (M-199 HEPES-buffered medium supplemented with 20% DSS). The Cryotop® containing the follicles was taken out of the liquid nitrogen and was immediately placed in 0.5 ml prewarmed TS for 1 min at room temperature, followed by two times 3 min in DS and two times 5 min in WS, both at room temperature. The survival rate of the warmed follicles were assessed by morphology.

2.6. Cx43 and TZP staining

The protocol for immunostaining of connexins was adapted from Bus et al. (2021) [25]. Follicles were fixed in 4% paraformaldehyde for 20 min at RT. They were washed in PBS + 1 mg/ml BSA and permeabilized with 1% Triton X-100 (Sigma) and 0.2% Tween 20 (Sigma) in PBS overnight at 4°C. Subsequently, follicles were blocked with a solution consisting of 10% normal goat serum and 0.2% Tween 20 prepared in PBS, at 4 °C for 1h and then incubated with rabbit anti-Cx43 polyclonal antibody (1:268, C6219, Sigma) diluted in blocking solution at 4 °C overnight. Afterwards, they were washed and incubated with goat anti-rabbit secondary antibody conjugated with FITC (1:250, A16105, Invitrogen) for 2h at RT. To visualize filamentous actin (F-actin) and consequently TZPs because they are composed of F-actin, follicles were washed and incubated with Alexa Fluor™ 568 Phalloidin, (1:40, A12380, Thermo Fisher Scientific) for 1 h at RT. Follicles were counterstained with 10 µg/ml Hoechst for 10 min, washed and mounted with Antifade mounting medium (Life Technologies) on microscope slides. Images were obtained using a Leica SP8 confocal microscope equipped with a 405 nm Diode laser (to detect the Hoechst nuclear stain in blue), an Argon laser used at 488 nm (connexins in green) and a DPSS laser used at 561 nm (to visualize TZP staining in red). For Cx43 and TZP analysis, a 60× water immersion (N.A. 1.20) objective was used and image acquisition settings were kept constant for the recordings of all follicles. Z-stacks were taken with a 0.36 μM interval through the complete follicle. The expression of Cx43 was classified as positive (present) or negative (absent). The follicles that showed connexin expression in at least ¾ of GCs through the complete follicle were evaluated as Cx43 positive. The organization of the TZPs was classified as 0: total absence (no physical connections between the oocyte and granulosa cells [no TZPs visible]); 1: partial absence (gaps between the oocyte and granulosa cells [no dense staining of F-actin]); 2: complete (no contact loss between oocyte and granulosa cells [i.e. dense F-actin staining]) (categorization of TZPs was adapted from [26]. Negative controls were obtained by omission of the primary antibodies for Cx43 and omission of TZP staining.

2.7. RNA extraction and cDNA synthesis

Total RNA from tissues were extracted using the RNeasy Micro kit (Qiagen, the Netherlands) according to the manufacturer's protocol using the DNase digestion extraction protocol. To avoid RNA disintegration, samples were stored in Trizol (Life Technologies, Belgium) at -80 °C until the conversion into cDNA was performed. Reverse transcription was performed on 15 µL RNA extraction with the use of the iScript advanced cDNA synthesis kit for RT-qPCR (Bio-Rad Laboratories, Belgium) according to the manufacturer's protocol with a reaction volume of 20 µL. The cDNA concentrations were determined using Qubit 2.0 fluorometric quantification method (Life Technologies). All cDNA samples were stored at −80 °C until qPCR analysis.

2.8. Quantitative real-time PCR

To ensure quality, MiQE guidelines were pursued [27, 28]. Twelve candidate reference genes were used from the human GeNorm kit 12 SYBR Green detection (PrimerDesign, UK) (Table 1). The stability of these reference genes were calculated using a computer algorithm called GeNorm [24, 26] which compares expression variation between each of the candidate reference genes. Reference genes with the lowest *M*-values were considered to be the most stable and therefore the most suitable reference genes in these experimental setting. GeNorm recommends the use of two or three reference genes as a valid and accurate method of normalization.

Primers for Cx43 were designed and subsequently commercially obtained from Thermo Fisher (Table 2). They were tested for their optimal annealing temperature. For normalization of the target gene expression the geometric mean of the 3 candidate reference genes (EIF4A2, 18S and ACTB) were used. RT-qPCR were performed with a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad Laboratories) using iTaq Universal Sybr Green Supermix (Bio-Rad Laboratories). Each quantitative PCR reaction included 15 µL master mix and 5 ng/5 µL cDNA and was performed triplet at the optimal annealing temperature in hard-shell low-profile thin wall 96-well skirted PCR plates, sealed with adhesive film. No template (water) was used as negative control. Thermo cycling conditions were as followed: 3 min at 95 °C followed by 40 cycles of 10 sec at 95 °C followed by 1 min at annealing temperature of 60 °C. To assess the specificity of amplification, a melting curve was carried out on all samples.

All values mentioned hereafter are the normalized values to the mean of both EIF4A2, 18S and ACTB for each sample. A software program, qbasePLUS version 3.4 (Biogazelle BE, Belgium) was used to process the data.

Table 1. Candidate references genes (GeNorm kit, PrimerDesign)

Reference gen	
ACTB	Actin beta
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
UBC	Ubiquitin C
B2M	Beta-2-Microglobulin
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
RPL13A	Ribosomal protein L13a
18S	Eukaryotic 18S rRNA
CYC1	Cytochrome c1
EIF4A2	Eukaryotic translation initiation factor 4A isoform 2
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
TOP1	Topoisomerase (DNA) I
ATP5B	ATP synthase

Table 2. Target gen primer.

Target	Primer sequence
Cx43	Forward: GGAGATGAGCAGTCTGCCTTTC
	Reverse: TGAGCCAGGTACAAGAGTGTGG

2.9. Statistical analyses

For the immunochemistry experiments, Cx43 expression (binary, present vs. absent) and transzonal projection network organization (complete, partial absent vs. absent) were considered a dependent categorical variable. Treatment group was considered an independent categorical variable (categorical: untreated control, vitrified). Potential differences in Cx43 expression and TZP network between groups were analyzed using a Fisher exact test. Differences between groups were considered to be significant when the P value is < 0.05. All statistical analyses were performed using IBM SPSS version 24® (USA).

For the RT-qPCR experiments, Cq values were normalized to the 3 identified reference genes and analyzed using the ANOVA and paired t-test on fold change gene expression using the inbuilt statistical software in qBasePLUS (Biogazelle, version 3.4). Gene expression was studied comparing vitrified and non-vitrified isolated follicles. $P<0.05$ were considered to be statistically significant.

3. Results

3.1. Immunochemistry

3.1.1. Follicle yield

A total of 1665 follicles were isolated from 20 ovarian cortex fragments of approximately 5 × 5 mm. Follicle yield varied considerably between cortex fragments, as already well investigated [30]. The mean ± SD was 83.25 ± 66.20. Intact primordial, primary and early secondary follicles were selected (Fig. 2, 3). Damaged follicles were not included in the experiments (Fig. 4).

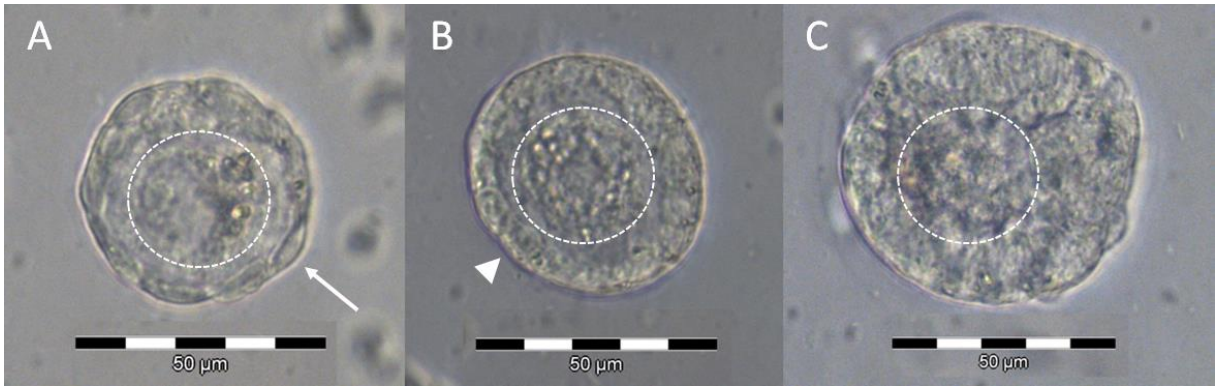

Fig. 2 Light microscopic morphological aspects of different follicle stages upon isolation. A: Primordial follicle. B: Primary follicle. C: Secondary follicle. White arrow indicates flattened granulosa cells, white arrow head indicates cuboidal granulosa cells, white dotted line indicates the oocyte

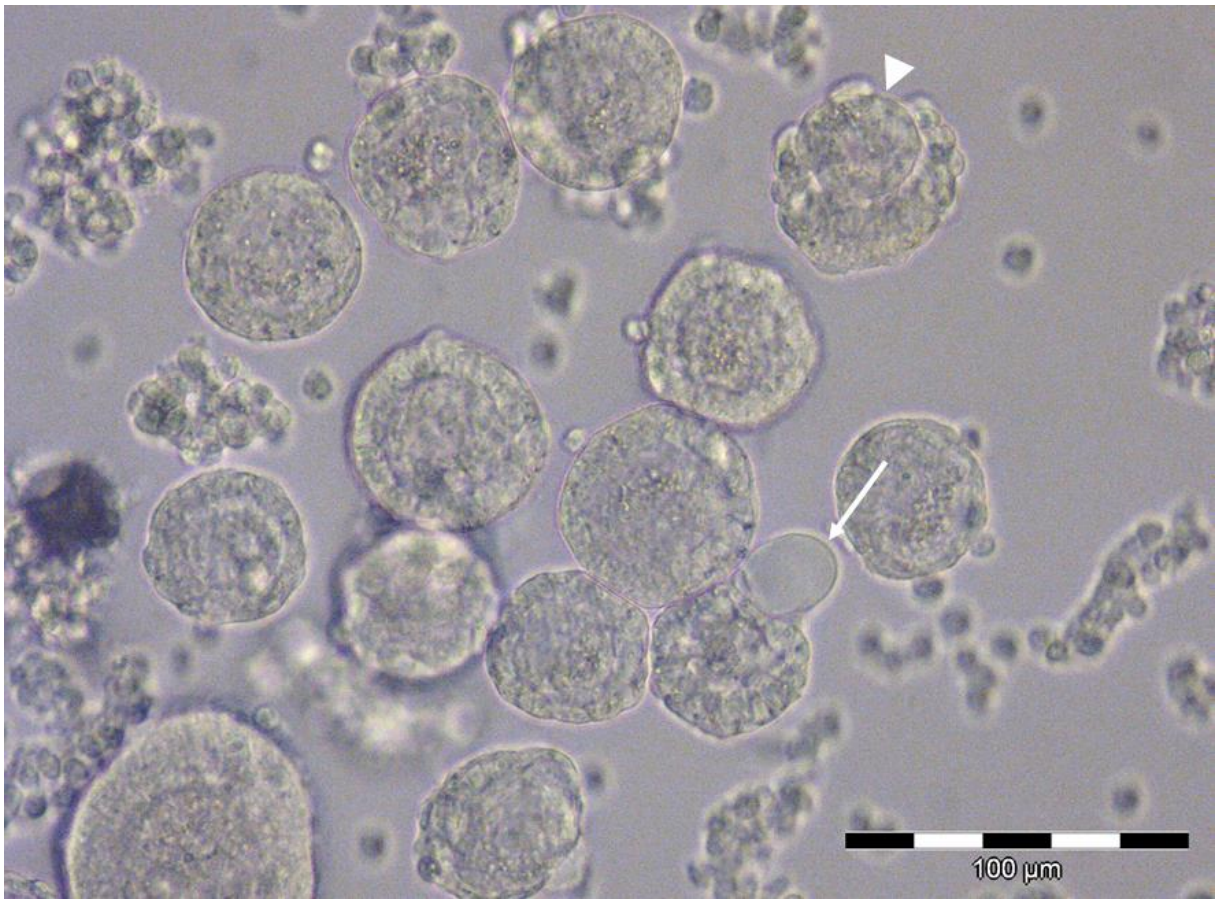

Fig. 3 Isolated human PAFs. The vast majority was presented by primary follicles with an oocyte surrounded by one layer of cuboidal granulosa cells and intact basal membrane. The white arrow indicates an extruded oocyte. The white arrowhead indicates a damaged follicle as the oocyte is not completely surrounded by granulosa cells

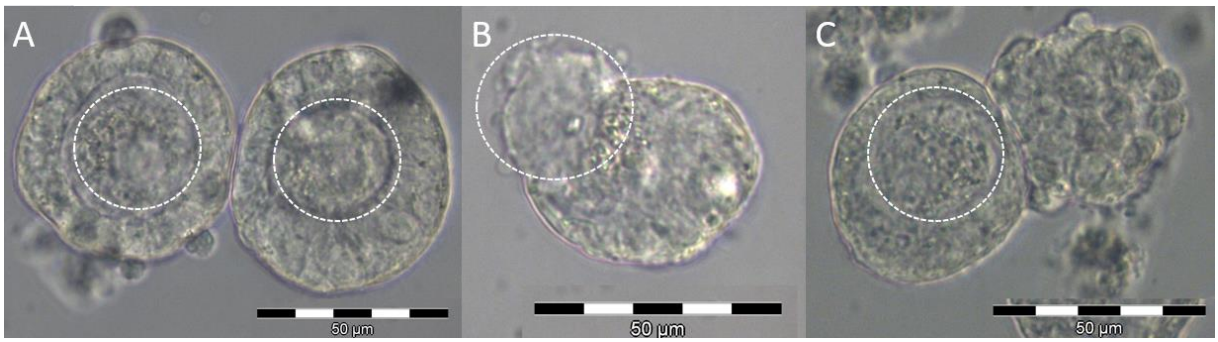

Fig. 4 Isolated human PAFs. A: Intact primary and primary transitioning to secondary follicle. B: Follicle with an extruded oocyte. C: Intact and damaged follicle; the oocyte of the damaged follicle is not clearly visible, granulosa cells are disorganized and are not surrounded by a clearly visible intact basal membrane. White dotted line indicates the oocyte

3.1.2. Follicle survival post warming based on morphology

Post warming, 68,8% of the retrieved follicles (n=846) showed a well preserved morphology. They maintained an intact basal membrane and central oocyte surrounded by granulosa cells (Fig. 5). 31,2%

of follicles failed to survive through the vitrification process and had morphologies consistent with cryo-injury, such as collapsed oocyte, dark granulosa cells, extruded granulosa cells indicating basement membrane disruption or clear space between oocyte and granulosa cells that indicated complete disconnection between the cells (Fig. 5).

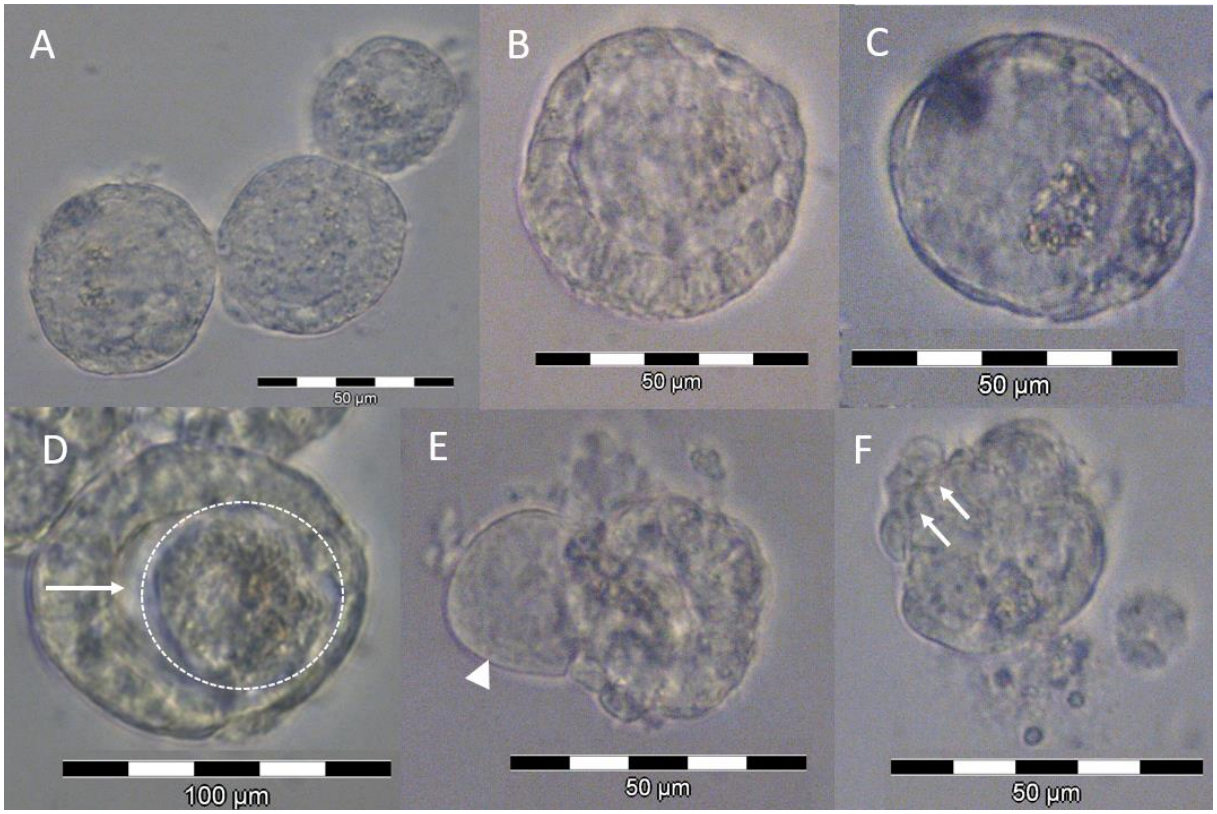

Fig. 5 Human isolated PAFs after vitrification and warming. A,B,C: Intact follicles with a central oocyte and adjacent granulosa cells, and an intact basal membrane. D: Space between oocyte and granulosa cells (indicated by white arrow, white dotted line indicates the oocyte). E: Follicle with an extruded oocyte (white arrow head). F: Basal membrane disruption with extruded granulosa cells (white arrows)

3.1.3. Cx43 and TZP staining

Cx43 and TZPs could be detected in all PAF stages before and following vitrification. In figures 6 and 7, from each group, one follicle was depicted as representative for the whole group. From the morphologically intact follicles that were fixed immediately upon isolation 99.2% (130 out of 131) stained positive for Cx43. From the morphologically intact follicles that were stained for TZPs (n=131), 96.2% (n=126) showed a complete TZP network and 3.8% (n=5) showed partial absence of TZPs. In 95.4% (n=125) of cases, both TZPs and Cx43 were preserved. From the vitrified morphologically intact follicles 88% (221 out of 251) stained positive for Cx43. From the morphologically intact follicles that were stained for TZPs (n=251), 98.4% (n=247) showed a complete TZP network and 1.6% (n=4) showed partial absence of TZPs. In 86.5% (n=217) of cases, vitrification resulted in the preservation of both

TZPs and Cx43. There was a significant difference (P=0.000) in Cx43 expression between untreated and vitrified follicles. On the other hand, there was no significant difference (P=0.157) in organization of the TZP network between untreated and vitrified follicles. Follicles that were morphologically damaged as assessed by light microscopy, were not selected for assessments of follicle integrity upon isolation or following vitrification and warming. Examples are shown in Fig. 8.

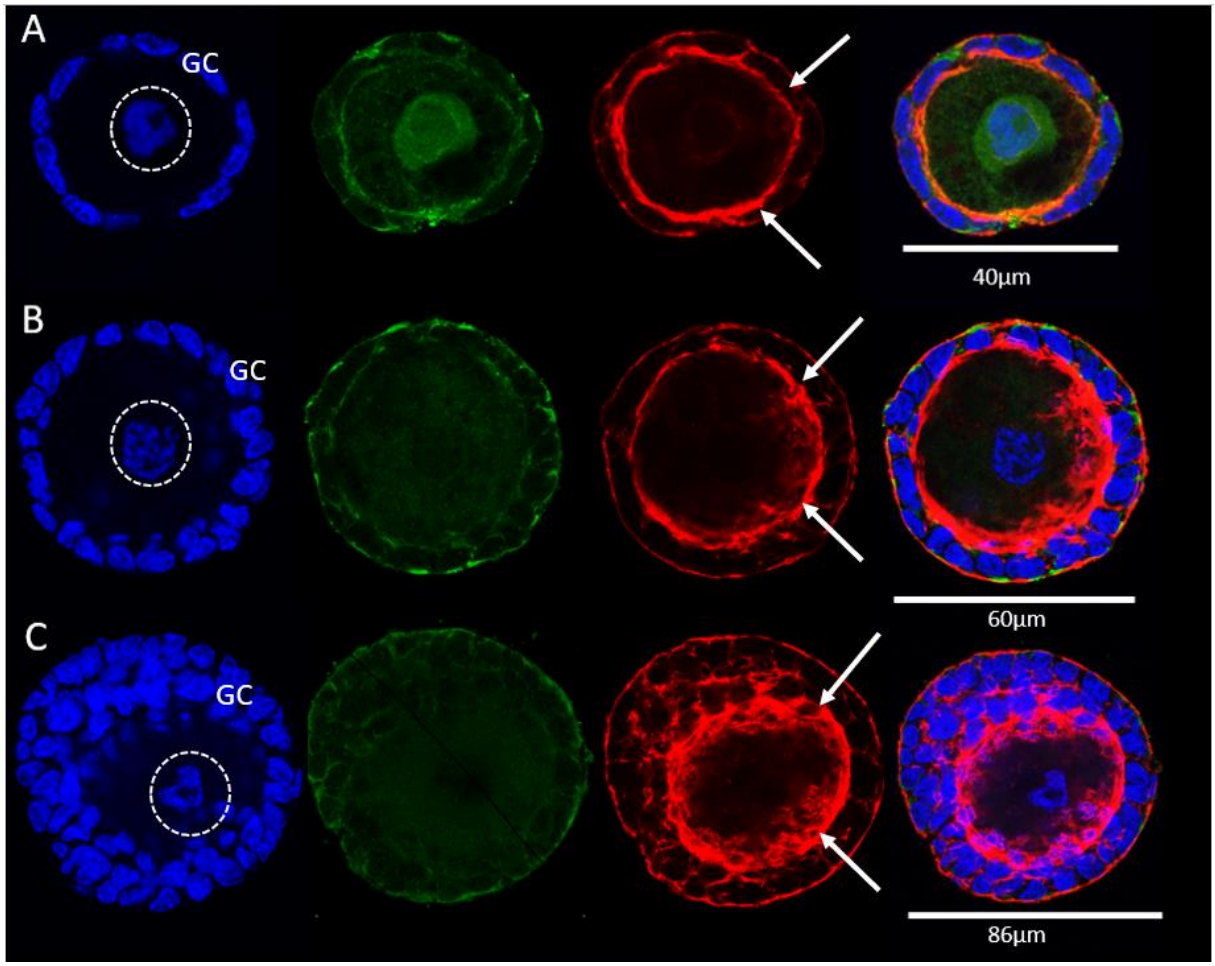

Fig. 6 Confocal images (single confocal plane) of expression and localization of Cx43 and TZPs in all PAF stages upon isolation. A: Primordial follicle; B: Primary follicle; C: Secondary follicle. Hoechst nuclear staining in blue, Cx43 staining in green, and Alexa Fluor™ 568 Phalloidin staining actin-rich TZPs in red; merged picture shows an overlay of the three stains. White dotted line indicates the oocyte. GC: granulosa cells. TZPs are indicated by arrows

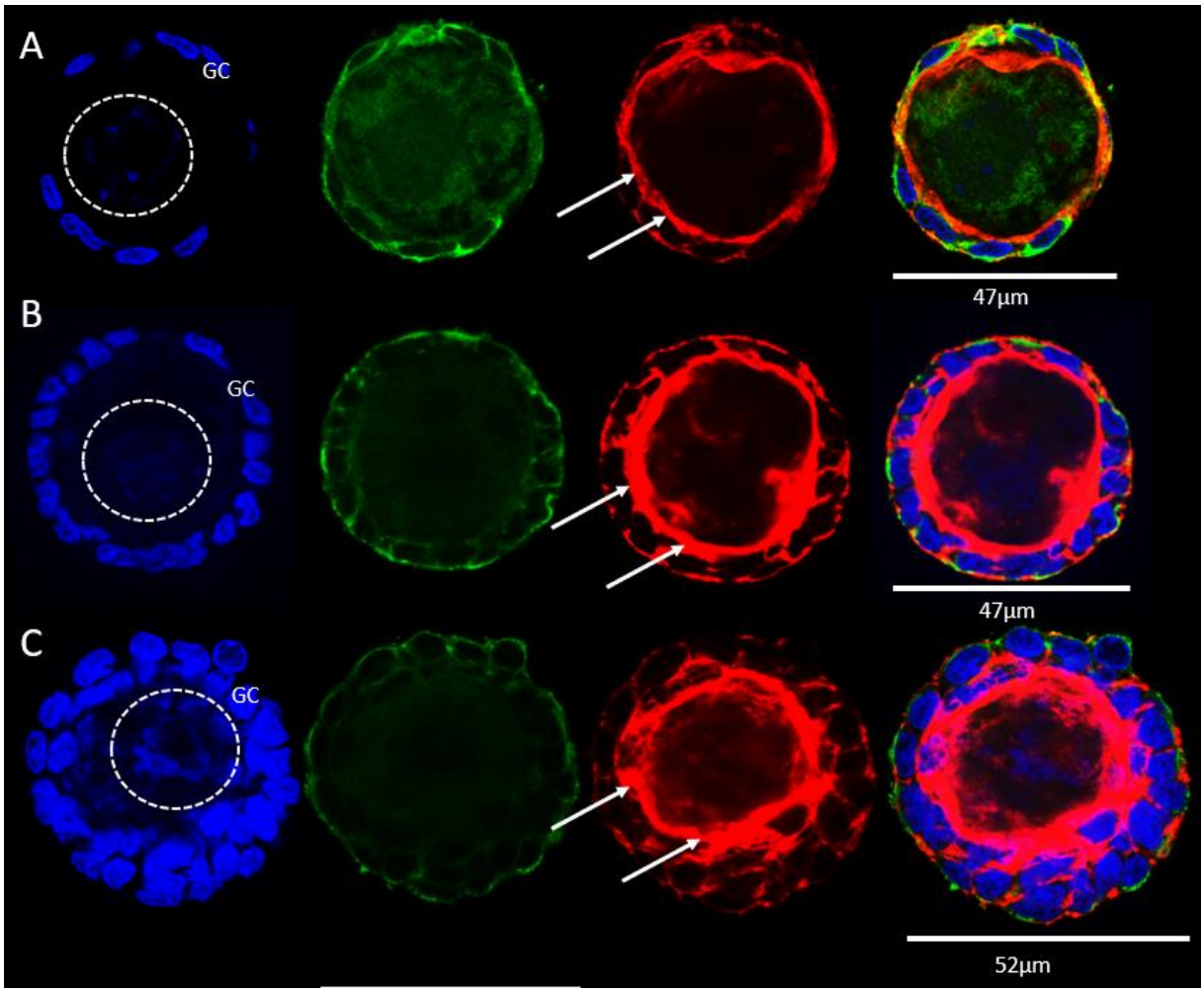

Fig. 7 Confocal images (single confocal plane) of expression and localization of Cx43 and TZPs in all PAF stages following vitrification and warming. A: Primordial follicle; B: Primary follicle; C: Secondary follicle. Hoechst nuclear staining in blue, Cx43 staining in green, and Alexa Fluor™ 568 Phalloidin staining actin-rich TZPs in red; merged picture shows an overlay of the three stains. White dotted line indicates the oocyte. GC: granulosa cells. TZPs are indicated by arrows

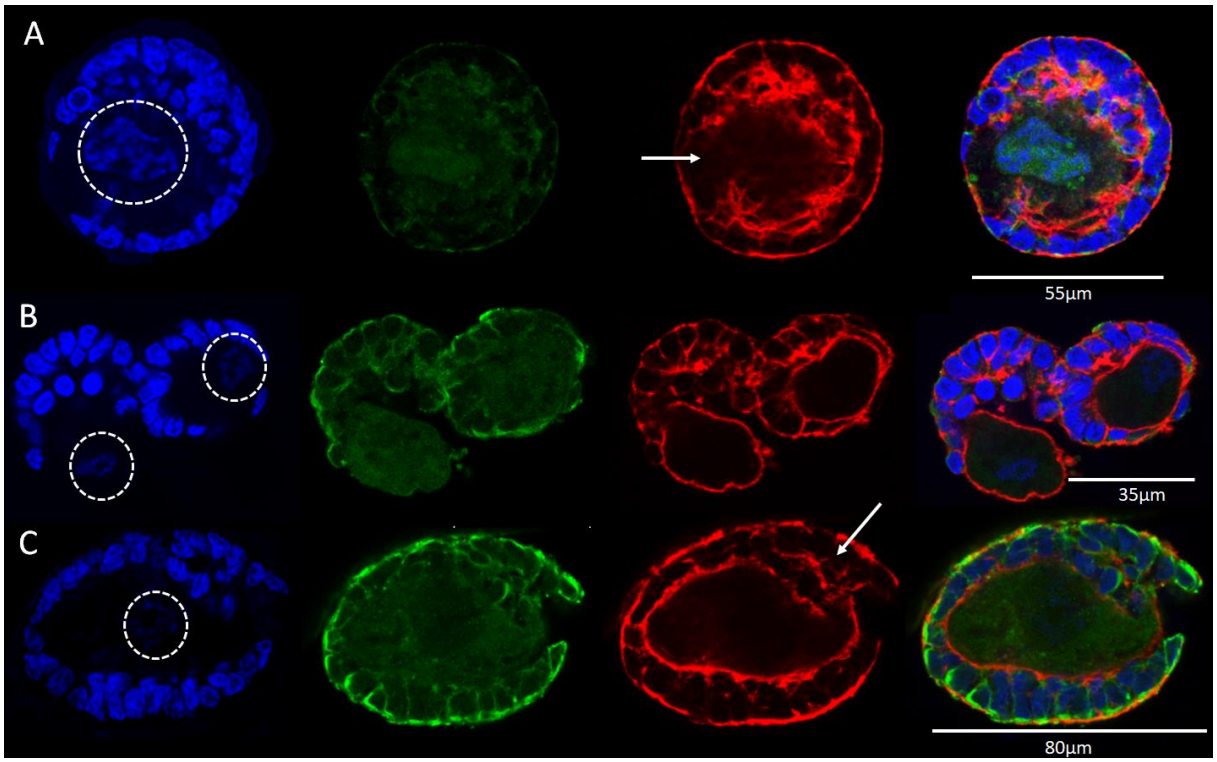

Fig. 8 Confocal images (single confocal plane) of expression and localization of Cx43 and TZPs in damaged PAFs as assessed by gross morphology A: Early secondary follicle upon isolation, staining negative for Cx43, TZP network was disrupted (arrow). B: Two follicles post vitrification and warming. The left follicle shows an extruded oocyte while the right follicle remained intact. C: Primary/beginning secondary follicle upon isolation, positive Cx43 staining, TZP network is disrupted (arrow). This follicle was hit by a glass capillary during manipulation, the cut is can clearly be seen. White dotted line indicates the oocyte

3.2. Gene expression

3.2.1. Reference genes

A total of 1169 follicles were isolated from 34 ovarian cortex fragments from 6 patients. Follicle yield varied between cortex fragments, the mean ± SD was 194.8 ± 79.1. Only intact primordial, primary and early secondary PAFs were included. Of these 1169 follicles, a number of 599 follicles were used for vitrification and warming (treated group). The overall post warming survival rate was 76.8%. A number of 570 follicles were used in the untreated group or control. After cDNA extraction, a mean ± SD of 7.9 ± 3.2 ng/µL cDNA was obtained for the treated group and 10.1 ± 7.0 ng/µL cDNA for untreated group. In this experimental setting, EIF4A2, 18S and ACTB were determined as the three most stable references genes with a M-value of 0.984 (Fig. 9).

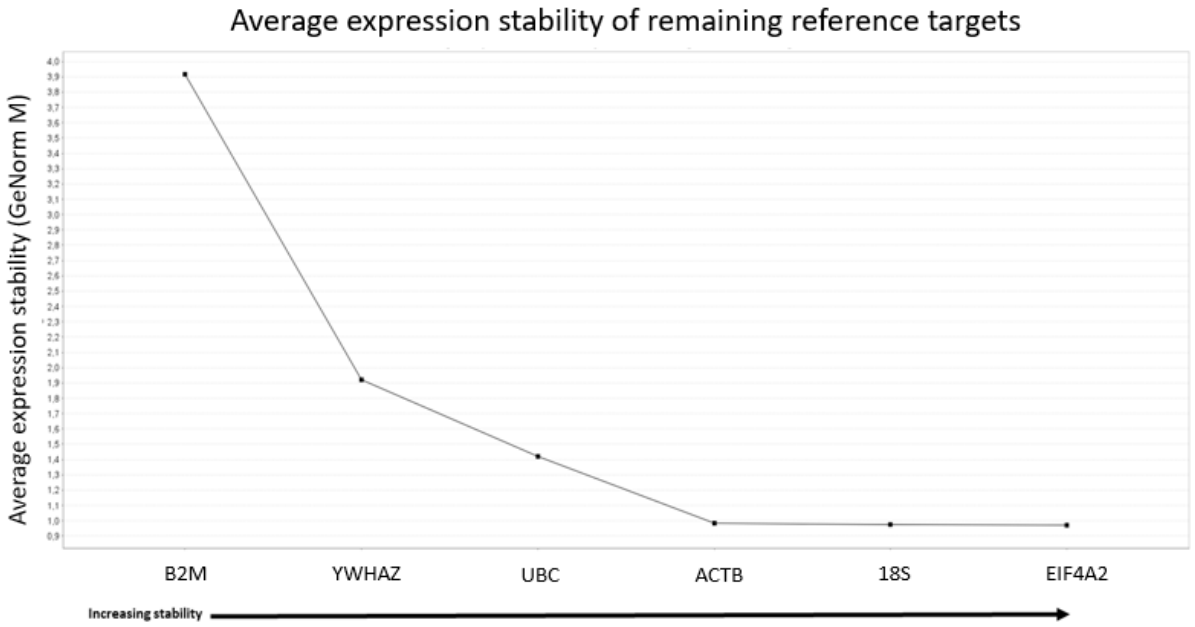

Fig. 9 Ranking of reference genes based on average expression stability values (M-values). The graph shows the average expression stability value (M) for each reference gen ranked according to increasing stability with the most stable genes (lowest M values) on the right (GeNorm 3.4)

3.2.2. Target gene; Cx43

A total of 919 follicles were isolated from 26 ovarian cortex fragments from the same 5 patients that were used for the immunochemistry section. Follicle yield varied between cortex fragments, the mean ± SD was 183.8 ± 24.3. Only intact primordial, primary and early secondary follicles were included. Of these 919 follicles, 519 were used for vitrification and warming (treated group). The overall post warming survival rate was 75.5%. A number of 400 follicles were used in the untreated group. After cDNA extraction, a mean ± SD of 6.5 ± 0.2 ng/µL cDNA was obtained for the treated group and 7.3 ± 1.1 ng/µL cDNA for untreated group.

3.2.3. Relative mRNA expression for Cx43

There was no difference (P = 0.9) in Cx43 mRNA expression between untreated and vitrified follicles (Fig. 10).

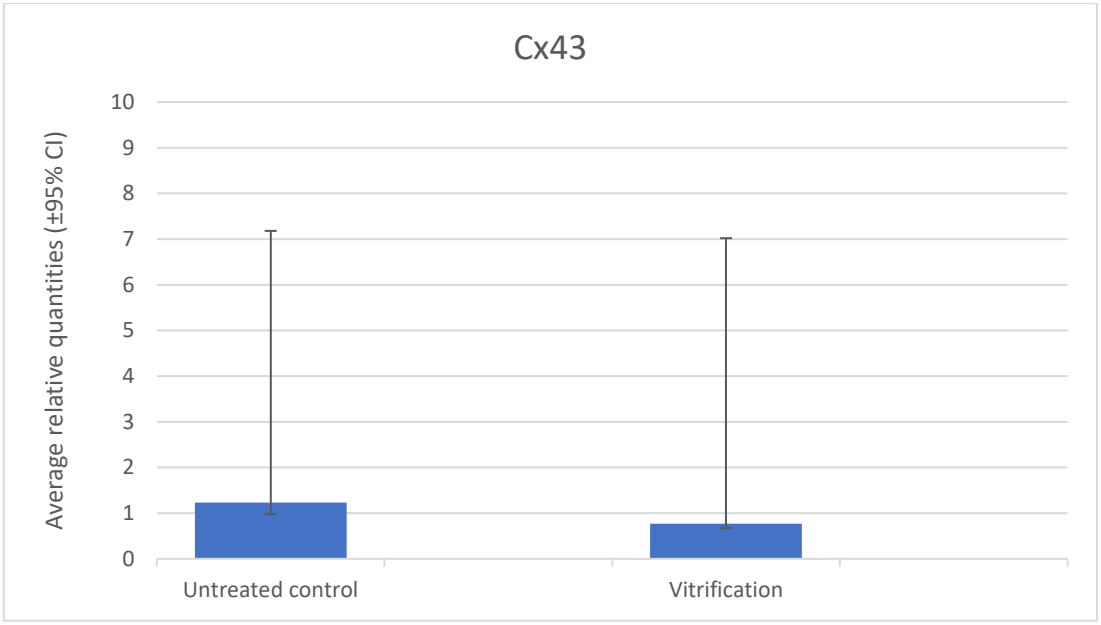

Fig. 10 Relative mRNA expression in untreated control group and vitrified isolated follicles for Cx43. No significant differences were found between both groups in gene expression of Cx43 ($P > 0.05$)

4. Discussion

These data are the first to demonstrate that it is possible to visualize Cx43 and TZPs in isolated human PAFs before and following vitrification and warming, using an Irvine Scientific Vitrification Kit for human oocytes and embryos. The detection and descriptive analysis of immunofluorescent labeled connexins and TZPs can provide an indication about their future developmental capacity post warming.

For the first aim of this study, we assessed the preservation of gross morphology of human PAFs upon isolation and following vitrification and warming. Vitrification of isolated human PAFs is a very innovative concept and at present no (established) vitrification protocols are available. Therefore, human PAFs were vitrified using established protocols for human oocyte vitrification. Already in the first 2 minutes of contact with equilibration solution, follicles shrank and swelled again. In the vitrification solution follicles also shrunk before they were loaded on a straw and plunged in LN_2. During warming, in the first droplet of thawing solution, the follicles appeared almost translucent and irregular in shape. Only when they were washed in the dilution solution and were equilibrated in the first droplet of washing solution, follicles regained their original shape. Therefore, a sufficiently long washing protocol is important for follicle recovery. For the vitrification procedure, a Cryotop® vitrification device was used. Several follicles could be loaded on the fine strip of transparent film which was easy to use. However, for application in clinical practice, which would involve the cryopreservation of hundreds to thousands of follicles, this technique is too laborious and not suitable for bulk

vitrification of PAFs. Post warming, a 73.7% survival rate was obtained as assessed by morphology. It was already demonstrated that increased vacuolization in the cytoplasm and disruption of cell-to-cell contacts between the germinal and somatic cell compartments are the main signs of cryodamage [31, 32]. Therefore, the visualization and quantification of cell-cell contacts as Cx43 and TZPs should be performed to provide an indication about future follicle developmental capacity post warming.

For the second aim of this study, we visualized Cx43 and TZPs in isolated human PAFs following vitrification and warming. Several studies, mostly on animal models and dealing with ovarian tissue, have been conducted on the expression of connexins and TZPs and most of them demonstrated that cryopreservation might cause damage to these membrane proteins, although the results are not consistent [25]. Our results show that Cx43 expression in vitrified isolated follicles (88%) was lower compared to follicles that were fixed upon isolation (99.2%) (P=0.00). Vitrified follicles showed a complete TZP network in 98.4% of cases, compared with 96.2% in follicles fixed upon isolation (P=0.157). Given that the presence of Cx43 and an intact TZP network is relatively high in both groups, morphology gives a good indication on follicle survival and integrity of cell connections.

For the third aim of this study, we measured mRNA expression levels of gap junction protein alpha 1 (Gja1; Cx43). There was no difference (P=0.900) in Cx43 mRNA expression between untreated and vitrified follicles. This contrasts to Cx43 protein expression as assessed by immunostaining, where a significant difference was found between the untreated and vitrified group. Therefore, quantification instead of categorization of immunostaining would be the preferable option. In the current experimental setting, we were unable to analyse Cx37. High Cq values and absence of melting curves originated from too low Cx37 gene expression in the sample. In order to analyse the gene expression of Cx37, a higher amount of cDNA input is necessary which was not possible in the current study design. In a study of Grosbois at al. (2019) [33], GJA4 mRNA was also not detected in human early staged isolated follicles. This can be explained by the very low expression levels of Cx37 in early human PAFs as demonstrated by Kristensen at al. (2015) [34].

For this study, frozen ovarian tissue was used. Earlier studies have shown no difference in follicle viability and developmental competence between fresh or frozen-thawed tissue [35-37], however caution is advised. Although morphologically normal follicles were selected, they might be affected by the freezing procedure in a way that it is only visible on a longer term. Therefore, this study should be repeated with fresh OT to confirm our findings. Moreover, as most follicles from frozen/thawed OT appear to survive the vitrification protocol, a potential application could be the vitrification of remaining follicles for future use. This applies for example if follicles from thawed OT fragments for

transplantation or an experiment were not all used (lack of place for transplantation, more follicles than necessary for an experiment, etc.).

In conclusion, we visualized Cx43 and TZPs and measured Cx43 mRNA in isolated human PAFs upon isolation and following vitrification and warming, which suggests the maintenance of communication between the oocyte and the somatic companion cells in most morphologically normal follicles. Isolated human PAFs were successfully vitrified and warmed using Cryotop®. However, it is necessary to develop a high throughput system that can be used for clinical purposes. In addition, effects of enzymatic isolation and vitrification, warming and culture should be investigated on the long-term to assure the production of developmentally competent and epigenetically normal oocytes.

References

1. Detti, L., D.C. Martin, and L.J. Williams, *Applicability of adult techniques for ovarian preservation to childhood cancer patients.* Journal of assisted reproduction and genetics, 2012. **29**(9): p. 985-995.
2. Rodriguez-Wallberg, K.A. and K. Oktay, *Options on fertility preservation in female cancer patients.* Cancer treatment reviews, 2012. **38**(5): p. 354-361.
3. Hyman, J.H. and T. Tulandi, *Fertility preservation options after gonadotoxic chemotherapy.* Clinical Medicine Insights: Reproductive Health, 2013. **7**: p. CMRH. S10848.
4. Leung, W., et al., *Late effects of treatment in survivors of childhood acute myeloid leukemia.* Journal of Clinical Oncology, 2000. **18**(18): p. 3273-3279.
5. Lee, S.J., et al., *American Society of Clinical Oncology recommendations on fertility preservation in cancer patients.* Journal of clinical oncology, 2006. **24**(18): p. 2917-2931.
6. Agarwal, S.K. and R.J. Chang, *Fertility management for women with cancer.* Oncofertility Fertility Preservation for Cancer Survivors, 2007: p. 15-27.
7. Donnez, J., et al., *Ovarian cortex transplantation: time to move on from experimental studies to open clinical application.* Fertility and sterility, 2015. **104**(5): p. 1097-1098.
8. Medicine, P.C.o.t.A.S.f.R., *Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion.* Fertility and sterility, 2019. **112**(6): p. 1022-1033.
9. de Figueiredo, J.R., et al., *Advances in in vitro folliculogenesis in domestic ruminants.* Animal Reproduction, 2019. **16**(1): p. 52.
10. Fisch, B. and R. Abir, *Female fertility preservation: past, present and future.* Reproduction, 2018. **156**(1): p. F11-F27.
11. Dolmans, M.-M., *From isolated follicles to the artificial ovary: why and how?* Current Opinion in Endocrine and Metabolic Research, 2021.
12. Motta, P.M., et al., *Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy.* Archives of histology and cytology, 1994. **57**(4): p. 369-394.
13. Larsen, W., *Biological implications of gap junction structure, distribution and composition: a review.* Tissue and Cell, 1983. **15**(5): p. 645-671.
14. Simon, A.M., et al., *Female infertility in mice lacking connexin 37.* Nature, 1997. **385**(6616): p. 525-529.
15. Winterhager, E. and G.M. Kidder, *Gap junction connexins in female reproductive organs: implications for women's reproductive health.* Human reproduction update, 2015. **21**(3): p. 340-352.
16. Gittens, J.E., et al., *Interplay between paracrine signaling and gap junctional communication in ovarian follicles.* Journal of Cell Science, 2005. **118**(1): p. 113-122.
17. Gittens, J.E. and G.M. Kidder, *Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries.* Journal of cell science, 2005. **118**(21): p. 5071-5078.
18. Veitch, G.I., et al., *Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface.* Journal of cell science, 2004. **117**(13): p. 2699-2707.
19. Anderson, E. and D.F. Albertini, *Gap junctions between the oocyte and companion follicle cells in the mammalian ovary.* The Journal of cell biology, 1976. **71**(2): p. 680-686.
20. Kidder, G.M. and A.A. Mhawi, *Gap junctions and ovarian folliculogenesis.* REPRODUCTION-CAMBRIDGE-, 2002. **123**(5): p. 613-620.
21. da Silva, A.M.S., et al., *Connexin 37 and 43 gene and protein expression and developmental competence of isolated ovine secondary follicles cultured in vitro after vitrification of ovarian tissue.* Theriogenology, 2016. **85**(8): p. 1457-1467.
22. Vanhoutte, L., et al., *Effects of chilling on structural aspects of early preantral mouse follicles.* Biology of reproduction, 2004. **70**(4): p. 1041-1048.

23. Barrett, S.L., L.D. Shea, and T.K. Woodruff, *Noninvasive index of cryorecovery and growth potential for human follicles in vitro.* Biology of reproduction, 2010. **82**(6): p. 1180-1189.
24. Trapphoff, T., et al., *DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles.* Human Reproduction, 2010. **25**(12): p. 3025-3042.
25. Bus, A., et al., *Preservation of connexin 43 and transzonal projections in isolated bovine pre-antral follicles before and following vitrification.* Journal of Assisted Reproduction and Genetics, 2021. **38**(2): p. 479-492.
26. Lierman, S., et al., *Follicles of various maturation stages react differently to enzymatic isolation: a comparison of different isolation protocols.* Reproductive biomedicine online, 2015. **30**(2): p. 181-190.
27. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome biology, 2002. **3**(7): p. 1-12.
28. Hellemans, J., et al., *qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.* Genome biology, 2007. **8**(2): p. 1-14.
29. Hellemans, J. and J. Vandesompele, *Selection of reliable reference genes for RT-qPCR analysis*, in *Quantitative Real-Time PCR*. 2014, Springer. p. 19-26.
30. Schmidt, K., et al., *Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries.* Human Reproduction, 2003. **18**(6): p. 1158-1164.
31. Keros, V., et al., *Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue.* Human reproduction, 2009. **24**(7): p. 1670-1683.
32. Fabbri, R., et al., *Morphological, ultrastructural and functional imaging of frozen/thawed and vitrified/warmed human ovarian tissue retrieved from oncological patients.* Human Reproduction, 2016. **31**(8): p. 1838-1849.
33. Grosbois, J., et al., *Ultrastructure and intercellular contact-mediated communication in cultured human early stage follicles exposed to mTORC1 inhibitor.* Molecular human reproduction, 2019. **25**(11): p. 706-716.
34. Kristensen, S.G., P. Ebbesen, and C.Y. Andersen, *Transcriptional profiling of five isolated size-matched stages of human preantral follicles.* Molecular and cellular endocrinology, 2015. **401**: p. 189-201.
35. Abir, R., et al., *Pilot study of isolated early human follicles cultured in collagen gels for 24 hours.* Human Reproduction, 1999. **14**(5): p. 1299-1301.
36. Hovatta, O., et al., *Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture.* Human reproduction (Oxford, England), 1997. **12**(5): p. 1032-1036.
37. Oktay, K., et al., *Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue.* Fertility and sterility, 1997. **67**(3): p. 481-486.

CHAPTER 7A

TECHNICAL NOTES RELATED TO FOLLICLE CRYOPRESERVATION

Cryopreservation strategies of pre-antral follicles

1. Vitrification

Vitrifcation of human oocytes and embryos in different stages of development is a key element of daily clinical practice of *in vitro* fertilization treatments and for the preservation of fertility. The vitrification of isolated PAFs however, is a very young and small field of research. The procedures used to freeze isolated follicles are based on protocols developed for embryos and mature oocytes. To improve follicular survival rates, it is important to design cryopreservation protocols specifically for follicles.

1.1. A simplified protocol for the vitrification of isolated pre-antral follicles

One of the first aims of this doctoral thesis was to vitrify isolated bovine PAFs while maintaining their viability. A commercially available Vitrification Kit (IrvineScientific®, Alere Health BV. Tilburg, NL) for human oocytes and embryos was used in this experiment with a slightly modified protocol. We reduced the number of transfer steps and shortened the exposure times to CPAs as it did not hamper the survival rate of isolated bovine PAFs. We found this modification beneficial because of the reduced exposure to suboptimal temperature and osmolarity, as well as potentially toxic CPAs. In addition, the risk of losing follicles is reduced due to fewer transfer steps and the working time was decreased. Indeed, the vitrification procedure as a whole is time consuming as most of the duration is employed in a long (8–15minutes), gradual or direct exposure to equilibration CPA solution, which is followed by a short exposure to a more concentrated vitrification solution. This procedure results in an increased intra- and extra- cellular viscosity to a level that the liquid water molecules will solidify so quickly that they will not have time to arrange themselves into a crystalline structure. The duration of exposure to permeable CPAs is determined by several biophysical factors such as the membrane properties (cellular permeability to water and CPA), the types and concentration of CPA, the surface/volume ratio of the cells, and the rate of cooling and warming. Preliminary experiments exposing isolated secondary bovine PAFs to two standard CPAs (1-5 M EG and 1-5 M DMSO) for 5 minutes showed that category 1 follicles followed a typical shrink-swell curve initiated by volume changes caused by exposure to CPAs [1].

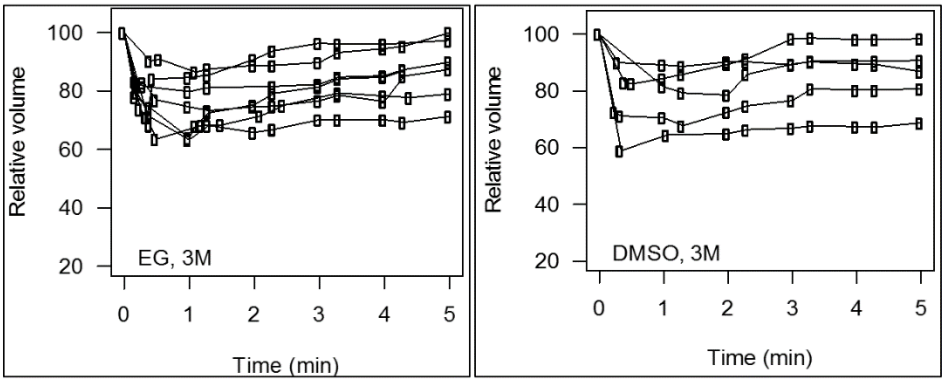

Fig. 1 Osmotically induced volume changes of isolated bovine early PAFs exposed to 3M EG (left) or DMSO (right) in isolation medium at 24 °C [2]

This work also indicated that the dehydration upon exposure occurred very fast: the point of minimum volume of the shrink-swell curve was reached within 2 minutes (Fig 1.) as also shown by Molina et al. (2019) [3] in human oocytes and zygotes. At that point, outflow of water caused by the external hyperosmotic solution and the subsequent permeation of low molecular weight penetrating CPAs is complete, leading to an osmotic equilibrium between intracellular and extracellular solutions. This shows that prolonging the exposure to the CPA solutions does not contribute to a higher protection level of CPAs as the optimal uptake level of CPAs is already reached. In a study performed on ovine primordial follicles, Amorim et al. (2006) [1] demonstrated that at 2 minutes of CPA exposure, all follicles were shrunken and had re-expanded to 90% or more of their original volume at 11 minutes after exposure to DMSO in all concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mol/L) and at 14 minutes after exposure to EG in almost all concentrations. However, our research demonstrated that an exposure time of 5 minutes to different concentrations of EG and DMSO was long enough for most bovine PAFs to re-expand to approximately 90% of their original volume.

When exposing follicles to CPAs, attention should be paid that changes in follicular volume do not exceed $\pm$ 30 - 40% [4, 5]. Indeed, severe variations in follicular volume can possibly lead to cell damage or even cell death or can sensitize the follicle to injuries during subsequent steps of the cryopreservation protocol. In order to reduce major volume changes, the exposure temperature of the CPA solution could be lowered [6] or two-step protocols could be used [7]. To gradually expose the follicles to higher concentrations of CPAs and avoid toxic or osmotic injuries, we merged the drop of culture medium with the follicles into the first drop of equilibration solution (7.5% DMSO, 7.5% EG) for 2 min. The follicles were then transferred to a second drop of equilibration solution for 2 min. Finally they were transferred to a drop of vitrification solution (15% DMSO, 15% EG, 0.5 M sucrose) for 30 s, where sucrose, as non-penetrating CPA, induces dehydration to reach an intracellular vitrified state and maintain a vitreous state during cooling and warming.

1.2. Increasing the efficiency of the vitrification protocol for isolated PAFs

In our earliest experiments **(Chapter 3)** HSV Straws® (HSV Kit, Groupe I.M.V. Technologies, Clemenceau, France) were used as a carrier. Follicles were individually or per group of maximum 5 follicles placed on a straw which was put in LN_2. Post-warming, a 87.5% follicle survival rate was obtained as demonstrated following Neutral Red staining. We, however, encountered a relatively high loss of follicles during transfer through different vitrification media, and at the moment that follicles were collected at warming. In equilibration and vitrification media follicles float in different directions and when they shrink and swell follicles tend to 'swirl' out of focus, they also become almost translucent, which made it very hard to follow follicles visually under the microscope. The collection of follicles in a very small volume (1 µL) of vitrification medium, which needs to be put on a straw, is therefore very difficult and made us decide to vitrify follicles individually or with only two in one time. At warming, we found that follicles sometimes stayed attached to the straw, or disappeared without knowing where they got lost. This way of working was very time-consuming and a relatively big loss of follicles could not be prevented, therefore an important objective became to make the vitrification procedure more efficient while maintaining follicle quality. This resulted in the use of a 3D culture system which facilitated the manipulation of isolated follicles **(Chapter 3)**. In a study of Bian et al. (2013) [8] human PAFs embedded in a 1.5% sodium alginate solution were successfully vitrified. In this way follicles were better protected to the harmful CPA concentrations used during vitrification. Moreover, follicular structure is better maintained in a 3D culture system as it better simulates the natural environment of the ovary. This enhances the preservation of the normal shape of the follicles. We did not detect a difference in follicle survival during short term culture between 2D or 3D culture systems (embedding in alginate beads). Follicle survival following vitrification and warming in a 3D system (45.9%) was lower compared to follicle survival following vitrification of non-embedded follicles using HSV straws (87.5%). However, when follicles were embedded, much less follicles were lost during the vitrification process. In addition, the transfer of follicles from one medium to another, was much easier. An alginate bead, which contained several follicles, was transferred in a self-made cup from a metal mesh cell strainer so that the individual manipulation of follicles was omitted.

1.3. Optimizing the changes for successful vitrification

During vitrification, liquid water is converted to a solid amorphous glass and not to ice crystals, which can form inside the cell during cooling and during warming. The formation of ice crystals can cause cell damage and will determine the viability of cells. Independent of the carrier device that determines the

cooling and/or the warming rate, the key of success in order to achieve a “glass-like” state depends on an optimal balance between the speed of cooling - rewarming (time and temperature) and the optimal cell dehydration and penetration of CPAs when they are exposed to concentrated hypertonic solutions [9]. It is supposed that ultra-rapid cooling and warming rates (as high as 20.000 °C – 30.000 °C/min) are mandatory during the vitrification process to reduce the risk of intracellular crystal formation and the resulting damage to the cell structures [10]. To achieve such high cooling rates, a very small volume of vitrification solution of less than 1 µl is deposited on open carrier devices, e.g., Cryotop [11], Vitriplug [12], open pulled straws [13], Cryoloop [14, 15] and copper electron microscopy grids [16] which are directly plunged into LN_2.

Martino et al. [16] used EM grids for the cryopreservation of bovine oocytes and reported a significantly higher survival rate for vitrified-warmed oocytes using grids as compared to oocytes frozen in straws. These results may be explained by the physical characteristics such as the ultrathermo- conductivity of the metal grid that results in about threefold higher cooling rates than that obtained with straws [17]. We might expect that cooling and warming rates are potentially as high for EM grids as for metal mesh cups **(Chapter 3)**. However on the other hand, embedding of PAFs in alginate beads increases the volume considerably, this may decrease the cooling rate, leading to a lower survival rate. Before transferring the cell strainer with beads to another medium or plunging it in LN_2, the vitrification solution was removed by dipping on an absorbent paper towel, minimizing its thermal mass and hence reducing vapour coat formation. This redresses the concern that the amount of vitrification solution around the samples might decrease the cooling rate due to the formation of a nitrogen vapour coat [18]. Furthermore, cell strainers are kept under the LN_2 surface by tweezers to prevent them from floating up and keep the cells immersed in the liquid nitrogen, which would also reduce formation of a vapour coat and increase the cooling rate.

During the vitrification process, the manipulation of PAFs embedded in alginate beads was greatly improved in terms of follicle losses and timewise, however, follicle survival decreased significantly. Therefore, in a next study we tried to retain the efficiency of the procedure and preserve follicle quality at the same time by vitrifying non-embedded PAFs using mini cell strainers **(Chapter 5)**. With the use of non-embedded follicles we aimed for better CPA permeation. We choose to work with cell strainers instead of self-made metal mesh cups because non-embedded follicles were very difficult to visualize on the thick dark mesh. Cell strainers are commercially available, have a convenient size and a small mesh size. However, cell strainers have relatively large working dimensions and thick walls and are made of plastic, a non-conductive material, which may negatively affect the cooling rate. Multiple follicles can be vitrified at the same time, as a mini cell strainer can hold many more follicles, whereas only a few follicles can be loaded into one straw. It was a much easier, quicker and simpler way to freeze PAFs. A difficulty experienced at warming was the retrieval of the follicles from the mesh, NR

staining however contributed to a higher visibility and served as viability assessment at the same time. Despite the ease of this technique, we encountered a relatively high follicle loss (the recovery rate varied between 33.3% and 69.2%), whether this is due to follicle shrinkage, follicle degeneration or hindered visibility by the mesh, needs to be elucidated. In addition, long term storage of PAFs in cell strainer is not desirable and needs further investigation. Following our search to more efficiently vitrify isolated PAFs while maintaining follicle quality, we can conclude that both techniques had their advantages but we cannot ignore their downsides. Further research is therefore required to establish a cryopreservation procedure that can be applied in a high throughput system while follicle health is maintained.

1.4. Influence of follicle quality and other factors influencing vitrification success

When it comes to vitrification we experienced that starting with follicles of good quality was very important for subsequent follicle survival. We observed that secondary category 1 follicles followed a typical shrink-swell curve initiated by volume changes caused by exposure to CPAs [1], whereas category 2 follicles did not show a typical shrink-swell curve and were less viable following vitrification. It is difficult to compare results of vitrification protocols between different laboratories and, even when using the same protocol, they are often conflicting. Vitrification protocols are influenced by the technical expertise and skills of the person carrying out the procedure. Next to the type and concentration of the CPAs, time and temperature are critical parameters for follicular survival, as are cooling and warming rates. These are difficult to maintain under perfect control, so differences in results can be expected even when using the same protocol.

2. Slow freezing

Next to vitrification we investigated the use of slow freezing, for its simplicity of use and inexpensiveness, on isolated PAFs but also on ovarian tissue. Whereas in Chapter 3 and 5 the bovine model was used, in Chapter 4 and 6, a shift towards the use of human material was made.

In order to cryopreserve isolated PAFs in a way that it is applicable in a high throughput system, we investigated the feasibility of PSF for isolated PAFs (using Nalgene's Mr. Frosty container). A high throughput system is important for clinical as well as research purposes. The vitrification of high numbers of isolated follicles is still very work and time consuming as hundreds to thousands of follicles are potentially isolated from a few ovarian cortex pieces. Therefore, the labor and the amount of CPAs needed make it very expensive. In a preliminary experiment we added secondary, category 1 bovine

follicles, after 2 days of culture to a cryovial with freezing medium consisting of 0.75 M DMSO and 0.75 M EG supplemented with 10% fetal bovine serum, 0,1 M sucrose and DMEM/F12. The cryovials were placed in the Mr. Frosty container overnight at -80 °C, transferred to liquid nitrogen at -196 °C and stored for 1-2 weeks. From a total number of 151 follicles that were frozen and thawed, we achieved a retrieval rate of 91% and a viability rate of 75% 24 hours following thawing (well preserved morphology and Neutral Red positive staining). Based on these results we may conclude that PSF is an effective and efficient way to cryopreserve bovine isolated PAFs, although effects on the long term still need to be investigated. However, when the same technique was applied to human isolated PAFs, following thawing, follicles appeared to be damaged by the irregular shape and dark color of the follicles. Follicles showed to be not viable by Neutral Red and Trypan Blue staining. The reason for the different outcome following PSF between bovine and human PAFs still needs to be clarified.

We aimed to assess the feasibility of passive slow freezing (PSF using Nalgene's Mr. Frosty container) as an alternative to controlled slow rate freezing (CSF using Air Liquide's Freezal™) for human OTC **(Chapter 4)**. PSF offers advantages compared to CSF as it is an inexpensive and uncomplicated technique. The application of PSF to OTC has shown that follicle growth *in vitro* and in mouse models is similar to that achieved with CSF, suggesting that PSF can be an interesting alternative device for OT slow freezing.

References

1. Amorim, C.A., et al., *Permeability of ovine primordial follicles to different cryoprotectants.* Fertility and sterility, 2006. **85**: p. 1077-1081.
2. de porte, H.F., *Permeability of bovine early pre-antral follicles to different cryoprotectant agents*, in *Master Thesis*. 2015.
3. Gallardo Molina, M., J.L. Sáenz Cuesta, and R. Risco, *Human oocytes and zygotes are ready for ultra-fast vitrification after 2 minutes of exposure to standard CPA solutions.* 2019.
4. Madden, P. and D. Pegg, *Calculation of corneal endothelial-cell volume during the addition and removal of cryoprotective compounds.* Cryo-letters, 1992. **13**(1): p. 43-50.
5. Newton, H., *The cryopreservation of ovarian tissue as a strategy for preserving the fertility of cancer patients.* Human reproduction update, 1998. **4**(3): p. 237-247.
6. Hunt, C.J., S.E. Armitage, and D.E. Pegg, *Cryopreservation of umbilical cord blood: 2. Tolerance of CD34+ cells to multimolar dimethyl sulphoxide and the effect of cooling rate on recovery after freezing and thawing.* Cryobiology, 2003. **46**(1): p. 76-87.
7. Woods, E., et al., *Osmometric and permeability characteristics of human placental/umbilical cord blood CD34+ cells and their application to cryopreservation.* Journal of hematotherapy & stem cell research, 2000. **9**(2): p. 161-173.
8. Bian, J., et al., *Vitreous cryopreservation of human preantral follicles encapsulated in alginate beads with mini mesh cups.* Journal of Reproduction and Development, 2013: p. 2012-157.
9. Leibo, S. and T.B. Pool, *The principal variables of cryopreservation: solutions, temperatures, and rate changes.* Fertility and sterility, 2011. **96**(2): p. 269-276.
10. Lane, M., et al., *Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique.* Fertility and sterility, 1999. **72**(6): p. 1073-1078.
11. Kuwayama, M., *All round vitrification of human oocytes and embryos.* J. Assist. Reprod. Genet., 2000. **17**: p. 477.
12. Stinshoff, H., et al., *Cryopreservation affects the quality of in vitro produced bovine embryos at the molecular level.* Theriogenology, 2011. **76**(8): p. 1433-1441.
13. Vajta, G., et al., *Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos.* Molecular Reproduction and Development: Incorporating Gamete Research, 1998. **51**(1): p. 53-58.
14. Lane, M., et al., *Containerless vitrification of mammalian oocytes and embryos.* Nat Biotechnol, 1999. **17**(12): p. 1234-6.
15. Mukaida, T., et al., *Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles.* Human Reproduction, 2003. **18**(2): p. 384-391.
16. Martino, A., N. Songsasen, and S. Leibo, *Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling.* Biology of reproduction, 1996. **54**(5): p. 1059-1069.
17. Steponkus, P., et al., *Cryopreservation of Drosophila melanogaster embryos.* Nature, 1990. **345**(6271): p. 170-172.
18. Wang, Y., et al., *Novel needle immersed vitrification: a practical and convenient method with potential advantages in mouse and human ovarian tissue cryopreservation.* Human reproduction, 2008. **23**(10): p. 2256-2265.

CHAPTER 7B

GENERAL DISCUSSION

TABLE OF CONTENTS

7.1. The advantages and disadvantages of collecting PAFs for FP purposes

About 90% of the total follicular reserve consists mainly of resting primordial follicles. The majority of these (99.9%) will never mature into preovulatory follicles [1], but rather perish at a premature stage along the developmental path. The stock of primordial and primary follicles thus represents an untapped potential, which could be cultivated for reproduction, preservation, or research purposes. PAFs can be further processed *in situ* or as isolated follicles. When the follicles remain in their natural environment, they are surrounded by paracrine growth factors and supported by a natural matrix. Many factors in this natural environment are still largely unknown. Transplanting cortical tissue back to the donor represents a possibility of restoring normal reproductive life for women, by re-establishing not only fertility but also the production of ovarian hormones. In some cases, transplanting cortical tissue back to the donor, can involve reintroduction of cancerous cells. Another drawback linked to transplantation of ovarian tissue is the extensive loss of PAFs due to delayed and deficient revascularization of the transplanted tissue [2, 3]. In addition, quantitative and qualitative assessments of the follicle population in ovarian tissue samples are difficult, if not impossible [4-6]. Indeed, PAFs are heterogeneously distributed and clustered throughout the ovarian cortex [7].
The second way of working with PAFs is to isolate them from the ovarium stroma. The choice to work on isolated follicles was inspired by the fact that ultimately, this will be the most rewarding strategy when dealing with FP in oncology patients as it rules out the risk of re-introducing malignant cells in former cancer patients with medium to high risk of ovarian metastasis, such as leukaemia [8, 9]. For these reasons, we concentrated mainly on the use of isolated follicles. In addition, working with isolated follicles has the advantage that individual follow-up of follicles is possible [10].

Cryopreservation is a crucial component in state of the art FP strategies [11]. PAFs are considered to be more resistant to cryo-injury than growing follicles because they only have a small amount of cold sensitive intra-cytoplasmatic lipid droplets, are less differentiated and their metabolism is relatively low, they only have a small amount of organelles and lack a zona pellucida, cortical granules and a meiotic spindle [12, 13]. Experiments carried out in mice, showed that it is possible to obtain normal offspring after isolated follicle cryopreservation and *in vitro* culture [14]. Studies reporting about the cryopreservation of isolated PAFs from large mammals and humans are however rather scarce.
As the oocyte in PAFs is arrested in the prophase, it may also be at a lower risk of cytogenic errors and has the possibility to repair sublethal damage during its prolonged growth phase. On the contrary, due to their size, water content, membrane permeability and nuclear maturation, (im)mature oocytes are known to be a difficult target for the current cryopreservation strategies [15]. Moreover, synchronizing

the nuclear and cytoplasmic maturation in these (im)mature oocytes and determining morphologic characteristics correlated with oocyte developmental quality, remain difficult [16].

The small size of PAFs (between 30 and 80 micrometers) greatly facilitates penetration of cryoprotectants [13, 17, 18]. Indeed, PAFs are very small, which on the contrary, greatly impedes their manipulation. Consequently, losses cannot be avoided during isolation, vitrification, warming and the evaluation processes like stainings [19, 20]. In this thesis we aimed to find solutions to facilitate manipulation and prevent big follicle losses. In this regard, we investigated the suitability of follicle embedding and cell strainers for the vitrification of follicles in bulk.

Up until now, complete *in vitro* culture of early-staged follicles is still not successful in large mammals, because of insufficient knowledge of suitable culture media that are necessary for all developmental steps in folliculogenesis [21]. Consequently, progress in vitrification strategies of isolated PAFs relies heavily on techniques that will hopefully be available in the near future such as an artificial ovary and an (multiple step) *in vitro* culture system.

7.2. Isolation and culture of PAFs; difficulties and opportunities

Follicle yield is dependent on the species of interest, but also on the reproductive age of the ovarian tissue. Follicles are more numerous and the isolation method tends to be easier in bovine neonatal and prepubertal ovaries than in bovine adult ovaries [22, 23]. Mechanical isolation is our method of choice for the isolation of PAFs from the bovine ovarian cortex which is assumed to cause the least detectable damage to the follicles [24-26]. The most important factor for preferring a mechanical or enzymatic digestion is the density of the ovarian collagen matrix [27]. Mechanical isolation of follicles has the advantage of preserving follicular integrity by maintaining the basal lamina. In addition, if mechanical damage occurs, its immediate visibility makes it easy to exclude those follicles. This in contrast to damage caused by enzymatic isolation procedures, which may remain undetectable by their morphology and may only appear when follicle's developmental capacity appears to be hampered.

Although not as abundant as primordial follicles, for all experiments we selected bovine primary follicles at isolation. After 2 days of *in vitro* culture, good quality follicles (as assessed by morphology) developed until the secondary stage while other follicles of lower quality degenerated. We found that when at day 2, only secondary category 1 follicles were selected for the experiments, an experiment can be repeatable. At the secondary stage, the classification 'Category 1' is a very good predictor for

follicle viability. However, this use of category 1 follicles only made it very difficult to obtain a sufficient number of follicles to set up or complete an experiment.

In order to retrieve a relative high number of category 1 follicles, the time from ovary collection to follicle culture seemed to be very critical for follicle survival. When culturing follicles, at least two 96-well plates were used. In this way, the plates could be filled alternately while the other plate was kept in the incubator. A maximum of 2 plates can be filled after one isolation procedure, as follicle quality decreased visibly during follicle selection, at least partly due to exposure to light and low temperatures. We discovered that a 30% retrieval of category 1 follicles from two plates, in which potentially 60 follicles are cultured, is high. Not only time plays a critical role, also other factors that cannot be controlled influence follicle quality, such as temperature, collection time of ovaries and transport time from abattoir to laboratory [28]. In addition, as the ovaries were obtained post mortem from cows of unknown origin, age and fertility status of the cows was also unknown, which will influence the ovarian reserve. Moreover, we noticed a larger follicle retrieval with a higher quality from beef cows in comparison to dairy cows. An explanation might be a presumable lower age of the beef cows at slaughter resulting in a larger ovarian reserve.

Human ovarian tissue is more stiff/dense compared to the bovine ovarian cortex and contains many collagen fibers which are in proportion the age [29, 30]. It is therefore difficult to isolate primordial follicles with mechanical method only. Ovarian cortex from transgender men is one of the few sources of human ovarian tissue for scientific research. It must be noted that an increased cortical stiffness in the most superficial part of the ovarian cortex following testosterone administration in transgender men compared to the non-exposed ovarian cortex of oncological patient was described [31]. More specifically, an increased subepithelial collagenization and stromal hyperplasia in the ovarian cortex after testosterone administration was described [32]. The altered stiffness particularly in the most superficial part of the cortex can be explained by the presence of androgen receptors in the ovarian surface epithelium [33]. Exposure of this epithelium to androgens results in DNA synthesis and in some cases a protection from cell death, leading to cell proliferation [34].

The most common adverse consequence of enzymatic isolation of follicles is that the basement membrane is degraded and the theca cell layer(s) are removed [22]. Although, this damage can be minimized by limiting enzyme exposure [35], the destruction of the basement membrane and theca layers can result in the spontaneous migration of GCs away from the oocyte, once the follicles are placed into culture. Obtaining viable isolated follicles is an absolute prerequisite for their further successful growth, either for culture or transplantation purposes. Several groups have investigated the impact of different enzymatic isolation protocols on human follicles [35-37]. Collagenase and Liberase

are commonly used for the enzymatic isolation of ovarian follicles. However, the drawback of collagenase, which is a crude preparation derived from Clostridium histolyticum, is that it might contain high endotoxin levels that could severely impair culture and grafting outcomes, as shown for pancreatic islets [38]. Moreover, collagenase shows substantial variations in effectiveness between batches [39], that may explain the discrepancies in results between different groups. In this respect, follicle isolation with Liberase DH provides a more standardized approach, as it uses a purified enzyme blend produced in GMP conditions and contains only negligible levels of endotoxins, therefore exhibiting lower lot-to-lot variability. Liberase DH contains dispase in high concentrations which is necessary concerning the dense stroma of human ovarian cortex. Indeed, dispase selectively cleaves fibronectin and collagen IV [40]. Because ovarian stroma is rich in these two elements [41, 42], Liberase DH appears to be the most appropriate choice of enzyme to isolate human PAFs. Dispase however does not cleave laminin [40], which is one of the main components of the basement membrane of human primordial follicles [43], it cleaves collagen IV [40], another essential constituent of the basement membrane of these follicles [43]. Hence, one can assume that follicles isolated during the first few minutes of enzymatic digestion could have their basement membrane progressively damaged over the course of 75 min of exposure to the enzyme [35]. Frequently DNase I is used during enzymatic isolation of human ovarian follicles to prevent clumping of cells. Cells in suspension may attach to one another and form clumps by the presence of free DNA and cell debris in the medium, which occurs following cell lysis. The sticky nature of DNA causes cells and other debris to aggregate into large clumps.

The use of three dimensional biodegradable culture systems like alginate or fibrin for early PAFs can tackle the problems associated with maintenance of 3D morphologic characteristics of the follicles *in vitro*. Follicles cultured via a two-dimensional approach are thought to lose their shape within 3 to 5 days, as the granulosa cells proliferate and spread across the surface of the plate [44]. This phenomenon is especially seen in larger animals and humans because of their larger oocyte and lengthy culture time to reach maturity. A consequence may be the dysfunction of gap junctions, which will hamper normal follicle development. In **Chapter 3** we compared 2D and 3D systems for short term culture of isolated bovine PAFs and concluded that the ones which were short term cultured in a 3D alginate bead culture system were of the same quality as compared to a 2D culture system, since no significant differences in viability and morphology were seen.

Gels can be modified, using varying concentrations and allow the addition of support cells, such as ovarian stromal cells, to better mimic the follicular microenvironment. Preliminary experiments showed that 2% (w/v) sodium alginate solution resulted in highest survival compared to 1.5%, which

made us decide to use a 2% alginate concentration. Gel concentration is inversely proportional to a follicle's potential for IVM and antrum formation [42, 45, 46]. Conversely, in larger animal species, such as human and nonhuman primates, it appears that the more rigid gel concentrations are more permissible to folliculogenesis [47-49]. In a study by Xu et al. [47], investigating 0.5% versus 0.25% alginate, follicular survival and growth were higher in the former. This may represent an affinity for a more rigid environment in early-stage primate follicles, which more closely resembles the more rigid ovarian stroma compared to mouse stroma.

Preliminary experiments also revealed that comparisons concerning the bead size showed a significant higher viability of PAFs cultured in beads with a size of 2.5 µL compared to 5 µL. Decreased viability of follicles in larger beads may result from reduced diffusion of nutrients to follicles in the center of the bead. The location of PAFs in the center or in the side of a bead may have an impact on development in larger beads, as nutrients may be in higher concentrations available for follicles located to the side of a bead. Taking up alginate, then follicles followed by alginate favors the chances to a centered location of PAFs, but is not a guarantee. An additional important advantage of alginate encapsulation is the quick and safe manipulation of follicles. We observed a reduced loss of follicles when encapsulated isolated follicles were cryopreserved compared to non-embedded isolated follicles.

Following up on McLaughlin et al. (2018) [50], we aimed to induce primordial follicle activation by culturing human cortical fragments. Cortex fragmentation disrupts the Hippo pathway, leading to increased expression of downstream growth factors and follicle growth [51, 52]. The PI3K/Akt pathway is considered the intracellular pathway where different extracellular factors involved in primordial follicle activation *in vivo* converge [53]. However, following 2 days of culture, follicles appeared to be dark and seemed degenerated. Therefore in **Chapter 6**, we decided to vitrify follicles immediately upon isolation, as those follicles looked morphologically intact and healthy by their color.

Considering the advances in the context of follicle culture, there is a need for comprehensive testing of oocytes derived by *in vitro* growth and maturation, as possible side effects may occur. The molecular and epigenetic programming and chromosomal health of *in vitro* derived MII oocytes, as well as the fertility of these gametes and evaluation of all aspects of the health, molecular and genetic normality and developmental competence of the embryos they produce, must be tested to quantify the risks of long-term culture [30].

7.3 Cryopreservation strategies for PAFs

Results obtained from studies on the cryopreservation of isolated PAFs have been scarce but encouraging. Isolated bovine follicles were successfully vitrified using HSV straws®, showing a high viability (87.5%) post thawing with no significant differences to follicles that were cultured in a 2D culture system (**Chapter 3**). However, considering the labor-intensive procedure and relatively low efficiency as many PAFs are lost during the vitrification procedure, vitrification in beads may be the method of choice in the future. As shown by our results, cryoresistance of the isolated follicles using the proposed vitrification protocol warrants additional research to improve survival rates. In addition, before cryopreservation of isolated follicles can be applied in clinical practice, additional research on a high throughput vitrification system and long-term effects on the cryopreserved follicles is needed.

7.4. Quality assessment of PAFs

The use of 'non-invasive' tools for viability testing is very useful by the means that they are reversible and follicle characteristics are not altered by the 'treatment'. Survival and follicle growth during short term *in vitro* culture can be used as a non-invasive tool for the evaluation of follicle *in vitro* viability. Individual follicle morphology and growth were light microscopically evaluated and allowed continuous evaluation over time. Based on the connection between the oocyte and the surrounding (pre-)granulosa cells and the microscopical integrity of the basal membrane, follicles were subdivided into three categories, with category 1 follicles showing the best morphological features. For an ongoing experiment and when follicles need to be processed further, morphologic assessment is one of the most important indications for immediate follicle viability. The assessment is easy and fast by light microscopy without any negative impact on the follicles, as long as the assessment is performed within a short time frame and follicles are quickly returned to the incubator. Nearly all category 1 follicles stained positive for Neutral Red, indicating that morphology is a good predictor for follicle viability.
Neutral Red, a water-soluble and non-toxic dye can serve as a non-invasive viability assay on the basis of its ability to diffuse through the plasma membrane and concentrate in the lysosomes of viable and metabolically active cells [54]. The reversibility of NR is beneficial when further growth and development need to be assessed or when other follicle assessment methods will be applied. In this regard viability can be tested and after follicle washing in NR free medium, other (invasive) follicle assessment methods can be applied. Neutral Red allowed us to successfully select viable follicles for vitrification experiments and improved follicle visibility for handling and assessment for example during a vitrification procedure. This is a great advantage as follicles are hard to visualize during equilibration in vitrification solutions. It is assumed that NR is not detrimental to tissue viability and

does not compromise subsequent *in vitro* follicle culture [54]. Other studies [10] also showed a non-detrimental effect of NR on isolated PAFs when an incubation time of 4 hours was used. Nevertheless, caution is advised when interpreting these results, because no data are available on oocyte development and subsequent embryo quality.

Unfortunately, there is a lack in the availability of noninvasive methods to assess viability and developmental capacity of PAFs in vitro, whether isolated or *in situ* [55]. Invasive quality assessment methods have the advantage to gather a lot more structural and functional information on follicle quality. However, following these kind of 'treatments', follicles cannot be used any further.

One method to assess morphology is through histology [56]. For this purpose, isolated follicles are individually embedded in Histogel® and subsequently embedded in paraffin. Paraffin sections are stained with hematoxylin-eosin (HE). In morphologically normal PAFs, GCs are well-organized in layers surrounding the oocyte and a distinguishable intact basement membrane can be observed. Degenerated follicles may show a retracted oocyte with or without a pyknotic nucleus. Layers of GCs remain unaltered or became disorganized and may often detach from each other and the basement membrane. Empty spaces or rupture of the basement membrane can also be observed.

However, damage that could occur in cellular organelles or membranes are not revealed by histological studies. Therefore, transmission electron microscopy (TEM) is recommended. Although TEM is technically more complex, time-consuming and expensive, it permits a deeper examination of the cells, identifying even slight damage in the follicle ultrastructure [57, 58]. We however, encountered great difficulties during the preparation of PAFs for TEM. The already small follicle shrinks even more during dehydration in ethanol (resulting in a follicular diameter of 20-30 µm), making it very difficult and time consuming (it takes hours) to find a single follicle back in the epon to make semi thin coupes. In order to have a bigger sample that can be easier found in the epon, we tried to concentrate individual PAFs as a group in the agar by using centrifugation. The results were however inconsistent and not very useful.

As morphological integrity does not guarantee follicular survival following thawing, viability stains can be used to get an indication on follicle viability. The fluorescent probe Calcein-AM is frequently used by many authors to assess viability of isolated follicles [36, 59, 60]. Through intracellular esterase activity, living cells convert the non-fluorescent cell-permeable Calcein-AM into a fluorescent calcein.

The visualization of connexins and transzonal projections using immunochemical methods is very informative as they play a crucial role in intercellular communication between different follicular components and are thus essential for further follicle development. Nevertheless, the validation of the antibodies and optimization of the protocols is certainly a long-term effort. In addition, the variable and by times low follicle retrieval rates make it difficult to gain fast progress. The small follicle size and multiple transfer steps between different media during the staining process complicate the

progression once more. However, advanced experience in follicle manipulation greatly improves the outcome and reduces follicles losses.

Currently, the best method to evaluate a cryopreservation procedure is transplantation of isolated PAFs [61] or OT **(Chapter 4)** by providing an adequate microenvironment for follicular survival and development.

7.5. Cell-cell connections in PAFs

Although outcome parameters are preferable non-invasive and reversible, these approaches of follicle quality do not always give sufficient guarantees for normal physiological function. In order to get more profound insights about the quality of follicles post cryopreservation, we investigated the physiological functionality of the follicles by means of the intercellular connections. Follicle growth and survival is dependent on communication between the GCs and the oocyte. Gap junctions (GJ) play a most important role in providing essential components to the oocyte during growth. Moreover, this type of intercellular communication among these cells allows coordinated cellular activity [62]. Gap junctions (GJs) are membrane channels that allow direct intercellular communication between adjacent cells. A single GJ comprises two hemichannels, and each hemichannel consists of six connexin proteins. Connexins are ubiquitously expressed in almost every mammalian cell. Although several connexins (Cx26, Cx30, Cx32, Cx37, Cx40, Cx43 and Cx45) have been detected in the ovary, it is Cx37 and Cx43 that have major influence on folliculogenesis [63]. Cx37 deficiency clearly demonstrated that disruption of GJ-mediated communication between oocytes and granulosa cells in mouse strongly influences the development of both the oocyte and GCs [64]. Ablation of Cx43 by Gja1 knockout abolishes the communication between granulosa cells, which are unable to form a multilayer of cells [65]. It was shown that Cx43 forms gap junctions between GCs, whereas Cx37 forms gap junctions between the oocyte and GCs in bovines and humans [66-69]. Demonstration of the presence of connexins will help to better assess the developmental capacity of follicles following cryopreservation, based on these results modifications can be implemented to vitrification protocols aiming to optimize PAF survival. The aim of **Chapter 5** was limited to the visualization of cell-cell connections in isolated PAFs. As the presence and functionality of these cell-cell connections should be further confirmed and quantified, in **Chapter 6** we aimed, next to the visualization, to demonstrate Cx43 at the gene transcription levels by using real-time quantitative PCR. In addition, functionality of gap junctions can be shown by the injection and transport of Lucifer yellow from the oocyte to the surrounding GCs [70].

We were not able to visualize Cx37 protein expression in isolated bovine and human PAFs, potentially due to the absence of Cx37, very low expression of Cx37 or the deficiency of reactivity of the antibody

for bovine and human Cx37. Also Grosbois et al. (2019) [67] could not detect Cx37 and GJA4 mRNA, encoding for the Cx37 protein in human isolated follicles. Kristensen et al. (2015) [71] demonstrated Cx43 as being the most abundant connexin in human follicles and Cx37 was shown to be expressed in very low levels throughout pre-antral human follicular growth. Thus, failure to detect Cx37 is probably related to the early developmental stage of the isolated follicles.

Other key structures that provide communication routes between the somatic cells and the oocyte are the transzonal projections (TZPs) [72]. A positive correlation between maintenance of the intra-follicular TZP network and oocyte growth has been demonstrated in cultured human PAFs [48]. TZPs are composed of F-actin and microtubules [73-75], which are very sensitive to changes in temperature and sheer stress and, as a consequence, vulnerable during cryopreservation [76]. It was demonstrated in human [67] and mouse [70] that thawed follicles in which a large percentage of the physical connections were destroyed could regrow and be maintained after culture.

We showed presence of Cx43 and TZPs in all isolated bovine and human PAF stages, from the primordial stage onwards. The presence of Cx43 and TZPs in vitrified-warmed bovine and human isolated PAFs suggest the maintenance of communication between the oocyte and the somatic companion cells following vitrification and warming.

7.6. From bovine model to the development of human FP strategies

The research described in this thesis aims to address some of the remaining challenges stated above in the development of (human) FP strategies. To progress in this field, it is necessary to apply different approaches and techniques to find optimal cryopreservation protocols and to assess follicle quality. The use of human tissue and gametes for research purposes is limited because of ethical and practical restrictions. Fortunately, the existing similarities between human and bovine reproductive pre-implantation physiology, makes the bovine model a worthy alternative reproductive research tool. For instance, cow, but also sheep ovaries are very similar to those of humans; they are almost the same size and show a similar texture and composition, as well as comparable follicle size and growth patterns [55, 77-80]. In bovine, folliculogenesis duration (development from the primordial to the ovulatory stage) is comparable to human, 180 days versus 205 days. Recently, bovine *in vitro* models are gaining more attention and are increasingly used in FP studies, especially those related to OT quality evaluation and cryopreservation, and follicle culture [55]. The main advantages of using the bovine model are that a large amount of material can be obtained from slaughterhouses and there is no need to create special

animal housing facilities. The main disadvantage is the unknown origin of the collected oocytes, such as age, reproductive status, and diseases of the reproductive tract [81].

It is difficult to obtain cortical OT from young patients for experimentation. Human OT used in our experiments was obtained from consenting female-to-male transsexual persons, ovarian tissue pieces were cryopreserved trough slow freezing (programmable freezer) and kept frozen in liquid nitrogen. Studies have shown no difference in viability and developmental competence between fresh or frozen-thawed tissue [82-84]. As most patients were taking testosterone treatment before tissue preservation, increased cortical stiffness should be taken into account. This may result in a longer incubation time needed to obtain a completely digested tissue compared to the non-exposed ovarian cortex. The follicles in these tissues have the potential to mature and grow as shown in previous studies [85, 86].

When biopsies are obtained for research from women over 35 years with gynecological pathologies, one need to consider that these tissues are inhomogeneous due to age-related follicle depletion. It is difficult to obtain cortical OT from young patients and also free from treatment (e.g. testosterone) for experimentation. Other possible sources of research material for culture are human fetal OT (therapeutic abortion material obtained from prenatal diagnostic units) and OT from consenting cancer patients [87]. This material can be obtained via collaboration with a prenatal diagnosis unit and through operative laparoscopists. Active recruitment of young and fertile tissue donors (e.g. at the time of Caesarean section) should be encouraged and the spare tissues and cells from IVF laboratories should be collected and properly stored for use in research.

Animals models can thus be used for preliminary tests. So far, the only reliable endpoints are pregnancy and birth. *In vitro* maturation from pre-antral follicles, followed by fertilization and birth of offspring has, however, only been successful in the mouse. Mouse models have the advantage that all physiological processes during the life cycle are accelerated, *in vitro* experiments can be shortened and it is a cheap resource as the housing facilities are small and easy manageable. The mouse model is the most useful and popular model for embryo manipulation and for post-implantation studies due to similar placentation processes compared to human [88, 89]. The physiological and morphological differences as it comes to ovarian function are considerable between mouse and human, therefore other models, like the bovine, are more suitable for studying pre-implantation embryology [90].

While extensive experience was gained in the processing of isolated bovine PAFs for all kind of purposes like isolation, culture, cryopreservation and follicle quality assessment methods, we aimed

to translate our expertise to human material. Immediately following isolation, the morphological aspects were quite similar for human and bovine PAFs (Fig. 2). The follicle yield from human tissue was variable between patients and between tissue fragments from the same patients, as already well known [7]. On average the follicle yield from one fragment (5 mm x 5 mm) of human ovarian cortex was much higher compared to one isolation from bovine ovaries (for which at least 10 fragments of the same size are used for mechanical isolation). The enzymatic isolation for human follicles is less labor intensive, but the incubation time in enzyme solution takes longer than the mechanical isolation for bovine follicles.

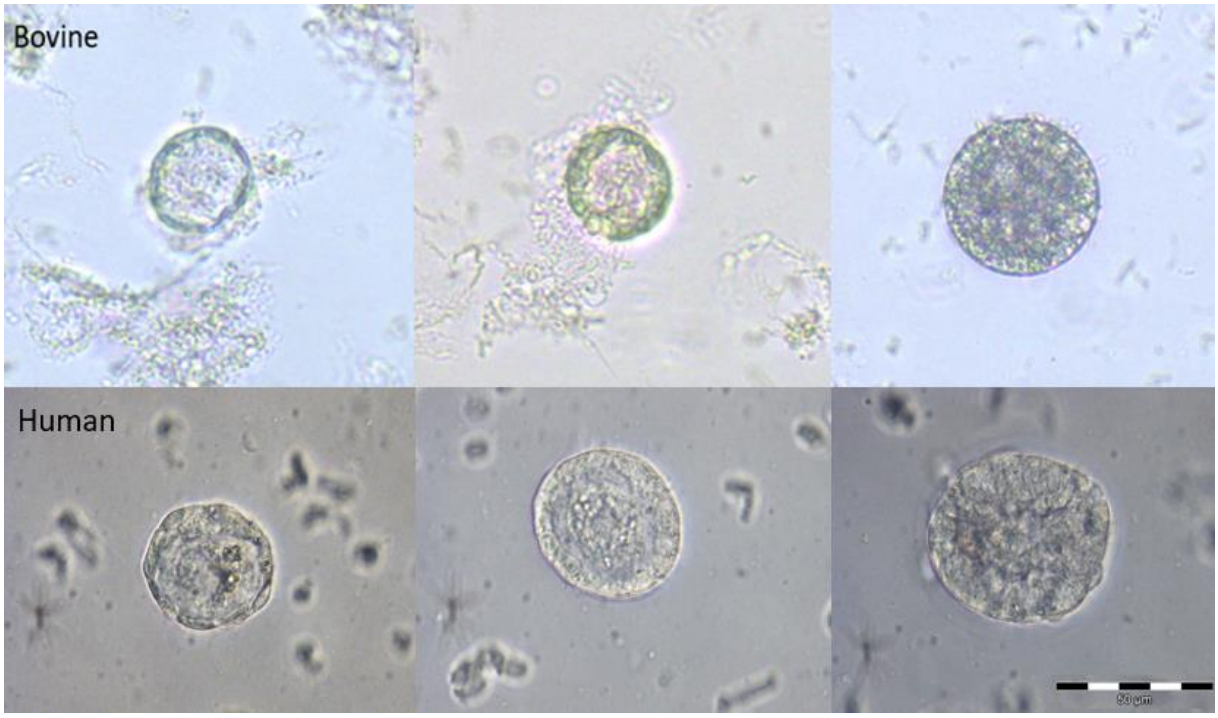

Fig. 2 Bovine and human PAFs immediately upon isolation. From left to right: primordial, primary and secondary follicle

When applying NR staining on human isolated follicles, we discovered some differences compared to bovine isolated PAFs. Immediately upon isolation, follicles with a good morphology, especially primary and secondary follicles did not stain positive for NR or showed a very weak staining. Primordial follicles stained generally positive for NR. Also following freezing and thawing, morphologically well preserved follicles stained negative for NR. However, when applying Trypan Blue staining to these follicles, not all follicles stained blue, indicating that these follicles were not dead. This suggests that isolated bovine and human follicles react differently to the vital stain NR. We also found different outcomes following PSF (Mr. Frosty). Bovine follicles showed a high survival rate (74%) while all human follicles showed a poor morphology and were dead as assessed by Trypan Blue staining. However, the implementation

of a 2 day culture period, allowed good quality bovine follicles to grow to the secondary stage and enhanced the selection of good quality follicles. As the selection of human follicles immediately upon isolation by means of morphological characteristics is more difficult, lower quality follicles could be selected for PSF. This may however not be the only explanation as all follicles were dead. When it comes to the optimization of the immunofluorescent staining of connexins, we encountered more difficulties to visualize connexins in isolated human PAFs. We found that a much longer permeabilization time (overnight vs. 20 min.) was necessary to achieve a good visualization of Cx43 in human isolated follicles. We could detect transzonal projections in the same manner for bovine and human isolated PAFs. Whether these differences can be explained by the use of the enzymatic isolation method or are inherent to the species, still needs to be elucidated. We may conclude that the bovine model is a very valuable model for human FP strategies, however, the translation of established techniques from one species to another, albeit quite similar, is not always straightforward.

References

1. Morita, Y. and J.L. Tilly, *Oocyte apoptosis: like sand through an hourglass.* Developmental biology, 1999. **213**(1): p. 1-17.
2. Martinez-Madrid, B., et al., *Chick embryo chorioallantoic membrane (CAM) model: a useful tool to study short-term transplantation of cryopreserved human ovarian tissue.* Fertil. and Steril., 2009. **91**(1): p. 285-292.
3. Van Eyck, A.-S., et al., *Both host and graft vessels contribute to revascularization of xenografted human ovarian tissue in a murine model.* Fertil. and Steril., 2010. **93**(5): p. 1676-1685.
4. Shaw, J., et al., *Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications.* Mol. and Cell. Endocrinol., 2000. **161**(1): p. 103-110.
5. Amorim, C., P. Gonçalves, and J. Figueiredo, *Cryopreservation of oocytes from pre-antral follicles.* Hum. Reprod. Update, 2003. **9**(2): p. 119-129.
6. Jorssen, E.P., et al., *Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension.* Theriogenology, 2014. **82**(3): p. 387-395.
7. Schmidt, K., et al., *Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries.* Human Reproduction, 2003. **18**(6): p. 1158-1164.
8. Dolmans, M.-M., *From isolated follicles to the artificial ovary: why and how?* Current Opinion in Endocrine and Metabolic Research, 2021.
9. Amorim, C.A. and A. Shikanov, *The artificial ovary: current status and future perspectives.* Future oncology, 2016. **12**(19): p. 2323-2332.
10. Kristensen, S.G., et al., *Isolation of pre-antral follicles from human ovarian medulla tissue.* Human Reproduction, 2011. **26**(1): p. 157-166.
11. Varghese, A.C., et al., *Cryopreservation/transplantation of ovarian tissue and in vitro maturation of follicles and oocytes: challenges for fertility preservation.* Reproductive Biology and Endocrinology, 2008. **6**(1): p. 1-10.
12. Hovatta, O., et al., *Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants.* Hum. Reprod., 1996. **11**(6): p. 1268-72.
13. Shaw, J.M., A. Oranratnachai, and A.O. Trounson, *Fundamental cryobiology of mammalian oocytes and ovarian tissue.* Theriogenology, 2000. **53**(1): p. 59-72.
14. Dela Pena, E., et al., *Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles.* Reproduction, 2002. **123**(4): p. 593-600.
15. Technology, S.f.A.R., *Mature oocyte cryopreservation: a guideline.* Fertility and sterility, 2013. **99**(1): p. 37-43.
16. Chen, S., et al., *Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing—a review article.* Molecular and Cellular Endocrinology, 2003. **202**(1-2): p. 101-107.
17. Depalo, R., G. Loverro, and L. Selvaggi, *In vitro maturation of primordial follicles after cryopreservation of human ovarian tissue: problems remain.* Medical and Pediatric Oncology: The Official Journal of SIOP—International Society of Pediatric Oncology (Societé Internationale d'Oncologie Pédiatrique, 2002. **38**(3): p. 153-157.
18. Gosden, R.G., et al., *Current perspective on primordial follicle cryopreservation and culture for reproductive medicine.* Human Reproduction Update, 2002. **8**(2): p. 105-110.
19. Camboni, A., et al., *Alginate beads as a tool to handle, cryopreserve and culture isolated human primordial/primary follicles.* Cryobiology, 2013. **67**(1): p. 64-69.
20. Rodrigues, A., et al., *Cryopreservation and short-term culture of isolated caprine primordial follicles.* Small Ruminant Research, 2005. **56**(1-3): p. 103-111.
21. Van den Hurk, R., et al., *Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes.* Human reproduction update, 2000. **6**(5): p. 457-474.

22. Figueiredo, J.R., et al., *Development of a combined new mechanical and enzymatic method for the isolation of intact preantral follicles from fetal, calf and adult bovine ovaries.* Theriogenology, 1993. **40**(4): p. 789-799.
23. Carambula, S., et al., *Effect of fetal age and method of recovery on isolation of preantral follicles from bovine ovaries.* Theriogenology, 1999. **52**(4): p. 563-571.
24. Roy, S.K. and G.S. Greenwald, *Methods of separation and in-vitro culture of pre-antral follicles from mammalian ovaries.* Human reproduction update, 1996. **2**(3): p. 236-245.
25. Lucci, C.M., et al., *Study of preantral follicle population in situ and after mechanical isolation from caprine ovaries at different reproductive stages.* Animal Reproduction Science, 1999. **56**(3-4): p. 223-236.
26. Itoh, T. and H. Hoshi, *Efficient isolation and long-term viability of bovine small preantral follicles in vitro.* In Vitro Cellular & Developmental Biology-Animal, 2000. **36**(4): p. 235-240.
27. Park, K.S., et al., *Effects of isolating methods (mechanical or enzymatical) on structure of pre-antral follicles in mouse.* Journal of assisted reproduction and genetics, 2005. **22**(9): p. 355-359.
28. Di Francesco, S., et al., *Influence of temperature and time during ovary transportation on in vitro embryo production efficiency in the buffalo species (Bubalus bubalis).* Italian Journal of Animal Science, 2007. **6**(sup2): p. 755-758.
29. Herraiz, S., C. Diaz-Garcia, and A. Pellicer, *Ovarian tissue cryopreservation: slow freezing*, in *Gonadal tissue cryopreservation in fertility preservation*. 2016, Springer. p. 53-77.
30. Smitz, J., et al., *Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation.* Human reproduction update, 2010. **16**(4): p. 395-414.
31. de Roo, C., *Ovarian tissue cryopreservation : insights in human ovarian tissue histology and culture.* PhD thesis, 2020.
32. Ikeda, K., et al., *Excessive androgen exposure in female-to-male transsexual persons of reproductive age induces hyperplasia of the ovarian cortex and stroma but not polycystic ovary morphology.* Human Reproduction, 2013. **28**(2): p. 453-461.
33. Al-Timimi, A., C. Buckley, and H. Fox, *An immunohistochemical study of the incidence and significance of sex steroid hormone binding sites in normal and neoplastic human ovarian tissue.* International journal of gynecological pathology: official journal of the International Society of Gynecological Pathologists, 1985. **4**(1): p. 24-41.
34. Edmondson, R., J. Monaghan, and B. Davies, *The human ovarian surface epithelium is an androgen responsive tissue.* British Journal of Cancer, 2002. **86**(6): p. 879-885.
35. Chiti, M.C., et al., *A modified and tailored human follicle isolation procedure improves follicle recovery and survival.* Journal of ovarian research, 2017. **10**(1): p. 1-9.
36. Lierman, S., et al., *Follicles of various maturation stages react differently to enzymatic isolation: a comparison of different isolation protocols.* Reproductive biomedicine online, 2015. **30**(2): p. 181-190.
37. Vanacker, J., et al., *Enzymatic isolation of human primordial and primary ovarian follicles with Liberase DH: protocol for application in a clinical setting.* Fertility and sterility, 2011. **96**(2): p. 379-383. e3.
38. Berney, T., et al., *Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis1.* Transplantation, 2001. **71**(1): p. 125-131.
39. McShane, P., et al., *Protease activity in pancreatic islet isolation by enzymatic digestion.* Diabetes, 1989. **38**(Supplement 1): p. 126-128.
40. Stenn, K.S., et al., *Dispase, a neutral protease from Bacillus polymyxa, is a powerful fibronectinase and type IV collagenase.* Journal of investigative dermatology, 1989. **93**(2): p. 287-290.

41. Berkholtz, C.B., et al., *Distribution of extracellular matrix proteins type I collagen, type IV collagen, fibronectin, and laminin in mouse folliculogenesis.* Histochemistry and cell biology, 2006. **126**(5): p. 583-592.
42. Gook, D.A., et al., *Follicle development following cryopreservation of human ovarian tissue.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2004. **113**: p. S60-S62.
43. Heeren, A.M., et al., *Development of the follicular basement membrane during human gametogenesis and early folliculogenesis.* BMC developmental biology, 2015. **15**(1): p. 1-13.
44. Green, L.J. and A. Shikanov, *In vitro culture methods of preantral follicles.* Theriogenology, 2016. **86**(1): p. 229-238.
45. West, E.R., et al., *Physical properties of alginate hydrogels and their effects on in vitro follicle development.* Biomaterials, 2007. **28**(30): p. 4439-4448.
46. Brito, I., et al., *Alginate hydrogel matrix stiffness influences the in vitro development of caprine preantral follicles.* Molecular reproduction and development, 2014. **81**(7): p. 636-645.
47. Xu, M., et al., *Encapsulated three-dimensional culture supports development of nonhuman primate secondary follicles.* Biology of reproduction, 2009. **81**(3): p. 587-594.
48. Xu, M., et al., *In vitro grown human ovarian follicles from cancer patients support oocyte growth.* Human Reproduction, 2009. **24**(10): p. 2531-2540.
49. Hornick, J., et al., *Isolated primate primordial follicles require a rigid physical environment to survive and grow in vitro.* Human reproduction, 2012. **27**(6): p. 1801-1810.
50. McLaughlin, M., et al., *Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system.* MHR: Basic science of reproductive medicine, 2018. **24**(3): p. 135-142.
51. Kawamura, K., et al., *Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment.* Proceedings of the National Academy of Sciences, 2013. **110**(43): p. 17474-17479.
52. Kawamura, K., N. Kawamura, and A.J. Hsueh, *Activation of dormant follicles: a new treatment for premature ovarian failure?* Current opinion in obstetrics & gynecology, 2016. **28**(3): p. 217.
53. Hsueh, A.J., et al., *Intraovarian control of early folliculogenesis.* Endocrine reviews, 2015. **36**(1): p. 1-24.
54. Chambers, E., et al., *In situ identification of follicles in ovarian cortex as a tool for quantifying follicle density, viability and developmental potential in strategies to preserve female fertility.* Human Reproduction, 2010. **25**(10): p. 2559-2568.
55. Langbeen, A., et al., *Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies.* Theriogenology, 2015. **84**(4): p. 477-489.
56. Jorssen, E.P., et al., *Morphologic characterization of isolated bovine early preantral follicles during short-term individual in vitro culture.* Theriogenology, 2015. **84**(2): p. 301-11.
57. Camboni, A., et al., *Preservation of fertility in young cancer patients: contribution of transmission electron microscopy.* Reproductive biomedicine online, 2008. **17**(1): p. 136-150.
58. Desai, N., et al., *A simple and efficient method for preparation of isolated ovarian follicles for transmission electron microscopy.* Journal of assisted reproduction and genetics, 2010. **27**(2): p. 97-101.
59. Dolmans, M.-M., et al., *Evaluation of Liberase, a purified enzyme blend, for the isolation of human primordial and primary ovarian follicles.* Human Reproduction, 2006. **21**(2): p. 413-420.
60. Vanacker, J., et al., *Should we isolate human preantral follicles before or after cryopreservation of ovarian tissue?* Fertility and sterility, 2013. **99**(5): p. 1363-1368. e2.
61. Aerts, J.M., et al., *Xenotransplantation by injection of a suspension of isolated preantral ovarian follicles and stroma cells under the kidney capsule of nude mice.* Fertility and sterility, 2010. **94**(2): p. 708-714.
62. Makabe, S., T. Naguro, and T. Stallone, *Oocyte–follicle cell interactions during ovarian follicle development, as seen by high resolution scanning and transmission electron microscopy in humans.* Microscopy research and technique, 2006. **69**(6): p. 436-449.

63. Kidder, G.M. and B.C. Vanderhyden, *Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence.* Canadian journal of physiology and pharmacology, 2010. **88**(4): p. 399-413.
64. Simon, A.M., et al., *Female infertility in mice lacking connexin 37.* Nature, 1997. **385**(6616): p. 525-529.
65. Ackert, C.L., et al., *Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse.* Developmental biology, 2001. **233**(2): p. 258-270.
66. Nuttinck, F., et al., *Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary.* Mol. Reprod. Dev., 2000. **57**(1): p. 60-6.
67. Grosbois, J., et al., *Ultrastructure and intercellular contact-mediated communication in cultured human early stage follicles exposed to mTORC1 inhibitor.* Molecular human reproduction, 2019. **25**(11): p. 706-716.
68. Zhang, Y., et al., *Transcriptome Landscape of Human Oocytes and Granulosa Cells Throughout Folliculogenesis.* bioRxiv, 2018: p. 285445.
69. Johnson, M.L., et al., *Expression of gap junctional proteins connexin 43, 32, and 26 throughout follicular development and atresia in cows.* Endocrine, 1999. **10**(1): p. 43-51.
70. Barrett, S.L., L.D. Shea, and T.K. Woodruff, *Noninvasive index of cryorecovery and growth potential for human follicles in vitro.* Biology of reproduction, 2010. **82**(6): p. 1180-1189.
71. Kristensen, S.G., P. Ebbesen, and C.Y. Andersen, *Transcriptional profiling of five isolated size-matched stages of human preantral follicles.* Molecular and cellular endocrinology, 2015. **401**: p. 189-201.
72. Albertini, D. and S. Barrett, *Oocyte-somatic cell communication.* REPRODUCTION-CAMBRIDGE-SUPPLEMENT-, 2003: p. 49-54.
73. Carabatsos, M.J., et al., *Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice.* Developmental biology, 1998. **204**(2): p. 373-384.
74. Albertini, D.F., et al., *Cellular basis for paracrine regulation of ovarian follicle development.* Reproduction, 2001. **121**(5): p. 647-653.
75. Albertini, D.F. and V. Rider, *Patterns of intercellular connectivity in the mammalian cumulus-oocyte complex.* Microscopy research and technique, 1994. **27**(2): p. 125-133.
76. Vanhoutte, L., et al., *Effects of chilling on structural aspects of early preantral mouse follicles.* Biology of reproduction, 2004. **70**(4): p. 1041-1048.
77. Newton, H., H. Picton, and R. Gosden, *In vitro growth of oocyte–granulosa cell complexes isolated from cryopreserved ovine tissue.* Reproduction, 1999. **115**(1): p. 141-150.
78. Oktay, K., G.G. Karlikaya, and B.A. Aydin, *Ovarian cryopreservation and transplantation: basic aspects.* Molecular and cellular Endocrinology, 2000. **169**(1-2): p. 105-108.
79. Adams, G., J. Singh, and A. Baerwald, *Large animal models for the study of ovarian follicular dynamics in women.* Theriogenology, 2012. **78**(8): p. 1733-1748.
80. Malhi, P.S., G.P. Adams, and J. Singh, *Bovine model for the study of reproductive aging in women: follicular, luteal, and endocrine characteristics.* Biology of reproduction, 2005. **73**(1): p. 45-53.
81. Santos, R.R., E.J. Schoevers, and B.A. Roelen, *Usefulness of bovine and porcine IVM/IVF models for reproductive toxicology.* Reproductive Biology and Endocrinology, 2014. **12**(1): p. 1-12.
82. Abir, R., et al., *Pilot study of isolated early human follicles cultured in collagen gels for 24 hours.* Human Reproduction, 1999. **14**(5): p. 1299-1301.
83. Hovatta, O., et al., *Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture.* Human reproduction (Oxford, England), 1997. **12**(5): p. 1032-1036.
84. Oktay, K., et al., *Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue.* Fertility and sterility, 1997. **67**(3): p. 481-486.
85. Soleimani, R., et al., *Xenotransplantation of cryopreserved human ovarian tissue into murine back muscle.* Human reproduction, 2010. **25**(6): p. 1458-1470.

86. Van den Broecke, R., et al., *Timing of FSH-stimulation and follicular development in cryopreserved human ovarian grafts.* Reproductive biomedicine online, 2002. **4**(1): p. 21-26.
87. Sadeu, J.C., et al., *Morphological and ultrastructural evaluation of cultured frozen–thawed human fetal ovarian tissue.* Fertility and sterility, 2006. **85**: p. 1130-1141.
88. Rossant, J., *A mouse is not a cow.* Nature, 2011. **471**(7339): p. 457-458.
89. Moreno-Moya, J.M., et al., *Complete method to obtain, culture, and transfer mouse blastocysts nonsurgically to study implantation and development.* Fertility and sterility, 2014. **101**(3): p. e13.
90. Ménézo, Y.J. and F. Hérubel, *Mouse and bovine models for human IVF.* Reproductive biomedicine online, 2002. **4**(2): p. 170-175.

CHAPTER 8

CONCLUSIONS

AND

FUTURE PERSPECTIVES

The research presented in this thesis shows that the vitrification of isolated PAFs is a promising fertility preservation strategy. This is particularly relevant for cancer patients at moderate-to-high risk of ovarian metastasis, as due to the possibility of reimplanting malignant cells that could lead to recurrence of the primary disease, transplantation of cryopreserved OT should not be performed.

Isolated PAFs can survive the cryopreservation process and maintain their intercellular connections crucial for further development. Different vitrification techniques were evaluated for follicle survival and applicability in clinical practice. We however, found that all evaluated techniques, next to their advantages also had their downsides (Fig. 1). Concerning the quality assessment pre and post cryopreservation, several methods were investigated. We found that not all outcome parameters could be easily translated from bovine species to human. For example NR staining could not be used for human isolated PAFs upon isolation. Also the immunocytochemistry staining protocol for determination of connexins needed adaptations.

Animal *in vitro* models such as the bovine that rely on unlimited sources of research material and which are largely free of ethical or moral restrictions, have an important role in contributing to make faster progress in the development of these FP strategies. However, before this very interesting alternative can be applied in practice and will help cancer patients to preserve their fertility, a long road is still ahead.

The following associated future perspectives can be outlined:

8.1. Need for a maturation system

One of the first and most urgent needs when developing FP strategies based on the use of isolated PAFs, is a system to mature them. At present, a system to grow PAFs until a mature oocyte is not available yet. Currently two strategies are being investigated to obtain mature oocytes from isolated PAFs, namely: the transplantation of an artificial ovary [1-3] that contains matrix-embedded isolated follicles and the application of a successful IVM system [4-6] for PAFs. In addition, three-dimensional (3D) bioprinting of tissue-engineered constructs and prototype organs for regenerative medicine is one of the most rapidly evolving and promising areas of biotechnology [7]. To date, the development of bioprinting technology completed the first stage since successes of using the strategy for constructing and assembling tissues and organs have been demonstrated. The progress of a printed artificial ovary is currently ongoing, and the first successful animal experiments are known [8]. Human clinical trials are still far away, however at this stage, it is of high importance to identify and solve several important ethical and regulatory issues associated with artificial ovary 3D bioprinting.

Researchers are coming closer to a world where complete IVG of human oocytes is possible. But, the end point of any IVG system is to produce developmentally competent and epigenetically normal

oocytes and therefore future research needs to focus on optimizing each of the stages and gaining further understanding of the epigenetic status of IVG oocytes and of any embryos formed [9].

8.2. Need for a cryopreservation system that cryostores PAFs in bulk

Since it is shown that isolated PAFs survive the vitrification and warming process, it is necessary to develop a high throughput system that can be used for clinical purposes. From only one ovarian cortex fragment (5 mm x 5 mm) hundreds of PAFs can be yielded, vitrification of all recovered PAFs would be very labor and time consuming. Next to that, a large loss of follicles should be taking into account during the process of maturation. Also during the 'normal' *in vivo* situation more than 99% of all PAFs perish through apoptosis along the developmental path. Therefore, a large number of PAFs should be cryopreserved because it is expected that many PAFs are needed to obtain one mature oocyte capable of producing a normal healthy embryo.

8.3. Assessment of the functionality of cell-cell connections

As the presence of connexin proteins only, as demonstrated by immunocytochemistry or qPCR, is no guarantee for flawless GJ communication because the dysfunction of GJs may only show in later folliculogenesis [10], GJ function tests using dye coupling are of great additional value. GJ function can be tested by using a tracer molecule that easily passes the intercellular channels as extensively reviewed by Hanani [11]. Although Lucifer Yellow (LY) was introduced several decades ago as a tracer, it is still considered as a valuable method to document GJ permeability [12]. While several methods exist to load LY into cells [11], intracellular injection by micro-pipetting seems particularly suited for large cells such as the oocyte. Following LY injection in the centrally positioned oocyte, the tracer will be passed on to the surrounding pre-granulosa cells if functional GJs are present [13].

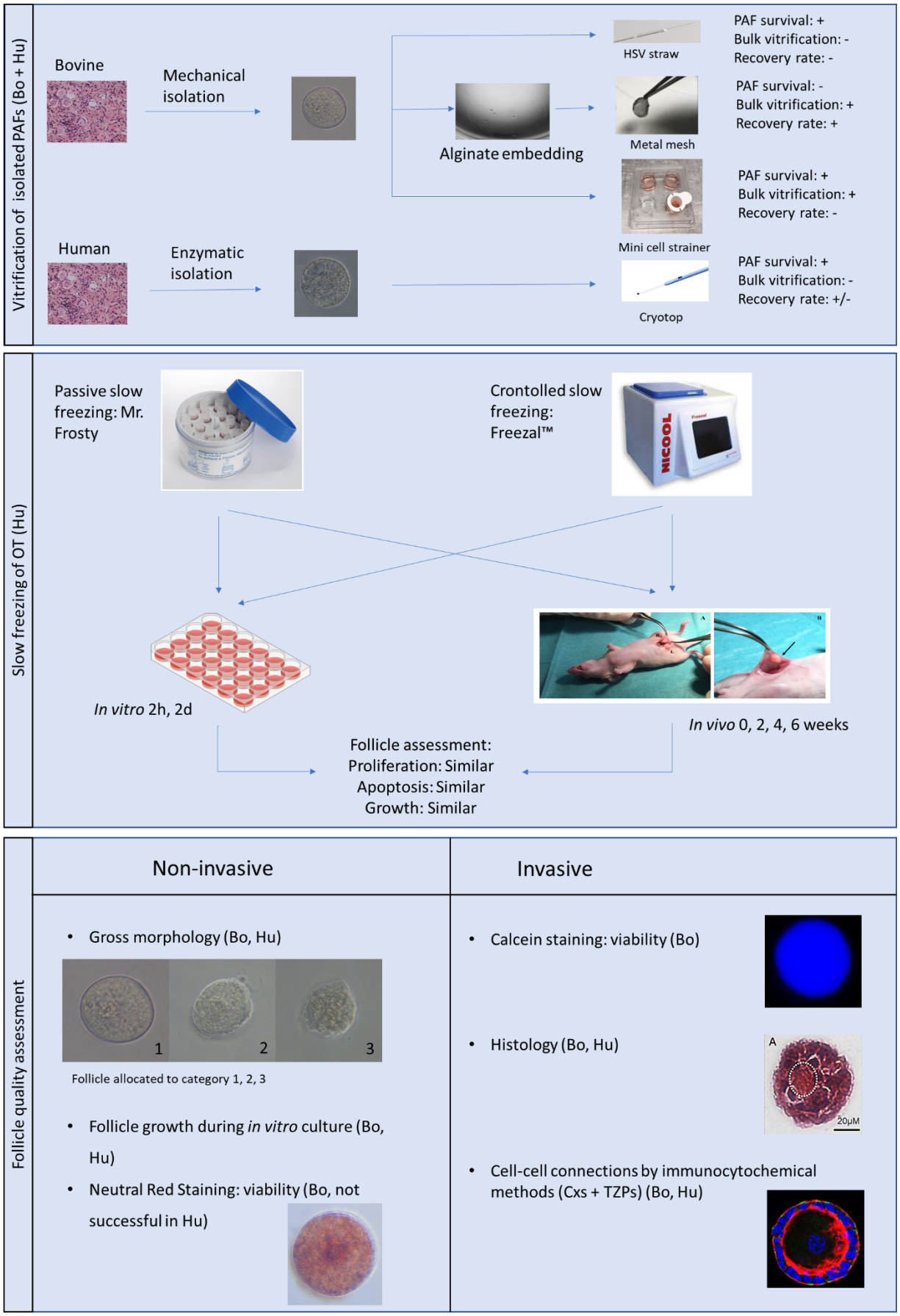

Fig. 1: Overview illustrating the different cryopreservation techniques and outcome parameters investigated in this thesis

References

1. Amorim, C.A. and A. Shikanov, *The artificial ovary: current status and future perspectives.* Future oncology, 2016. **12**(19): p. 2323-2332.
2. Dolmans, M.-M., *From isolated follicles to the artificial ovary: why and how?* Current Opinion in Endocrine and Metabolic Research, 2021.
3. Paulini, F., et al., *Survival and growth of human preantral follicles after cryopreservation of ovarian tissue, follicle isolation and short-term xenografting.* Reproductive biomedicine online, 2016. **33**(3): p. 425-432.
4. Telfer, E.E., *Future developments: in vitro growth (IVG) of human ovarian follicles.* Acta obstetricia et gynecologica Scandinavica, 2019. **98**(5): p. 653-658.
5. Green, L.J. and A. Shikanov, *In vitro culture methods of preantral follicles.* Theriogenology, 2016. **86**(1): p. 229-238.
6. McLaughlin, M., et al., *Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system.* MHR: Basic science of reproductive medicine, 2018. **24**(3): p. 135-142.
7. Kirillova, A., et al., *Bioethical and legal issues in 3D bioprinting.* International Journal of Bioprinting, 2020. **6**(3).
8. Laronda, M.M., et al., *A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice.* Nature communications, 2017. **8**(1): p. 1-10.
9. Anckaert, E., M. De Rycke, and J. Smitz, *Culture of oocytes and risk of imprinting defects.* Human reproduction update, 2013. **19**(1): p. 52-66.
10. Winterhager, E. and G.M. Kidder, *Gap junction connexins in female reproductive organs: implications for women's reproductive health.* Human reproduction update, 2015. **21**(3): p. 340-352.
11. Hanani, M., *Lucifer yellow–an angel rather than the devil.* Journal of cellular and molecular medicine, 2012. **16**(1): p. 22-31.
12. Chen, Y.-C., et al., *Transforming growth factor-β1 up-regulates connexin43 expression in human granulosa cells.* Human Reproduction, 2015. **30**(9): p. 2190-2201.
13. Barrett, S.L., L.D. Shea, and T.K. Woodruff, *Noninvasive index of cryorecovery and growth potential for human follicles in vitro.* Biology of reproduction, 2010. **82**(6): p. 1180-1189.

SUMMARY

Nowadays, an emerging need for (human) fertility preservation (FP) strategies is present, in particular when young girls and women are confronted with cancer treatment. The strategies in this thesis focus on the use of pre-antral follicles (PAF) as, for prepubertal girls and women whose cancer treatment cannot be postponed, there are no other options. More specifically, this thesis focusses on the use of isolated follicles to provide a safe option for cancer patients who are at risk of the reintroduction of malignant cells. The research described aims to contribute to the current knowledge on predominantly the vitrification of isolated PAFs and follicle survival following warming. In a final step, knowledge and skills obtained by using a bovine model were translated to human ovarian follicles.

Chapter 1 gives an overview of the state of the art of the possible applications of early PAFs for FP strategies. Girls and women affected by chemo/radiotherapy induced premature ovarian failure surviving cancer, are the largest and still growing group of interest. In addition, as fertility preservation is not only a concern in humans, the preservation of genetic material from endangered animal species or animals with important genetic traits will also greatly benefit from the development of alternative FP strategies. Further on, this Chapter reviews and summarizes the currently available techniques in female reproductive medicine and points out its remaining limitations. Human research material is scarce due to ethical and practical constraints. Infinitely available, ruminant material on the other hand seems a worthy alternative to use as an animal model.

Chapter 2 summarizes the main concerns that led to the specific aims of this thesis being discussed separately in specific chapters.

As individual follicle cryopreservation techniques are still labor-intensive and a substantial proportion of isolated follicles are lost during handling and after warming, in **Chapter 3** we investigated the feasibility of alginate embedding to increase vitrification efficiency. Mechanically isolated bovine pre-antral follicles were 1) cultured in 2% alginate beads (3D system) and vitrified in beads using mesh cups (3DVIT), 2) cultured in 2% alginate beads (3DCUL), 3) cultured in 96-well plates (2D system) and vitrified using High Security Vitrification straws® (2DVIT), and 4) cultured in a 2D system (2DCUL). No differences were observed in viability between follicles cultured (short term) in a 2D or 3D system. Follicles vitrified embedded in alginate showed a low viability (45.9%) as assessed by calcein and Neutral Red staining. Non-embedded follicles vitrified using straws displayed a high viability (87.5%) and follicles with a well preserved morphology. Although the viability of vitrified embedded follicles was too low, embedding in alginate allowed to handle follicles more efficiently; i.e. without excessive manipulation and thus less labor-intensive in combination with a reduced loss of follicles during the vitrification procedure.

We assessed the feasibility of passive slow freezing (PSF using Nalgene's Mr. Frosty container) as an alternative to controlled slow rate freezing (CSF using Air Liquide's Freezal™) for human ovarian tissue cryopreservation (OTC) (**Chapter 4**). Two studies, one *in vitro* (after 2 h and 2 days of culture) and another one *in vivo* (2, 4 and 6 weeks xenotransplantation in Balbc/nude mice), were conducted where the validity of PSF to freeze OT was assessed and compared to that of CSF. Folliculogenesis was assessed by means of the evaluation of follicle activation (PCNA and Ki-67) and apoptosis index (caspase-3 and TUNEL) in the former, and follicle growth (haematoxylin/eosin staining) and fibrosis index (Masson's trichrome staining) in the latter. The application of PSF to OTC has shown that follicle growth *in vitro* and in mouse models is similar to that achieved with CSF. The proliferation markers, the apoptosis rate and fibrosis were acceptable in both techniques. This pre-clinical evidence has shown that PSF can be an easy, cost-effective low-risk (as assessed by the EuroGTP-II ART) alternative to CSF for cryopreservation of human OT.

During the vitrification process, the manipulation of PAFs embedded in alginate beads was greatly improved in terms of follicle losses and timewise, however, follicle survival decreased significantly (Chapter 3). Therefore, in **Chapter 5** we aimed to retain the efficiency of the procedure and preserve follicle quality at the same time by vitrifying non-embedded PAFs using mini cell strainers. To determine follicle survival we visualized and observed the precise location of Connexin 43 (Cx43) and transzonal projections (TZPs) using immunocytochemical methods before and following vitrification. Indeed, disruption of cell-to-cell contacts between the germinal and somatic cell compartments are the main signs of cryodamage and these cell-cell connections are crucial for further follicle development. Cx43 and TZPs were present in all PAF stages. The group fresh follicles (assessed immediately upon isolation), showed a higher percentage of follicles that were positive for Cx43 (91.7%) than the follicles that were vitrified (77.4%). All follicles that were cultured for 2 days were Cx43 positive (100%). Follicles cultured for 4 days (65.8%) (P=0.002) showed the lowest percentage of follicles that were Cx43 positive. The percentages of follicles with the presence or (partial) absence of a TZP network were shown to be very heterogenous between follicles in different treatment groups. In this Chapter we demonstrated the maintenance of communication between the oocyte and the somatic companion cells following vitrification and warming. Furthermore, in view of the challenges that still need to be overcome regarding follicular retrieval and manipulation, we were able to use a simpler and much more workable cryopreservation method, by means of mini cell strainers. Vitrification in cell strainers has important advantages, as manipulation is much easier and time is saved because follicles are vitrified in group.

In **Chapter 6** we translated our experience in follicle isolation, vitrification and immunofluorescent staining obtained with bovine material to human material. Human follicles were enzymatically isolated and morphologically good follicles were vitrified using a Cryotop® vitrification device (Kitazato, Japan). We demonstrated Cx43 (immunostaining + qPCR) and TZPs (immunostaining) in all PAF stages which were assessed immediately upon isolation and following vitrification and warming. The identification of Cx43 and TZPs indicate the presence of these proteins which are essential for follicle development.

The overall results were profoundly discussed in **Chapter 7**, the conclusions of this thesis were summarized and suggestions for future research were provided in **Chapter 8**.

SAMENVATTING

Het stijgend aantal vrouwelijke patiënten dat kanker en de ermee samenhangende behandelingen overleeft is de directe aanleiding voor een sterke toename in de interesse voor strategieën die de fertiliteit herstellen en/of behouden. De strategieën die onderzocht werden in deze thesis concentreren zich op het gebruik van pre-antrale follikels die de basis of reserve vormen van de vrouwelijke reproductiviteit. Pre-antrale follikels bevinden zich in de buitenste rand van de eierstok, de zogenaamde cortex. Het is belangrijk op te merken dat voor prepuberale meisjes en vrouwen van wie de kankerbehandeling niet uitgesteld mag worden, de preservatie van pre-antrale follikels de enige optie is, omdat eicellen niet gecollecteerd kunnen worden. Meer specifiek focussen we op het gebruik van geïsoleerde follikels, omdat dit een veilige optie is voor kankerpatiënten die het risico lopen op reïntroductie van maligne kankercellen na terugplaatsen van ovarieel weefsel. Het onderzoek beschreven in deze thesis wil een bijdrage leveren aan de kennis omtrent vitrificatie van voornamelijk geïsoleerde follikels en follikeloverleving na opwarmen met het boviene model als relevant voorbeeld voor de humane reproductie.

Hoofdstuk 1 geeft een overzicht van de mogelijke indicaties om fertiliteitsbehoudende strategieën toe te passen met gebruik van vroege pre-antrale follikels. Meisjes en volwassen vrouwen die een kankertherapie overleven en van wie mogelijk de follikels aangetast zijn door de behandeling zijn de grootste en nog steeds groeiende doelgroep. Een andere indicatie is het behoud van de genetische diversiteit, vooral bij diersoorten die met uitsterven bedreigd zijn, of omwille van de hoge genetische waarde m.b.t. productiekenmerken van individuele dieren. Deze inleiding geeft daarenboven een overzicht van de beschikbare technieken die gebruikt worden in de humane geassisteerde voortplanting. Humaan onderzoeksmateriaal is omwille van ethische en praktische redenen slechts beperkt beschikbaar voor wetenschappelijk onderzoek. Het boviene model wordt voorgesteld als een waardig alternatief door verschillende fysiologische en anatomische overeenkomsten tussen koe en vrouw wat betreft de reproductie. Daarenboven is er een oneindige voorraad aan onderzoeksmateriaal beschikbaar in de slachthuizen.

In **Hoofdstuk 2** worden de specifieke doelstellingen van deze thesis nader omschreven.

Het cryopreserveren van individuele follikels is zeer arbeidsintensief en een aanzienlijk deel van de geïsoleerde follikels gaat verloren tijdens manipulatie en opwarmen. Daarom hebben we in **Hoofdstuk 3** de mogelijkheid om follikels in alginaat in te bedden onderzocht om zo de vitrificatie efficiëntie te vergroten. Mechanisch geïsoleerde boviene pre-antrale follikels werden 1) gecultiveerd in 2% alginaat (3D systeem) en gevitrificeerd in alginaat met mesh cups (3DVIT), 2) gecultiveerd in 2% alginaat (3DCUL), 3) gecultiveerd in 96-well platen (2D systeem) en gevitrificeerd met strootjes (HSV straws®)

(2DVIT), en 4) gecultiveerd in een 2D systeem (2DCUL). We zagen geen verschil in viabiliteit tussen follikels die gecultiveerd (korte duur) werden in een 2D cultuur systeem en follikels die gecultiveerd werden in een 3D cultuur systeem. Follikels die gevitrificeerd werden in alginaat vertoonden een lage viabiliteit (45.9%) na calceïne en Neutral Red kleuring. Niet ingebedde follikels die gevitrificeerd werden met strootjes vertoonden een hoge viabiliteit (87.5%) en de morfologie van de follikels werd niet beschadigd. Hoewel de viabiliteit van gevitrificeerde follikels te laag was, maakte het inbedden van follikels in alginaat het wel mogelijk om follikels efficiënter te manipuleren. Follikels kunnen namelijk in groep behandeld en verplaatst worden, waardoor de vitrificatie procedure minder arbeidsintensief is en er bovendien minder follikels verloren gaan.

In **Hoofdstuk 4** onderzochten we de mogelijkheid om humaan ovarieel weefsel te cryopreserveren door passief traag bevriezen (*passive slow freezing (PSF)* gebruik makend van Nalgene's Mr. Frosty) in plaats van gecontroleerd traag bevriezen (*controlled slow feezing (CSF)* gebruik makend van een programmeerbare vriezer Air Liquide's Freezal™). Twee studies, één *in vitro* (2 uur en 2 dagen cultuur) en één *in vivo* (2, 4 en 6 weken xenotransplantatie in Balbc/naakte muizen) werden uitgevoerd en de validiteit van PSF om ovarieel weefsel in te vriezen werd bepaald en vergeleken met CSF. Folliculogenese werd bepaald door middel van de beoordeling van follikel activatie (PCNA en Ki-67) en apoptose index (caspase-3 en TUNEL) in de *in vitro* studie en follikel groei (hematoxiline/eosine kleuring) en fibrose index (Masson's trichrome klering) werden geëvalueerd in de *in vivo* studie. De toepassing van PSF bij het cryopreserveren van humaan ovarieel weefsel heeft aangetoond dat follikelgroei *in vitro* en in het muismodel gelijkaardig waren aan CSF. De follikelproliferatie, apoptose ratio en mate van fibrose waren acceptabel voor beide technieken. Dit preklinische bewijs heeft aangetoond dat PSF een gemakkelijk en kostenefficiënt laag-risico (zoals beoordeeld door EuroGTP-II ART) alternatief kan zijn voor CSF voor de cryopreservatie van humaan ovarieel weefsel.

Het inbedden van PAFs in alginaat zorgde ervoor dat tijdens de vitrificatie procedure de manipulatie van PAFs verbeterd was wat betreft follikelverlies en het was bovendien tijdbesparend. De follikeloverleving was echter significant gedaald (Hoofdstuk 3). Daarom, richtten we ons in **Hoofstuk 5** op het behoud van de efficiëntie van de procedure en het behoud van follikelkwaliteit door niet-ingebedde PAFs te vitrificeren in mini cell strainers. Om follikeloverleving te bepalen hebben we Connexine 43 (Cx43) en transzonale projecties (TZPs) gevisualiseerd en de precieze locatie bepaald met immunocytochemische methoden zowel voor als na vitrificatie. Onderbreking van cel-cel contacten tussen granulosacellen en de eicel zijn namelijk de hoofdtekenen van cryoschade en deze cel-cel contacten zijn cruciaal voor de verdere folliculaire ontwikkeling. Cx43 en TZPs waren aanwezig in pre-antrale follikels in alle stadia. De groep verse follikels (beoordeeld direct na isolatie) vertoonde

een hoger percentage follikels die positief waren voor Cx43 (91.7%) dan de follikels die werden gevitrificeerd (77.4%). Alle follikels die gecultiveerd werden voor 2 dagen waren Cx43 positief (100%). Follikels die gecultiveerd werden voor 4 dagen vertoonden het laagste percentage Cx43 positieve follikels (65.8%) (P=0.002). De percentages van follikels met de aanwezigheid of (partiële) afwezigheid van een TZP netwerk bleek zeer heterogeen tussen follikels in verschillende behandelingsgroepen. In dit Hoofdstuk toonden we het behoud van communicatie tussen de eicel en de somatische cellen aan na vitrificatie en opwarmen. Bovendien, wat betreft de uitdagingen die nog overwonnen moeten worden op het vlak van follikelcollectie en manipulatie, waren we in staat om een simpeler en meer werkbare cryopreservatie techniek toe te passen, namelijk met mini cell strainers. Vitrificatie in cell strainers bood belangrijke voordelen, zoals gemakkelijke manipulatie en besparing van tijd doordat follikels in groep werden gevitrificeerd.

In **Hoofdstuk 6** vertaalden we onze ervaring in follikelisolatie, vitrificatie en immunofluorescentie kleuringen opgedaan met het bovien model naar humaan materiaal. Humane follikels werden enzymatisch geïsoleerd en morfologisch intacte follikels werden gevitrificeerd met een Cryoptop® vitrificatie systeem (Kitazato, Japan). We hebben Cx43 (middels immunofluorescentiekleuring en qPCR) en TZPs (middels immunofluorescentiekleuring) in follikels in alle pre-antrale stadia kunnen aantonen zowel na isolatie als na vitrificatie en opwarmen. The identificatie van Cx43 en TZPs duiden op de aanwezigheid van deze eiwitten die essentieel zijn voor verdere follikelontwikkeling.

De algemene resultaten zijn nogmaals grondig besproken en gekaderd in de Algemene Discussie (**Hoofdstuk 7**). Mogelijke onderwerpen voor toekomstig onderzoek werden behandeld in **Hoofdstuk 8**.

CURRICULUM VITAE

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Participating in the society and working together with all strata of the society has given me a wider view of the world. I learned to look at things from another point of view and to admire habits from other people in my environment.

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Lab techniques
Work with bovine: isolation, characterization, evaluation, culture and vitrification of pre-antral follicles. *In vitro* production of bovine embryos. Vitrification of bovine oocytes.
Work with human: isolation, characterization, evaluation, culture and vitrification of pre-antral follicles.
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Cryopreservation: human and bovine pre-antral follicles/ ovarian tissue: vitrification/warming, slow freezing/thawing, protocol optimization.
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International publications

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Bus A, Langbeen A, Martin B, Leroy J, Bols P. Is the pre-antral ovarian follicle the 'holy grail' for female fertility preservation? (2019) *Animal reproduction science*, 207:119-130.

Bus A, Szymanska K, Pintelon I, Leroy J, Leybaert L, Bols P. Preservation of connexin 43 and transzonal projections in isolated bovine pre-antral follicles before and following vitrification (2021) *Journal of Assisted Reproduction and Genetics*, *38*(2), 479-492.

Lierman S*, **Bus A***, Andries S, Trias E, Bols PEJ, Tilleman K. Passive slow freezing is an efficacious and cost-effective alternative to controlled slow freezing for ovarian tissue cryopreservation (2021) *Cryobiology,* 100:164-172. *Equally contributed

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- International Congress on Animal Reproduction, Tours, France, 26-30 June 2016.

Poster: Vitrification of isolated bovine pre-antral follicles using a commercial vitrification kit.

- International Society for Fertility Preservation Congress, Vienna, Austria, 16-18 November 2017.

Poster: A comparison between 2d and 3d systems for the vitrification of isolated bovine pre-antral follicles.

- Annual congress Vereniging voor Fertiliteitsstudies, Tilburg, The Netherlands, 12 October, 2018.
- European College of Animal Reproduction Symposium, Vienna, Austria, 4-6 July 2019.

Poster: The use of alginate beads for the vitrification of isolated bovine pre-antral follicles.

- International Society for Fertility Preservation Congress, New York, US, 14-16 November 2019.

Poster: Gap junction protein connexin-43 expression before and after vitrification of isolated bovine pre-antral follicles.

BIBLIOGRAPHY

International publications

Bus A, van Hoeck V, Langbeen A, Leroy J, Bols P. Effects of vitrification on the viability of alginate encapsulated isolated bovine pre-antral follicles (2018) *Journal of Assisted Reproduction and Genetics,* 35(7):1187-99.

Bus A, Langbeen A, Martin B, Leroy J, Bols P. Is the pre-antral ovarian follicle the 'holy grail' for female fertility preservation? (2019) *Animal reproduction science*, 207:119-130.

Bus A, Szymanska K, Pintelon I, Leroy J, Leybaert L, Bols P. Preservation of connexin 43 and transzonal projections in isolated bovine pre-antral follicles before and following vitrification (2021) *Journal of Assisted Reproduction and Genetics*, *38*(2), 479-492.

Lierman S*, **Bus A***, Andries S, Trias E, Bols PEJ, Tilleman K. Passive slow freezing is an efficacious and cost-effective alternative to controlled slow freezing for ovarian tissue cryopreservation (2021) *Cryobiology,* 100:164-172. *Equally contributed

Poster publications

Baetens E, Andries S, **Bus A**, De porte HFM, Leroy JLMR, Bols PEJ. (2015). The use of Neutral Red as a viability indicator hampers *in vitro* development of semi-nude bovine oocytes to the blastocyst stage. (Abstract). Proceedings of the 29th Annual Meeting of the Brazilian Embryo Technology Society and 31st Meeting of the European Embryo Transfer Association. Anim Reprod, p 727.

De porte HFM, Andries S, **Bus A**, Langbeen A, Baetens E, Leroy JLMR, Bols PEJ. (2015). Osmotic challenge of bovine early pre-antral follicles with different cryoprotectant agents. (Abstract). Proceedings of the 29th Annual Meeting of the Brazilian Embryo Technology Society and 31st Meeting of the European Embryo Transfer Association. Anim Reprod, p 603.

Bus A, Merckx E, Andries S, Leroy JLMR, Bols PEJ. Vitrification of isolated bovine pre-antral follicles using a commercial vitrification kit. International Congress of Animal Reproduction (ICAR), Tours, France, 26-30 June 2016.

Bus A, Leroy JLMR, Bols PEJ. A comparison between 2d and 3d systems for the vitrification of isolated bovine pre-antral follicles. International Society for Fertility Preservation (ISFP), Vienna, Austria, 16-18 November 2017.

Bus A, Leroy JLMR, Bols PEJ. The use of alginate beads for the vitrification of isolated bovine pre-antral follicles. European College of Animal Reproduction (ECAR), Vienna, Austria, 4-6 July 2019.

Bus A, Szymanska KJ, Leroy JLMR, Leybaert L, Bols PEJ. Gap junction protein connexin-43 expression before and after vitrification of isolated bovine pre-antral follicles. International Society for Fertility Preservation (ISFP), New York, US, 14-16 November 2019.

International research activities

December 2015 — Traineeship in Electron Microscopy; 2 week training period regarding the preparation of early pre-antral follicles for transmission electron microscopy
7-19 December 2015
Department of Veterinary Clinical And Animal Sciences
Section for Anatomy & Biochemistry
University of Copenhagen
Supervised by Prof. Dr. Poul Hyttel

June 2016 — Summer school Multiscale Imaging for the Study of Reproduction
21-24 June 2016
INRA Center Val de Loire
Organized by Prof. Dr. Anne Duittoz

Co-supervisor Bachelor and Master Theses

2014-2015 — E. Baetens
Master in de Biomedische wetenschappen
Vitrificatie van mature boviene eicellen en de gevolgen op de ontwikkelingscompetentie tot blastocysten *in vitro*

2015-2016 — I. Goetschalckx
Bachelor in de Biomedische Laboratoriumtechnologie
Het opstellen van een vitrificatieprotocol voor boviene pre-antrale follikels

2016-2017 — G. Verbraak
Bachelor in de Biomedische Laboratoriumtechnologie
Het inbedden van geïsoleerde boviene pre-antrale follikels in alginaat beads

2016-2017 — J. Cobbaut
Bachelor in de Biochemie en Biotechnologie
De invloed van de morfologie op de viabiliteit en omvang van de pre-antrale follikel.

2017-2018 — R. Vermeiren
Bachelor in de Biomedische Laboratoriumtechnologie
Het vitrificeren van ingebedde pre-antrale follikels in vloeibare stikstof

2018-2019 — W. Bertels
Bachelor in de Biomedische Laboratoriumtechnologie
Optimalisatie van een protocol voor de vitrificatie van pre-antrale follikels ingebed in alginaat beads

2018-2019 — J. Cobbaut
Master in de Biochemie en Biotechnologie
Localization of connexin based cell contacts in (isolated) bovine pre-antral follicles

2019-2020	L. Rose Bachelor in de Biomedische Laboratoriumtechnologie Het identificeren van connexine 43 en transzonale projecties in boviene pre-antrale follikels voor en na vitrificatie
2020-2021	C. van Reeth Master in de Biomedische Wetenschappen Optimization of pre-antral ovarian follicle retrieval and cryopreservation through passive slow freezing

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